THESIS

REMOVING SEMINAL PLASMA IMPROVES SEX-SORTING OF BOVINE SPERM

Submitted by

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ABSTRACT

REMOVING SEMINAL PLASMA IMPROVES

SEX-SORTING OF BOVINE SPERM

Experiments for this thesis evaluated how characteristics of bovine ejaculates affect efficiency of sorting X- from Y-chromosome bearing sperm by flow cytometry/cell sorting, and further, document that removal of seminal plasma improves efficacy of sex-sorting bovine sperm. In Experiment I, ejaculates were collected by artificial vagina, two each from 10 bulls with an average of one hour between collections. Semen was centrifuged to separate sperm from seminal plasma, and then sperm were re-diluted to $160x10^6$ sperm per ml with staining TALP (Tyrode's albumin, lactate, pyruvate) and 0, 5, 10 or 20% seminal plasma from the same ejaculate or reciprocally from first/second ejaculates. Following incubation with Hoechst 33342, sperm were sorted and analyzed with a flow cytometer/cell sorter. The % live-oriented sperm was higher for treatments with 0% seminal plasma (64.4%) than 5 (59.6%), 10 (59.0%) and 20% (57.8%) seminal plasma ($p<0.01$). The % live-oriented sperm was higher for second (63.0%) than first ejaculates (56.2%). Sort rate was higher for samples with 0% seminal plasma ($p<0.05$). The percentages of membrane-impaired sperm were lower for 0% (16.5%) than 5 (21.9%), 10 (23.6%) or 20% (23.4%) seminal plasma ($p<0.003$), and for second

ejaculates (18.2%) compared to first ejaculates (25.9%). Whether seminal plasma was from first versus second ejaculates had no effect ($p>0.1$). Effects of seminal plasma originating from bulls different from those whose sperm were sorted was evaluated in Experiment II. Semen collection and initial analysis were performed as in Experiment I and for all successive experiments. Seminal plasma from ejaculates of 6 bulls (3 that sorted well; 3 that sorted poorly) used in Experiment I was used with sperm collected for Experiment II as well as seminal plasma from sperm donors. Sperm were stained with Hoechst 33342 as in Experiment I, but with 10% seminal plasma in staining TALP. Only seminal plasma from one bull resulted in differences for any sorting parameters; 31% of sperm were membrane-impaired with seminal plasma from this bull compared to 16-19% for seminal plasma from other bulls ($p<0.05$). Sort rate was decreased ($3.02x10^3$ sperm per sec) compared to a range of $3.64\text{-}3.97x10^3$ sperm per sec with the other seminal plasmas ($p<0.05$). This may have been due to contamination of the original semen sample, since this effect was only seen with one of six bulls.

Experiment III investigated effects of adding bovine serum albumin (BSA) during staining with Hoechst 33342 using semen from 10 bulls. BSA was added at 0, 0.3 or 0.9% to staining TALP with either 0 or 10% seminal plasma. Although BSA has been shown to be beneficial to sperm, there was a possibility that BSA would bind Hoechst 33342, thereby reducing sorting efficiency. There was no evidence of either effect; however, sperm with 0% seminal plasma had higher % live oriented cells (65%) than sperm incubated with 10% seminal plasma (61%; $p<0.05$). In addition, samples containing 0%

seminal plasma had only 16% membrane-impaired sperm compared to 20% for samples containg 10% seminal plasma ($p<0.05$).

Experiment IV explored current industry dogma that ejaculates with initial sperm concentrations of $<10^9$ sperm per ml sort poorly. Seminal plasma volume was added or removed to create sperm concentrations of 0.7, 1.4 and $2.1x10^9$ sperm per ml, replicated with 10 ejaculates. Samples were sorted at 0h or stored at 16°C and sorted at 4h. While sorting parameters and sperm quality deteriorated from 0 to 4h, there was no interaction between storage time and sperm concentration; means presented are averaged over 0 and 4h. The % live-oriented sperm was higher for samples stored at $2.1x10^9$ sperm per ml (66.4%) than $1.4x10^9$ sperm per ml (64.1%) or $0.7x10^9$ sperm per ml (62.7%; $p<0.01$). The % membrane-impaired sperm was lower for samples containing $2.1x10^9$ sperm per ml (15.1%) than $1.4x10^9$ per ml (17.0%) or $0.7x10^9$ per ml (18.0%; $p<0.01$). The X sort rate was lower for samples of $0.7x10^9$ per ml ($3.45x10^3$ sperm per sec) than for samples containing $1.4x10^9$ and $2.1x10^9$ sperm per ml (3.85 and $3.94x10^3$ sperm per sec; $p<0.05$). Sperm stored at high concentration in seminal plasma (low seminal plasma when diluted for staining and sorting) resulted in superior sorting.

For Experiment V, sperm from 10 bulls were diluted to 0.7, 1.4 and $2.1x10^9$ sperm per ml using staining TALP containing 0 or 10% seminal plasma and stored for 1h. After bulk sorting, sperm were cryopreserved in 20% egg yolk TRIS extender with 6% glycerol. Post-thaw analysis was performed by flow cytometry for % membrane compromised sperm, and by computer assisted sperm analysis for motility. Samples containing 0% seminal plasma had greater % live-oriented cells (54.0%) than samples

containing 10% seminal plasma (50.3%). Samples with 0% seminal plasma also had lower % membrane-impaired sperm than samples containing 10% seminal plasma (18.8% vs. 22.2%; $p<0.01$). Post-thaw motility was higher for sperm incubated with 0% seminal plasma (41.0%) than for sperm incubated with 10% seminal plasma (35.5%; $p<0.05$). No differences were observed for sperm stored at 0.7, 1.4 or $2.1x10^9$ when all samples contained 0 or 10% seminal plasma during storage and staining. Therefore, when combined with Experiment IV, seminal plasma, not initial sperm concentration, impairs sort efficiency.

Experiment VI evaluated various combinations of sperm, seminal plasma, and Hoechst 33342 concentrations during staining. Two ejaculates were collected from 11 bulls on different days. After seminal plasma removal, sperm were re-suspended in TALP at 160 or $240x10^6$ sperm per ml with 0 or 10% seminal plasma. Hoechst 33342 was added for final concentrations of 49, 65 or 81µM. Staining sperm with 0% seminal plasma resulted in higher % live-oriented cells (57.4% vs. 53.7%) and higher sort rates ($3.60x10^3$sperm per sec vs. $3.28x10^3$sperm per sec) compared to sperm in 10% seminal plasma (both $p<0.01$). There was an interaction between sperm concentration and H33342 concentration for ability to separate X and Y populations and for sort rate. Using 65µM H33342 was sufficient to optimally stain $160x10^6$ sperm per ml, while $240x10^6$ sperm required 81µM H33342 to reach similar degrees of separation (peak to valley ratio) and sort rates. The optimal combination for staining bull sperm was 0% seminal plasma, $160x10^6$ sperm per ml, and 65µM H33342.

In conclusion, removing seminal plasma resulted in an 11% increase in sort rate, 14% fewer membrane impaired sperm at sorting, and a 17% increase in post-thaw motility with only the addition of a 15 min centrifugation step to current procedures. In addition, ejaculates with low initial sperm concentration were found to sort just as well as ejaculates with high initial sperm concentration when seminal plasma was removed; therefore, nearly all ejaculates can be utilized for sex-sorting if seminal plasma is removed.

