Animal (2014), **8:s1**, pp 160–164 © The Animal Consortium 2014
doi:10.1017/S1751731114000202

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Update on sexed semen technology in cattle

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(Received 8 November 2013; Accepted 11 January 2014; First published online 28 March 2014)

The technology in current use for sexing sperm represents remarkable feats of engineering. These flow cytometer/cell sorters can make over 30 000 consecutive evaluations of individual sperm each second for each nozzle and sort the sperm into three containers: X-sperm, Y-sperm and unsexable plus dead sperm. Even at these speeds it is not economical to package sperm at standard numbers per inseminate. However, with excellent management, pregnancy rates in cattle with 2 million sexed sperm per insemination dose are about 80% of those with conventional semen at normal sperm doses. This lowered fertility, in part due to damage to sperm during sorting, plus the extra cost of sexed semen limits the applications that are economically feasible. Even so, on the order of 2 million doses of bovine semen are sexed annually in the United States. The main application is for dairy heifers to have heifer calves, either for herd expansion or for sale as replacements, often for eventual export. Breeders of purebred cattle often use sexed semen for specific matings; thawing and then sexing frozen semen and immediately using the few resulting sexed sperm for in vitro *fertilization is done with increasing frequency. Beef cattle producers are starting to use sexed semen to produce crossbred female replacements. Proprietary improvements in sperm sexing procedures, implemented in 2013, are claimed to improve fertility between 4 and 6 percentage points, or about 10%.*

Keywords: sexed semen, cattle, fertility, economics

Implications

Over the next few years, it is unlikely that any fundamentally new technology will become available for sexing sperm commercially. However, incremental improvements in flow cytometer/cell sorting procedures almost certainly will result in faster speeds of sexing sperm and improved fertility. However, fertility of sexed semen is unlikely to exceed 90% of unsexed semen for most situations for several years, so profitable applications for cattle will be limited. Longer term prospects are encouraging; since sperm are evaluated one at a time, some defective sperm are discarded during sexing, and this may become more sophisticated, increasing fertility.

Introduction

Most companies and cooperatives selling bovine semen in the United States sell sexed semen from a subset of their bulls. For reasons to be explained later, it is impossible to know the precise volume of sexed semen sales, but a consensus figure is on the order of 2 million doses annually, averaged over beef and dairy sires and domestic and export sales. This represents something $<5\%$ of bovine semen sales volume.

To interpret the sexed semen industry, it will be useful to consider a bit of history plus some intellectual property issues. Sexing sperm is still a developing technology, so changes in the product continue to be made, such as improving fertility, increasing accuracy of sexing, adding flexibility to delivery systems and reducing costs.

Intellectual property issues

There is only one useable, reasonably accurate method of sexing mammalian sperm available, flow cytometry/cell sorting. This equipment measures the amount of DNA in sperm; bovine X-sperm have about 4% more DNA than bovine Y-sperm. A patent for this technology was submitted in 1991 by the United States Department of Agriculture with Dr Lawrence Johnson as inventor, and related technology has been licensed and sub-licensed to various entities over the years. That patent has now expired, but dozens of other patents on improvements and variations have continued to be filed such that in the United States and many other countries, most of the intellectual property associated with sexed semen as currently used commercially is owned and

[a] The author served as Scientific Director of XY Inc. as part of his faculty responsibilities at Colorado State University, and has continued to be a consulting member of the Scientific Advisory Committee for XY Inc. and Sexing Technologies. However, he never had a financial interest in any of the commercial entities that developed and marketed sexed semen.

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https://doi.org/10.1017/S1751731114000202 Published online by Cambridge University Press

controlled by Sexing Technologies or related companies located in Navasota, Texas. This technology is licensed to several companies worldwide or used by employees of Sexing Technologies who operate in close proximity to where bulls that produce the semen at the major bull studs are housed. Due to the competitive nature of the bovine semen industry, the information about how many doses of semen are sexed at any given location, or the exact contractual parameters are not publicly available. For these reasons, the numbers of doses of semen sexed, the current accuracy of sexing, and the prices charged for sexing sperm must be estimated indirectly. The exact technology currently in use is similarly proprietary, although in principle it likely does not differ substantively from what has been published recently (Garner *et al.*, 2012).

