

# Biological and practical lessons associated with the use of sexed semen

Simon P. de Graaf<sup>1</sup>, Tamara Leahy<sup>1</sup> and Ramakrishnan Vishwanath<sup>2</sup>

<sup>1</sup>Faculty of Veterinary Science, The University of Sydney NSW 2006 Australia; <sup>2</sup>Sexing Technologies, 22575 State Hwy 6 Sth, Navasota, Texas, USA

## Summary

Numerous biological and practical lessons associated with the use of sexed semen have been learnt in the 15 years since it was released onto the commercial market. Over this time, concerns regarding the safety of sex-sorted spermatozoa have been largely allayed through the birth of several million pre-sexed calves, but the major issue that remains is that of reduced fertility. Numerous studies have demonstrated that the process of flow cytometric sorting and subsequent cryopreservation causes reduced *in vitro* and *in vivo* fertility of sexed semen, which cannot be wholly compensated for by increasing the number of spermatozoa inseminated. As a result of this diminished fertilising capacity, the use of sex-sorted bull spermatozoa was traditionally only recommended for insemination of heifers, not lactating, superovulated and/or synchronised animals due to the low fertility obtained. However, recent changes to the processing methods used during and after flow-sorting are showing the fertility of 'sexed semen' is improving for the first time in over a decade of commercial activity. With continued research and development of methods to improve the conditions under which spermatozoa are sorted and frozen and concurrent advancements in sorter technology, it is anticipated that the fertility and application of sexed semen will continue to increase.

## Introduction

It is now almost three decades since sex-preselection of offspring was proven possible with the birth of a litter of female rabbit kits (Johnson *et al.* 1989). This remarkable achievement was made possible by a modified flow cytometer able to separate spermatozoa into X and Y chromosome bearing populations based on their difference in DNA content. Since this breakthrough, this technology has been used to produce pre-sexed offspring in over a dozen species (rabbits (Johnson *et al.* 1989), pigs (Johnson 1991), cattle (Cran *et al.* 1993), sheep (Catt *et al.* 1996), humans (Levinson *et al.* 1995), horses (Buchanan *et al.* 2000), buffaloes (Presicce *et al.* 2005), elk (Schenk & DeGroff 2003), dolphins (O'Brien & Robeck 2006), dogs

(Meyers *et al.* 2008), cats (Pope *et al.* 2008), deer (Gao *et al.* 2010) and goats (Bathgate *et al.* 2013)), and sex-sorting has become a multi-million dollar commercial enterprise throughout the international cattle industry.

This commercial success has been realised by the expenditure of considerable time, money and effort to improve not just the speed and efficiency of flow cytometric sorting, but also the viability and functional competence of the sex-sorted sperm populations. Research and development saw the two major limitations of sex-sorting by flow cytometry, 1) the speed at which sperm cells can be separated, and 2) the quality of spermatozoa following sorting and frozen storage, improved to levels acceptable for the commercial viability of the technology in dairy cattle. To achieve this a delicate balance was struck, whereby the number of spermatozoa in a dose of sexed semen was small enough to ensure profitability of production, yet retained sufficient functionally competent cells to achieve reasonable levels of fertility following artificial insemination. These developments saw Cogent UK (XY Inc.'s first licensee) release to market the first commercial straw containing 2.1 million frozen sexed bull spermatozoa for standard artificial insemination (AI) only 10 years after the aforementioned births of pre-sexed rabbits following surgical oviductal AI with fresh sex-sorted spermatozoa. Today, the technology is now available to a global cattle market through numerous XY Inc. licensees as well as 19 other laboratories operated by Sexing Technologies who are the worldwide license holder.

Since the first commercial straw of sexed semen was produced there have been various incremental improvements to sorting technology, such as the development of the orientating nozzle, and sperm processing techniques (reviewed by Sharpe & Evans (2009), Evans (2010) and Rath *et al.* (2013)), but by and large relatively little has changed. Indeed several million pre-sexed calves have been produced in this time using doses of 'sexed semen' almost identical in terms of the number of spermatozoa contained within and, most importantly, fertility to that which was first reported for sexed, frozen-thawed bull spermatozoa some 15 years ago. That said, much has been learnt in this time about the fertility and function of sexed spermatozoa; the extent and source of damage induced by sorting and freezing (from collection to insemination), the effects of sperm number and insemination technique as well as species specific phenomena. Insights into the safety and efficacy of the technique have also been made. With an emphasis on ruminants (particularly cattle), the aim of this review is to discuss these biological and practical lessons associated with the use of 'sexed semen' as well as examine the most recent research and development that may see some of these traditionally held lessons overturned in the near future.

### **Economic lessons**

Considering the commercial focus of sex-sorting research over the past 2-3 decades it is useful to briefly consider the economics of sex-sorting and the sale of sexed semen in order to frame our understanding of the subsequent biological and practical lessons that have been learnt.

