In *Vitro* Characterisation of Fresh and Frozen Sex-Sorted Bull Sperm

Abridged Title: In-Vitro Assessment of Sex-Sorted Bull Sperm

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Abstract

The objective of this study was to compare the *in vitro* characteristics of fresh and frozen non-sorted (NS) and sex-sorted (SS) bull sperm (both X- and Y-sorted). Experiment 1: Semen was collected from Holstein Friesian bulls (n = 10; 1 - 2 ejaculates per bull), each ejaculate was split across 4 treatments and processed into 0.25 mL straws as follows: (i) NS fresh at 3 million (M) sperm per straw, (ii) X-SS frozen at 2M sperm per straw, (iii) X-SS fresh at 2M sperm per straw, (iv) X-SS fresh at 1M sperm per straw. A fifth treatment (v) of NS frozen sperm at a dose of 20M sperm per straw was sourced from co-operating animal breeding centres, from previously frozen ejaculates (n = 3) of the same bulls. Experiment 2: Semen was collected from Aberdeen Angus bulls (n = 4; 1 - 2 ejaculates per bull), and each ejaculate was split across 4 treatments as follows: (i) NS fresh 3M sperm per straw; (ii) Y-SS fresh at 1M sperm per straw; (iii) Y-SS fresh at 2M sperm per straw; and (iv) X-SS fresh at 2M sperm per straw. As per Experiment 1, NS frozen straws at 20M sperm per straw were sourced from previously frozen ejaculates (treatment v). One straw per bull per treatment (all five treatments, both fresh and frozen) were assessed *in vitro* on Days 1, 2 and 3 post-sorting (Day 0 = day of sorting) for progressive linear motility (PLM), acrosomal status, and oxidative stress. In Experiment 1, the two X-SS fresh treatments had higher levels of agglutination in comparison to the NS fresh treatment (38.3 ± 8.68% and 8.0 ± 1.64% respectively; mean ± SEM; $P < 0.001$) and this high agglutination of SS fresh treatments was

also observed in Experiment 2 ($P < 0.001$). In both Experiments, the NS frozen treatments had the greatest PLM ($P < 0.05$). Both fresh and frozen SS treatments had higher levels of viable acrosome intact sperm compared to NS frozen treatments ($P < 0.01$; Experiment 1). In both Experiments, NS sperm exhibited higher levels of superoxide anion production compared to SS sperm ($P < 0.05$). In conclusion, this study highlights that while sex-sorting results in a sperm population with reduced oxidative stress, fresh semen diluents must be tailored for SS fresh sperm to avoid excessive levels of sperm agglutination at low dilution rates.

Keywords: Sexed semen, Agglutination, Bovine, Gender bias, Oxidative stress

Introduction

The use of flow cytometry to sex-sort bull sperm is currently the only sperm-sorting technology available that gives a strong and reliable bias in offspring gender, typically in the order of 90% (Garner *et al.* 2013). Widespread use of this technology has the potential to revolutionise the dairy breeding industry, whereby X-sex-sorted (SS) sperm of dairy breeds could be used to produce replacement heifers from the highest genetic merit cows with the remainder being bred to Y-SS sperm of beef breeds with the shortest-gestation length such as Aberdeen Angus (Hohenboken 1999; Cromie *et al.* 2014). However, despite this technology being commercially available for over a decade, SS sperm still only represents a small percentage of the artificial insemination (AI) market (Seidel 2014). The use of SS frozen sperm has primarily been limited to use on heifers (Borchersen and Peacock 2009; DeJarnette *et al.* 2009; Frijters *et al.* 2009) due to concerns over reduced fertility rates in cows (Seidel

and Schenk 2008; Dejarnette *et al.* 2011; Healy *et al.* 2013). This situation appears to be changing, however, with improved sorting technologies, and the gap between the fertility of conventional non-sorted (NS) semen and SS semen is closing as assessed by scanned pregnancy rate; (de Graaf *et al.* 2014) and non-return rates; (Xu 2014; as reviewed by Vishwanath 2014).

The reasons for lower fertility with SS frozen sperm are multi-factorial, and have been attributed to a combination of reduced number of sperm per straw (typically 15-20 million [M] vs. 2.1M sperm per straw for NS frozen and SS frozen sperm, respectively) as well as sperm damage during the sex-sorting and freezing processes. During sex-sorting sperm undergo stresses such as exposure to Hoechst stain (Garner and Seidel Jr 2008), laser exposure (Garner 2006), centrifugation (Shekarriz *et al.* 1995) and sorting pressure (Suh *et al.* 2005), while, reduced fertility has primarily been attributed to mechanical stresses during the sorting process (Garner *et al.* 2013). Furthermore, the sperm's natural defences against lipid peroxidation are diminished as a result of the high dilution factor during sorting (Espinosa-Cervantes and Cordova-Izquierdo 2012). Dilution can disrupt the pH/Osmolarity equilibrium which could affect membrane stability (Gosalvez *et al.* 2011), such as early capacitation and the ability to undergo the acrosome reaction thus affecting fusogenic properties with the oocyte (Maxwell and Johnson 1997; Watson 2000; Gosalvez *et al.* 2011).

The use of SS fresh sperm may be a viable alternative and has the potential to yield higher fertility than SS frozen sperm as sperm are not subjected to the insult of freezing post-sorting as well as the added benefit of requiring fewer sperm per straw (Butler *et al.* 2014a; Xu 2014). However, there are a small number of published studies describing the *in vitro* quality

of SS fresh bull sperm (Mocé *et al.* 2006; Klinc *et al.* 2007; Blondin *et al.* 2009; Gosalvez *et al.* 2011; Bucci *et al.* 2012). Blondin et al. (2009) compared SS and NS (fresh and frozen) sperm in *in vitro* fertilization (IVF) and found that freezing, and not sexing, had a more significant negative effect on semen quality. The same study reported that the sex-sorting process selects better quality sperm by eliminating sperm with compromised DNA. Klinc et al. (2007) reported no difference in the motility or membrane stability of fresh SS and NS sperm and in a field trial with a small number of females reported that fresh SS bull sperm can maintain its fertile lifespan for up to 72 h post-sorting. In contrast, Bucci et al. (2012) compared several *in vitro* capacitation-related factors in both SS and NS fresh bull and boar sperm and concluded that the sex-sorting process resulted in de-stabilization of the sperm membrane. Mocé et al. (2006) working with both fresh and frozen bull sperm, reported higher viability in SS sperm compared to NS sperm within both fresh and frozen treatments. Interestingly, they also demonstrated that although fresh SS and NS sperm respond similarly to the induction of the acrosome reaction (using PC12), frozen SS bull sperm exhibited an accelerated acrosome reaction compared to NS frozen sperm, under non acrosome reaction-inducing conditions. Taken together, this growing body of evidence suggests that SS fresh sperm are less compromised than SS frozen sperm.

