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Removing seminal plasma improves bovine sperm sex-sorting

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SUMMARY

Bull ejaculates with sperm concentrations of less than 1 billion sperm sort poorly for sex chromosomes, but whether this is because of the sperm concentration or the concomitant seminal plasma content has not been elucidated. Experiments were conducted to determine why ejaculates with lower sperm concentrations sort poorly and develop a protocol to increase sorting efficiency. In Experiment I, spermatozoa at 160 or 240 $\times$ 10^6 sperm/mL were stained at 49, 65 or 81 μM Hoechst 33342 with 0 or 10% seminal plasma and then sex-sorted. In Experiment II, seminal plasma was adjusted to create samples with sperm concentrations of 0.7, 1.4 and 2.1 $\times$ 10^9 sperm/mL, prior to sex-sorting. In Experiment III, spermatozoa were diluted to 0.7, 1.4 and 2.1 $\times$ 10^9 sperm/mL using TALP containing 0 or 10% seminal plasma prior to sex-sorting and cryopreservation. In Experiment I, the optimal staining combination was 160 $\times$ 10^6 sperm/mL stained with 65 μM Hoechst 33342 and no seminal plasma. In Experiment II, the percentages of membrane-impaired sperm were lower for sample concentrations of 2.1 $\times$ 10^9 sperm/mL (15%) than for samples at 1.4 $\times$ 10^9 (17%) or 0.7 $\times$ 10^9 sperm/mL (18%; $p < 0.01$). The X sort rate was slower for samples stored at 0.7 $\times$ 10^9 sperm/mL (3.45 $\times$ 10^3 sperm/sec) than for samples stored at 1.4 $\times$ 10^9 and 2.1 $\times$ 10^9 sperm/mL (3.85 and 3.94 $\times$ 10^3 sperm/sec, respectively; $p < 0.05$). In Experiment III, samples containing 0% seminal plasma had higher percentages of live-oriented cells (54 vs. 50%; $p < 0.05$), fewer dead sperm (19 vs. 22%; $p < 0.01$) and higher post-thaw motility (41 vs. 35%; $p < 0.05$) than samples containing 10% seminal plasma. Ejaculates with high sperm concentrations result in superior sorting because these samples have less seminal plasma during staining than ejaculates with lower initial sperm concentrations as all samples are diluted to 160 $\times$ 10^6 sperm/mL for staining. Therefore, sorting efficiency appears to be affected by seminal plasma concentration, not by the original sperm concentration.

INTRODUCTION

Typical sperm concentrations, in bull ejaculates, range between 5 $\times$ 10^8–2.5 $\times$ 10^9 sperm/mL. This range in sperm concentration is a result of the method of collection, collection frequency, sexual preparation, bull age, bull testes size, and individual bull variability. In addition, spermatozoa from some ejaculates are difficult to sex-sort by flow cytometry, especially ejaculates containing sperm concentrations of less than 1 billion sperm/mL. Therefore, these ejaculates are sometimes concentrated by centrifugation, with partial removal of seminal plasma, or they are simply not utilized for sex-sorting. One question that arises is whether sperm concentration, or seminal plasma content, affects the efficiency of sex-sorting sperm.

Accessory sex gland fluids are added to sperm and epididymal fluid upon ejaculation. This seminal plasma contains ions, sugars and proteins involved in sperm viability, acrosome stability, membrane protection, prevention of oxidative stress, and support of sperm motility (Moura *et al.*, 2007). In natural mating, spermatozoa remain in seminal plasma for only a short period of time, as ejaculates are diluted upon entering the female reproductive tract. This is especially true for species such as cattle, where the fertilizing spermatozoa traverse the cervix, removing them from the seminal plasma. In situations where spermatozoa are going to be cryopreserved or sex-sorted, however, spermatozoa can remain in the seminal plasma for many hours, in the diluted form. While this has not proven to be detrimental to bovine sperm (Graham, 1994; Maxwell *et al.*, 1996), for porcine and equine sperm, seminal plasma is detrimental to sperm survival during cryopreservation and extended cold storage (Pursel & Johnson, 1975; Jasko *et al.*, 1991; Brinsko *et al.*, 2000). In some species, such as sheep, seminal plasma has been found to be beneficial, and removing seminal plasma from ram spermatozoa causes a rapid decline in sperm viability (Ashworth *et al.*, 1994).

Sex-sorting bovine sperm introduces a new set of obstacles to sperm survival, but many protocols used for this technique are derived from conventional sperm cryopreservation protocols. While current procedures work, improvements might be made that cater to the needs of the highly stressed, sexed spermatozoa that may not be necessary or even desirable for conventional sperm processing for cryopreservation. Bovine sperm used for conventional sperm cryopreservation are typically exposed to seminal plasma for less than 1 h before dilution, while spermatozoa for sex-sorting can be held, undiluted, for hours in seminal plasma. Shannon (1965) found that spermatozoa stored at 5°C for several days lived 1.5 times longer when seminal plasma was removed from sperm samples, indicating that seminal plasma may influence the sex-sorting process where spermatozoa are stored for several hours, more than it does conventional cryopreservation.

One objective of these experiments was to determine the effect of sperm concentration during storage between collection and staining the spermatozoa for sex-sorting; spermatozoa were stored in seminal plasma only, without seminal plasma or at controlled levels of seminal plasma. In addition, the effect of sperm concentration, Hoechst 33342 concentration, and seminal plasma content were analyzed to find an optimal combination of these for sorting spermatozoa and for sperm survival. These objectives were developed to answer the main question, whether seminal plasma concentration or sperm concentration affects efficacy of sex-sorting bovine sperm.