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CHAPTER I

Literature Review

Purpose

There are many potential benefits to separating X-and Y-bearing spermatozoa to choose the sex of offspring at conception, but current technology also has limitations that, when resolved, will lead to a more efficacious process. Sex-sorting of spermatozoa is commercially viable mainly in the cattle industry, although sperm from other livestock species such as sheep, swine and horses are being sorted on a small scale. Sorting sperm allows producers to make faster genetic advances in their herds as well as lower cost of production by producing only the more economically valuable sex, such as heifers in the dairy industry. Sorting is also valuable for exotic and/or endangered species, such as the elephant, where normal sex ratios result in unwanted, dangerous males (Seidel, 2003). Sorting of human sperm allows selection for females in cases of sex-linked genetic disease. Although there are many benefits, sorting sperm is still an expensive process that impairs the ability of sperm to fertilize an oocyte due to a multitude of mechanical and chemical stresses.

Current sexing technologies are 85-95% accurate for the selected sex, and there has been no evidence of increased calf abnormalities with sexed sperm (Tubman et al., 2004). Unfortunately, fertility is decreased with use of sexed semen as seen in

pregnancy rates reported by Norman et al. (2010), where mean conception rates for dairy heifers was 56% for conventional semen and 39% for sexed semen, and by DeJarnette et al. (2009) who found conception rates with sexed semen about 80% of those with conventional semen. The reason for decreased fertility with sexed semen is still an enigma, and is one of the areas where improvements need to be made. The other area for improvement is efficiency of the sex sorting process, which will be addressed in this thesis.

History

For millennia mankind has been trying to select sex of offspring at conception. Although there have been many theories over time, including Democritus' suggestion that the right testis produced males and the left testis produced females (Gordon, 1979), only quite recently has a scientifically proven, practical way been available to skew offspring sex ratio. While some mild distortions in male to female ratios occur with natural mating, artificial insemination, embryo transfer, and in vitro fertilization, none of these practices skew the ratio to more than 53% of one sex or the other on average (Garner and Seidel, 2008). Sex of embryos and fetuses can be determined by biopsy, PCR and ultrasound (Hasler and Garner, 2011), but as these methods can only be utilized post-conception, embryos of the undesired sex are either not transferred or aborted, resulting in a waste of time and money.

Sex chromosomes were first described by Guyer (1910), and work was done by multiple groups over the years to find a method of sex pre-selection. In 1971 a conference entitled "Sex ratio at Birth-Prospects for Control" was held at Pennsylvania

State University to explore advances made in the biological sciences that might be applied to a possible sexing technology. No substantial advances were identified at this conference.

But the topic of a sexing technology was not forgotten and, in 1981, faculty at Colorado State University were contacted by Mr. William Goddard of Warwick Land Co., Providence, RI, USA regarding investing in sperm sexing research. At that time, faculty were not aware of any potentially feasible approaches. But Drs. Rupert Amann and George Seidel did agree to organize a symposium, Prospects for Sexing Mammalian Sperm, to discuss possibilities with a variety of people from multiple disciplines. The results of the symposium identified breakthroughs necessary for developing technologies based on the difference between DNA content of an X- versus a Y-bearing sperm.

Flow cytometry was developed in the 1960's, and with addition of cell sorting, allowed separation of large populations of cells based on optical properties (Kamentsky and Melamed, 1967). The technology became commercially available in the 1970's about the time Moruzzi described the differences in chromatin content between X- and Y- bearing spermatozoa for multiple species (1979). These two scientific breakthroughs permitted development of a sex-selecting technology.

Dr. Daniel Pinkel from the Lawrence Livermore National Laboratory (LLNL) developed a beveled injection needle for the flow cytometer that oriented the flat head of most sperm with the flat side perpendicular to the laser to increase accuracy of measurements of DNA content. In most domestic species there is only a 3-4% difference

between the DNA content of X- and Y-bearing sperm; therefore, a high degree of accuracy is necessary for detecting differences in fluorescence (Johnson, 2000). A coordinated effort between Oklahoma State University, USDA, and LLNL was then able to prove the usefulness of flow cytometry in determining DNA content differences in sperm of cattle, sheep, pigs and rabbits. The only problem was that sperm were killed in the process due to the use of a non-membrane permeable stain, which required destroying the sperm membrane. Dr. Lawrence Johnson was able to use the fluorochrome Hoechst 33342, a membrane permeable fluorescent dye to stain sperm and keep them alive. The sperm sorted with Hoechst 33342 staining were then used to produce live offspring from sex-sorted rabbit sperm at the USDA Beltsville Research Center, resulting in 81% male offspring with the Y-chromosome bearing sperm fraction and 94% female offspring with the X-chromosome bearing sperm fraction (Johnson et al. 1989). In August 1992, Johnson was granted a U.S. patent entitled Beltsville Sperm Sexing Technology (US Patent#5,135,759).

Since that time, the sperm sorting process has been improved with the advent of high-speed flow cytometers and a new nozzle orienting system. The high-speed flow cytometers allow for the separation of up to 8000 X- and Y-bearing sperm per second with greater than 90% purity (Sharpe and Evans, 2009). Orientation of the sperm cells is important due to a flattened ovoid shape and compacted chromatin; therefore, the flat side of the head needs to be oriented toward the detector to optimize fluorescence and correctly quantify DNA content. The new nozzle orienting system described by Rens et

al. (1998) improved the percent correctly oriented sperm to approximately 60% whereas the beveled nozzle only had 25-30% correctly oriented cells.

Several factors affecting throughput and efficiency of sorting sperm could be improved, including: measurement resolution, cell orientation and statistical and timing aspects. Measurement resolution refers to the ability to resolve X- and Y-bearing populations. There are both biological and mechanical aspects that alter resolution, including: a range of 3.7-4.2% difference between fluorescence of X- and Y-bearing sperm in cattle (Garner et al., 1983), fluidic instability, laser noise, opaqueness of fluid, and fluorescence collection efficiency (Sharpe and Evans, 2009). Resolution is typically displayed as a histogram with a double Gaussian distribution with greater resolution seen with greater distinction between the two peaks. Currently, two photodetectors (0 and 90°) at a 90° angle to each other are used to assess sperm head orientation and fluorescence as they move past the laser. Suggestions have been made that adding two more detectors at 45° and 135° may increase the current 60% sufficiently oriented cells by 15-20% (Sharpe and Evans, 2009).

Statistical and timing aspects are important to the rate of sperm sorting. Sperm must enter in single file at sufficient distance not to interfere with each other when the fluorescence is measured. Once sperm DNA content is defined, the droplet containing an individual sperm must be given a charge. Any asynchrony during this process causes high coincidence rates, discarding sperm that were sortable. Timing can be partially controlled by the pressure, which regulates the speed at which the fluid is moving through the system. Normal operating pressure is 40 psi. Higher pressures (50 psi) have

been attributed to increased sperm mortality and membrane impairment as well as lowered fertility and decreased rates of IVF (Suh et al., 2005; Schenk et al., 2009; Barceló-Fimbres et al. 2010). Improvements have also been made in the electronics of the system. The digital systems are capable of continuous measurement and improved sort efficiency, so that over 40% of the live sperm that enter are sorted (Sharpe and Evans, 2009). Due to both fragility of the sperm cell and expense of the sexing process, optimization of every aspect is necessary.

Sex Sorting Sperm – Process

Sperm are stained using Hoechst 33342, and red food coloring (FD&C Red #40) then is added to quench the Hoechst 33342 in cells with damaged membranes. These cells are eventually discarded because they are not viable. Sperm are then pumped past a Vanguard^{TM} diode-pumped solid state laser system to cause excitation of Hoechst 33342. The resulting fluorescence from each individual cell is quantified by a digital photomultiplier tube. The stream of sperm is broken into individual droplets by a vibrator, and each droplet is given a charge based on the observed fluorescence of the sperm within. Droplets pass through an electrical field, and are pulled toward their opposite charge, creating one of three streams, X-bearing sperm, Y-bearing sperm, and uncharged containing no, dead, ill-oriented, or two sperm; these are discarded.