A recent field study from New Zealand evaluated X-SS fresh sperm (1M sperm per straw) against NS fresh sperm (1.25 to 2M sperm per straw) in over 50,000 cows and reported a reduction in fertility, measured via non return rate, of just 3.8-4% following insemination with SS fresh sperm (Xu 2014). This is encouraging, as SS fresh sperm at 1M sperm per straw would enable more than twice as many insemination doses to be obtained per ejaculate compared to traditional SS frozen sperm, leading to a potentially cheaper product, without the marked reduction in fertility in cows. The *in vitro* and *in vivo* fertility of NS fresh sperm

following storage for a number of days has been well documented (Vishwanath and Shannon 2000; Al Naib *et al.* 2011; Murphy *et al.* 2013; Murphy *et al.* 2015); however, it is unclear if these procedures are suitable for storing SS fresh sperm and if differences exist between X- and Y-SS sperm during *in vitro* storage. Therefore, understanding the physiology of SS fresh sperm under field conditions is critical to designing diluents for extension of SS sperm at low concentrations. With this in mind, the objective of this study was to assess the *in vitro* quality of X- and Y-SS sperm processed fresh or frozen from the same ejaculate and compare this with conventional NS fresh and frozen sperm from the same bulls. Uniquely, this *in vitro* characterisation is strengthened by the availability of conception rate data (Experiment 1 only) from a large scale sexed semen field trial.

Materials and Methods

Experimental Design

Experiment 1:

The aim of this experiment was to assess the *in vitro* quality of X- SS and NS sperm processed as fresh or frozen. Semen was collected at two commercial AI centres from Holstein Friesian bulls (*Bos taurus*; n = 10; denoted A to J) of proven fertility using an artificial vagina (1-2 ejaculates per bull; 18 ejaculates collected in total). All ejaculates processed had a minimum normal morphology and motility of 80% and 75%, respectively. Undiluted ejaculates were transported in a temperature regulated box (20 ^{o}C) within 3 h to a Sexing Technologies laboratory to be X-SS according to standard procedures with a targeted purity for the X sperm fraction of 90%. Sex sorting was performed on a high speed flow cytometer at 40 psi with a 70 µm nozzle and vanguard laser set at 200 mW. The event rate

was controlled between 20,000 and 21,000 events per second (Burroughs *et al.* 2013). Post-sorting, sperm from individual ejaculates were processed as follows: (i) NS fresh at 3 M sperm per straw (ii) X-SS frozen at 2M sperm per straw, (iii) X-SS fresh at 2M sperm per straw, (iv) X-SS fresh at 1M sperm per straw, and (v) NS frozen at 20M sperm per straw. Treatments (i) to (iv) were split from the same ejaculate on the day of collection and Treatment (v) was sourced through two AI centres from previously collected ejaculates of the same bulls. The sperm number for the NS and SS frozen and NS fresh treatments were based on industry standards. As there have been only a limited number of studies using SS fresh sperm, two concentrations were used (1M and 2M). Previous work by our group has demonstrated that the *in vitro* quality of NS fresh sperm can be influenced by sperm concentration (Murphy et al., 2013). All fresh and frozen media used for processing NS fresh, SS fresh and SS frozen semen were proprietary media supplied by Sexing Technologies (Navasota, Texas, USA). The SS frozen semen media was a Tris-egg yolk buffer containing 6% glycerol which has been optimised over many years to provide a benign environment to hold and freeze sperm in low concentrations and is now part of the SexedULTRA suite of media (R. Vishwanath, Sexing Technologies, Navasota, Texas, USA, personal communication). The fresh semen medium was a new medium containing HEPES buffer and additives that was developed initially for NS sperm but had been adapted to work with SS fresh sperm for this project (R. Vishwanath, Sexing Technologies, Navasota, Texas, USA, personal communication). NS frozen semen was processed in a commercial freezing media, BULLXcell (IMV Technologies, L'Aigle, France).

All fresh treatments were transported and stored at room temperature until time of assessment on each day, whereas frozen treatments were frozen using routine procedures and stored under liquid nitrogen. *In vitro* analyses were first carried out the morning post-sorting (Day 1;

Day 0 = day of sorting), at which time ejaculates ranged from 15 h to 33 h post-sorting (24 ± 1.3 h; mean ± s.e.m) . One straw per treatment was assessed *in vitro* at 24 h intervals (range 23-25 h) on Day 1, 2 and 3 post-sorting for progressive linear motility (PLM; indicative of a functional motile sperm population), acrosomal status (intact acrosome required for fertilization), and oxidative stress (high levels are indicative of stressed sperm leading to diminished sperm function). The remaining straws were distributed amongst AI technicians as part of a field trial (approximately 15,000 inseminations), for which the preliminary conception rate data based on 3,943 ultrasound pregnancy scans have been reported in Butler et al. (2014a, 2014b).