Brief history of sexed semen

The history of development of commercial sexed semen was summarized by Garner and Seidel (2008). Briefly, the basic technology was developed in the early 1980s at the United States Department of Energy's Lawrence-Livermore Laboratory in California using procedures that required demembraning sperm, resulting in non-viable sperm (Garner *et al.*,1983). Procedures then were greatly improved so that sperm remained live and fertile post-sorting; the research was spearheaded by Dr Lawrence Johnson at the United States Department of Agriculture in Beltsville, Maryland (Johnson *et al.*, 1989). Procedures were further improved by efforts at Colorado State University and made practical for use for artificial insemination. Most of the research in Colorado was funded by the company XY Inc., which acquired a license from the US Government for the intellectual property regarding the sexing technology developed in Beltsville. The first commercial license for AI was granted by XY Inc. to Cogent in the United Kingdom in 2002. In 2007, XY Inc. was sold to the group in Texas, who greatly broadened commercialization, and who have continued to improve semen processing procedures and the equipment for sexing sperm.

The technology and delivery systems

Procedures for sexing sperm have been described numerous times (e.g. Johnson and Welch, 1999; Seidel and Garner, 2002; Garner *et al.*, 2012). The equipment used is impressive in speed and accuracy, but also is expensive and, for practical purposes, unavailable except in the context of contracting or licensing sperm sexing services from Sexing Technologies. The current technology suffers from one overriding limitation; each sperm must be evaluated and sorted individually as they exit a nozzle. Most systems are set up with two nozzles operating in parallel like a two-cylinder engine; other configurations of additional nozzles have been tested, and some years ago the Monsanto Corporation even worked on sperm sexing equipment with four banks of four nozzles as the configuration. Nevertheless, to my knowledge, none of these more complex configurations is in routine commercial use. However, it is inevitable that the current two-nozzle systems will be superseded with configurations that increase output.

There are physical limits in how many sperm can be evaluated accurately, one at a time, as they are passing by a detector and exiting a nozzle (Garner *et al.*, 2012). Currently the speed of passage is around 80 km/h during which about 30 000 sperm can be evaluated per second under ideal conditions. The percent actually sexed of those evaluated depends on semen quality and desired accuracy. A fringe benefit of sexing sperm is that dead and dying sperm, those with compromised cell membranes, are discarded, but sorter capacity is spent in evaluating these sperm. On the accuracy issue, up to 50% more sperm can be sorted per unit time at 75% accuracy than at 90% accuracy; 95+% accuracy is possible, but that is slow, and therefore, expensive. Note that there always is a confidence interval on accuracy, so machines usually are set slightly higher than advertised accuracy; for example 91%, but an individual straw may range from 89% to 93% of the desired sex. One other factor to keep in mind is with low numbers of inseminations, there will be disappointments in practice. For example, at 90% accuracy, there is a 19% chance that any two consecutive calves will not be of the desired sex.

To illustrate typical sorter performance at 90% accuracy with 10% membrane-compromised sperm, 32 000 sperm may be evaluated per second, with 3000 discarded as dead or dying sperm, 1000 discarded due to technical issues such as two sperms per droplet or their being too close to each other in the stream to get accurate information, 8000 not evaluated because of not being appropriately oriented for accurate measurement because the flat aspect of the sperm was not facing the detector, and another 4000 not being clearly X- or Y-sperm due to biological and procedural variability. Thus, in this example, only 16 000 sperm actually get sorted per second, or 8000 of each sex, which represents 25% of the ejaculate for each sex. Unfortunately, the problem does not end there because the sorted sperm are very dilute and must be concentrated by centrifugation, and a few percent of sperm are lost in that process. Also, some sperm are lost in the 'dead space' of the equipment and used for quality control. Packaging 2 million sperm per insemination dose under the above ideal conditions would produce about 14 straws of sexed semen of each sex per hour.

Discarding >75% of sperm is one of the reasons that owners of bulls whose semen is in high demand are reluctant to sex that semen. Because sexed semen is routinely packaged at only 2 million sexed sperm per dose as the current industry standard, much lower numbers than routinely used for unsexed semen, the total doses produced from one ml of semen may not be much different whether sexed or not.