The equipment, labour and maintenance involved in separating spermatozoa into X and Y chromosome bearing populations is profoundly expensive. Running costs aside, one must remember that the construction of a sorting facility is no small undertaking. Prior to 2004, most of the costs were all met by each individual licensee holders of the technology. However, since this point in time when Inguran LLC (dba Sexing Technologies) acquired the global rights to this technology, the economic considerations for sex-sorting cattle semen have changed. Rather than provide artificial insemination companies with standalone licenses, Sexing Technologies now operate their own sperm sexing laboratories in 19 locations worldwide under a fee for

service model ([www.sexingtechnologies.com](http://www.sexingtechnologies.com)). This change has meant the capital cost of acquiring flow cytometers and the ongoing operating costs are no longer borne by the artificial breeding companies. Instead, Sexing Technologies provides a customised service to each of these breeding organisations (J Moreno, CEO Sexing Technologies, 2014, personal communication).

Nevertheless, sperm-sexing still entails a substantial cost which must be passed on to the end user as a cost premium for purchase of a sexed straw of semen. Obviously, the aforementioned capital costs are built into the fee for service that Sexing Technologies charge each breeding company, in addition to those inherent to the time consuming process of sex-sorting. Indeed, processing inefficiencies are a key factor driving the cost premium associated with a dose of sexed spermatozoa. Current single-head flow cytometers (the most recent multi-head machines will be discussed in the latter section on recent developments), while markedly faster than earlier models, are only able to sort spermatozoa at around 6000-8000 spermatozoa of each sex (with 90% accuracy) per second. While this appears remarkably fast considering each spermatozoon is evaluated sequentially, it is quite slow when a conventional dose of bull semen is 10-20 million total spermatozoa. The cost of labour is a substantial percentage of the price premium associated with sexed semen. Numerous studies have been conducted to quantify the economic value of sexed spermatozoa, usually in beef and dairy cattle enterprises (Taylor *et al.* 1988, Amann 1999, Hohenboken 1999, Seidel 2003a,b). As these investigators attest, sex-preselection will not be profitable for all livestock producers to use, or for all breeding companies to offer. Price premium influences the uptake of sex preselection technology and the reduced fertility associated with sex-sorted spermatozoa inseminated at low doses from virtually all tested species (Garner 2006), with the exception of sheep (de Graaf *et al.* 2007c, Beilby *et al.* 2009) and possibly goats (Bathgate *et al.* 2013), has a detrimental effect on its commercial viability. Considerable research has been conducted to understand why fertility of sex-sorted spermatozoa is reduced and develop methods for its subsequent enhancement.

### **Biological and practical lessons**

#### *The fertility of sex-sorted bull spermatozoa is compromised*

Fertility has been recognised as an issue since the first offspring were produced using sex-sorted spermatozoa (Johnson *et al.* 1989). Reduced litter size (rabbits (Johnson *et al.* 1989), pigs (Johnson 1991)), retarded embryo development (rabbits (McNutt & Johnson 1996), cattle (Merton *et al.* 1997, Lu *et al.* 1999)) and higher embryo mortality (rabbits (McNutt & Johnson 1996), pigs (Bathgate *et al.* 2008)) have all been characteristic traits of sex-sorted spermatozoa. Sexed spermatozoa have consistently been reported to have diminished conception and pregnancy rates whether artificially inseminated fresh (sheep (Cran *et al.* 1997), cattle (Seidel *et al.* 1999a), pigs (Grossfeld *et al.* 2005)) or after freezing and thawing (cattle (Seidel *et al.* 1999b, Frijters *et al.* 2009)). As a general rule, sexed bull spermatozoa are expected achieve 75-80% of the fertility of conventional non-sexed, frozen-thawed semen (Schenk *et al.* 2009). Reduced fertility of sexed, compared to non-sexed, frozen bull semen was first documented in the late 1990s (Seidel *et al.* 1999b) and continues to be reported today (DeJarnette *et al.* 2009, Norman *et al.* 2010, Healy *et al.* 2013). In the majority of the early studies, direct comparisons between sex-sorted and non-sorted spermatozoa were confounded by a difference in the number of motile spermatozoa inseminated between the two treatment groups. However, in more recent times as sorting efficiency has continued to improve, attempts have been made to elucidate whether the decrease in fertility observed following AI of sexed spermatozoa is due to reduced inseminated sperm numbers or reduced fertility of sex-sorted spermatozoa. Unfortunately, it does not appear

reduced fertility of sex-sorted spermatozoa can be compensated for by increasing the dose of spermatozoa inseminated to the levels used in conventional AI programs. Marginal increases in fertility have been achieved (in some sires) by increasing the total number of bull spermatozoa inseminated to 3.5 or 5 million (DeJarnette *et al.* 2008, DeJarnette *et al.* 2010). Another study with several thousand inseminations comparing AI of 10 million total sexed or conventional spermatozoa failed to demonstrate comparable fertility between the two groups (44% and 60% conception rates for AI of 10 million total sexed or non-sexed spermatozoa, respectively; DeJarnette *et al.* 2011). Efforts to improve the fertility of sex-sorted bull spermatozoa via the use of deep intrauterine (Kurykin *et al.* 2007) insemination of the uterine horn ipsilateral to ovulation and/or various methods of fixed time synchrony prior to AI (Sá Filho *et al.* 2012) have likewise failed to overcome the fundamental fertility problem outlined above. Indeed, it is still recommended that lactating dairy cows not be inseminated with sexed semen due to the extremely low fertility that will most likely result. However, it should be noted that this is due to the additive effect of the compromised fertility of the modern dairy cow, rather than any specific deleterious interaction between this sperm type and the lactating cow as was originally thought (DeJarnette *et al.* 2008, DeJarnette *et al.* 2009).