Experiment 2:

Currently, the greatest demand for sexed semen is for X-SS Holstein sperm but there is a growing demand for Y-SS sperm from short gestation early maturing beef breeds for use on the dairy herd. The aim of this experiment was to evaluate the *in vitro* characteristics of Y-SS sperm processed and stored as fresh sperm under field conditions and compare this to X-SS fresh sperm and to NS fresh and frozen sperm. Bull semen was collected, transported and processed as described above from Aberdeen Angus bulls (n = 4; denoted K to N) of proven fertility (1-2 ejaculates per bull; 15 ejaculates assessed in total). All ejaculates processed had a minimum normal morphology and motility of 80% and 75%, respectively. Transport and sorting were carried out as previously described with a target purity of 90%. Treatments were sorted and stored in the appropriate Sexing Technologies diluents as follows; (i) NS fresh at 3M sperm per straw; (ii) Y-SS fresh at 1M sperm per straw; (iii) Y-SS fresh at 2M sperm per straw; and (iv) X-SS fresh at 2M sperm per straw and (v) NS frozen at 20M sperm per straw, sourced from previous ejaculates of the same bulls. Only one concentration of X-SS fresh

sperm was used as the main focus of the experiment was to characterise Y-SS fresh sperm. Proprietary diluents were used for NS fresh, SS fresh and frozen sperm while BULLXcell was the diluent used for NS frozen sperm. *In vitro* analysis was carried out as per Experiment 1; however, no field inseminations were carried out using this semen.

Progressive Linear Motility and Agglutination

Frozen straws were thawed at 37 ^{o}C for 30 sec and incubated in a heated block, at 37 ^{o}C while fresh straws were stored at room temperature until use and heated to 37 ^{o}C in a heated block prior to being assessed, within 20 min of initial incubation. PLM was assessed for each treatment by placing a 5 µL sample on a pre-warmed glass slide overlain with a pre-warmed coverslip. Sperm were assessed under a phase-contrast microscope (400x) by one experienced evaluator. Two counts were taken, where 50 motile sperm were assessed in each count and the number of motile sperm swimming in a progressive linear fashion was recorded (Kiernan *et al.* 2013).

The incidence of sperm agglutination were assessed using nigrosin-eosin staining (sperm:stain ratio of 1:1; 0.068 M water-soluble nigrosin, 0.014 M water-soluble eosin and 0.116 M sodium citrate; Kiernan et al., 2013). A smear was made with 10 µL of the sperm stain mixture on a glass slide and allowed to dry. Sperm were viewed under a phase-contrast microscope (1000x) by one experienced evaluator and two counts were taken, where 50 events were assessed in each count and the average of these counts was recorded. An event was described as the occurrence of a single sperm cell or an agglutinated mass of sperm. The number of sperm per agglutinated mass was also recorded. Agglutination percentage was

calculated as the number of sperm cells present in 50 agglutinated masses as a percentage of the total sperm population assessed in the 50 events as per the following equation (No. of sperm in agglutinated masses)/(Total No. of sperm counted) x 100.

Flow Cytometric Analysis of Acrosome Integrity and Oxidative Stress

Flow cytometry assessment of sperm for specific intracellular markers of acrosome integrity and oxidative stress was used as it can objectively quantify large numbers of sperm in a short period of time. Samples were diluted to a concentration of 3 x 10^5 sperm per mL in Biggers, Whitten and Whittingham medium (BWW; Koppers et al., 2008) and were analysed on a flow cytometer (Guava easyCyte 6HT-2L, Merck Millipore, Billerica, MA, USA) equipped with both a Krypton Laser (642 nm) and an Argon Laser (488 nm). Appropriate single colour controls were prepared to establish the respective fluorescent peaks of the individual stains. These were used in conjunction with the forward scatter (FSC) and side scatter (SSC) signals to discriminate sperm from debris (Supplemental Figures 1 and 2). Fluorescent events were recorded using GuavaSoft (Version 2.7; Merck Millipore) and all variables were assessed using logarithmic amplification. In each sample 10,000 gated events were captured.

Acrosome status was assessed using the fluorescent stain Alexa Fluor 647 PNA (AF647; Ex/Em 650/658; Life Technologies, Carlsbad, CA, USA) and a method adapted from Murphy (2015). AF647 consists of an Alexa Flour 647 fluorochrome conjugated with Lectin PNA from *Arachis hypogaea* (peanut). Briefly AF647 was added to 500 µL of sperm diluted to 3 x 10^5 sperm per mL in BWW medium to give a final concentration of 6 µg/ mL and was incubated in the dark at 37 ^{o}C for 15 min. AF647 fluoresces in the presence of the enzyme acrosin, which is exposed upon the loss of the acrosomal cap. Following this incubation period the fluorescent stain Propidium Iodide (PI Ex/Em 535/617 nm; Life Technologies,

Carlsbad, CA, USA) was added to the sample at a final concentration of 12 µM and incubated in the dark for 5 min at 37 ^{o}C. PI is selectively taken up by membrane compromised cells thus indicating a loss of viability. The fluorescence of AF647 was analysed via the Red2 (664/20 BP) detector and PI via the Yellow detector (583/26 BP), no compensation was needed. The percentage of viable sperm with intact acrosomes was calculated as the percentage of AF647 negative cells of the PI negative population as initially gated based on controls, FSC and SSC (Figure 1).

The generation of the superoxide anion was assessed using the fluorescent stain MitoSOX Red (Ex/Em ~510/580 nm; Life Technologies, USA) and a method adapted from Kiernan et al. (2013). Briefly, diluted samples were incubated at 37 ^{o}C in the presence of MitoSOX Red (4 µM) in the dark for 15 min. MitoSOX Red is an intracellular stain that fluoresces in the presence of the superoxide anion. Following this, the nucleic dead stain SYTOX Green (Ex/Em 504/523 nm; Life Technologies, Carlsbad, CA, USA) was added to give a final concentration of 0.25 µM and again, incubated at 37 ^{o}C in the presence of MitoSOX Red in the dark for 15 min. SYTOX Green works in a similar manner to PI as the stain will only penetrate cells with compromised membranes. The fluorescence of MitoSOX Red was analysed via the Red (690/50 BP) detector and SYTOX Green via the Green detector (525/30 BP), minor computed compensation was carried out. The presence of superoxide was calculated as the percentage of MitoSOX Red positive of the SYTOX Green negative population as initially gated based on controls, FSC and SSC (Figure 2).