Sex Sorting Sperm – Staining

The use of the lipophilic cell membrane permeable dye Hoechst 33342 allowed sexing of sperm by flow cytometry to become a viable technology, because sperm were able to survive the staining process, plus this dye had no mutagenic properties (Garner,

2009). Hoechst 33342 is a bis-benzimidazole fluorescent dye that binds selectively to A-T base pairs in the minor groove of double stranded DNA of live cells with great affinity. Therefore, the rate limiting step in creating the DNA-Hoechst 33342 complex is movement of Hoechst 33342 by way of an unmediated diffusion transport mechanism across the sperm cell plasma membrane. The pK_a of Hoechst 33342 is 7.45 (Weisenfeld, 2007); therefore, movement into the cell is optimized with staining media at pH 7.45. Staining typically takes place at pH 7.2 due to concerns regarding sperm viability when left at an alkaline pH for extended periods of time. Temperature also affects Hoechst 33342 movement. Current procedures stain sperm at 34.5°C while optimal Hoechst 33342 movement would occur at temperatures nearer to 41°C (Weisenfeld, 2007). Again, sperm survival is the reason for using depressed temperature as sperm vitality is greatly impaired above body temperature. Hoechst 33342 is excited with ultraviolet light at 350nm and emits light at 460nm, giving the sperm cells a blue fluorescence when viewed with fluorescence microscopy.

Characteristics of Semen

Semen is composed of two components: spermatozoa and seminal plasma. Spermatozoa are produced in the testes, combined with epididymal fluid, and then accessory sex gland fluid upon ejaculation. The seminal plasma provides a vehicular environment for spermatozoa that is conducive to maintaining viability, inducing progressive motility and inhibiting capacitation from time of ejaculation until dilution occurs in the female reproductive tract. With natural mating, spermatozoa do not

remain in seminal plasma for any great length of time, but are merely transported in seminal plasma.

Evaluation of Spermatozoa

Sperm are composed of a head and a flagellum with a total length of 68 to 74 microns in the bull (Salisbury et al., 1978). The head contains the genetic material which is composed of a condensed haploid genome that contains either an X or a Y sex chromosome. This genetic package is surrounded by a plasma membrane. Covering the anterior portion of the head is the acrosome, which originated from the Golgi complex of the spermatid, and contains enzymes necessary for fertilization. The mid-piece of a sperm flagellum contains mitochondria necessary for production of ATP, and, therefore, maintenance of cell life and motility. The principal piece or tail is responsible for motility, which is necessary to traverse portions of the female reproductive tract as well as during fertilization. Basically, the sperm cell is a “self-propelled DNA delivery vehicle” whose main function is delivering the male’s genetic input to the egg for fertilization.

After production in the testis, sperm are transported to the epididymis to begin maturation, which includes changes in the plasma membrane as well as acquisition of motility. Potential fertilizing ability of sperm is assessed by motility and morphology upon ejaculation. Typically, in bulls, progressive motility of >60% of sperm at collection is considered acceptable. Motility is typically assessed post-thaw as a quality control measurement, and ≥35% progressively motile sperm is acceptable. Motility is required for fertilization of an ovum, but is not necessarily an indicator of whether or not a sperm

can fertilize an ovum, since sperm can lose fertilizing capacity before losing motility, and abnormal sperm may be motile but unable to fertilize an ovum (Bazer et al., 1993).

Morphology can also be indicative of fertilizing capacity and is assessed by visual microscopic inspection. While most semen contains at least a few abnormal sperm, greater than 30% abnormal sperm is generally associated with decreased fertility. Sperm morphology is often classified as three categories: normal, primary abnormalities and secondary abnormalities. Primary abnormalities are typically associated with defects incurred during spermatogenesis and consist of double headed sperm or heads of an abnormal shape. Secondary abnormalities are typically associated with defects incurred during epididymal transit and include coiled or bent tails as well as proximal cytoplasmic droplets. Some abnormalities can be caused by improper semen handling such as bent principal pieces due to cold shock (Salisbury et al., 1978). Abnormal morphologies can be caused by a variety of factors, including: genetics, environmental temperature, health and stress.

Acrosomal integrity is also an indicator of the fertilizing ability of a sperm cell. The acrosome contains hydrolyzing and proteolytic enzymes necessary for penetration of the zona pellucida of the oocyte, and must be intact for spermatozoa to be capable of fertilization. Capacitation occurs as a precursor to the acrosome reaction, and is defined as a destabilization process which includes loss of cholesterol from the plasma membrane, removal of seminal plasma proteins from the sperm surface and an increase in intercellular pH (Senger, 2005). *In vivo* capacitation occurs in the uterus and oviduct in female reproductive tracts and is followed by the acrosome reaction upon interaction

with the ovum. During cryopreservation (Wheeler and Seidel, 1987) and possibly flow sorting of spermatozoa, capacitation is partially induced in a portion of sperm, which leads to cell death or irreversible damage before sperm even reach the oviduct, impairing fertilization rates.

While the optimal measure of sperm fertility is fertilization of an oocyte, sperm viability can be determined using microscopic observation, computer assisted sperm analysis (CASA) and flow cytometric analysis. Microscopic observation is useful for determining sperm motility and sperm abnormalities, and is done by simply placing a semen sample on a slide and viewing sperm. CASA is used for motility as well but is also useful for determining more detailed sperm movement parameters such as velocity and linearity of movement. CASA is performed with a specialized computer program that when calibrated, can quickly and efficiently analyze hundreds of sperm. Sperm also can be evaluated post-thaw; when stained with fluorescent stains, interference from the large molecules normally found in egg yolk and milk based extenders can be avoided. Flow cytometric analysis is useful for determination of live versus membrane-impaired (dead) sperm, and measurement of acrosome integrity with the use of specific fluorescent markers (Gillan et al., 2005).

Seminal Plasma

Seminal plasma is an enigmatic fluid with some properties similar to those found in other body fluids, but also with properties that are quite different. Seminal plasma is composed of the secretions produced by the accessory sex glands and epididymis of the male. The bull has four main accessory sex glands; seminal vesicles, ampullae, prostate

and bulbo-urethral gland, as do the ram and stallion; the boar does not have substantial ampullae. The secretions produced by these glands contain substances not found at such a high concentration anywhere else in the body (Mann, 1964). Citric acid, fructose, phosphorylcholine, ergothioneine, inositol and glycerylphosphorylcholine are among the molecules found in seminal plasma (Salisbury, 1978). Most of the fructose and citric acid are produced by the seminal vesicles of the bull, and vesicular fluid contains a high concentration of potassium and a low concentration of sodium. In some bulls, secretions from the seminal vesicles can be yellow in color due to a high content of riboflavin (Salisbury, 1978). The epididymal fluid contains no fructose and has a high potassium:sodium ratio as well as a high glycerolphosphorylcholine content. Proteins identified in seminal plasma of bulls have roles in capacitation, sperm membrane protection, prevention of oxidative stress, anti-microbial activity, initiating sperm motility, the acrosome reaction and sperm-oocyte interaction (Moura et al., 2007).