The use of sexed semen in MOET programs is also not recommended. This is due to the reduced fertilisation rates in superovulated heifers inseminated with sexed spermatozoa (Sartori *et al.* 2004), particularly when low (2 million spermatozoa) insemination doses are used (Schenk *et al.* 2006) or cows are used as donors (Kaimio *et al.* 2013). Problems with fertilisation often have a flow on effect to the number of transferable embryos produced, so it is unsurprising to discover fewer transferable embryos are produced following AI with sex-sorted spermatozoa when compared with donors inseminated with non-sexed semen (Larson *et al.* 2010, Soares *et al.* 2011). There is the occasional contrary report in the literature, but such studies usually contain relatively small animal numbers (An *et al.* 2010).

When considered as a whole, the clear lesson of these fertility results is that factors other than sperm number control the fertility of sexed bull spermatozoa, and that the process of flow cytometry itself must significantly alter their functional capacity (Frijters *et al.* 2009, Rath *et al.* 2013). Only the most recent advances in flow-sorted sperm processing (described at the end of this review) show evidence of amelioration of these functional problems and a subsequent reduction in the fertility gap between conventional and sexed bull semen (R W Lenz, T B Gilligan, J M deJarnette, M Utt, L Helser, K M Evans, C Gonzalez, J F Moreno & R Vishwanath 2014, unpublished observations).

#### *The in vitro function of bull spermatozoa is compromised*

Functional studies *in vitro* found the sorting process results in a more advanced membrane state which resembles *in vitro* capacitation (evidenced by CTC analysis and protein tyrosine phosphorylation) (Bucci *et al.* 2012). Interestingly, these capacitation-like changes were more prominent in bull spermatozoa than boar spermatozoa (Bucci *et al.* 2012). Sorted spermatozoa have also demonstrated inferior motility characteristics (velocity and amplitude of lateral head displacement assessed by computer assisted sperm analysis (bull (Suh *et al.* 2005), sheep (de Graaf *et al.* 2006)), and ability to penetrate cervical mucus (de Graaf *et al.* 2006) compared with non-sorted spermatozoa. Sorted ram spermatozoa also bind in fewer numbers to oviduct epithelial cell monolayers (OECMs) *in vitro* (de Graaf *et al.* 2006) and detach more rapidly than non-sorted spermatozoa (Hollinshead *et al.* 2003). This finding suggests that binding/release within the oviduct (Gillan *et al.* 2000), thought to play a significant role in successful fertilisation (Hunter 1995), may be impaired by sorting, possibly due to the advanced state of the membrane.

It is important to note that despite these aforementioned stressors, sex-sorting is also a highly discriminating technology. The orientating forces of the nozzle selects morphologically normal spermatozoa and counterstaining with a non-toxic red food dye quenches Hoechst 33342 fluorescence in non-viable cells, allowing this population to be gated to waste during sorting. The resulting population exhibits some improved sperm parameters (e.g. membrane integrity and total motility) compared to their non-sorted counterparts (ram (de Graaf *et al.* 2006), bull (Underwood *et al.* 2009b)) and some diminished traits (e.g. capacitation-like changes). The combination of these functional changes makes sorted spermatozoa a unique cell population with a reduced fertilising lifespan (except in sheep (Beilby *et al.* 2009)) which accounts for the aforementioned reduction in fertility.

*In vitro embryo production (IVP) with sex-sorted bull spermatozoa is impaired and the resultant embryos may have altered properties*

Due to the relatively small number of spermatozoa required, the use of sex-sorted spermatozoa for *in vitro* fertilisation (IVF) systems has long been thought a highly efficient means of utilising sex-preselection technology. While some studies report similar rates of cleavage and blastocyst formation between embryos produced from sexed and non-sexed sperm treatments (Xu *et al.* 2009, Carvalho *et al.* 2010), the majority of investigators report some reduction in blastocyst yield when sex-sorted bull spermatozoa are used (Merton *et al.* 1997, Lu *et al.* 1999, Bermejo-Álvarez *et al.* 2008, 2010). Detailed analysis of developmental kinetics has also shown delayed onset of first cleavage in embryos fertilised by sex-sorted spermatozoa (Bermejo-Álvarez *et al.* 2010) as well as alterations to blastocyst structure (Palma *et al.* 2008) and developmentally important gene transcripts in some studies (Morton *et al.* 2007), but not others (Bermejo-Álvarez *et al.* 2010). In sheep, gene transcripts are altered in embryos fertilised by sex-sorted spermatozoa *in vivo*, but not *in vitro*, for reasons which are yet to be determined (Beilby *et al.* 2011). These *in vitro* results tell a similar story to those conveyed in the previous section; namely, that the sorting process alters the sperm cell and this may impede its ability to fertilise the egg and produce a viable embryo. While a recent study did report similar calving rates following the transfer of *in vitro* embryos produced with non-sorted and sex-sorted spermatozoa, the number of animals used was relatively low (Rasmussen *et al.* 2013).