METHODS AND MATERIALS

Ejaculate collection and initial analysis

First ejaculates were collected with an artificial vagina from dairy bulls that had been collected at least once in the week prior to collection for the study and were housed at Sexing Technologies, Inc (Navasota, TX, USA). Raw semen was processed by determining the initial sperm concentration (Nucleocounter® SP-100™, ChemoMetec, Allerod, Denmark), percent motile sperm (subjective), percent morphologically normal sperm, and pH. Antibiotics were added as recommended by Certified Semen Services (National Association of Animal Breeders, Columbia, MO, USA). All ejaculates were assessed by one evaluator, and only ejaculates with greater than 60% motile sperm and 70% morphologically normal sperm were accepted for further use. All semen samples were centrifuged at 1000 ***g*** for 15 min to remove seminal plasma, by aspiration. The recovered seminal plasma was clarified by an additional 15 min of centrifugation at 2000 ***g***. Fewer than one percent of spermatozoa from the original ejaculate were removed with the seminal plasma.

Sperm storage

In Experiment II, four sperm concentrations: initial, 0.7, 1.4 and 2.1×10^9 sperm/mL were created by seminal plasma removal or addition (from the same ejaculate). Samples (0.5 mL) were stored in 1.5 mL Eppendorf tubes at 16°C. At 0 and 4 h, subsamples were taken for staining and sorting.

In Experiment III, sperm concentrations of 0.7, 1.4 and 2.1×10^6 sperm/mL were created by addition of staining TALP (pH 7.4; Schenk *et al.*, 1999) and 0 or 10% clarified seminal plasma. Spermatozoa were stored for 1 h before preparation for staining and sorting.

Sperm staining

In Experiment I, Hoechst 33342 (H33342; Molecular Probes, Eugene, OR, USA) was added to create final concentrations of 49, 65 or 81 μM in sperm samples diluted to 160 or 240×10^6 sperm/mL with staining TALP containing 0 or 10% seminal plasma. Spermatozoa were incubated for 45 min in a 34.5°C water bath to facilitate H33342 movement into cells. Spermatozoa were then diluted to 80×10^6 sperm/mL by adding an equal volume of TALP (pH 5.5) + 4% egg yolk + 0.002% food-coloring dye (red TALP; FD&C Red #40, Sensient Technologies Corporation, St. Louis, MO, USA) or by addition of an equal amount of TALP (3 : 1 red TALP, staining TALP) in order to maintain the pH in the samples stained at 240×10^6 sperm/mL and then kept for 5 min before sorting.

In Experiments II and III, H33342 was added to spermatozoa that had been diluted to 160×10^6 sperm/mL, using staining TALP for a final H33342 concentration of 65 μM.

Sperm sorting

Spermatozoa were sorted on a high-speed flow cytometer (XY Sperm Sorter II, XY, Inc, Navasota, TX, USA) with sheath fluid at 40 psi and laser at 175 mW. Event rates were held between 20,000 and 21,000 events/sec. Responses were recorded based on sorting 50,000 spermatozoa for % live-oriented cells, X sort rate, the percent membrane-impaired sperm, and the percent X-bearing spermatozoa collected.

Freezing/thawing

Sorted spermatozoa were only cryopreserved in Experiment III. Approximately 12 million bulk sorted (both X-and Y-bearing sperm) spermatozoa were collected into tubes containing TRIS buffer + egg yolk (Schenk *et al.*, 1999). Collection tubes were placed in a cold room at 5°C for at least 90 min before adding an equal volume of TRIS buffer + 12% glycerol (6% final glycerol concentration) added in two equal aliquots, 15 min apart. After addition of the second aliquot, tubes were centrifuged for 20 min at 850 ***g***. The supernatant was decanted, and sperm concentration of the remaining pellet was determined using a Nucleocounter. TRIS buffer + 6% glycerol + 20% egg yolk was then added to create a final sperm concentration of 10^7 sperm/mL for packaging into 0.25 mL straws. Straws were placed on racks in liquid nitrogen vapor (−120°C) for 25 min, and then plunged into liquid nitrogen (−196°C) for storage.

Frozen straws were shipped to Colorado State University (Fort Collins, CO, USA) for analysis. Straws were thawed in a 37°C water bath for 20 sec and randomly expelled into numbered tubes to remove bias from evaluators. Spermatozoa were analyzed by flow cytometry and computer-assisted sperm analysis (CASA). For flow cytometry analysis, sperm were stained with propidium iodide (1 mg/mL), FITC-PNA (1 mg/mL), and then incubated for 5 min (Purdy & Graham, 2004). Spermatozoa were diluted with 500 μL of TALP, filtered through a 20 μm mesh, and analyzed, using a MoFlo™ High-Performance Cell Sorter (Dako, Fort Collins, CO, USA) to determine the percentages of live, acrosome-reacted (Hoechst 33342 and FITC-PNA positive), live, non-acrosome reacted (only Hoechst 33342 positive), and membrane-impaired (propidium iodide positive) spermatozoa. The percentage of motile spermatozoa were determined, using CASA (IVOS, Hamilton Thorne Biosciences, Beverly, MA, USA),

evaluating a minimum of 200 cells treatment in eight fields of view. System parameters for these analyses were as follows: 100 frames acquired at 60 frames/sec; minimum contrast = 70, minimum cell size = 4 pixels; lower average path velocity (VAP) cut-off = 10 μm/sec; lower straight-line velocity (VSL) cut-off = 40 μm/sec; VAP cut-off for progressive cells = 40 μm/sec and straightness = 75%.