Statistical Analysis

Data were examined for normality of distribution, tested for homogeneity of variance and transformed where appropriate. Data for sperm acrosomal status and PLM (both from

Experiment 1) were transformed using a Log_{10} transformation and a power transformation, (lambda = 1.0), respectively. Motility, agglutination, acrosome status and oxidative stress data were analysed using the general linear model repeated-measures procedure with a compound symmetry covariance structure in the Statistical Package for the Social Sciences (SPSS; version 22.0, IBM, Chicago, IL). The final model included the main effects of treatment, day, bull, treatment × day and treatment × bull. Where an effect was significant, a Bonferroni post hoc test was carried out to compare data groups of interest. Data presented in this paper are presented as the non-transformed values; however, all P-values were calculated using the transformed data where required. All data are presented as means ± SEM.

Results

Experiment 1

Agglutination

The two X-SS fresh treatments exhibited higher levels of agglutination compared to all other treatments ($P < 0.001$; Figure 1). There was no effect of day (Days 1-3) or day × treatment interaction. A significant bull × treatment interaction ($P < 0.05$) was evident in the X-SS fresh treatments. The bull with the highest overall level of agglutination (Bull F) had 65.4 ± 3.5% and 74.5 ± 2.1% sperm agglutinated in the X-SS fresh 1M and 2M treatments, respectively, whereas the bull with the lowest levels of agglutination (Bull I) had 31.3 ± 6.1% and 66.1 ± 4.0% sperm agglutinated in X-SS fresh 1M and 2M treatments, respectively.

Progressive Linear Motility

There was an effect of treatment on PLM ($P < 0.01$). PLM was highest in the NS frozen treatment and was statistically higher than the two X-SS fresh treatments on all assessment days (Table 1). It should be noted that PLM was assessed on the motile population of sperm

and may have been skewed by the high levels of agglutination in the SS fresh treatments. There was no effect of bull, day or day × treatment interaction for PLM.

Acrosome Status

The NS frozen treatment had the lowest percentage of viable acrosome intact sperm in comparison to all of the X-SS treatments (Table 2). All of the fresh treatments, both SS and NS, showed similar levels of viable acrosome intact sperm ($P > 0.05$). There was no bull × treatment interaction; however, there was an effect of bull on the level of viable acrosome intact sperm ($P < 0.01$), indicating variability between animals in acrosomal status. There was no effect of day (Days 1-3) on the percentage of viable acrosome intact sperm.

Oxidative Stress

NS treatments, both fresh and frozen, exhibited a higher percentage of sperm positive for superoxide (74.8 ± 5.90% and 52.8 ± 1.89%, respectively; Figure 2) compared to other treatments (< 45%; $P < 0.001$). On Day 1, the NS fresh treatment had a greater proportion of sperm positive for superoxide (76.0 ± 4.0%) compared to X-SS fresh 1M and 2M treatments (40.5 ± 3.6% and 34.2 ± 2.4% on Day 1, respectively; $P < 0.001$). There was no effect of day but there was a treatment by day interaction on superoxide production (Figure 2; $P < 0.05$).

Experiment 2

Agglutination

The trends observed for agglutination in Experiment 1 were mirrored in Experiment 2. Higher levels of agglutination were observed in the three SS fresh treatments compared to the NS treatments ($P < 0.001$; Figure 1). There was a day × bull interaction ($P < 0.01$) as some

bulls exhibited an increased level of agglutination with increasing day of storage. There was no effect of day or day by treatment interaction on agglutination.

Progressive Linear Motility

The NS frozen treatment had higher overall PLM than the Y-SS fresh 2M treatment across all days ($P < 0.05$; Table 3). Despite the high levels of agglutination in the fresh SS treatments all X- and Y-SS treatments had mean PLM scores ranging from 40-70 %. There was no effect of either bull or day (Days 1-3) on PLM.

Acrosome Status

There was no effect of treatment on the percentage of viable acrosome intact sperm, and no significant interactions were detected between bull, day or treatment, in any combination (Table 4). Day of storage had no effect on the percentage of viable acrosome intact sperm.

Oxidative Stress

NS treatments, regardless of storage type, exhibited a higher proportion of sperm positive for superoxide anion ($P < 0.05$; Figure 2). NS treatments had 63-73% of the viable population positive for the superoxide anion, whereas, only 25-40% of the SS sperm were positive for the superoxide anion. In addition, there was no effect of bull, day or day × treatment interaction.

Discussion

The main findings of this study were that (i) sex-sorting resulted in a more functional sperm population with reduced oxidative stress and (ii) excessive agglutination in highly diluted fresh SS treatments indicates the need for a diluent tailored for SS fresh sperm. This is the

first published study to assess the *in vitro* characteristics of SS fresh (X- and Y-SS) and frozen bull sperm from the same ejaculate. While there was no difference in the *in vitro* quality of X- and Y-SS sperm sorted from the same ejaculate processed as fresh sperm, SS fresh sperm exhibited significantly higher levels of agglutination compared to the other treatments, regardless of whether they were X- or Y-SS. Irrespective of the way in which it was processed, SS sperm had a lower percentage of sperm positive for oxidative stress (superoxide anion) compared to the NS treatments.

The incidence of sperm agglutination in the SS and NS frozen treatments was within the normal range (< 10%), in agreement with findings of Carvalho *et al.* (2010). In the current study, however, there were abnormally high levels of agglutination in all of the SS fresh treatments, irrespective of whether they were X- or Y-SS. Sperm agglutination has been known, from previous studies of domestic mammals, to be due to a variety of factors including media composition (Harayama *et al.* 2000), removal of sperm surface anti-agglutinin (Lindahl and Sjoblom 1981; Yang *et al.* 2012) as well as centrifugation and washing (Dott and Walton 1960; Suzuki and Nagai 2003). However, the same sorting process was used for the SS frozen and fresh treatments, yet agglutination was only an issue in the latter, suggesting that either the freezing process dissociates any agglutination or the diluent used in freezing prevents agglutination in the SS frozen treatment. In agreement with the present study, Yang et al. (2012) reported that the agglutination of NS fresh sperm, stored in an egg yolk diluent, had no adverse effect on sperm functionality in terms of acrosome integrity, but did interfere with sperm oocyte interactions during IVF. However, it should be noted that the correlation between IVF success and field fertility is tenuous. In the current study, the conception rates for the SS frozen, SS fresh 2M and SS fresh 1M treatments (Experiment 1) were approximately 87, 80 and 75% of the conventional fresh treatment,