*The cause of diminished functional capacity of sex-sorted spermatozoa is multifactorial*

As for the precise cause of alterations to the sexed cell, the possibilities remain many and varied and are most likely cumulative. They may be the result of high dilution during the sorting process, known to negatively impact on the percentage of live and motile spermatozoa (Ashworth *et al.* 1994, Catt *et al.* 1997a). Reintroducing seminal plasma after sorting negates some of these deleterious effects suggesting that lower concentrations of protective seminal lipids and proteins (highly diluted during sorting) to be the cause (Catt *et al.* 1996, Maxwell *et al.* 1997, Maxwell *et al.* 1998, Leahy *et al.* 2009). As a result, supplementation of collection media with 1% seminal plasma is standard practice in modern boar sorting protocols (Grossfeld *et al.* 2005). However, the protective effects of seminal plasma during sorting are species-specific (Leahy & de Graaf 2012) and exposure of bull spermatozoa to seminal plasma prior to sorting reduced the efficiency of the technique, but the potential benefits of post-sort supplementation were not tested (Burroughs *et al.* 2013).

Apart from dilution, the decrease in sexed sperm viability and membrane status seen post-sorting may be the result of any number of stressors to which spermatozoa are exposed

prior to, during, or after passage through the flow cytometer. These include the addition of the fluorochrome Hoechst 33342, exposure to 351 and 364 nm of ultraviolet laser light, high temperatures during incubation, pressure changes, physical stress and shear forces and electrostatic gating (Seidel & Garner 2002). Stepwise comparison of the stages of sorting found that the mechanical stress of transit through the machine (without H33342 staining or laser illumination) resulted in the largest increase in the percentage of damaged spermatozoa ((18.6% above non-sorted, unstained controls; (Seidel & Garner 2002)). Exposure to UV laser illumination, staining with H33342 or both stressors combined increased sperm damage a further 6.8, 3.6 and 0.3%, respectively. Studies demonstrating the beneficial effects of decreasing sorting pressure from 50 to 40 psi on motility characteristics (Suh *et al.* 2005), embryo development (Campos-Chillon & de la Torre 2003) and fertility (Suh *et al.* 2005) further emphasise that deterioration of sexed sperm function is largely due to mechanical/physical stress during transit through the flow cytometer. However, oxidative stress from repeated electric charging and electrostatic deflection (3000 V) is also thought to play a role (Rath *et al.* 2013).

*Safety concerns over the use of sexed semen have been largely allayed*

Concerns were also raised over potential cytotoxic and/or mutagenic damage to gametes through the use of ultraviolet activated fluorochromes. Hoechst 33342 is a reported inhibitor of DNA synthesis, and a cause of chromosomal aberrations, in hamster lung cells (Durand & Olive 1982). An investigation suggesting H33342 and UV laser light caused an increase in chromosomal aberrations of sperm nuclei microinjected hamster eggs (Libbus *et al.* 1987) added weight to this argument. Further research suggests that H33342 does not impair chromatin of the mammalian spermatozoon (Garner 2009) probably due to H33342 binding to the minor helical groove of the DNA rather than intercalating between the base pairs (Amann 1999) like many other nucleic acid staining dyes (Schenk *et al.* 1999). In any case, Catt *et al.* (1997b) discovered no increase in endogenous DNA nicks after exposure of human spermatozoa to H33342 stain and/or ultraviolet light. Sperm Chromatin Structure Assay (SCSA) of bovine spermatozoa revealed that H33342, laser illumination, and both stressors combined increased DNA fragmentation cumulatively by only 2.04, 1.50 and 1.01% on top of that already caused by the mechanical insult of flow cytometry (1.77% above that of non-sorted unstained controls (Garner *et al.* 2001)). More recent studies have shown frozen-thawed sexed bull spermatozoa initially display similar levels of DNA fragmentation when compared to non-sorted spermatozoa, but rapidly deteriorate to become more fragmented following 72h of incubation (Gosalvez *et al.* 2011). Nevertheless, this damage does not appear to be extensive enough to cause any genotoxic effects in any resultant progeny to date (Parrilla *et al.* 2004).

Perhaps the strongest case for the safety of the technique lies in the several million animals born using sexed spermatozoa, seemingly without alteration to phenotype beyond natural levels (Amann 1999, Tubman *et al.* 2004). Admittedly, even if phenotypes did differ between parent and offspring, unless the mutation had an obvious effect it would most likely go unnoticed due to the difficulty inherent in detecting a random genetic change. Perhaps this uncertainty allows concern to remain over the safety of sex-sorting, particularly for human application (Ashwood-Smith 1994, Johnson & Schulman 1994, Munne 1994). Even the remotest of possibilities that the genome is being altered invoke serious ethical and philosophical dilemmas, which contribute in part to the reticence of some to utilise sexing technology.