DNA fragmentation

During Experiment III, sperm samples for DNA fragmentation analysis were collected during the sex-sorting process: pre- and post-storage (1 h at room temperature), pre-sort, post-sort, and post-thaw. Samples of 100 μL were stored in 1.5 mL Eppendorf tubes at 34°C in a water bath. At 0 and 24 h of incubation, subsamples were frozen immediately at −20°C to stop progression of DNA damage. Sperm DNA fragmentation was analyzed using Sperm-Halomax® kit (Halotech DNA, Madrid, Spain). Upon thawing, 5 μL of sperm sample was mixed with 5 μL of low melting point agarose. The mixture was placed upon pre-treated slides, covered with a coverslip and refrigerated at 4°C for 5 min. After coverslip removal, slides were covered with lysing solution for 5 min, and then washed with distilled water for 5 min at room temperature. Slides were washed in a series of ethanol baths and stained using a 1 : 1 ratio of SybrGreen 20× fluorochrome and Vectashield® Mounting Medium. Fluorescence microscopy was used for subjective analysis of chromatin dispersion halos around sperm heads, with small, compact heads indicating intact DNA. Three hundred spermatozoa were analyzed per bull per treatment, and the percentage of spermatozoa with fragmented DNA calculated.

Degree of X- and Y-sperm distribution separation

Calculations for split were based on sort data recorded on 100,000 sperm cells per treatment per bull. From the bimodal histogram showing the distance between the X- and Y-bearing sperm populations, the height of the valley divided by the average height of the two peaks was subtracted from 1.0, and then multiplied by 100. A higher percentage indicates a higher degree of resolution between X- and Y-bearing sperm populations.

Statistical analyses

Experiment I was a factorial design of 3 Hoechst 33342 concentrations by 2 sperm concentrations by 2 seminal plasma contents by 2 breeds with 22 ejaculates (2 ejaculates each from Jersey, $n = 7$; Holstein, $n = 4$) for replication. Bull was nested within breed and a considered a random effect. Experiment II was a factorial design of 4 sperm concentrations by 2 time points (0 and 4 h storage) with 10 bulls (Jersey, $n = 7$; Holstein, $n = 2$; Brown Swiss, $n = 1$) for replication. Experiment III was a factorial design of 3 sperm concentrations by 2 seminal plasma contents with 10 bulls (Jersey, $n = 6$; Holstein, $n = 4$) for replication. DNA fragmentation was analyzed as 3 sperm concentrations by 2 seminal plasma contents by 2 incubation times (0 and 24 h) with 6 of the bulls (Jersey, $n = 3$; Holstein, $n = 3$) from Experiment III for replication. Data for all experiments were subjected to a mixed model ANOVA with bulls considered a random effect. Main effects and first-order interactions were considered in all models. For Experiment I, second-order interactions that included bull nested within the breed were also considered. Least squares means are presented; linear contrasts were used for Experiment II. Least squares means are presented with Tukey's HSD test used for multiple comparison tests. Residual plots showed no obvious need for transformation.

RESULTS

Experiment I

Multiple concentrations of spermatozoa and H33342 were explored in this experiment with 0 or 10% seminal plasma during staining. Spermatozoa incubated with 0% seminal plasma had higher percentages of live-oriented cells (56 vs. 53%) as well as higher sort rates (3.47 vs. 3.15 × 10^3 sperm/sec) than sperm incubated with 10% seminal plasma ($p < 0.04$; Table 1). The percentages of membrane-impaired spermatozoa were lower for samples incubated with 0% seminal plasma (15 vs. 19%) than for samples containing 10% seminal plasma ($p < 0.04$).

There was an interaction between H33342 dye concentration and sperm concentration for split and sort rate (Table 2). Sperm samples incubated at a sperm concentration of 160 × 10^6 sperm/mL reached a maxima for both split and sort rate when stained

Table 1 Main effects of sex-sorting, including the percentage of live cells that were correctly oriented for sorting (Live-oriented Cells), the rate at which X-bearing sperm were collected (X-Sperm Sort Rate), the percentage of X-bearing sperm in the original population that were collected (X-Sperm Collected), the percentage of membrane impaired sperm in X-bearing population after sex-selection (Membrane-Impaired Cells), and the degree that X- and Y-bearing sperm populations were able to be separated due to differences in fluorescence intensity (Split); when bovine sperm were incubated with either 0 or 10% seminal plasma. Means are averaged across dye and sperm concentrations for 22 ejaculates in Experiment I

Seminal plasma	Live-oriented cells (%)	X-sperm sort rate 10^3 cells/sec	Membrane-impaired cells (%)	X-sperm collected (%)	Split (%)
0%	56^a	3.47^a	15^a	39^a	27^a
10%	53^b	3.15^b	19^b	37^a	24^a
SEM	3	0.21	2	1	3

[ab]Means different superscripts differ ($p < 0.05$) within responses.