Ejaculate Characteristics:

Concentration, Volume and pH

The pH for bovine semen upon ejaculation ranges from 6.4 to 7.8 (Mann, 1964) with most normal ejaculates being between 6.5-6.9 (Salisbury et al. 1978). The pH of an ejaculate will increase immediately due to loss of carbon dioxide followed by a decrease due to lactic acid accumulation, primarily from the breakdown of fructose. Ejaculates with a greater sperm concentration will have a pH that falls faster and more drastically due to the increased production of lactic acid by more spermatozoa. Too great of a pH change in either the acidic or alkaline direction can be detrimental to the fertilizing

potential of sperm. A low intracellular pH can be detrimental to the capacitation process (Storey, 2008). A high pH can rarely be caused by a bacterial contamination or a high dead sperm rate that causes an increase in ammonia (Salisbury et al. 1978), which is detrimental to remaining viable sperm. An alkaline pH can also be an indication of inflammation of the epididymis.

Color of bull semen is usually a milky white but can also have a yellowish hue, especially in dairy breeds such as Jersey and Brown Swiss due to high riboflavin content produced by the seminal vesicles (Salisbury et al. 1978).

Volume of ejaculates varies greatly between bulls and even for an individual bull. Health, age, sexual preparation and testes size can all affect ejaculate volume. Mean ejaculate volumes for mature bulls average 5-6 ml but volumes can range from 1-15 ml (Salisbury et al. 1978).

Sperm concentration can vary greatly from ejaculate to ejaculate, primarily due to frequency of collection, sexual preparation, health, age, testes size and season of the year. Sperm concentrations can range from zero with azoospermia to over 3 billion sperm per ml with most average ejaculates being reported between 1 and 1.5 billion sperm per ml (Salisbury et al. 1978).

Collection Methods

Semen for sex-sorting is either collected via artificial vagina or electro-ejaculation. Most bulls are trained to mount a steer so that an artificial vagina is slid onto the erect penis by a technician before ejaculation. Electro-ejaculation is often used with bulls that only will be collected once or twice, when there is not enough time to

train them, or for bulls with feet, leg or back problems. Ejaculates collected via electro-ejaculation usually have a greater volume, a more dilute sperm concentration and a higher pH than if collected with an artificial vagina due to direct stimulation of accessory sex glands (Austin et al., 1961).

Dilution Effect

The 'dilution effect' has been described extensively by Mann (1964) as a reduction in spermatozoa viability with dilution in a simple saline media. While the reasons for this effect are not fully understood, changes in ion exchange and alteration of cell components may play a role. The extent to which the dilution effect is seen varies between species, but typically, sperm appear to enter senescence, the first stages of which can be reversed. Later stages, however, begin to resemble apoptosis and are not reversible. Sperm senescence can also occur during long term storage where dilution has not occurred and is hastened by any added stress such as cold shock. The dilution effect is most apparent when only a saline solution was used to dilute sperm. There is a short period of increased activity followed by permanent loss of motility, metabolism and fertilizing capacity (Mann, 1964).

Addition of certain molecules and ions can help dissipate the dilution effect as seen by an increase in motility and metabolism. In bull sperm, a combination of potassium and magnesium ions without calcium ions was shown by Lardy and Phillips (1943) to maximize motility. Phosphate is beneficial, mainly as buffer for the lactic acid produced by active sperm, although a high phosphate concentration can decrease motility. Sulfates prevent the first stages of senescence, which include swelling of the sperm and rearrangement of the plasma membrane. Addition of glucose or fructose

provides an energy source. Egg yolk and milk have both been used successfully to alleviate the dilution effect; antibiotics usually are added to inhibit bacterial growth (Mann, 1964).

There are species differences in how much the dilution effect actually harms sperm. Rams seem to be especially susceptible, but the initial sperm concentrations of ram ejaculates are much higher than those of bulls, boars or stallions. Therefore, a much greater dilution factor is used to bring sperm to an appropriate concentration for cryopreservation or sorting. Bull sperm appear to be less susceptible to dilution, especially in certain extenders and culture media.

Basically, the dilution effect is dependent on whether or not sperm are provided with an environment conducive to maintaining viability. Most extenders in use today allow sperm to retain not only their motility, but their fertilizing ability and membrane integrity when kept at concentrations $<10^6$ sperm per ml for hours, even following the trauma incurred during flow-sorting of sperm (Maxwell and Johnson, 1999).

Seminal Plasma & Bull Sperm

The effects of seminal plasma on bull sperm are still unclear, especially with sperm that have undergone the sorting process. Some reports show that seminal plasma improves sperm viability (Garner et al. 2001), but these are usually in an extreme dilution situation. Most reports show that, for bulls, the presence or absence of seminal plasma makes no difference in the viability of sperm cells (Graham, 1994; Maxwell et al., 1997) . The extender that is used to replace the seminal plasma has a

role in evaluating the importance of seminal plasma since in most studies sperm survival in an extender is compared to survival in seminal plasma.

The “dilution effect” can be attenuated with the addition of seminal plasma when sperm are at concentrations less than 80 million sperm per ml. Garner et al. (2001) demonstrated that adding seminal plasma to diluted sperm sample increases the percentage of motile sperm compared to no seminal plasma. While sperm motility decreased with decreasing sperm concentrations, the magnitude of loss of viability was less when seminal plasma was added, indicating that addition of seminal plasma to highly diluted sperm may be beneficial.

Baas et al. (1983) found that when seminal plasma was added to immotile bull sperm that had been washed in a 7% Ficoll diluent (containing glucose, glycerol, glycine, catalase and antibiotics), the seminal plasma restored motility, but the duration of motility decreased with increasing concentrations of seminal plasma. After expose to seminal plasma, motility could not be restored once lost, by addition of bovine serum albumin.

Graham (1994) described no effect on motility when ejaculated or epididymal bull sperm were washed and re-suspended in either seminal plasma or a modified Tyrode’s medium after cooling to 5°C or after thawing. Maxwell et al. (1997) demonstrated that the addition of 0, 10 or 20% seminal plasma to the extender prior to sorting as well as to the medium for collection post-sorting did not have a significant influence on motility or acrosome integrity of bull sperm, especially in comparison to ram and boar sperm. Earlier findings by Seidel et al. (1997) support Maxwell’s findings in

that pregnancy rates were not different in heifers inseminated with sexed sperm when 5% seminal plasma was added to collection medium.

Seminal Plasma in Other Species

Viability and motility of ram sperm has repeatedly been shown to improve in the presence of seminal plasma for both fresh and frozen sperm as reviewed by de Graaf et al. (2008) and Ashworth et al. (1994). With use of the Beltsville Thawing Solution (BTS), Catt et al. (1997) demonstrated that viability and motility could be maintained with or without seminal plasma, which suggests that the extender used is important. Addition of up to 50% seminal plasma to BTS pre-sort and to collection media post-sort was shown by Maxwell et al. (1997) to improve percentages of live, motile and acrosome-intact ram spermatozoa. Catt et al. (1997) demonstrated that in ram sperm, increased motility by adding 10% seminal plasma may in part be due to decreased agglutination of sperm.

With boar sperm, seminal plasma has been found to be both beneficial and toxic depending on the situation. Seminal plasma aided in developing a resistance to cold shock, but can be toxic at higher concentrations with sperm that are frozen (Pursel and Johnson, 1975). Adding seminal plasma to boar sperm post-thaw improved motility, pregnancy rates and litter size compared to normal frozen-thawed semen (Garcia et al. 2010). Maxwell et al. (1997) demonstrated that adding 10% seminal plasma pre-sorting and to post-sort collection medium improved motility and acrosome integrity, although this study also concluded that >20% seminal plasma resulted in high rates of sperm death.