Essentially all of the sexed semen sold for AI in the US is frozen. There is anecdotal information from New Zealand that fertility of sexed, unfrozen semen, processed similarly to New Zealand procedures with unsexed semen, is excellent. Because fertility of frozen bovine semen that is thawed and refrozen is usually severely compromised, sexing frozen semen is only done if it can be used without refreezing.

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The latter approach works quite well in conjunction with *in vitro* fertilization (IVF), and this is widely practiced by one company.

Because of the above considerations, the equipment for sexing sperm is usually located in close proximity to the bulls providing semen. In a few cases, fresh, unsexed semen, slightly diluted with an extender, is collected mid-afternoon and shipped by overnight courier to sites with cell sorters for sexing semen the following morning.

A frequent logistical problem is that insufficient sorter power is available to sex all sperm in an ejaculate within a reasonable period of time, for example 7 h. Having semen waiting to be sexed results in decreased quality. For example, if two, two-nozzle sorters are available for 7 h at 30 000 sperm/s per nozzle, one would use about 3 billion sperm; it is not unusual for a bull to produce more than twice that number of sperm per ejaculate. A common approach to this situation is to use part of an ejaculate for sexing, and then process the remainder of the ejaculate for conventional frozen semen sales. Garner *et al.* (2012) further explain the complex procedures for preparing sperm for sexing such as adding antibiotics, staining for up to 45 min with the DNA-binding dye Hoechst 33342, concentrating the sexed sperm by centrifugation, adding extender including the cryoprotectant glycerol, holding the sperm at low temperature for several hours before freezing, packaging in straws, etc. All this gets to be logistically demanding, especially because parts of the process are done in batches at approximately hourly intervals.

The very high costs of the sperm sexing equipment create pressure to use the equipment around the clock. Although this has moderated over the years, there have been situations in which sorters have run 24/7, necessitating up to four teams of employees per week. Collecting semen three times per day also has put stress on bull handlers. Modifications often are made so that two collection times per 24 h are made to work.

Fertility

Fertility of sexed semen in cattle is lower than control, unsexed semen for two reasons: fewer sperm per insemination dose and damage to sperm from the sorting process. Frijters *et al.* (2009) estimated that two-thirds of the decrease is due to fewer sperm, and the balance is due to damaged sperm. Although few other experiments have been done to partition the causes of the low fertility, the consensus is that for most bulls, both factors are involved in lowered fertility.

There is considerable variation in fertility from bull to bull at low doses of both unsexed (Den Daas *et al.*, 1998) and sexed (DeJarnette *et al.*, 2008, 2010 and 2011) sperm per inseminate. However, evidence for a sexed *v.* unsexed sperm × bull interaction for fertility is weak or absent; the same holds for a sperm dose × sexed/unsexed sperm × bull interaction. Thus, fertility of bulls using low doses of unsexed sperm usually is a fairly good indicator of relative fertility using low doses of sexed sperm. A caveat in this conclusion is that the studies cited above likely excluded bulls at the low end of acceptable fertility with unsexed, frozen semen.

The nature of the damage to sperm has not been explained adequately by research, but there are many possibilities, including stretching the sperm tail as droplets are formed at the nozzle opening, and continued binding of the Hoechst 33342 to sperm post fertilization, slowing down progression of the first cell cycle between fertilization and the first cleavage (Seidel, 2012). An unsettling point is that the damage appears to be only partially compensable by increasing sperm numbers per dose (DeJarnette *et al.*, 2008, 2010 and 2011), although there is one study in which pregnancy rates in heifers were virtually identical with 10 million sexed or unsexed sperm per dose (Schenk *et al.*, 2009).

Fertility with doses of 2 million sexed sperm is usually in the range of 70% to 85% of controls at normal sperm numbers in well managed herds, which often equates to about 10 percentage points lower fertility. Easily the largest cost of using sexed semen is decreased fertility (Seidel, 2003). The most clearly documented change in procedures to improve this situation has been to lower pressure in the sorting equipment (Suh *et al.*, 2005; Schenk *et al.*, 2009). Importantly, calves resulting from sexed semen appear to be normal (Tubman *et al.*, 2004), although there is some evidence of higher neonatal mortality in the few bull calves that are of the 'wrong' sex when using X-sorted sperm (DeJarnette *et al.*, 2009).