Table 2 Two-way means for sex-sorting effects, including the percentage of live cells that were correctly oriented for sorting (% Live-oriented Cells), the rate at which X-bearing sperm were collected (X-Sperm Sort Rate), and the degree that X- and Y-bearing sperm populations were able to be separated due to differences in fluorescence intensity (Split); when bovine sperm were incubated at different sperm concentrations and different concentrations of Hoechst 33342 prior to sex-sorting. Means are averaged across 0 and 10% seminal plasma treatments for 22 ejaculates in Experiment I

Response	Hoechst 33342 (μM)	Sperm concentration		Average
		160 × 10^6 sperm/mL	240 x 10^6 sperm/mL	
% Live-oriented cells	49	54 ± 2.7^b	48 ± 2.7^a	51 ± 2.6^a
	65	57 ± 2.7^b	55 ± 2.7^b	56 ± 2.6^b
	81	56 ± 2.7^b	56 ± 2.7^b	56 ± 2.6^b
X-sperm sort rate (10^3 cells/sec)	49	3.27 ± 0.22^b	2.38 ± 0.22^a	2.83 ± 0.21^a
	65	3.47 ± 0.22^c	3.22 ± 0.22^b	3.48 ± 0.21^b
	81	3.68 ± 0.22^c	3.56 ± 0.22^{bc}	3.62 ± 0.21^b
Split (%)	49	27 ± 4.0^{bc}	3 ± 4.0^a	15 ± 3.2^a
	65	37 ± 4.0^c	15 ± 4.0^{ab}	26 ± 3.2^b
	81	35 ± 4.0^c	35 ± 4.0^c	35 ± 3.2^c

[abc]Column means±SEM with different superscripts differ ($p < 0.05$) within responses.

with 65 μM H33342, while the samples stored at 240×10^6 sperm/mL required 81 μM H33342 for optimum splits. Samples stained at 85 μM H33342 exhibited split and sort rates that were similar for both sperm concentrations, as were the percentages of live-oriented cells and X-bearing sperm collected. The percentages of membrane-impaired sperm were similar across all H33342 and sperm concentration combinations ($p > 0.1$).

Bull breed was considered in the analysis, and an interaction found between breed and dye concentration (Table 3). Spermatozoa from Jersey bulls exhibited higher percentages of live-oriented spermatozoa at 49 and 65 μM H33342 (56 and 58%) compared to spermatozoa from Holstein bulls (46 and 53%), while Holstein and Jersey sperm samples stained with 81 μM H33342 exhibited similar percentages of live-oriented sperm (58 vs. 55%, respectively). Spermatozoa from Jersey and Holstein bulls had similar percentages of membrane-impaired spermatozoa ($p > 0.05$). Spermatozoa from Jersey bulls sorted better at every dye concentration (3.96×10^3 sperm per sec) compared to spermatozoa from Holstein bulls (3.28×10^3 sperm per sec) when stained with 81 μM H33342 ($p < 0.05$). Split samples were also greater for Jersey bull sperm at all dye concentrations. The average age of Jersey and Holstein bulls was similar, 26 and 28 months, respectively.

Experiment II

The effect of sperm concentration was explored in this experiment by adjusting sperm numbers in seminal plasma. Samples were stored in only seminal plasma at four sperm concentrations: initial (original ejaculate sperm concentration), 0.7, 1.4 and 2.1×10^9 sperm/mL. These concentrations were chosen as an incremental spread over the natural ejaculate sperm concentration range; initial ejaculate sperm concentration was a control. Sort responses for the initial ejaculates and samples reconstituted to 1.4×10^9 sperm/mL were similar. This was expected as 1.4×10^9 sperm/mL was similar to the average initial sperm concentration of the ejaculates (1.2×10^9 sperm/mL). Therefore, initial sperm concentration (control) was removed from the analysis when testing for linearity effects with linear contrasts. There was no interaction between 0 and 4 h times and sperm concentration. Means are presented in Table 4.

The percentages of live-oriented cells exhibited a positive linear effect with sperm concentration ($p < 0.01$). In addition, as sperm concentration increased (seminal plasma was more dilute during staining), there was an increase in the percentage of live-oriented cells. Sperm concentration also had a positive linear effect on sort rate, with samples containing higher sperm concentrations (>1246×10^6 sperm/mL) sorting more efficiently than samples containing 700×10^6 sperm/mL ($p < 0.01$). Sperm concentration had a negative linear effect on the percentage of membrane-impaired spermatozoa ; therefore, there were lower percentages of membrane-impaired spermatozoa at higher sperm concentrations ($p < 0.01$). There were no linear effects of sperm concentration on the percentages of X-sperm collected or on split, nor were there any quadratic effects on any response ($p > 0.1$). Overall, this indicates greater ability to sort spermatozoa when spermatozoa were stored at higher sperm concentrations.

Experiment III

Effects of storing spermatozoa for 1 h in the presence of 0 or 10% seminal plasma were explored in this experiment. The ability to sort spermatozoa and post-thaw responses were similar at all three sperm concentrations ($p > 0.1$; Table 5). However,

Table 3 Two-way means for sex-sorting responses for breed by Hoechst concentration when spermatozoa from 7 Jersey bulls and 4 Holstein bulls were treated with three different concentrations of Hoechst 33342 prior to being sex-sorted. Responses include the percentage of live cells that were correctly oriented for sorting (Live-oriented Cells), the rate at which X-bearing sperm were collected (X-sperm sort rate), the percentage of X-bearing sperm in the original population that were collected (X-sperm collected), and the degree that X- and Y-bearing sperm populations were able to be separated because of differences in fluorescence intensity (Split). Means are averaged across 0 and 10% seminal plasma treatments for 22 ejaculates in Experiment I