Effects of Bovine Serum Albumin

Addition of bovine serum albumin (BSA) enhances sperm motility in multiple species, including stallions, bulls, rams and boars. (Kreider et al., 1985;Harrison et al., 1978). This is believed to be due to the protection from lipid peroxidation that BSA provides. Serum albumin is a major antioxidant in the blood that binds iron, decreasing its availability for membrane lipid peroxidation, as well as trapping free radicals (Fukazawa et al., 2005). BSA also strongly binds bacterial endotoxins at a neutral pH, helping to remove any toxins that may be present in semen (Hanora et al., 2005).

BSA facilitates capacitation by binding the sperm plasma membrane and adsorption of the cholesterol onto the BSA molecule. This removal of cholesterol changes the composition of the membrane to a higher phospolipid/cholesterol ratio (Blank et al. 1976). When utilized at low enough concentrations, the benefits of BSA are still seen without induction of capacitation. Therefore, BSA can be used effectively at low concentrations in semen extenders while higher concentrations are used for *in vitro* fertilization where capacitation is desired.

In a study comparing diluents used for stallion sperm during staining for flow-sorting, Gibb et al. (2011) demonstrated that a diluent with 1% BSA was superior to skim milk for separation of X- and Y-bearing sperm populations with no significant difference in sperm motility after 45 or 90 min in the diluent. This suggests that addition of BSA to diluents currently used for staining sperm prior to sorting may improve sperm viability.

Variation Between Bulls

One of the greatest challenges in studying semen is the variation between males of any species. Each male has different characteristics, and there is individual to individual variation in how spermatozoa respond to stresses such as dilution, cooling, cryopreservation, flow sorting, and centrifugation. While some uniformity between ejaculates can be created by manipulation of collection methods, frequency of collection, and sexual preparation, every ejaculate will still be unique (Mann, 1964).

CHAPTER II

Effects of seminal plasma on sex sorting bovine sperm

INTRODUCTION

The ablity to choose the sex of offspring at conception has long been desired, but the technology to do so has only been available for 20 years, and for less than 10 years commercially. Unfortunately, separation of X-and Y-bearing bovine sperm by flow cytometry is still an inefficient process, in which only about one third of sperm that pass through the flow cytometer are collected as live, sexed sperm with an accuracy of 90% for the sex selected (Garner and Seidel, 2003). To improve efficiency and, therefore, decrease the cost of the sex sorting process, each step of the process needs to be re-analyzed. Current industry dogma is that ejaculates of $<10^9$ sperm per ml sort poorly. It has been hypothesized that seminal plasma may be the reason for this reduction in efficiency.

Seminal plasma is the fluid added to sperm during epididymal transport and ejaculation and is produced by the epididymis and accessory sex glands. During normal mating, sperm spend a relatively short period of time in seminal plasma originating from the accessory sex glands, but when processing sperm for cryopreservation or sex sorting, sperm may spend hours or even days in diluted seminal plasma. Seminal plasma is composed of proteins, sugars, and ions necessary for maintaining sperm viability,

inhibiting sperm capacitation, protecting sperm membranes, and initiating sperm motility (Moura et al., 2007).

The presence of seminal plasma has been described as not being necessarily beneficial, or detrimental, to bovine sperm survival during storage, cryopreservation or sex-sorting (Garner et al., 2001; Graham, 2004; Maxwell et al., 1997). However, this has not been studied thoroughly in the context of sexing bovine sperm. Therefore, these experiments explore the effects seminal plasma exerts on sperm during the sex-sorting process.

MATERIALS AND METHODS

Semen Collection and Initial Analysis

First ejaculates were collected by artificial vagina from dairy and beef bulls on a normal collection schedule housed at Sexing Technologies, Inc (Navasota, TX, USA). For Experiment I, second ejaculates were also collected an average of one hour after first ejaculates. Raw semen was processed by determining initial sperm concentration (Nucleocounter® SP-100™, ChemoMetec, Allerod, Denmark), percent progressively motile sperm, percent morphologically normal sperm, and pH. Antibiotics were added as recommended by Certified Semen Services. All ejaculates were evaluated by the same person, and only ejaculates with greater than 60% motile sperm and 70% morphologically normal sperm were accepted. All semen was centrifuged at 1000 x g for 15 min, and seminal plasma was removed by aspiration. Less than 1% of sperm were discarded with the seminal plasma. Seminal plasma was clarified by an additional 15 min of centrifugation at 2000 x g. Sperm were re-suspended in staining TALP (Schenk et al.,

1999) and/or seminal plasma to appropriate concentrations. For Experiment II, a semen sample was removed before centrifugation for a control.

Experimental Design

For Experiment I, sperm were diluted to $160x10^6$ sperm per ml with staining TALP and 0, 5, 10 or 20% seminal plasma from either the same ejaculate or the other ejaculate from the same bull (Table 2.1). For Experiment II, frozen seminal plasma collected during Experiment I was thawed in a 34.5°C water bath and stored at 4°C during the experiment. All treatments were diluted to $160x10^6$ sperm per ml by adding staining TALP to: control semen, sperm with no seminal plasma, sperm with 10% seminal plasma from the same ejaculate, and sperm with 10% seminal plasma from bulls used during Experiment I (Table2.2). Seminal plasma bulls were chosen from Experiment I based on whether the ejaculate sort efficiency was good or poor. For Experiment III, sperm were diluted to $160x10^6$ sperm per ml using staining TALP with 0 or 10% seminal plasma and 0, 0.3 or 0.9% bovine serum albumin (Fraction V).

Storage

Sperm were only stored in Experiment III. Sperm were stored in 1.5 ml Eppendorf tubes at $1.4x10^9$ sperm per ml at 16°C without seminal plasma. Sperm samples were taken at 0 and 4h of storage for staining and sorting.

Staining

For all experiments, Hoechst 33342 (Molecular Probes, Eugene, OR, USA) was added at a final concentration of 65µM, and sperm were incubated for 45 min in a 34.5°C water bath to facilitate Hoechst 33342 movement into sperm cells. The pH of the

sperm samples was taken before and after incubation. After staining, sperm were diluted to $80x10^6$ sperm per ml by adding an equal amount of staining TALP containing 0.002% food-coloring dye (FD&C Red #40, Sensient Technologies Corporation, St. Louis, MO, USA) to quench Hoechst 33342 fluorescence in membrane impaired sperm.

Sorting

Sorting analysis was performed on a high speed flow cytometer (XY Sorter II, XY Inc, Navasota, TX, USA) at 40 psi with a Vanguard™ (Spectra Physics Lasers-North America, Santa Clara, CA, USA) laser set at 200 mW. The event rate was controlled between 20,000 and 21,000 events per sec. Treatments were sorted in a different random order for each bull. Responses recorded include (Figure 2.1): % live-oriented cells (live sperm that are correctly oriented toward the photodetectors), X Sort Rate (number of X-bearing sperm collected per second), % membrane-impaired sperm (food dye quenched the Hoechst 33342), and % X collected (% of the presumed X-bearing population that were correctly oriented was collected).

Statistical Analyses

Calculations for split were based on 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 multiplied by 100. A higher percentage indicates a higher degree of resolution between X- and Y-bearing sperm populations.