respectively, (Butler *et al.* 2014a). This represents an improvement in fertility of frozen SS sperm compared to that reported by DeJarnette *et al.* (2009) but was significantly lower than the 95% that Xu (2014) reported for SS fresh sperm. The Xu (2014) study which was carried out over the years 2011 to 2013 inclusive, used XY SS sperm in 2011 and 2012 and SexedULTRA™ sperm in 2013 (R. Vishwanath, Sexing Technologies, Navasota, Texas, USA, personal communication), while the current study used SexedULTRA™ sperm. Another key difference between the studies is that Xu (2014) used the Caprogen™ diluent while in the current study Sexing Technologies own proprietary media (not disclosed) was used for all the fresh sperm treatments and may at least in part explain the differences in observed fertility rates. While, there are no direct comparisons of agglutinated sperm and field fertility in the literature, it is likely that the high levels of agglutination in this study compromised *in vivo* fertility.

The negative effects of the SS process on PLM of sperm were observed in both experiments. In particular, it was noteworthy that the NS fresh 3M treatment had consistently higher PLM compared to the SS fresh sperm treatments. This higher PLM was mainly attributed to the high occurrence of agglutination within the SS fresh treatments. A number of studies have reported that SS fresh sperm has similar motility characteristics to NS fresh sperm in bulls (Klinc *et al.* 2007), boars (del Olmo *et al.* 2013) and stallions (Balao da Silva *et al.* 2013), but those studies did not report the high incidence of agglutination that was observed in this study.

The NS frozen treatment had a significantly higher percentage of viable acrosome-reacted sperm when compared to all the SS treatments in Experiment 1 (both fresh and frozen) but this trend was not repeated in Experiment 2. Overall, the levels of viable

385 acrosome reacted sperm in both SS and NS treatments were comparable to other studies
386 (Mocé *et al.* 2006; Pons-Rejraji *et al.* 2009) and was not affected by duration of storage. This
387 is in agreement with Klinc et al. (2007) who reported a higher proportion of NS fresh sperm
388 with damaged acrosomes (12%) compared to SS fresh sperm (6.2%) and this was not affected
389 by duration of storage when stored for up to 48 h at 15 ^{o}C.

390

391 As by-products of cellular respiration, ROS are required for inter- and intra-cellular
392 signalling (Rivlin *et al.* 2004; Aitken *et al.* 2012), however, ROS above basal levels can have
393 detrimental effects on sperm function (Guthrie and Welch 2012). The lower levels of
394 oxidative stress in the SS treatments suggest that sex-sorting results in better quality sperm.
395 This would suggest that the sex sorting process may remove the major sources of ROS such
396 as leukocyte contamination (human; Sikka 1996) and immature and/or morphologically
397 abnormal sperm (bull; Al-Makhzoomi *et al.* 2008; Shojaei Saadi *et al.* 2013: human; Agarwal
398 *et al.* 2003; De Vos *et al.* 2003; Walczak-Jedrzejowska *et al.* 2013). Mammalian sperm can,
399 however, spontaneously produce ROS such as superoxide anion (O'Flaherty *et al.* 2003),
400 hydrogen peroxide (Alvarez *et al.* 1987) and nitric oxide (Chatterjee and Gagnon 2001). At
401 low concentrations, these ROS play an important role in sperm physiological processes, such
402 as capacitation and the acrosome reaction in the bull (Breininger *et al.* 2010) and human (de
403 Lamirande *et al.* 1997) and also signalling during fertilization as seen in the bull (Morado *et*
404 *al.* 2013) and mouse (Kodama *et al.* 1996). In excessive concentrations, however, ROS cause
405 ATP depletion leading to insufficient axonemal phosphorylation (Bansal and Bilaspuri 2011),
406 lipid peroxidation as well as loss of motility and viability (Sikka 1996; Bansal and Bilaspuri
407 2011) of mammalian sperm.

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The *in vitro* results of the current study demonstrate that sex sorting results in a more functional sperm population. Although this is in contrast to some previous studies (Suh et al., 2005, Mocé et al., 2006, Carvalho et al., 2010), other, more recent studies have reported improvements in fertility to SS sperm (de Graaf et al., 2014, Xu, 2014; as reviewed by Vishwanath 2014). SS fresh sperm has immense commercial potential as it can be used at much lower sperm numbers than SS frozen sperm without the same reduction in fertility rates (Xu, 2014) and is especially attractive in seasonal grass-based production systems, such as in Ireland and New Zealand, where NS fresh sperm is already in use. However, in this study there were abnormally high levels of head-to-head agglutination in the SS fresh treatments, which may explain the reduced fertility rates of the respective treatments in the corresponding field trial (Butler, 2014a). Clearly this illustrates that semen diluents for SS fresh sperm need to be carefully optimised to ensure agglutination of sperm is at a minimum and does not compromise its success.

Conclusion

In conclusion, this study characterises the *in vitro* quality of X- and Y-SS bull sperm from the same ejaculate, processed as fresh or frozen and stored under field conditions. The results show that sex sorting can result in a more functional sperm population in terms of lower levels of oxidative stress. However, the high levels of agglutination observed in the fresh SS treatments clearly demonstrates the need to optimise the composition of semen diluents for SS fresh sperm. The use of sex-sorted (SS) fresh sperm has the potential to yield higher pregnancy rates than SS frozen sperm as sperm are not subjected to the insult of cryopreservation post sorting.

Competing Interests

Juan F Moreno is co-chief excutive officer of Sexing Technologies.

Authors' Contributions

Shauna Holden completed the laboratory work with the help of Craig Murphy. Juan Moreno, Stephen Butler and Andrew Cromie designed and implemented the corresponding field trial. Sean Fair and Patrick Lonergan designed the *in vitro* experiments and were the lead supervisors of Shauna Holden.