		Hoechst 33342 concentration (μM)			
Response	Breed	49	65	81	SEM
Live oriented cells (%)	Jersey	56^{b}	58^{b}	58^{b}	3
	Holstein	46^{a}	53^{b}	55^{b}	4
X-sperm sort rate (10^3cells/sec)	Jersey	3.37^{b}	3.81^{c}	3.96^{c}	0.26
	Holstein	2.29^{a}	3.15^{b}	3.28^{b}	0.34
X-sperm collected (%)	Jersey	37^{b}	42^{b}	43^{b}	1
	Holstein	31^{a}	37^{b}	38^{b}	2
Split (%)	Jersey	22^{ab}	34^{bc}	44^{c}	4
	Holstein	9^{a}	18^{ab}	26^{bc}	5

[abc]Means without common superscripts differ ($p < 0.05$) within responses.

Table 4 Sex-sorting responses (mean and SEM), including the percentage of live cells that were correctly oriented for sorting (Live-oriented Cells), the rate at which X-bearing sperm were collected (X-sperm sort rate), the percentage of X-bearing sperm in the original population that were collected (X-sperm collected), the percentage of membrane impaired sperm in X-bearing population after sex-selection (membrane-impaired cells), and the degree that X- and Y-bearing sperm populations were able to be separated as a result of differences in fluorescence intensity (Split); when bovine sperm, from 10 bulls, were incubated at different sperm concentrations for 0 or 4 h prior to sorting

Response	Sperm concentration (10^6 sperm/mL)	Incubation time		Average
		0 h	4 h	
Live oriented cells (%)	Initial (1246)	67 ± 0.7	61 ± 0.7	64 ± 0.5
	700	66 ± 0.7	60 ± 0.7	63 ± 0.5[a]
	1400	67 ± 0.7	62 ± 0.7	64 ± 0.5
	2100	69 ± 0.7	64 ± 0.7	66 ± 0.5
	Average	67 ± 0.4	62 ± 0.4[b]	64
X-sperm sort rate (10^3 sperm/sec)	Initial (1246)	3.97 ± 0.14	3.63 ± 0.14	3.80 ± 0.10
	700	3.73 ± 0.14	3.17 ± 0.14	3.45 ± 0.10[a]
	1400	4.05 ± 0.14	3.64 ± 0.14	3.85 ± 0.10
	2100	4.10 ± 0.14	3.77 ± 0.14	3.94 ± 0.10
	Average	3.96 ± 0.07	3.55 ± 0.07[b]	3.76
Membrane-impaired sperm (%)	Initial (1246)	14 ± 0.9	20 ± 0.9	17 ± 0.6
	700	15 ± 0.9	21 ± 0.9	18 ± 0.6[a]
	1400	14 ± 0.9	20 ± 0.9	17 ± 0.6
	2100	13 ± 0.9	17 ± 0.9	15 ± 0.6
	Average	14 ± 0.4	20 ± 0.4[b]	17
X-sperm collected (%)	Initial (1246)	38 ± 1.3	38 ± 1.3	38 ± 0.9
	700	38 ± 1.3	36 ± 1.3	37 ± 0.9
	1400	38 ± 1.3	38 ± 1.3	38 ± 0.9
	2100	39 ± 1.3	37 ± 1.3	38 ± 0.9
	Average	38 ± 0.6	37 ± 0.6	38
Split (%)	Initial (1246)	35 ± 3.5	34 ± 3.5	34.5 ± 2.5
	700	30 ± 3.5	28 ± 3.5	29.0 ± 2.5
	1400	37 ± 3.5	38 ± 3.5	37.5 ± 2.5
	2100	38 ± 3.5	32 ± 3.5	35.0 ± 2.5
	Average	35 ± 1.8	33 ± 1.8	34

[a]There were linear effects of sperm concentration for average % live-oriented cells, X sort rate, and % membrane-impaired sperm ($p < 0.05$).
[b]Differs from 0 h storage ($p < 0.01$).

Table 5 Main effects of sex-sorting and post-thaw responses for bovine sperm from 10 bulls incubated at different sperm concentrations and with 0 or 10% seminal plasma prior to sex-sorting. Sex sorting responses reported include the percentage of live cells that were correctly oriented for sorting (Live-oriented Cells), the rate at which X-bearing sperm were collected (X-Sperm Sort Rate), the percentage of X-bearing sperm in the original population that were collected (X-Sperm Collected), the percentage of membrane impaired spermatozoa in X-bearing population after sex-selection (Membrane-Impaired Cells), and the degree that X- and Y-bearing sperm populations were able to be separated as a result of differences in fluorescence intensity (Split). After spermatozoa were cryopreserved and then thawed analyses included the percentages of spermatozoa possessing intact plasma and acrosomal membranes (live AR intact sperm), spermatozoa possessing intact plasma membranes but damaged acrosomal membranes (live AR damaged sperm), spermatozoa without intact plasma membranes (membrane-impaired sperm), and the percentages of motile sperm (motile sperm)

	Sex-sorting responses					Post-thaw responses (%)			
Treatment	Live- oriented cells (%)	X-sperm sort rate (10^3sperm/sec)	Membrane- impaired sperm (%)	X-sperm collected (%)	Split (%)	Live AR intact sperm	Live AR damaged sperm	Membrane- impaired sperm	Motile sperm
Seminal plasma									
0%	54[a]	3.55[a]	19[a]	42[a]	38[a]	39[a]	3[a]	61[a]	41[a]
10%	50[b]	3.20[b]	22[b]	41[a]	36[a]	37[a]	2[a]	62[a]	35[b]
SEM	3	0.25	4	1	7	3	1	4	3
Sperm conc. (10^6 cells/mL)[c]									
700	53	3.47	20	42	40	39	3	59	38
1400	53	3.37	21	41	37	37	2	62	38
2100	51	3.30	21	41	35	38	3	62	38
SEM	3	0.25	4	1	7	3	1	4	3

