Evolution of cervine, caprine and ovine sex-sorted semen processing

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Introduction

It has been three decades since the first publication showing that flow cytometry was a reliable method to separate X and Y chromosome bearing sperm based on their difference in DNA content (Johnson *et al.,* 1989). Ten years after that publication, the first commercial straw containing 2.1 x 10^6 frozen sex-sorted bovine sperm was released to the market for standard artificial insemination (Garner and Seidel, 2008). Several improvements have happened in the flow cytometry technology since that first commercial straw was released, such as the introduction of orienting nozzles, digital processing, multiple headed sorters, and automation, in a new generation of faster and more efficient sperm sorters known as Genesis (Sharpe and Evans, 2009; Evans, 2010). Significant enhancements in sperm handling, preparation for sorting and media composition have also allowed for sperm quality and conception rates of sex-sorted sperm to reach levels that are comparable with non-sorted (conventional) semen (Vishwanath *et al*., 2014; Vishwanath, 2014; González-Marín *et al.,* 2018; de Graaf *et al*., 2014). The result is a complete overhaul of the conditions under which sperm is processed and sorted, known commercially as SexedULTRA™. Today, beef and dairy cattle sperm sorting laboratories are operating commercially in more than 25 locations, in 14 countries, with an annual production of more than eight million straws. In the past few years, small ruminant industries have been progressively testing and implementing the sperm sex-sorting technology for application in their specific environments. The demand for sheep and goat products has increased considerably worldwide since these small ruminants are easily managed, require a relatively small initial investment and their short generation interval lends itself to a fast return on investment for farmers. Also, dairy goat production keeps drawing the attention of producers due to the health benefits of milk and the popular cheeses and dips made from it. On the other hand, cervine industries have been slowly integrating sex-sorted sperm into their artificial insemination practices for antler trophy hunting and genetic improvement of the herds. Sex-sorted sperm will allow these growing industries to produce optimal proportions of males and females, improve herd management and increase the rate of genetic progress.

Keywords: Sex-sorted sperm, fertility, small ruminants

Cervine sex-sorted sperm – the bucks win

The captive deer breeding industry has experienced an important period of growth worldwide in recent years (Garde *et al*., 2006; Gao *et al*., 2011). There has been an increased trend toward deer gaming farms where the main financial profits rely on antler trophies. Given that males have the highest economic value, sex-sorted sperm represents significant management cost-savings.

In vitro sperm quality studies comparing post-thaw motility and DNA fragmentation kinetics of sex-sorted and conventional sperm of red and white-tailed deer have shown equal or better semen characteristics for the sex-sorted samples (Kjelland *et al*., 2011).

Commercial production of cervine sex-sorted sperm started in 2009 in the headquarters for STGenetics in Navasota, TX. Since then, there has been a steady market for both fresh and frozen sex-sorted sperm in white-tailed deer in the United States, amounting to many thousands of straws produced every year. *In vitro* quality analysis of white-tailed deer sperm including total visual motile sperm, acrosome integrity, computer assisted sperm analysis (CASA) for total and progressive motile and gender purity is part of the commercial production routine, and demonstrates equal or better semen characteristics for the sex-sorted compared to conventional samples (Table 1).

Table 1. Number of fresh and frozen straws produced for white-tailed deer in Navasota, TX in the past four years. Percent visual motile, intact acrosomes, CASA motile and progressive, and gender purity were measured 20 minutes after thawing the straws for frozen semen and at day 1 after sorting for fresh semen.

		Straws produced	% Visual Motile	% Intact Acrosomes	% CASA Motile	% CASA Prog.	% Gender Purity
2014	**Conventional frozen**	2588	55.1	70.5	55.6	46.4	
	Sex-sorted frozen	7537	59.9	73.8	71.2	65.8	91.5
	Sex-sorted fresh	439	61.6	78.2	70.5	52.5	91.5
2015	**Conventional frozen**	2197	56.1	70.4	65.2	37.5	
	Sex-sorted frozen	8676	59.0	72.3	66.5	38.8	92.1
	Sex-sorted fresh	826	63.5	79.1	77.0	55.3	90.3
2016	**Conventional frozen**	1466	58.8	70.5	63.4	44.5	
	Sex-sorted frozen	9347	60.7	77.1	68.4	42.7	91.3
	Sex-sorted fresh	699	74.9	87.3	88.1	63.5	90.7
2017	**Conventional frozen**	1455	57.5	71.0	62.7	43.2	
	Sex-sorted frozen	7354	62.5	79.3	71.1	43.4	90.9
	Sex-sorted fresh	837	74.1	86.6	87.5	62.7	90.6
TOTAL	**Conventional frozen**	7706	56.9	70.6	61.7	42.9	
	Sex-sorted frozen	32914	60.5	75.6	69.3	47.7	91.5
	Sex-sorted fresh	2801	68.5	82.8	80.8	58.5	90.8