Experiment I had a factorial design of 4 seminal plasma concentrations by 2 sperm ejaculates by 2 seminal plasma ejaculates with 10 bulls (beef breeds, *n=4;* dairy

breeds, *n=6*) for replication. Since this was not a complete block design (there was no corresponding seminal plasma ejaculate with 0% seminal plasma), the experiment was analyzed first as a 4 seminal plasma by 2 sperm ejaculate design, then a 3 seminal plasma by 2 sperm ejaculate by 2 seminal plasma ejaculate design. Experiment II was a factorial design of 9 treatments with 11 bulls (Jersey, *n=8*; Holstein, *n=2*; Brown Swiss, *n=1*) for replication. Experiment III had a factorial design of 3 BSA levels by 2 seminal plasma contents by 2 time points (0 and 4h storage) with 10 bulls (Jersey, *n=7*; Holstein, *n=2*; Brown Swiss, *n=1*) for replication.

Data collected for all experiments was subjected to a mixed model ANOVA with bulls considered a random effect. Main effects and first order interactions were considered in all models. Least squares means are presented with Tukey's HSD test used for multiple comparison tests. Residual plots showed no obvious need for transforming data.

RESULTS

In Experiment I, effects of different seminal plasma concentrations during staining were explored as well as differences between first and second ejaculates (Table 2.3). There was no effect ($p>0.1$) for any response whether seminal plasma originated from the first or second ejaculate, so these were pooled. Incubating sperm in 0% seminal plasma resulted in the highest % live-oriented cells (64%) compared to sperm in 5, 10, and 20% seminal plasma (60, 59, and 58%, respectively; $p<0.05$). Sperm incubated with 0% seminal plasma sorted at a higher rate ($p<0.05$) than sperm incubated with 20% seminal plasma (4.26 vs. 3.61×10^3 sperm per sec); neither was different ($p>0.05$) from

sperm incubated with 5 and 10% seminal plasma. There was an interaction between % seminal plasma and ejaculate for % membrane-impaired sperm ($p<0.05$); there were fewer membrane-impaired sperm in samples containing 0% seminal plasma and in second ejaculates (Figure 2.2). Seminal plasma did not affect split or % X collected. The pH before incubation was highest in samples containing 0% seminal plasma (7.35) and decreased with increasing levels of seminal plasma (Table 2.3). Second ejaculates were better for sorting, as they displayed higher % live-oriented cells (63 vs. 57%) and survivability of sperm (18 vs. 25% membrane-impaired sperm) compared to first ejaculates ($p<0.05$).

Experiment II

Effects of seminal plasma from 6 bulls in addition to the sperm donating bull were explored in this experiment (Table 2.4). The 6 bulls used as seminal plasma donors were chosen from Experiment I based on whether they had good or poor sort efficiency with their own sperm. The control sperm were not centrifuged, and seminal plasma was not manipulated. “Own” is the seminal plasma from the sperm donating bull with 10% seminal plasma during staining and “none” is sperm with all seminal plasma removed. For each of the other treatments with seminal plasma from other bulls, sperm were stained in 10% seminal plasma.

Only seminal plasma from bull Poor3 had any significant effect on any response. Seminal plasma from this bull induced greater sperm membrane damage (31%) than seminal plasma from other bulls (16-19%; $p<0.05$). In addition, sort rate was lower for sperm incubated in seminal plasma from this bull ($3.02x10^3$ sperm per sec) versus

seminal plasma from other bulls (3.64-3.97x10^3 sperm per sec; $p<0.05$). There was no effect of seminal plasma origin on split.

Experiment III

Effects of adding additional BSA during staining were explored in this experiment (Table 2.5). Adding 0, 0.3 and 0.9% BSA to staining TALP medium, which already contained 0.3% BSA, did not affect any of the sperm responses.

Treatments had either 0 or 10% seminal plasma in addition to the BSA additions. Sperm incubated with 0% seminal plasma had higher % live oriented cells (65%) than sperm incubated with 10% seminal plasma (61%; $p<0.05$). Sperm incubated with 0% seminal plasma also had only 16% membrane impaired sperm compared to 20% when incubated in 10% seminal plasma ($p<0.05$). There was no interaction between BSA concentration and seminal plasma for any response.

DISCUSSION

Seminal plasma is typically not removed in the commercial sperm sex-sorting industry, due to practicality and an understanding that seminal plasma does not harm bovine sperm when present during cryopreservation or sex sorting (Graham, 1994; Maxwell et al. 1997). This series of experiments explored if seminal plasma may be affecting the sorting process through analysis of responses measured during flow cytometric sperm sorting.

In Experiment I, seminal plasma was added to sperm in a range of concentrations (0, 5, 10 and 20%) that represent what would be present when ejaculates within a normal sperm concentration range are diluted for staining. All sperm were diluted to

$160x10^6$ sperm per ml for sorting; therefore, an initial sperm concentration of $2.4x10^9$ sperm per ml will have about 7% seminal plasma during staining, while $700x10^6$ sperm per ml will have about 23% seminal plasma.

As seen in Table 2.3, X Sort Rate decreased with addition of seminal plasma, indicating that seminal plasma may be interfering with sorting efficiency. Sperm also survived better with seminal plasma removed (Figure 2.2); therefore, sperm quality was improved and more live sperm were available to be sorted. As expected, second ejaculates were more efficient to sort, most likely due to increased quality in the second ejaculate, and a lower susceptibility to the presence of seminal plasma, which was seen with an average of 38% more live cells. The differences between first and second ejaculates can be reduced by collecting bulls more often (Everett et al., 1978). Seminal plasma has been shown to have different characteristics when collected from first or second ejaculates (Seidel and Foote, 1969). In this experiment those differences appeared to have no effect on sex-sorting efficiency.

The pH during staining of sperm is important to facilitate the movement of Hoechst 33342 (H33342) into the sperm cell and the nucleus, while still keeping the sperm membrane stable. A pH of 7.45 optimizes H33342 membrane permeation (Weisenfeld, 2007), but as seen in Figure 2.3, the highest medium pH was 7.35, and pH decreased during incubation. Therefore, it is possible that increasing the pH of the staining medium may improve sort efficiency. Removal of seminal plasma also causes an increase in pH (Figure 2.3).

In Experiment II, origin of seminal plasma was explored by exchanging seminal plasma between bulls. There was only one bull whose seminal plasma caused a significant decrease in sorting responses (Table 2.4). This may have been due to contamination of the original semen sample, since this effect was only seen with one bull. Sperm from that particular bull also sorted poorly in Experiment I with the first ejaculate, but sperm from the second ejaculate sorted normally. Seminal plasma from the first ejaculate was used in this experiment. Whether or not there are substantive seminal plasma effects between bulls would require further studies across a wider population of bulls. There was no detectable benefit of seminal plasma from ejaculates that sorted well.

Within this experiment control sperm were not centrifuged, while all other treatments were centrifuged, and since control sperm exhibited similar sperm survival (Table 2.4), centrifugation at 1000 x g for 15 min was not damaging to sperm membranes.

In Experiment III, addition of BSA during staining was investigated. BSA improves sperm motility and provides protection from lipid peroxidation, leading to overall higher sperm quality (Harrison et al. 1978). Due to possible binding of H33342 to BSA, there was also the possibility that BSA would interfere with H33342 binding sperm DNA. There was no evidence of either effect as there were no differences in sort responses across this range of BSA concentrations. Evidence that BSA is beneficial to sperm may have been more apparent if sperm from this experiment had been cryopreserved, thawed and analyzed for motility and acrosome integrity. Addition of more BSA, or addition of

BSA during storage, may be necessary to see effects. Improved sort efficiency and sperm survival were seen when seminal plasma was removed (Table 2.6).

Seminal plasma has been shown to be an inhibitor of long term sperm survival by Shannon and Curson (1972) who found that sperm stored at $10x10^6$ sperm per ml with 0% seminal plasma in Caprogen + egg yolk lived 1.5 times longer than sperm stored with 10% seminal plasma. A similar effect of seminal plasma was seen in these experiments even though sperm were only subjected to seminal plasma for 5 hours at most before sex-sorting. Sperm may not respond adversely when left with seminal plasma for less than an hour as is typical (before dilution steps) with cryopreservation of bovine sperm, but the longer exposure found with the sex-sorting process may harm sperm survival.