The only evidence which may be cause for concern has come from recent studies which have tracked pregnancy outcome over several thousand or million inseminations. Four separate groups of authors have now highlighted a higher incidence of stillbirth in calves produced

from sexed semen (DeJarnette *et al.* 2009, Norman *et al.* 2010, Healy *et al.* 2013, Rath *et al.* 2013). Interestingly, with the exception of Healy *et al.* (2013), the increased rate of stillbirth was isolated to male calves. One theory is that Y-chromosome spermatozoa might be included in the X-chromosome bearing sorted population due to an aneuploid condition (DeJarnette *et al.* 2009). Clearly this is an issue worthy of further investigation, particularly if the technology is ever to be re-launched for human application.

Whether the risks of mutation and genomic change are real or imagined it remains prudent and practical to use the minimum amount of dye (and laser power) that allows consistent resolution between X and Y sperm populations. The implementation of quasi continuous wave lasers c. 2004, while also improving operational efficiency, reduce the time each spermatozoon is exposed to the laser beam in addition to allowing a reduction in the amount of H33342 needed to attain X-Y resolution (Schenk *et al.* 2005, Sharpe & Evans 2009). Overall laser power has also been reduced although this has not necessarily improved the developmental rate or quality of embryos derived from sexed spermatozoa (Guthrie *et al.* 2002).

#### *Liquid storage of spermatozoa prior to sorting decreases sorting efficiency and post-sort sperm function*

The concern has always been that relatively few sex-sorting facilities exist due to establishment and maintenance costs, but this situation has begun to change with a number of facilities established in recent years. In the last five years alone, new facilities have been established in New Zealand, Australia, Germany, France, Continental US, and Brazil. Nonetheless, in many places sorters are still often located a considerable distance from the male(s) and/or female(s) used in the sex-preselection program. After sorting, this issue has been largely overcome by many years of development to adapt cryopreservation protocols to the peculiarities of sex-sorted spermatozoa. However, before sorting, an effective transport solution which does not involve animal translocation has proven more difficult to develop.

Liquid storage of spermatozoa destined for sorting within 8 h for gorilla (O'Brien *et al.* 2002), 14-17 h for dolphin (O'Brien & Robeck 2006), 18 h for stallion (Lindsey *et al.* 2005) or 24 h for ram (Hollinshead *et al.* 2004b) post-collection has been considered, resulting in pregnancies when inseminated into animals with (dolphin (O'Brien & Robeck 2006)) and without (horse (Lindsey *et al.* 2005)) post-sort cryopreservation. In cattle, liquid storage for 24 h prior to sex-sorting and freeze-thawing demonstrated acceptable levels of *in vitro* motility and acrosome integrity *in vitro* (Underwood *et al.* 2009a) but resulted in poor pregnancy rates (7.3% compared to 57.4% for control frozen spermatozoa; (Underwood *et al.* 2010a)). The poor result was achieved despite the use of double inseminations at 12 and 20 h post onset of standing oestrus (sperm number) and a reasonable total sperm dose (4 or 8 million total spermatozoa). These results indicate that the combination of these three technologies results in significant damage to spermatozoa, possibly through stressors in the handling process and aging of spermatozoa due to extended processing times.

If the sire is deceased, or transport cannot be relied upon to deliver semen in a timely fashion, cryopreservation is a far more attractive sperm storage option. As a result, sex-sorting from previously frozen samples has been the focus of much research over the past decade.

Initial attempts to sex-sort bull spermatozoa that has been previously frozen proved successful, but low sort rates, as well as delayed blastocyst development following IVF, were observed (Stap *et al.* 1998, Lu *et al.* 1999). Further development of the technique to improve sort speed and sperm viability (O'Brien *et al.* 2003) culminated in the production of lambs following the transfer of *in vitro* embryos produced with frozen-thawed, sex-sorted

and re-frozen thawed ram spermatozoa (Hollinshead *et al.* 2004a). Frozen-thawed, sex-sorted, re-frozen-thawed ram spermatozoa were also shown to have similar fertility to sexed ram spermatozoa that had been sorted from fresh semen or even non-sorted controls, when intrauterine insemination was performed (de Graaf *et al.* 2007b). This was seemingly the result of motility, acrosome integrity, viability and mitochondrial activity in frozen-sorted-re-frozen samples, while lower than fresh-sorted-frozen, being significantly higher than non-sorted, frozen spermatozoa (de Graaf *et al.* 2006). Since spermatozoa were inseminated directly into the uterus the reduced velocity and mucus penetrating ability of sexed spermatozoa observed *in vitro* (de Graaf *et al.* 2007b) were inconsequential to *in vivo* function under the conditions tested. Such successes stimulated similar research in cattle, but to date results in this species have been less encouraging.