[ab]Means without common superscripts differ ($p < 0.05$) within responses for seminal plasma treatments.
[c]No differences within sperm concentration treatment set ($p > 0.1$).

samples incubated in 0% seminal plasma exhibited higher percentages of live-oriented cells (54 vs. 50%, respectively) and a higher sort rate (3.55 vs. 3.20 × 10^3 sperm/sec; respectively) than samples incubated in 10% seminal plasma ($p < 0.05$). The percentages of membrane-impaired spermatozoa were also lower for spermatozoa incubated with 0% seminal plasma (19%) than for spermatozoa incubated with 10% seminal plasma (22%; $p < 0.05$), indicating better sperm survival when seminal plasma is removed. The percentages of motile sperm after cryopreservation were higher for spermatozoa incubated with 0% seminal plasma (41%) than for spermatozoa incubated with 10% seminal plasma (35%; $p < 0.05$). No interactions between sperm concentration and seminal plasma level were seen for any response ($p > 0.1$).

Storing and staining spermatozoa without seminal plasma was beneficial to both sorting and post-thaw responses. Sperm concentration did not affect sperm sorting when the seminal plasma content was held constant across sperm concentrations, indicating that seminal plasma content, not sperm concentration, affects sort efficiency.

DNA fragmentation

DNA fragmentation was analyzed for samples taken from six bulls during Experiment III. Interactions existed ($p < 0.001$) for sperm concentration by hour of storage and seminal plasma by hour of storage; therefore, two-way means are presented in Table 6, for seminal plasma by hour of storage. DNA fragmentation was higher at 24 h of incubation for sperm stored at 2.1 × 10^9 sperm/mL (17%) than for sperm stored at 0.7 × 10^9 sperm/mL (9%) from samples taken prior to sorting ($p < 0.05$) There was no interaction between sperm concentration and presence of seminal plasma. Presence of 10% seminal plasma resulted in higher DNA fragmentation over time compared to spermatozoa incubated with 0% seminal plasma ($p < 0.05$). Increasing sperm concentration also resulted in increasing rates of DNA fragmentation at 24 h of incubation (Fig. 1). All of these effects disappeared after sorting, which removed essentially all spermatozoa exhibiting DNA fragmentation.

Table 6 Two-way means for the percentages of bovine sperm exhibiting DNA fragmentation, (using the Sperm-Halomax® procedure), when sperm from six bulls were incubated for 0 or 24 h in the presence of 0 or 10% seminal plasma

Seminal plasma (%)	Incubation time (hr)	Pre-storage (%)	Post-storage (%)	Pre-sort (%)	Post-sort (%)	Post-thaw (%)
0%	0	5.82[a]	4.46[a]	3.89[a]	0.03[a]	0.66[a]
10%	0	6.81[a]	6.27[a]	5.44[a]	0.03[a]	0.80[a]
0%	24	13.88[a]	11.92[b]	12.72[b]	0.37[b]	0.92[a]
10%	24	26.09[b]	23.08[c]	15.69[b]	0.08[a]	1.09[a]
SEM		4.30	1.27	1.26	0.05	0.17

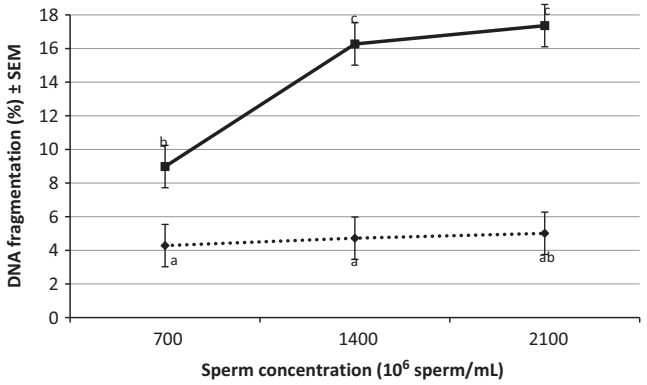
[abc]Column means with different superscripts differ ($p < 0.05$).

DISCUSSION

Initial sperm concentration of ejaculates has been a primary criterion for whether or not an ejaculate is used for sex-sorting because of the fact that ejaculates having sperm concentrations with less than 1 billion sperm/mL do not sort efficiently. In Experiment II, sperm concentration did affect the efficiency of spermatozoa sorted when the seminal plasma was not manipulated, and data in Table 4 show that samples containing higher sperm concentrations resulted in higher sperm sort rates and more membrane-intact sperm that were correctly oriented. This agrees with what has been observed in the industry. An ejaculate having 2.1 × 10^9 sperm/mL will contain approximately 7% seminal plasma after dilution for staining, compared to an ejaculate having 0.7 × 10^9 sperm/mL, which will contain approximately 23% seminal plasma during staining. This leads to the question of whether it is sperm concentration, or the amount of seminal plasma, that affects sorting efficiency.