The event rate of the flow cytometer was held constant at 20,000 to 21,000 events per second for these studies. For commercial sperm sexing, the event rate is often higher. For some responses (e.g. % membrane-impaired sperm) event rate has little effect. But for others responses (e.g. % X-bearing sperm collected) it is unclear if treatment effects would be enhanced or diminished if the sort rate would have been subjectively optimized, as is done in commercial practice.

Seminal plasma was affecting sex sorting efficiency of sperm. With removal, sort rates increased and there were more live sperm. While the mechanisms as to why seminal plasma influences sort efficiency were not studied, it could be hypothesized that the opaqueness of seminal plasma interferes with the ability of the flow cytometer to measure fluorescence, the proteins of seminal plasma interfere with Hoechst 33342

movement, or removing seminal plasma results in more live sperm and having a greater proportion of live sperm increases sort efficiency.

Table 2.1. Experimental design with sperm and seminal plasma from first and second ejaculates and seminal plasma concentration- Experiment I

Seminal Plasma (%)	Sperm Ejaculate	Seminal Plasma Ejaculate
0	1	NA
0	2	NA
5	1	1
5	1	2
5	2	1
5	2	2
10	1	1
10	1	2
10	2	1
10	2	2
20	1	1
20	1	2
20	2	1
20	2	2

Table 2.2. Experimental design with seminal plasma from 7 bulls- Experiment II

Treatment	Seminal Plasma (%)	Seminal Plasma Origin
Control	Control	Sperm Bull
Own	10	Sperm Bull
None	0	NA
Good1	10	**Good1**
Good2	10	**Good2**
Good3	10	**Good3**
Poor1	10	**Poor1**
Poor2	10	**Poor2**
Poor3	10	**Poor3**

Table 2.3. Main effect means for sex-sorting responses for seminal plasma concentration and sperm from first versus second ejaculates – Experiment I (10 bulls)

Seminal Plasma/ Sperm Ejaculate	% Live Oriented Cells	X Sort Rate (10^3 sperm /sec)	% Membrane -Impaired Sperm	% X Collected	Split	pH Pre-Incubation	pH Post-Incubation
0%	64^{a}	4.26^{a}	17^{a}	43^{a}	31^{a}	7.35^{a}	7.28^{a}
5%	60^{b}	4.02^{ab}	22^{b}	43^{a}	35^{a}	7.32^{ab}	7.25^{a}
10%	59^{b}	3.90^{ab}	24^{b}	43^{a}	35^{a}	7.29^{b}	7.24^{a}
20%	58^{b}	3.61^{b}	23^{b}	42^{a}	32^{a}	7.23^{c}	7.17^{b}
SEM	±3.4	±0.31	±3.4	±1.1	±5.0	±0.03	±0.02
First	57^{A}	3.81^{A}	25^{A}	43^{A}	33^{A}	7.30^{A}	7.23^{A}
Second	63^{B}	4.08^{A}	18^{B}	43^{A}	34^{A}	7.30^{A}	7.23^{A}
SEM	±3.3	±0.30	±3.5	±0.6	±4.5	±0.03	±0.02

Means within columns and treatment sets without common superscripts differ ($p<0.05$).

Table 2.4. Means of sex-sorting responses when seminal plasma was exchanged between bulls- Experiment II (11 bulls)

Treatment (Seminal Plasma Bull)	% Live Oriented Cells	X Sort Rate (10^3sperm/sec)	% Membrane-Impaired Sperm	% X Collected	Split
Control	64[a]	3.67[a]	18[a]	35[a]	33[a]
Own	62[a]	3.64[a]	18[a]	34[a]	36[a]
None	65[a]	3.95[a]	16[a]	34[a]	35[a]
Good1	62[a]	3.91[a]	18[a]	34[a]	36[a]
Good2	65[a]	3.83[a]	16[a]	34[a]	36[a]
Good3	65[a]	3.97[a]	16[a]	34[a]	41[a]
Poor1	61[a]	3.78[a]	19[a]	32[a]	39[a]
Poor2	64[a]	3.88[a]	17[a]	35[a]	43[a]
Poor3	52[b]	3.02[b]	31[b]	27[b]	37[a]
SEM	±2.8	±0.22	±2.7	±1.5	±4.8

[ab] Means without common superscripts differ ($p<0.05$).
Treatments are: control (unmanipulated seminal plasma), none (no seminal plasma), own and bulls (10% seminal plasma)

Table 2.5. Main effect means for sex-sorting responses when BSA was added during staining– Experiment III (10 bulls)

BSA	% Live Oriented Cells	X Sort Rate (10^3 sperm/sec)	% Membrane-Impaired Sperm	% X Collected	Split
0.00%	63[a]	3.83[a]	19[a]	40[a]	37[a]
0.30%	63[a]	3.86[a]	18[a]	39[a]	37[a]
0.90%	63[a]	3.76[a]	18[a]	39[a]	35[a]
SEM	±1.4	±0.11	±1.2	±1.0	±3.3

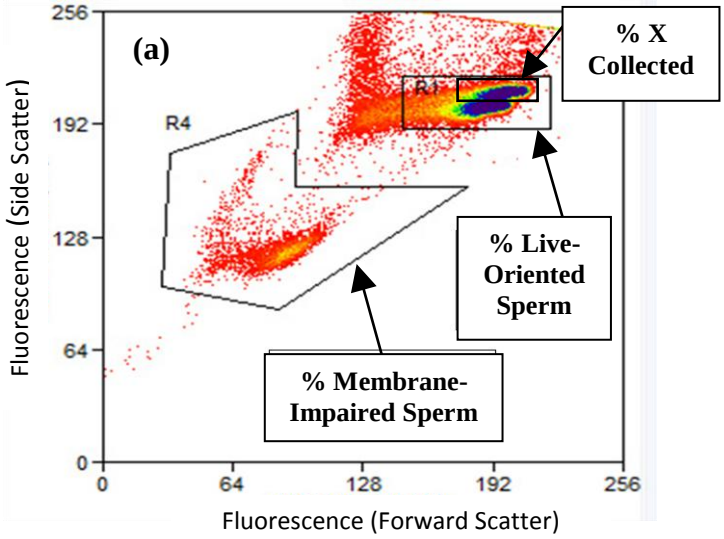
[a] No differences (p>0.1).