In a proof of concept study, 3 pregnancies were achieved (3/74) after insemination of frozen, sexed, frozen bull spermatozoa to oestrus synchronised heifers but no live births were recorded (0/74) (Underwood *et al.* 2010a). In the same study, conventionally frozen semen displayed acceptable fertility (39/68 pregnant, 36/68 calved). Further *in vitro* tests found the sorting procedure improved the function of frozen-thawed bull spermatozoa, most likely due to the elimination of membrane-damaged sperm during the sorting process, with frozen-sorted spermatozoa retaining high motility (66.5%) and acrosome integrity/membrane viability (64.9%) 24 h after sorting (Underwood *et al.* 2009b). However, after re-cryopreservation (double freezing) spermatozoa had lower total motility and velocity compared to non-sorted frozen-thawed spermatozoa.

In an attempt to compensate for the rapid decline in motility exhibited by frozen sexed frozen spermatozoa, this sperm type was inseminated ( $4 \times 10^6$  spermatozoa) close to the site of fertilisation (mid-horn of heifers;  $n = 20$ ) around the time of ovulation (within 6h). Again, the pregnancy rate of non-sorted frozen-thawed spermatozoa (6/8; 75%) differed significantly from that of frozen-thawed sex-sorted re-frozen-thawed spermatozoa (0/12; 0%) (Underwood *et al.* 2010b). In a further experiment, one pregnancy (1/7; 14.3%) was recorded when a dose of  $10 \times 10^6$  frozen-sexed-frozen spermatozoa was inseminated (Underwood *et al.* 2010b), which provides proof of concept, but is a long way from the commercialisation of this sperm type. For the time being, it is restricted to use in IVP systems where it appears to perform with similar ability to conventionally frozen spermatozoa (Underwood *et al.* 2010c).

It remains unknown why frozen-sexed-frozen spermatozoa can achieve acceptable *in vivo* fertility in sheep (de Graaf *et al.* 2007b), but not cattle. A current hypothesis is that ram spermatozoa show greater resistance to the handling-induced damages of the sorting and freezing processes, and these are the subject of previous review (de Graaf *et al.* 2009). This hypothesis would account for the many differences in function and fertility between sex-sorted bull spermatozoa and sex-sorted ram spermatozoa reported over the last decade. As alluded to throughout this review, sex-sorted, frozen-thawed ram spermatozoa are now recognised to have comparable if not superior fertility to that of non-sorted, frozen-thawed controls (de Graaf *et al.* 2009). Whether inseminated in superovulated ewes (de Graaf *et al.* 2007a), in non-superovulated ewes at very low doses (1 million motile (de Graaf *et al.* 2007c, Beilby *et al.* 2009)) or even when frozen both prior to and following sex-sorting (de Graaf *et al.* 2007b), flow-sorted ram spermatozoa result in similar or higher fertilization and/or lambing percentages compared with non-sorted spermatozoa. These results highlight a further lesson from the use of 'sexed semen': sorting affects the spermatozoa of each species in a different way and will impact on their subsequent fertility depending on the species specific context in which they are used.



## Recent developments and future prospects in sperm sexing

### *Fertility of sexed bull spermatozoa*

Encouragingly, recent unpublished research by Sexing Technologies indicates some of the issues associated with the use of sexed spermatozoa such as dramatically reduced fertility and function may soon be ameliorated to a significant degree.

A concerted effort has been made in the last few years to understand the biochemistry of media being used for processing of sex-sorted spermatozoa, which has resulted in substantial changes in the composition of media used in several stages of the sorting process. The aim of these changes was to provide a benign environment that accommodates all these changes in pH and temperature that occur during the various steps of processing and sorting, but retains sperm integrity through the process. The end result is a process called SexedUltra<sup>®</sup>, which is a complete overhaul of the media and conditions under which semen is processed and sorted. Using the new generation Genesis I flow cytometers and software (Cytonome/ST LLC; [www.cytonomest.com](http://www.cytonomest.com)), the SexedUltra<sup>®</sup> processing method was compared to the traditional XY processing methods developed in the late 1990s and early 2000s. Semen from 8 bulls was distributed to over 40 herds and inseminated into several thousand Holstein heifers. As can be seen from the results in Table 1, this trial provides recent evidence of an improvement in the fertility of sexed, frozen-thawed bull semen.

**Table 1.** Fertility of sex-sorted, frozen-thawed bull spermatozoa processed using traditional XY protocols or the new SexedUltra<sup>®</sup> method and inseminated into Holstein dairy heifers. Scanned pregnancy data from R W Lenz, T B Gilligan, J M deJarnette, M Utt, L Helser, K M Evans, C Gonzalez, J F Moreno & R Vishwanath 2014, unpublished observations.