On 1 October 2013, Sexing Technologies announced that licensees have recently changed to procedures shown to improve fertility 4 to 6 percentage points over previous procedures, documented by thousands of inseminations. This would be about a 10% boost in fertility. The nature of the changes was not specified and remains proprietary. A published study by Klinc *et al.* (2007) indicated that addition of certain antioxidants to sperm during the sorting process was very beneficial to fertility under European condition, but numbers of inseminations were limited. In any case, fertility of sexed semen likely will continue to improve with further research (Rath *et al.*, 2013). In addition, procedures to optimize timing of AI for sexed semen with ovulation synchronization procedures are being thoroughly researched. One study from Missouri (Thomas *et al.*, 2013) showed a remarkable increase in fertility with sexed semen by using estrus-detection patches affixed anterior to the tailhead with an appointment breeding program if AI was delayed 20 h for those females with inactivated patches. Apparently sexed, frozen semen does not maintain fertility after extended time in the female reproductive tract as well as frozen, unsexed semen. From other studies (e.g. Schenk *et al.*, 2009) a consensus has evolved that it is best to delay AI 6 to 12 h relative to what has been optimized for unsexed semen over the years.

Applications

By far, the main application of sexed semen in the US is to breed dairy heifers to have heifer calves. The base fertility level in lactating dairy cows is much lower than in heifers, so even though the percentage decrease in fertility does not

https://doi.org/10.1017/S1751731114000202 Published online by Cambridge University Press

differ greatly between cows and heifers, the extra cost of using sexed semen per female calf produced is much higher with cows. For example, a 20% decrease in fertility with a 60% base to 48% pregnant results in twice as many calves than a 30% base reduced to 24%. Other advantages with heifers having heifer calves include less dystocia because female calves average about 2.5 kg lighter at birth than male calves (Tubman *et al.*, 2004) and decreasing generation interval. With any rational breeding program for genetic improvement, on average, the youngest animals in the herd are the best genetically, so calves resulting from first calf heifers are ideal replacements.

There are two main reasons for wanting more heifer calves: herd expansion and sale of heifers to others. At any given time, a fair number of dairy herds are expanding, and there are several important reasons for expanding from within the herd. The main reason over the years has been to build on known genetics, but recently, biosecurity issues have become almost as important, for example including managing Johne's disease. A third reason can be economic, being able to raise replacements at lower cost than purchasing them.

Selling more replacement heifers as a reason for using sexed semen comes with financial risk. The problem is that others may have the same idea, resulting in a glut of heifers in the market. Sexed semen was in fact invoked as a cause of dramatic decreases in replacement heifer prices in 2009. There are two problems with this interpretation. The first is that milk prices in the United States had dropped by nearly 50%, so it was the worst of times to buy replacement heifers. The second issue was that sexed semen was just starting to be used at substantive levels about that time (Norman *et al.*, 2010), and it takes 2½ years from inseminating with sexed semen to having heifers several months from calving, which was where falling prices were most notable.

It is obvious that use of sexed semen could easily result in 'boom and bust' cycles of prices for heifers. My suspicion is that bred heifer prices currently are lower in the United States than they would be without sexed semen. However, prices for bred dairy heifers have not been disastrous over the past several years for several reasons. Perhaps the main one is that there has been a huge export market for US dairy heifers, particularly if pregnant using sexed semen. Numbers of heifers exported have exceeded 100 000/year, which has continued greatly to support heifer prices. There also has been a strong domestic market for bred Jersey heifers because of the continued demand for high-protein milk for making cheese.

A final factor maintaining dairy heifer prices has been historically high prices for cull cows driven by demand for ground beef. Thus dairy farmers could cull problem cows readily and replace them with the available first-calf heifers, with most of the cost recouped from sale of the larger culled cows. The US market for bred dairy heifers will not be supported indefinitely by factors such as export demand and high cull cow prices, so sexed semen will inevitably produce considerable surpluses of heifers at times and consequent low prices (DeVries *et al.*, 2008). Farmers need to be aware of this reality.

Sexed semen is routinely used via AI by breeders of purebred cattle for specific, high-value matings, in some cases with superovulation. However, yields of transferable quality embryos can be reduced by half in cows and somewhat less in heifers (Schenk *et al.*, 2006; Kaimio *et al.*, 2013). Nevertheless, this still makes excellent economic sense because recipient resources are not wasted to produce calves of the less valuable sex. There is anecdotal evidence that use of sexed semen from certain bulls for superovulated cows results in unacceptable yields of tranferrable quality embryos. Although limited in scope, sexed semen is beginning to be used by commercial beef cattle producers to produce crossbred females for replacements without superovulation and embryo transfer.