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References

Agarwal, A., Saleh, R.A., and Bedaiwy, M.A. (2003) Role of reactive oxygen species in the pathophysiology of human reproduction. *Fertil Steril* 79(4), 829-43

Aitken, R.J., Jones, K.T., and Robertson, S.A. (2012) Reactive Oxygen Species and Sperm Function—In Sickness and In Health. *J Androl* 33(6), 1096-1106

Al-Makhzoomi, A., Lundeheim, N., Håård, M., and Rodríguez-Martínez, H. (2008) Sperm morphology and fertility of progeny-tested AI dairy bulls in Sweden. *Theriogenology* 70(4), 682-691

Al Naib, A., Ward, F., Kelly, A.K., Wade, M., Marti, J.I., and Lonergan, P. (2011) Effect of duration of storage at ambient temperature on fertilizing ability and mucus penetration ability of fresh bovine sperm. *Theriogenology* 76(6), 1070-5

Alvarez, J.G., Touchstone, J.C., Blasco, L., and Storey, B.T. (1987) Spontaneous Lipid Peroxidation and Production of Hydrogen Peroxide and Superoxide in Human Spermatozoa Superoxide Dismutase as Major Enzyme Protectant Against Oxygen Toxicity. *J Androl* 8(5), 338-348

Balao da Silva, C.M., Ortega Ferrusola, C., Morillo Rodriguez, A., Gallardo Bolanos, J.M., Plaza Davila, M., Morrell, J.M., Rodriguez Martinez, H., Tapia, J.A., Aparicio, I.M., and Pena, F.J. (2013) Sex sorting increases the permeability of the membrane of stallion spermatozoa. *Anim Reprod Sci* 138(3-4), 241-51

Bansal, A.K., and Bilaspuri, G.S. (2011) Impacts of Oxidative Stress and Antioxidants on Semen Functions. *Vet Med Int* 2011, 686137

Blondin, P., Beaulieu, M., Fournier, V., Morin, N., Crawford, L., Madan, P., and King, W.A. (2009) Analysis of bovine sexed sperm for IVF from sorting to the embryo. *Theriogenology* 71(1), 30-38

Borchersen, S., and Peacock, M. (2009) Danish A.I. field data with sexed semen. *Theriogenology* 71(1), 59-63

Breininger, E., Cetica, P.D., and Beconi, M.T. (2010) Capacitation inducers act through diverse intracellular mechanisms in cryopreserved bovine sperm. *Theriogenology* 74(6), 1036-1049

Bucci, D., Galeati, G., Tamanini, C., Vallorani, C., Rodriguez-Gil, J.E., and Spinaci, M. (2012) Effect of sex sorting on CTC staining, actin cytoskeleton and tyrosine phosphorylation in bull and boar spermatozoa. *Theriogenology* 77(6), 1206-1216

Burroughs, C.A., Graham, J.K., Lenz, R.W., and Seidel, G.E., Jr. (2013) Seminal plasma effects on sex-sorting bovine sperm. *Theriogenology* 79(3), 551-7

Butler, S.T., Hutchinson, I.A., Cromie, A.R., and Shalloo, L. (2014a) Applications and cost benefits of sexed semen in pasture-based dairy production systems. *Animal* 8(Supplement 1), 165-172

Butler, S.T., Hutchinson, I.A., and Cromie, A.R. (2014b) Preliminary results from a field trial to evaluate sexed semen in dairy cows and heifers. In 'Agricultural Research Forum', Tullamore, Ireland

Carvalho, J.O., Sartori, R., Machado, G.M., Mourão, G.B., and Dode, M.A.N. (2010) Quality assessment of bovine cryopreserved sperm after sexing by flow cytometry and their use in in vitro embryo production. *Theriogenology* 74(9), 1521-1530

Chatterjee, S., and Gagnon, C. (2001) Production of reactive oxygen species by spermatozoa undergoing cooling, freezing, and thawing. *Mol Reprod Dev* 59(4), 451-458

Cromie, A.R., Kearney, F., Evans, R., and Berry, D.P. Genomics for Pedigree and Cross-bred Beef Cattle Populations; Some experiences from Ireland. In '10th World Congress of Genetics Applied to Livestock Production', 2014, Vancouver, Canada,

de Graaf, S.P., Leahy, T., and Vishwanath, R. (2014) Biological and practical lessons associated with the use of sexed semen. In 'Ninth International Symposium on Reproduction in Domestic Ruminants.' Leicestershire)

de Lamirande, E., Jiang, H., Zini, A., Kodama, H., and Gagnon, C. (1997) Reactive oxygen species and sperm physiology. *Rev Reprod* 2(1), 48-54

De Vos, A., Van De Velde, H., Joris, H., Verheyen, G., Devroey, P., and Van Steirteghem, A. (2003) Influence of individual sperm morphology on fertilization, embryo morphology, and pregnancy outcome of intracytoplasmic sperm injection. *Fertil Steril* 79(1), 42-48

Dejarnette, J.M., Leach, M.A., Nebel, R.L., Marshall, C.E., McCleary, C.R., and Moreno, J.F. (2011) Effects of sex-sorting and sperm dosage on conception rates of Holstein heifers: is comparable fertility of sex-sorted and conventional semen plausible? *J Dairy Sci* 94(7), 3477-83

DeJarnette, J.M., Nebel, R.L., and Marshall, C.E. (2009) Evaluating the success of sex-sorted semen in US dairy herds from on farm records. *Theriogenology* 71(1), 49-58

del Olmo, D., Parrilla, I., Gil, M.A., Maside, C., Tarantini, T., Angel, M.A., Roca, J., Martinez, E.A., and Vazquez, J.M. (2013) Handling of boar spermatozoa during and after flow cytometric sex-sorting process to improve their in vitro fertilizing ability. *Theriogenology* 80(4), 350-6

Dott, H.M., and Walton, A. (1960) Effects of dilution and washing on ram spermatozoa studied by the flow dialysis technique. *J Reprod Fertil* 1, 350-67

Espinosa-Cervantes, R., and Cordova-Izquierdo, A. (2012) Sexing sperm of domestic animals. *Trop Anim Health Prod* 45(1), 1-8

Frijters, A.C., Mullaart, E., Roelofs, R.M., van Hoorne, R.P., Moreno, J.F., Moreno, O., and Merton, J.S. (2009) What affects fertility of sexed bull semen more, low sperm dosage or the sorting process? *Theriogenology* 71(1), 64-7