Method of processing sexed bull spermatozoa	Number of inseminations	Scanned pregnancy rate
XY	3384	41.6% <sup>a</sup>
SexedUltra <sup>®</sup>	3546	46.1% <sup>b</sup>

Values without common superscripts differ significantly ( $P < 0.01$ )

Substantial trials conducted in New Zealand (Livestock Improvement, New Zealand) with sex-sorted bull spermatozoa that have not undergone subsequent cryopreservation also show promising fertility results. Dairy cattle breeding in New Zealand is seasonal and in a period of 16 weeks (September to December) around 4 million dairy cattle are inseminated predominantly with fresh semen (Vishwanath & Shannon 2000), so this system lends itself well for an intensive study on this particular type of sexed spermatozoa. The results of these trials indicate that sexed bull spermatozoa (1 million total spermatozoa/dose), when not frozen after sorting but instead inseminated within 48 h after initial collection, exhibit 94-96% of the fertility of non-sexed, liquid stored spermatozoa (1 to 2 million total spermatozoa/dose; Table 2). Remarkably, this result with unfrozen sexed spermatozoa was in lactating dairy cows rather than dairy heifers. Future studies to confirm this finding outside of the New Zealand production setting will be of great interest.

Combined, these latest fertility results are quite encouraging as the fertility of 'sexed frozen semen' has improved by about five percentage points compared with the previous method of processing and 'fresh sexed semen' is around 95% of that of fresh conventional semen in large scale field studies in New Zealand. It will be interesting to see if these results can be applied on the world scale. The very minor decrease in fertility observed with 'fresh sexed semen' suggests that the overall process of dilution, staining and sex sorting *per se* is only marginally detrimental in the tested context. It could be that the bulk of the loss in fertility outlined earlier

**Table 2.** Non Return Rate (NRR; 2-24 day) of sex-sorted and non-sorted, liquid stored semen inseminated into dairy cows within 48h after semen collection. (Z Xu and S Ballinger 2013, unpublished observation, Livestock Improvement Corporation (LIC), New Zealand, used with permission)

Year	Number of inseminations	2 – 24 day NRR <sup>1</sup>		Sex-sorted NRR / non-sorted NRR
		Sex-sorted <sup>2</sup>	Non-sorted <sup>3</sup>	
2011	8600	65.0%	69.0%	94.0%
2012	12000	67.0%	69.5%	96.4%
2013	14000	65.2%	68.0%	95.8%

<sup>1</sup>Non Return Rate

<sup>2</sup>1 million total sex-sorted, liquid-stored spermatozoa/AI dose

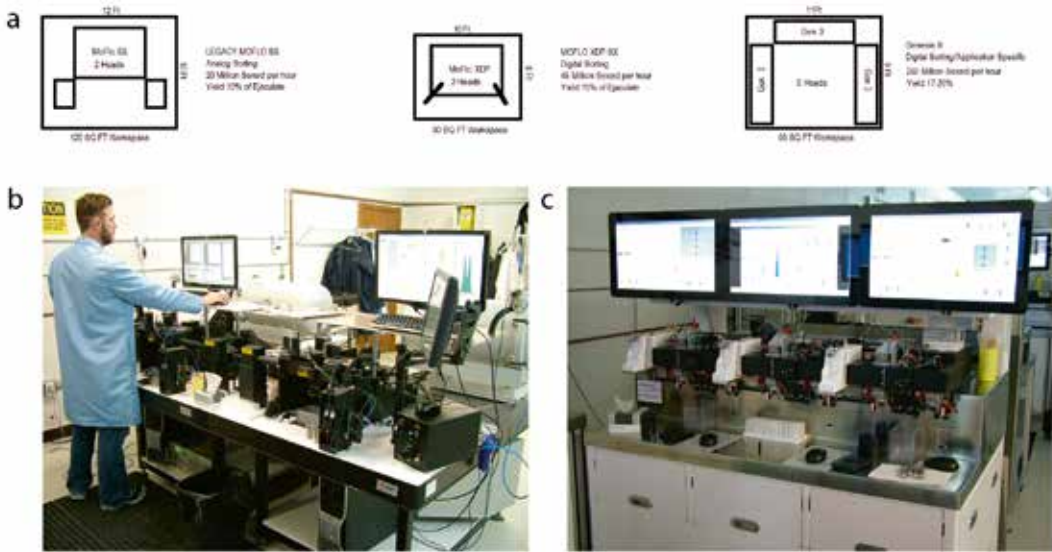
<sup>3</sup>1 to 2 million total non-sorted, liquid-stored spermatozoa/AI dose

in this review occurs mainly during the freezing and thawing process, but further testing in other settings is required for this hypothesis to be confirmed. Additionally, improvements made with the SexedUltra® process shows that there is potential to reduce the gap in fertility between sexed frozen and conventional frozen semen. No doubt further opportunities exist to enhance sperm protection during the cryopreservation process to allow for greater number of sperm to survive post thaw and improve the probability of fertilisation subsequent to AI. This is an area which deserves considerable attention in future research studies.