Similar sorting efficiencies were observed, in Experiment III, for spermatozoa in all sperm storage concentrations, when seminal plasma content was controlled at either 0 or 10% (Table 5). However, sort rates were 11% higher for samples stored with no

Figure 1 Percentages of bovine sperm exhibiting DNA fragmentation (using the Sperm-Halomax® procedure), prior to sorting, when spermatozoa from six bulls were stored for either 0 (-◆-) or 24 (-■-) h at three different sperm concentrations. [abc]Means with different superscripts differ ($p < 0.05$) when averaged over 0 and 10% seminal plasma.



seminal plasma compared to samples containing 10% seminal plasma. There was also a 14% decrease in spermatozoa having compromised cell membranes in samples containing no seminal plasma. Not only did spermatozoa sort more efficiently when seminal plasma was removed, but the percentage of motile spermatozoa also increased by 17%. Similarly, Tibary *et al.* (1990) reported increased post-thaw sperm motility in samples where the seminal plasma had been removed. Therefore, seminal plasma impairs the ability of bull sperm to be sex-sorted and cryopreserved.

Some sperm DNA fragmentation is present in most ejaculates with variation among bulls, and high levels of fragmentation are considered detrimental to sperm fertility (García-Macías *et al.*, 2007). DNA fragmentation can be induced by oxidative stress, cell apoptosis, and failures in histone-protamine replacement; DNA fragmentation also increases with time after ejaculation. In the data collected, there was a significant decrease in DNA fragmentation ($p < 0.05$) as sperm progressed through the different steps of the sex-sorting process before actual sorting. This causes concern for the meaning of the assay used, since fragmentation would be expected to increase over time. Another concern is that DNA fragmentation, as measured, virtually disappeared when membrane-compromised spermatozoa were discarded. However, this does agree with conclusions made by Gosálvez *et al.* (2011) who found 63% of DNA fragmented sperm were removed by sex-sorting compared to neat semen. This means that only dead spermatozoa exhibit DNA fragmentation or the reagents simply reached the DNA more easily in membrane-compromised sperm. Sperm concentration may also affect the accuracy of this assay, as samples with higher sperm concentrations also had higher fragmentation, not only for the sperm concentrations considered in the study but in the change in sperm concentrations over the collection times. For example, the pre-storage samples were at 0.7, 1.4 and 2.1 $\times$ 10^9 sperm/mL while pre-sort samples were at 80 $\times$ 10^6 sperm per mL, and post-sort samples had <1 $\times$ 10^6 sperm per mL.

During sex-sorting, spermatozoa are exposed to high dilution environments for extended times. Mann (1964) extensively described the 'dilution effect' where sperm survival is greatly impaired by dilution from the initial ejaculate concentration when diluted in a simple saline solution. With the extenders/diluents used currently, this effect has been greatly reduced and allows for survival of spermatozoa at concentrations of <10^6 sperm/mL for hours between sex-sorting and cryopreservation. However, dilution may still be an issue. Determining whether sperm membranes are impaired just before adding glycerol, which would be after hours of the spermatozoa being at a low concentrations, may give insight into how spermatozoa are responding to their dilute environment, since only live sperm should be in the sample after sorting.

Seminal plasma was shown by Shannon (1965) to have long term effects on sperm survival. When spermatozoa were stored at 5°C, and then incubated at 37°C to determine livability, spermatozoa with 0% seminal plasma lived 1.5 times longer after 2 days of storage and 3 times longer after 7 days of storage. Spermatozoa for cryopreservation are typically exposed to seminal plasma for less than 1 h before dilution, so effects of seminal plasma typically are not seen. But spermatozoa undergoing sex-sorting can be exposed for hours, and seminal plasma can have a greater impact, as was seen in these experiments. It could be hypothesized that storing bovine sperm to be used for sex-sorting without seminal plasma would be beneficial when incubating for even longer periods than the 5 h studied.

The increase in sperm survival seen when seminal plasma was removed could be due to removal of acrosomal enzymes that were released into seminal plasma from dead sperm. Acrosomal enzymes such as hyaluronidase are present in seminal plasma, and at increasing concentrations over time (Foulkes & Watson, 1975). High levels of glutamic-oxalacetic transaminase, another acrosomal enzyme, have been shown to correlate with decreased fertility (Pace & Graham, 1970; Breeuwsma, 1972). These enzymes could be acting adversely on the living sperm, causing a cascade during which more sperm die and more acrosomal enzymes are available to act on the remaining live sperm. Therefore, removing seminal plasma would result in the higher percentages of membrane-intact sperm seen in these experiments.

Movement of H33342 into the nucleus is one of the most important parts of the sex-sorting process. Many factors impact efficiency of H33342 infiltration and binding to DNA, including: temperature, pH, dye concentration, sperm membrane permeability, diluent components, and sperm concentration (Garner, 2009). Studying of all these factors simultaneously would be nearly impossible. Therefore, dye concentration and sperm concentration were chosen for this study with removal of seminal plasma to determine whether the seminal plasma affects H33342 movement. Having 0% seminal plasma in the sample improved sorting, but this was probably the same influence as was seen in earlier experiments, as there was no interaction between seminal plasma and H33342 concentration. However, there was an interesting interaction between dye and sperm concentrations. At the lower sperm concentrations, sorting responses were optimized when staining with 65 μM H33342, while the higher sperm concentrations required 81 μM H33342. The clearest example of this was with the split response, which could indicate when spermatozoa are reaching the saturation point for dye (Table 2).