Garner, D.L. (2006) Flow cytometric sexing of mammalian sperm. *Theriogenology* 65(5), 943-957

Garner, D.L., Evans, K.M., and Seidel, G.E. (2013) Sex-sorting sperm using flow cytometry/cell sorting. In 'Spermatogenesis.' pp. 279-295. (Springer)

Garner, D.L., and Seidel Jr, G.E. (2008) History of commercializing sexed semen for cattle. *Theriogenology* 69(7), 886-895

Gosalvez, J., Ramirez, M.A., Lopez-Fernandez, C., Crespo, F., Evans, K.M., Kjelland, M.E., and Moreno, J.F. (2011) Sex-sorted bovine spermatozoa and DNA damage: II. Dynamic features. *Theriogenology* 75(2), 206-11

Guthrie, H.D., and Welch, G.R. (2012) Effects of reactive oxygen species on sperm function. *Theriogenology* 78(8), 1700-8

Harayama, H., Liao, P.C., Gage, D.A., Miyake, M., Kato, S., and Hammerstedt, R.H. (2000) Biochemical characterization of sialoprotein "anti-agglutinin" purified from boar epididymal and seminal plasma. *Mol Reprod Dev* 55(1), 96-103

Healy, A.A., House, J.K., and Thomson, P.C. (2013) Artificial insemination field data on the use of sexed and conventional semen in nulliparous Holstein heifers. *J Dairy Sci* 96(3), 1905-14

Hohenboken, W.D. (1999) Applications of sexed semen in cattle production. *Theriogenology* 52(8), 1421-33

576
577 Kiernan, M., Fahey, A., and Fair, S. (2013) The effect of the in vitro supplementation of
578 exogenous long-chain fatty acids on bovine sperm cell function. *Reprod, Fert Develop* 25(6),
579 947-954

580
581 Klinc, P., Frese, D., Osmers, H., and Rath, D. (2007) Insemination with Sex Sorted Fresh
582 Bovine Spermatozoa Processed in the Presence of Antioxidative Substances. *Reprod Domest*
583 *Anim* 42(1), 58-62

584
585 Kodama, H., Kuribayashi, Y., and Gagnon, C. (1996) Effect of Sperm Lipid Peroxidation on
586 Fertilization. *J Androl* 17(2), 151-157

587
588 Lindahl, P.E., and Sjoblom, P. (1981) On mechanisms of head-to-head association in bovine
589 spermatozoa. *Biol Reprod* 25(1), 29-43

590
591 Maxwell, W.M.C., and Johnson, L.A. (1997) Chlortetracycline analysis of boar spermatozoa
592 after incubation, flow cytometric sorting, cooling, or cryopreservation. *Mol Reprod Dev*
593 46(3), 408-418

594
595 Mocé, E., Graham, J.K., and Schenk, J.L. (2006) Effect of sex-sorting on the ability of fresh
596 and cryopreserved bull sperm to undergo an acrosome reaction. *Theriogenology* 66(4), 929-
597 936

598
599 Morado, S., Cetica, P., Beconi, M., Thompson, J., and Dalvit, G. (2013) Reactive oxygen
600 species production and redox state in parthenogenetic and sperm-mediated bovine oocyte
601 activation. *Reproduction* 145(5), 471-478

602
603 Murphy, C., Fahey, A.G., Shafat, A., and Fair, S. (2013) Reducing sperm concentration is
604 critical to limiting the oxidative stress challenge in liquid bull semen. *J Dairy Sci* 96(7),
605 4447-54

606
607 Murphy, C., Holden, S.A., Murphy, E.M., Cromie, A.R., Lonergan, P., and Fair, S. (2015)
608 The impact of storage temperature and sperm number on the fertility of liquid-stored bull
609 semen. *Reprod Fert Develop* (In Press)

610
611 O'Flaherty, C., Beorlegui, N., and Beconi, M.T. (2003) Participation of superoxide anion in
612 the capacitation of cryopreserved bovine sperm. *Int J Androl* 26(2), 109-114

613
614 Pons-Rejraji, H., Bailey, J.L., and Leclerc, P. (2009) Cryopreservation affects bovine sperm
615 intracellular parameters associated with capacitation and acrosome exocytosis. *Reprod Fertil*
616 *Dev* 21(4), 525-37

617

618 Rivlin, J., Mendel, J., Rubinstein, S., Etkovitz, N., and Breitbart, H. (2004) Role of hydrogen
619 peroxide in sperm capacitation and acrosome reaction. *Biol Reprod* 70(2), 518-22

620
621 Seidel, G.E., Jr., and Schenk, J.L. (2008) Pregnancy rates in cattle with cryopreserved sexed
622 sperm: effects of sperm numbers per inseminate and site of sperm deposition. *Anim Reprod*
623 *Sci* 105(1-2), 129-38

624
625 Seidel, G.E.J. (2014) Update on sexed semen technology in cattle. *Animal* 8(Supplements1),
626 160-164

627
628 Shekarriz, M., DeWire, D.M., Thomas, A.J., Jr., and Agarwal, A. (1995) A method of human
629 semen centrifugation to minimize the iatrogenic sperm injuries caused by reactive oxygen
630 species. *Eur Urol* 28(1), 31-5

631
632 Shojaei Saadi, H.A., van Riemsdijk, E., Dance, A.L., Rajamanickam, G.D., Kastelic, J.P., and
633 Thundathil, J.C. (2013) Proteins associated with critical sperm functions and sperm head
634 shape are differentially expressed in morphologically abnormal bovine sperm induced by
635 scrotal insulation. *J Proteomics* 82, 64-80

636
637 Sikka, S.C. (1996) Oxidative stress and role of antioxidants in normal and abnormal sperm
638 function. *Front Biosci* 1, e78-86

639
640 Suh, T.K., Schenk, J.L., and Seidel, G.E., Jr. (2005) High pressure flow cytometric sorting
641 damages sperm. *Theriogenology* 64(5), 1035-48

642
643 Suzuki, K., and Nagai, T. (2003) In vitro fertility and motility characteristics of frozen–
644 thawed boar epididymal spermatozoa separated by Percoll. *Theriogenology* 60(8), 1481-1494