Information on fertility of sex-sorted deer sperm is very vague. Some reports point towards the resilience of deer sperm to withstand the sorting process and maintain good fertility. Fertility trials with red deer using 3 x 10^6 sex-sorted sperm on two separate ranches showed that pregnancy results were similar using conventional and sex-sorted sperm (Brigans *et al*., 2010). Other studies show a slightly lower fertility of Y sorted sperm when using Iberian red deer (Anel-Lopez *et al*., 2017), although pregnancy rates were significantly higher when hinds were inseminated closer to ovulation induction, so lower fertilities could be attributed to the need of devising synchronization protocols for this specific species when using sex-sorted sperm samples (Anel-Lopez *et al*., 2018). There are no published reports on the application of SexedULTRA™ in deer semen, but personal communication from the sorting laboratory confirms that, when the proper breeding management and synchronization protocols are used, sex-sorted frozen cervine sperm presents pregnancy rates of ~93-95% to those of conventional (70% vs 74%), and fresh sex-sorted sperm is achieving average conception rates 5-8% better than those of conventional. Gender purity in the field is 92-95% (Personal communication. Jared Templeton, Global Production Manager. STGenetics).

Caprine sex-sorted sperm – the kids matter

Goat sperm sex-sorting has become a recent interest, especially as the dairy goat industry continues to expand. In 2013, one of the few publications regarding sex-sorted goat semen reported successful sorting and birth of kids after laparoscopic intrauterine artificial insemination (LAI) with about 32 x 10^6 sperm per insemination of either sex-sorted or conventional sperm. In this report, fertility was lower for sex-sorted sperm, but the success of the technique was demonstrated (Bathgate *et al*., 2013).

The magnitude of the DNA content between the sex-determining gametes varies among species. The average difference between X and Y sperm DNA content in bovine sperm is 3.8% (Garner, 2006) while, for caprine, this difference is closer to 4.3%, so the separation of X and Y chromosome bearing sperm using flow cytometry is not a problem when using ram sperm. Also, the implementation of SexedULTRA™ procedures and straw freezing, have resulted in a successful commercialization of caprine sex-sorted sperm that started at the end of 2015. Since then, over 11,000 sex-sorted straws have been produced for LAI purposes with an average post-thaw visual motile sperm of 60%, a CASA total and progressive motile of 67% and 59% respectively, intact acrosomes of 74% and a gender purity of 93%.

In small scale field trials in Waco, TX, 75 does were divided into two groups and inseminated with sex-sorted fresh (2 x 10^6 sperm per insemination) or sex-sorted frozen sperm (4 x 10^6 sperm per insemination). Pregnancy rates were 57% for fresh and 49% for frozen sperm. These results are comparable to conventional semen used in the same farm (Personal communication. Earl Peacock, Owner. Premiere Semen).

Further trials in Camperdown (Australia) with 150 does split into three groups and inseminated using LAI with conventional frozen semen at 20 x 10^6 sperm per insemination and sex-sorted frozen sperm at 4 and 2 x 10^6 sperm per insemination demonstrated no difference in pregnancy rates between conventional and sex-sorted sperm, and between the two types of sex-sorted sperm doses (Personal communication. Ponneelan Ganesan, Laboratory Manager. Sexing Technologies Australia).

Most caprine inseminations occur trans-cervically, so the current challenge is to be able to deliver a good fertile dose of sex-sorted semen that can be used for this purpose. Therefore, researchers are investigating the optimal dose for trans-cervical insemination in sheep. Contemporaneous ejaculates from four high genetic value bucks were processed as conventional semen (208 straws) and sex-sorted sperm at concentrations of 4 x 10^6 (160 straws) and 8 x 10^6 (287 straws). Sex-sorted sperm presented an average post-thaw visual motile sperm of 62%, intact acrosomes of 77% and a gender purity of 93%, and has now been released to the field for trans-cervical insemination.

Ovine sex-sorted sperm - the exception to the rule

The use of sex-sorted sperm has genetic, management and financial benefits for dairy, wool and/or meat sheep production. Catt et al. (1996) presented the first report using ram sex-sorted sperm, where 85 conventional, 92 female-sorted and 74 male-sorted ram sperm were injected into *in vitro* matured sheep oocytes and placed into the oviducts of 28 estrous sheep. One pregnancy was diagnosed by ultrasonography after 55 days from an oocyte injected with 'male-sorted' sperm. Besides this initial study, the use of IVF and ICSI is not commercially relevant for the ovine industry, so research has been focused on sex-sorted, fresh and frozen sperm to be used in LAI.

The first pregnancies after LAI with sex-sorted frozen-thawed sperm were achieved using low numbers of sperm per dose (2-4 x 10^6). The overall pregnancy rate for ewes inseminated with sex-sorted sperm was half that of conventional controls (140 x 10^6 sperm; Hollinshead *et al.*, 2002). Further testing of *in vitro* quality parameters of sex-sorted ram spermatozoa showed a reduced total and progressive motility and a tendency towards premature capacitation in sex-sorted sperm compared to conventional (Hollinshead et al. 2003). Combining these findings suggested that sex-sorted ram spermatozoa had a reduced fertilizing lifespan, which would explain the decrease in fertility. In subsequent field experiments, the fertility problems were shown to be partly improved by increasing the number of sex-sorted sperm per insemination (Hollinshead et al. 2003), but this was not a viable solution considering the commercial imperative to minimize the number of sex-sorted sperm per LAI dose.