Breed differences between Jersey and Holstein bulls were seen in Experiment I. The difference in H33342 fluorescence between X- and Y-bearing sperm, for Holstein sperm of 3.98%, while that difference for Jersey sperm is 4.24% (Garner & Seidel, 2008). This information indicates spermatozoa from Jersey bulls should sort more efficiently than spermatozoa from Holstein bulls. The data

presented in Experiment I support this, as sperm from Jersey bulls sorted more optimally than spermatozoa from Holstein bulls. H33342 binds to adenine-thymine base pairs, which may be more prevalent on the Jersey X chromosome or the Holstein Y chromosome, and would account for the differences in fluorescence between the two breeds. The breed by dye concentration interaction supports observed practices where extra H33342 is often added to Holstein sperm to improve sort efficiency. Changes in protocols may need to be made based on bull breed to optimize efficiency.

Implementing seminal plasma removal into sex-sorting procedures not only improved sort rates and number of live sperm collected per ejaculate, but spermatozoa were also of higher quality post-thaw. An added benefit is that ejaculates having low sperm concentrations sort just as well as more concentrated ejaculates. The only intervention into current procedures is to add a 15 min centrifugation step, which is a minimal cost compared to the benefits of an 11% increase in sort rates and 17% increase in post-thaw motility. While it could be hypothesized that some of the highest quality spermatozoa are being removed with seminal plasma, data indicate 14% fewer membrane-impaired spermatozoa at sorting. Furthermore, less than 1% of sperm were lost in the discarded seminal plasma.

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REFERENCES

Ashworth PJ, Harrison RA, Miller NG, Plummer JM & Watson PF. (1994) Survival of ram spermatozoa at high dilution: protective effect of simple constituents of culture media as compared with seminal plasma. *Reprod Fertil Dev* 6, 173–180.

Breeuwsma AJ. (1972) The Inter-Relationship between Seminal Glutamic-Oxalacetic Transaminase and Fertility in Bulls. In: Proc 7th Int Congr Anim Reprod & A.I., Munich. pp. 2129-2132.

Brinsko SP, Crockett EC & Squires EL. (2000) Effect of centrifugation and partial removal of seminal plasma on equine spermatozoal motility after cooling and storage. *Theriogenology* 54, 129–136.

Foulkes JA & Watson PA. (1975) Hyaluronidase activity in seminal plasma as a method of assessing bull sperm integrity. *J Reprod Fertil* 43, 349–353.

García-Macías V, de Paz P, Martinez-Pastor F, A'Ivarez M, Gomes-Alves S, Bernardo J, Anel E & Anel L. (2007) DNA fragmentation assessment by flow cytometry and Sperm-Bos-Halomax (bright-field microscopy and fluorescence microscopy) in bull sperm. *J Androl* 30, 88–98.

Garner DL. (2009) Hoechst 33342: the dye that enabled differentiation of living X-and Y-chromosome bearing mammalian sperm. *Theriogenology* 71, 11–21.

Garner DL & Seidel GE Jr. (2008) History of commercializing sexed semen for cattle. *Theriogenology* 69, 886–895.

Gosálvez J, Ramirez MA, López-Fernández C, Crespo F, Evans KM, Kjelland ME & Moreno JF. (2011) Sex-sorted bovine spermatozoa and DNA damage: I. Static features. *Theriogenology* 75, 197–205.

Graham JK. (1994) Effect of seminal plasma on the motility of epididymal and ejaculated spermatozoa of the ram and bull during the cryopreservation process. *Theriogenology* 41, 1151–1162.

Jasko DJ, Moran DM, Farlin ME & Squires EL. (1991) Effect of seminal plasma dilution or removal on spermatozoa motion characteristics of cooled semen. *Theriogenology* 35, 1059–1067.

Mann T. (1964) *The Biochemistry of Semen and of the Male Reproductive Tract*. Barnes & Noble Inc, New York.

Maxwell WM, Welch GR & Johnson LA. (1996) Viability and membrane integrity of spermatozoa after dilution and flow cytometric sorting in the presence or absence of seminal plasma. *Reprod Fertil Dev* 8, 1165–1178.

Moura AA, Chapman DA, Koc H & Killian GJ. (2007) A comprehensive proteomic analysis of the accessory sex gland fluid from mature Holstein bulls. *Anim Reprod Sci* 98, 169–188.

Pace MM & Graham EF. (1970) The release of glutamic oxalacetic transaminase from bovine spermatozoa as a test method of assessing semen quality and fertility. *Biol Reprod* 3, 140–146.

Purdy PH & Graham JK. (2004) Effect of adding cholesterol to bull sperm membranes on sperm capacitation, the acrosome reaction, and fertility. *Biol Reprod* 71, 522–527.

Pursel VG & Johnson LA. (1975) Freezing of boar spermatozoa: fertilizing capacity with concentrated semen and a new thawing procedure. *J Anim Sci* 40, 99–102.

Schenk JL, Suh TK, Cran DG & Seidel GE Jr. (1999) Cryopreservation of flow-sorted bovine spermatozoa. *Theriogenology* 52, 1375–1391.

Shannon P. (1965) Contribution of seminal plasma, sperm numbers, and gas phase to dilution effects of bovine spermatozoa. *J Dairy Sci* 48, 1357–1363.

Tibary A, Graham EF, Asri A, Boukhliq R & Deyo R. (1990) Effect of dialysis or centrifugation on post-thaw motility and fertility of Santa Gertrudis bull semen collected by electroejaculation. *Theriogenology* 33, 733–739.