645
646 Vishwanath, R. SexedULTRA™ - Raising the fertility bar of sexed sorted semen. In 'Natl
647 Assoc Artif Breeders', 2014, Wisconsin,,

648
649 Vishwanath, R., and Shannon, P. (2000) Storage of bovine semen in liquid and frozen state.
650 *Anim Reprod Sci* 62(1-3), 23-53

651
652 Walczak-Jedrzejowska, R., Wolski, J.K., and Slowikowska-Hilczer, J. (2013) The role of
653 oxidative stress and antioxidants in male fertility. *Cent European J Urol* 66(1), 60-7

654
655 Watson, P.F. (2000) The causes of reduced fertility with cryopreserved semen. *Anim Reprod*
656 *Sci* 60–61(0), 481-492

657

Xu, Z. (2014) Application of liquid semen technology improves conception rate of sex-sorted semen in lactating dairy cows. *J Dairy Sci* 97(11), 7298

Yang, D.H., McMillan, A.G., Standley, N.T., Shannon, P., and Xu, Z.Z. (2012) Extracellular calcium is involved in egg yolk-induced head-to-head agglutination of bull sperm. *Theriogenology* 78(7), 1476-86

667 Table 1: Progressive linear motility (%) of Non-sorted (NS) and Sex-sorted (SS) sperm from

668 dairy bulls assessed on Days 1, 2 and 3 post sex sorting (Mean ± SEM; Experiment 1)

				Statistical Significance		
	Day 1	Day 2	Day 3	Treatment	Day	Treatment x Day
NS Fresh 3M	64.0 ± 5.51[ab]	63.5 ± 5.18[a]	67.2 ± 5.11[ab]	P<0.01	ns	ns
X-SS Frozen 2M*	59.3 ± 6.35[a]	66.2 ± 3.57[ab]	61.3 ± 6.71[a]			
X-SS Fresh 2M	48.8 ± 5.14[a]	53.3 ± 5. 11 [a]	51.8 ± 3.86[a]			
X-SS Fresh 1M	58.0 ± 4.02[a]	55.5 ± 3.22 [a]	56.3 ± 3.93[a]			
NS Frozen 20M*	71.8 ± 5.45[b]	77.4 ±5.93[b]	78.0 ± 5.61[b]			

669 NS frozen 20M treatment was from a different ejaculate to the other treatments; * Frozen

670 treatments were thawed daily just before analysis on each assessment day; ns = not

671 significant; [a,b] represents significant differences between treatments within each column

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687 Table 2: Viable acrosome intact sperm (%) of Non-sorted (NS) and Sex-sorted (SS) sperm

688 from dairy bulls assessed on Days 1, 2 and 3 post sex sorting (Mean ± SEM; Experiment 1)

	Day 1	Day 2	Day 3	Statistical Significance		
				Treatment	Day	Treatment x Day
NS Fresh 3M	94.3 ± 1.41 [a]	94.2 ± 1.65 [a]	93.0 ± 1.51 [ab]	P<0.01	ns	P=0.06
X-SS Frozen 2M*	97.1 ± 0.69 [a]	98.3 ± 0.54 [a]	97.5 ± 0.72 [a]			
X-SS Fresh 2M	96.5 ± 0.38 [a]	98.0 ± 0.67 [a]	96.8 ± 0.70 [ab]			
X-SS Fresh 1M	97.3 ± 0.59 [a]	97.8 ± 0.42 [a]	95.8 ± 0.97 [ab]			
NS Frozen 20M*	88.9 ±1.99 [a]	88.7 ± 1.86 [a]	88.7 ±1.82 [b]			

689 NS frozen 20M treatment was from a different ejaculate to the other treatments; * Frozen

690 treatments were thawed daily just before analysis on each assessment day; ns = not

691 significant; [a,b] represents significant differences between treatments within each column

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Table 3: Progressive linear motility (%) of Non-sorted (NS) and Sex-sorted (SS) sperm from Aberdeen Angus bulls assessed on Days 1, 2 and 3 post sex sorting (Mean ± SEM; Experiment 2)

	Day 1	Day 2	Day 3	Statistical Significance		
				Treatment	Day	Treatment x Day
NS Fresh 3M	59.7 ± 5.12 [ab]	62.7 ± 7.09 [ab]	74.4 ± 2.78 [a]	P<0.05	ns	P<0.05
Y-SS Fresh 1M	62.7 ± 4.78 [ab]	70.3 ± 6.33 [ab]	70.0 ± 5.22 [a]			
Y-SS Fresh 2M	52.7 ± 9.68 [a]	57.0 ± 6.08 [a]	48.7 ± 7.33 [b]			
X-SS Fresh 2M	61.0 ± 6.15 [ab]	60.0 ± 6.28 [ab]	40.0 ± 3.79 [bc]			
NS Frozen 20M*	87.5 ± 2.50 [b]	86.0 ± 2.10 [b]	84.4 ± 3.06 [a]			

NS frozen 20M treatment was from a different ejaculate to the other treatments; * Frozen treatments were thawed daily just before analysis on each assessment day; ns = not significant; [a,b,c] represents significant differences between treatments within each column

Table 4: Viable acrosome intact sperm (%) of Non-sorted (NS) and Sex-sorted (SS) sperm from angus bulls assessed on Days 1, 2 and 3 post sex sorting (Mean ± SEM; Experiment 2)

	Day 1	Day 2	Day 3	Statistical Significance		
				Treatment	Day	Treatment x Day
NS Fresh 3M	98.9 ± 0.27	99.0 ± 0.22	98.9 ± 0.29	ns	ns	ns
Y-SS Fresh 1M	99.3 ± 0.08	99.1 ± 0.12	99.2 ± 0.07			
Y-SS Fresh 2M	99.2 ± 0.19	99.1 ± 0.18	99.2 ± 0.12			
X-SS Fresh 2M	99.0 ± 0.06	98.7 ± 0.22	99.0 ± 0.26			
NS Frozen 20M*	98.7 ± 0.35	99.1 ± 0.23	98.9 ± 0.35			

NS frozen 20M treatment was from a different ejaculate to the other treatments; * Frozen treatments were thawed daily just before analysis on each assessment day; ns = not significant