A growing application is to combine IVF and sexed semen with registered, purebred herds, in which owners desire heifers (or in a few cases bulls) from specific matings for which sexed semen is unavailable. In these cases, a dose of frozen, unsexed semen is thawed, sexed, and used immediately for IVF, for which a few hundred thousand sexed sperm are plenty. These sexed sperm are of very high quality because the dead ones are removed in the sexing process; while this also occurs with conventional sexing of fresh semen, a high percentage of sperm then die during cryopreservation.

To make this application functional, oocytes are collected from the donor dam, sometimes during the first 3 months of pregnancy, and usually shipped by overnight courier in a portable incubator while oocytes are maturing. Timing of oocyte collection needs to be coordinated with shipping time. Oocytes from superstimulated cows require less maturation time than those from cows not treated with superstimulatory protocols.

In any case, the IVF is done at the same location as the sperm sorters are located and cultured embryos are either frozen, or more often, sent by portable incubators to the site housing recipients, often in the herd supplying the oocytes. This highly coordinated system currently is used for over 20 000 oocytes/year, with IVF services primarily provided by TransOva Genetics in Iowa, who obtained a license for such procedures. Sexing Technologies also provides such a service, but those currently are the only two providers in the United States both because of intellectual property issues and unavailability of equipment at other companies to sex sperm.

Much has been written and discussed about the possibilities of crossing dairy cows with sexed semen of beef breeds to produce steers. Use of sexed semen so that dairy heifers have heifer calves frees up matings of older cows that can be used for beef production. One contributing factor is that most Jersey bull calves currently have negative value at birth. A problem with all of these dairy beef possibilities is that under current US conditions, the lower fertility and extra cost of sexed semen, approximately double that of unsexed semen from the same bulls, make these applications uneconomical. What often is profitable, is to use unsexed semen from beef breeds for matings not needed for replacements. Widespread use of sexed semen to produce males for beef will require lower costs and especially higher fertility than provided by the currently available sexed semen products.

https://doi.org/10.1017/S1751731114000202 Published online by Cambridge University Press

Prospects for new methods of sexing sperm

Sex is the most important genetic trait, and eventually it is likely that procedures will be developed that do not require evaluation of each individual sperm in series. However, nature has imposed a series of formidable barriers to prevent the genotype (including sex chromosome composition) of sperm from affecting phenotype of sperm in terms of fertilizing ability within males (Seidel, 1999). Mendel's law of independent assortment, including a 50 : 50 sex ratio requires phenotypic identity of genetically different sperm, which makes it very difficult to distinguish X-sperm from Y-sperm. As indicated earlier, bovine X-sperm have about 4% more total DNA than Y-sperm. The X-sperm clearly are very slightly heavier on average than the Y-sperm, but distributions overlap, much like trying to sex humans by their height or weight. There is one convincing study using centrifugation to separate the smaller human Y-sperm from X-sperm (Koundouros and Verma, 2012), but their procedure is completely impractical plus the procedure almost certainly enriches for monosomies. It is the equivalent of saying humans more than 2 m tall likely are men, but this excludes being able to assign sex to >99% of men; this population also would have an excess of XYY men. There is one study using an immunological procedure for sexing bovine sperm that provides some evidence of efficacy (Sang *et al.*, 2011). However, it also is impractical for a number of reasons and possibly only works for the dead/dying sperm in the ejaculate.

One other approach is to use transgenic technology to create sperm that are sexable. Hermann *et al.* (1999) have produced a strain of mice that naturally has a 2 : 1 male to female sex ratio in progeny without sorting sperm. Currently, however, there is a reluctance to invest in transgenic food-producing animals, and making such transgenic cattle would be very expensive.

Although currently available, commercial sexed semen is not yet an ideal product, but improvements will continue to be made (Seidel, 2012; Rath *et al.*, 2013). While I have emphasized how sexed semen is produced and used in the United States, the situation is very similar in Canada and several other countries.

Acknowledgement

Several colleagues in industry and academia provided insightful suggestions for improving the manuscript.

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