The last few years has also seen an increase in the number of studies which have utilised sex-sorted bull spermatozoa in timed artificial insemination programs. That is, in synchronised rather than naturally cycling cattle. As previously mentioned, timed AI has not improved the fertility of sexed semen to the levels observed when conventional semen is used (Mallory *et al.* 2013, Sa Filho *et al.* 2013, Thomas *et al.* 2014). However, when used appropriately, sex-sorted spermatozoa in timed AI programs displays a comparable decline in fertility to that observed in naturally cycling animals (approximately 70% of conventional semen) (Macedo *et al.* 2013). The key to obtaining such levels of fertility in timed AI programs appears to be insemination later than normal in order to deposit semen closer to the time of ovulation (Sales *et al.* 2011). Interestingly, in suckled beef cows which fail to display signs of oestrus, sex-sorted spermatozoa perform equally as well as conventional semen (36% vs 37% pregnancy rates, respectively) if insemination of the former is delayed 20h (Thomas *et al.* 2014). Nonetheless, the same trial still demonstrated the compromised nature of sex-sorted spermatozoa as pregnancy rates achieved in animals that did display oestrus was 77% for conventional semen and 51% or 42% for sexed semen (inseminated at the same time as conventional or 20h later, respectively (Thomas *et al.* 2014)). These results suggest that with careful management sexed semen can be effectively utilised in timed artificial insemination programs in dairy and beef cattle.

#### *Advances in sperm-sorting technology*

Since the early MoFlo SX sperm sorters which had an overall sorting speed of 500 to 600 spermatozoa per second, sorting technology has continually improved as summarised in reviews by Sharpe & Evans (2009) and Evans (2010). Since these reports, a new generation of sorters with improved speeds and efficiency have been developed by Cytonome/ST LLC (Boston, USA) exclusively for sex sorting spermatozoa. These latest Genesis machines offer significant improvements in sort efficiency through improved speed, yield and reduced labour, not to mention other developments to reduce maintenance problems and other issues that have



**Fig. 1.** a) Diagrammatic representation of the evolution of high speed sperm-sorters. Physical footprint, number of sexed cells produced per hour and relative efficiency are shown for dual head Legacy MoFlo SX (analog processing), dual head MoFlo XDP (digital processing) and nine head GENESIS III machines. b) Dual head MoFlo XDP and c) GENESIS III sperm sorters shown in operation at Sexing Technologies laboratories in Navasota Texas. The GENESIS III triple head sorter differs greatly from the MoFlo machines as it has a compact modular layout, no sheath tanks as the sheath delivery is through a new fluid delivery system that is pressurised only before entry into the sorter, new software and high level automation for alignment, drop delay, sort speed and many other features. Photos courtesy K. M. Evans (Sexing Technologies), data and schematic diagrams courtesy K. M. Evans (Sexing Technologies) and Dr John Sharpe (CEO, Cytonome/ST LLC, Boston, USA), used with permission.

traditionally afflicted flow cytometers. This evolution of sorters and their relative efficiencies is shown schematically in Fig. 1a with operational MoFlo XDP and Genesis III machines illustrated in Fig. 1b and 1c, respectively.

Clearly, the ability to sex in excess of 250 million spermatozoa per hour from a bank of sorters greatly improves the scope of sperm sexing technology to deliver adequate numbers of spermatozoa to achieve reasonable fertility for most applications. Given sufficient development in sperm processing techniques, these advances in flow sorting may see growth of sexed semen in animal industries besides cattle. There is substantial research occurring with pigs, horses and deer and it is likely that there will be more reports on fertility trials in these species in the near future. The confluence of semen biochemistry research and sorter technology improvements should ensure a step change in both fertility and efficiency of the sex sorting process.

## Conclusions

Undoubtedly, the primary lesson associated with the use of sexed semen is that its fertility is to some degree compromised during the sorting process. That it is not more so, considering the dramatic ordeal which each sexed sperm cell has had to undergo, is testimony to the research efforts of the numerous investigators who have worked in this area over the past few decades and the resilience of the male gamete. Nevertheless, as a result of damage during the sorting

process the fertility of sex-sorted, frozen-thawed bull spermatozoa remains at approximately 75% that of conventional semen when inseminated in naturally cycling heifers. Early reports suggest similar reductions in fertility when used in timed AI programs. It is not recommended for use in lactating cows or superovulated females and cannot be effectively applied to spermatozoa that have been liquid or frozen stored prior to AI, unless the sexed spermatozoa are subsequently used for IVF. The most recent research demonstrates that the fertility issue in heifers can be partially overcome (85% fertility of non-sexed semen) by careful development of sorting media or largely overcome in lactating cows (95% fertility of non-sexed semen) if cells are not frozen following sorting. However, the latter solution is limited in its scope as the majority of users will still desire frozen straws. It remains to be seen if these current advances have improved fertility to the point that sexed bull semen can be used in the aforementioned non-recommended situations (e.g. MOET programs), but obviously they are a major step forward after 15 years without any reported improvement in fertility. The fact that the result with fresh sexed semen was in lactating dairy cows certainly opens another avenue for application of this technology and makes it more relevant to modern dairy production systems.

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### Conflicts of Interest

R Vishwanath is a current employee of Sexing Technologies. SP de Graaf and T Leahy declare that they have no conflicts of interest.

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