However, de Graaf *et al.* (2006) later reported that sex-sorted ram spermatozoa presented higher motility, viability, acrosome integrity and mitochondrial activity than non-sorted controls. *In vivo* studies supported these *in vitro* results, demonstrating that sex-sorted ram sperm result in similar or superior fertilization/lambing percentages than conventional controls (de Graaf *et al.*, 2007; Beilby *et al.*, 2009). It appeared that the sex-sorting process could select a functionally superior population of sperm in terms of both *in vitro* and *in vivo* function from the ejaculate, resulting in sex-sorted ram sperm with a superior fertilizing lifespan inside of the female reproductive tract compared with conventional sperm from the same ejaculate. Since that moment, ram sex-sorted sperm was considered an exception to the long-held rule that sex-sorting negatively impacted sperm function to an extent where fertility was compromised.

For the past three years, researchers at STGenetics have been working to make the sex pre-selection technology a commercially viable and effective reproductive management option for the sheep industry. The research performed has been focused on adapting SexedULTRA™ bovine sperm sorting procedures for ovine semen, and to replace the pellet freezing method (Evans and Maxwell, 1987) that was used in all other previous experiments using sex-sorted ovine sperm, since this method is not a commercially viable option.

In the first field trial (New Zealand, 2017), ejaculates from two rams were split and processed in one of two methods: conventional or sex-sorted. Conventional (CONV) semen was processed at 60 x 10^6 per dose and sex-sorted sperm was processed at a gender purity of 92% as fresh semen at 1 x 10^6 cells per dose (Fresh1M), fresh semen at 2 x 10^6 cells per dose (Fresh2M), cryopreserved in pellets at 3 x 10^6 cells per dose (Cryo3M) and cryopreserved in straws at 6 x 10^6 cells per dose (Cryo6M). Percent visual sperm motilities were analyzed after final dilution (0 h) and after 24 h of incubation at 18°C for sex-sorted fresh semen, and after thawing (0 h) and after 3 h incubation at 37°C for sex sorted cryopreserved semen. No differences ($P < 0.05$) were found in percent total motile between Fresh1M and Fresh2M at 0 h (73.0% vs 73.0%) or after 24 h (71.0% vs 71.0%). However, sperm motility was greater in fresh semen compared to Cryo3M at 0 h (73.0% vs 69.0%) and after incubation (71.0% vs 63.0%). Percent total motile sperm was the lowest in Cryo6M at 0 h (44.0%) and 3 h after incubation (43.0%). No statistical differences ($P < 0.05$) were found in the percentage of ewes lambing after insemination between treatments (Table 2). This study confirmed that sex-sorted ram sperm are equally fertile to conventional. In fact, 6 x 10^6 cryopreserved sex-sorted sperm and 2 x 10^6 sex-sorted fresh sperm presented numerically superior fertility when used in LAI than conventional sperm inseminated at higher concentrations. Additionally, no difference was observed between fresh and cryopreserved sex-sorted sperm, which would allow more flexibility once the product is commercialized. Finally, sorted sperm cryopreserved at 6 x 10^6 per straw presented numerically higher conception rates than sorted sperm cryopreserved at 3 x 10^6 pellet, which would ensure ease of use in the sorting laboratories and in the field.

Table 2. Pregnancy rate and lambing information after insemination of synchronized ewes with 60 x 10^6 total frozen-thawed conventional, 3 or 6 x 10^6 total frozen-thawed sex-sorted, and 1 or 2 x 10^6 total fresh sex-sorted ram sperm.

Type/dose of semen	**Ewes Inseminated**	**Lambed (%)**	**Born/ewes lambing**
Conventional Frozen-thawed 60x10^6	60	41.8	1.6
Sex-sorted Fresh 1x10^6	57	35.8	1.4
Sex-sorted Fresh 2x10^6	56	43.6	1.4
Sex-sorted Frozen 3x10^6 (pellet)	62	32.8	1.4
Sex-sorted Frozen 6x10^6 (straw)	51	56.1	1.3

It is likely that the promising results in sheep with sex-sorted sperm could in part be due to LAI procedures, where the sperm are placed at the tips of the uterine horns. More trials are needed at this time to determine optimal synchronization protocols and the minimum sex sorted sperm per dose that would allow a corresponding decrease in the associated cost per dose, but there is no question that this product could become an important breeding option at the elite stud level as well as the commercial farm level in the next couple of years.

The future of small ruminant sperm sorting

In the past decade, sheep and goat production has increased by about one-third due to their economic value as efficient converters of low-quality forages into quality meat, milk, and wool. The deer gaming farms have also experienced an important period of growth worldwide.

For these growing industries, it is imperative to use all available pregnancies to modify the offspring sex-ratio in order to generate productive animals (females or males), allowing for faster genetic progress and increased production while reducing wastage. Sperm sex-sorting by flow cytometry is the only reliable technology to separate X and Y chromosome bearing sperm based on their difference in DNA content. The technology has now been validated for all three species on the basis of laboratory analysis and live births, and incorporates modified flow cytometric sorting instrumentation and SexedULTRA™.

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