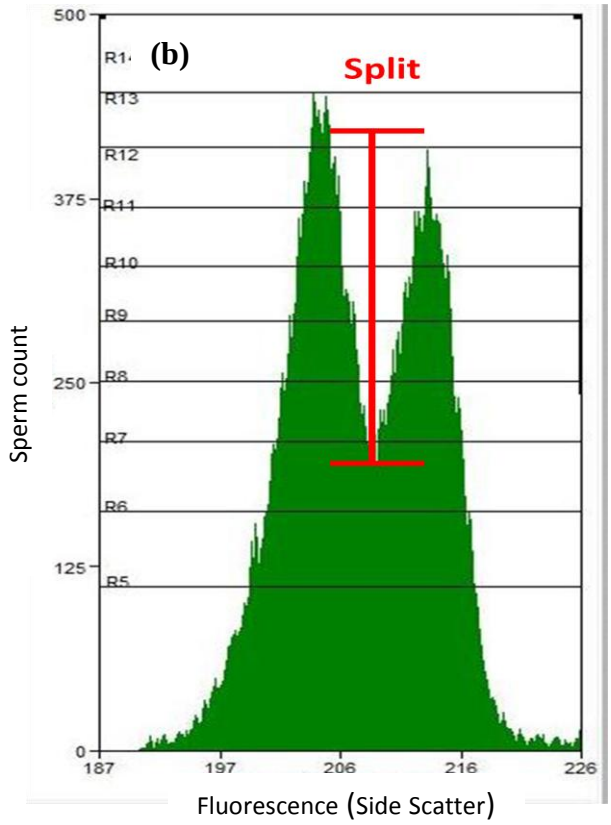
Table 2.6. Main effect means for sex-sorting responses for seminal plasma (0 and 10%) during staining- Experiment III (10 bulls)

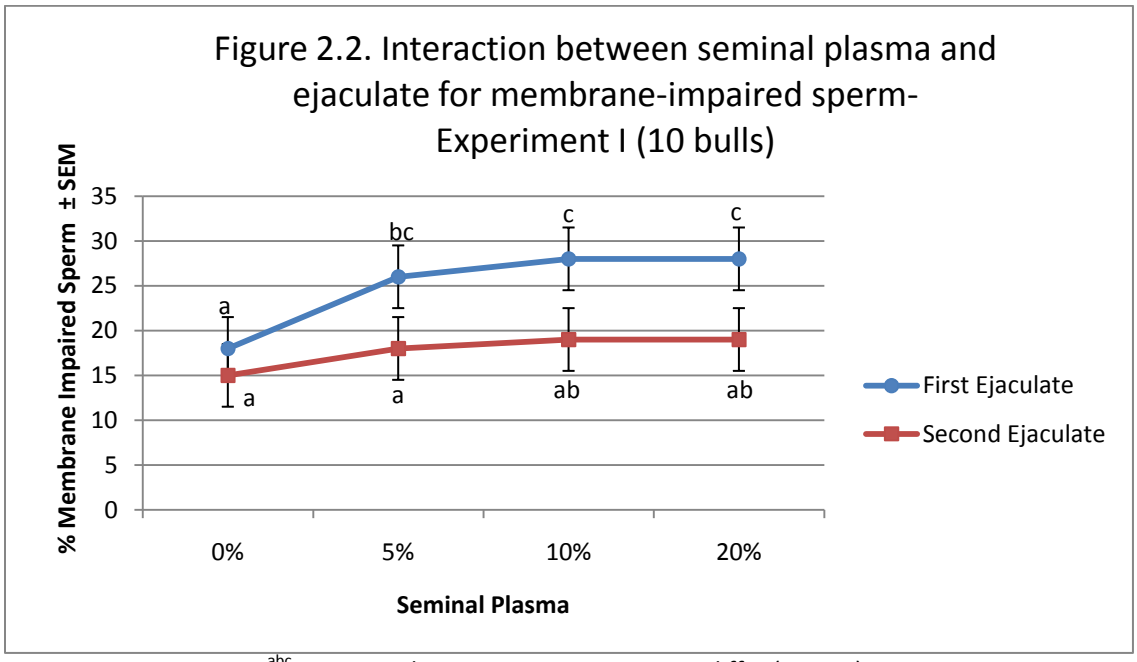
Seminal Plasma	% Live Oriented Cells	X Sort Rate (10^3 sperm/sec)	% Membrane-Impaired Sperm	% X Collected	Split
0%	65[a]	3.87[a]	16[a]	39[a]	34[a]
10%	61[b]	3.75[a]	20[b]	39[a]	38[a]
SEM	±1.4	±0.11	±1.2	±1.0	±3.3

[ab] Means within columns with different superscripts differ (p<0.05).

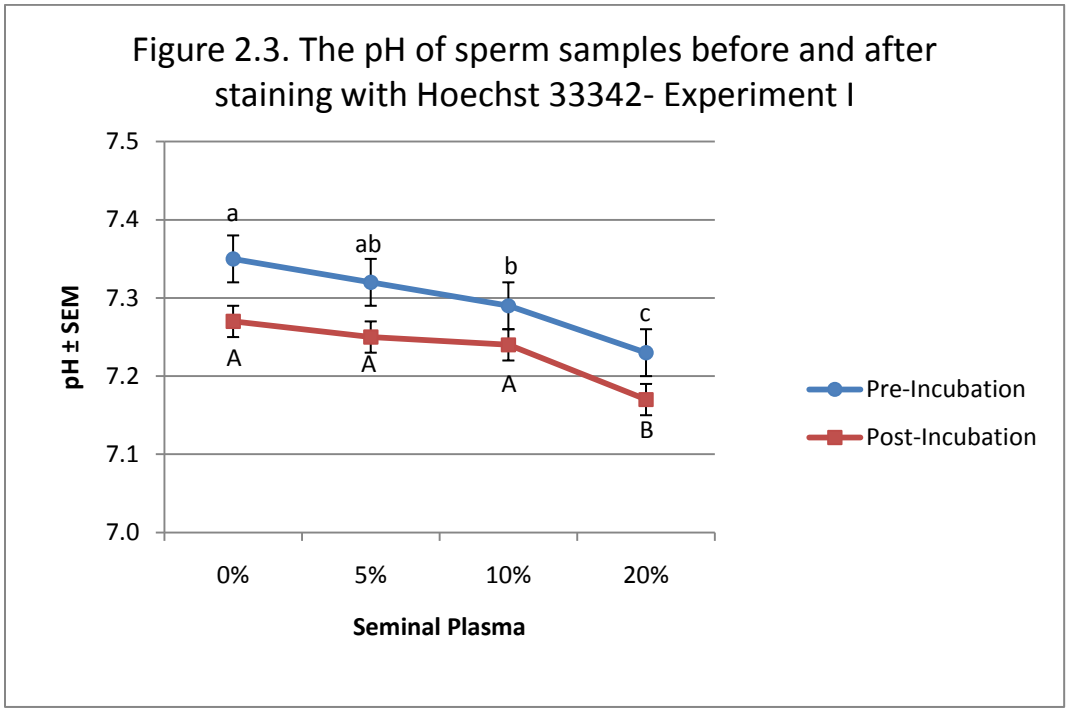
Figure 1. Histograms produced by a flow cytometer during sex-sorting







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



Means without common superscripts differ ($p<0.05$) within incubation time.

CHAPTER III

Removing seminal plasma improves bovine sperm sex-sorting

INTRODUCTION

Typical sperm concentrations of bull ejaculates range from $5x10^8 – 2.5x10^9$ sperm per ml. These sperm concentrations vary due to collection method, collection frequency, sexual preparation, age, testes size, and individual bull variability. Sperm from some ejaculates are difficult to sex-sort by flow cytometry, especially those with sperm concentrations less than 10^9 sperm per 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, and resulting 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, sperm remain in seminal plasma, mostly from accessory sex glands, 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 fertilizing sperm traverse the cervix, removing them from the seminal plasma. In situations where sperm are going to be

cryopreserved or sex-sorted, sperm can remain in seminal plasma for hours or even days in diluted form. While this has not proven to be detrimental to bovine sperm (Graham, 1994; Maxwell et al. 1997), for species such as porcine and equine sperm, seminal plasma has be found to be detrimental to sperm survival during cryopreservation and extended cold storage (Pursel and 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 sperm causes a rapid decline in sperm viability (Ashworth et al. 1994). The effect of seminal plasma is also dependent on diluents and extenders used (Maxwell and Johnson, 1999).

Sex-sorting bovine sperm induces a whole new set of obstacles to sperm survival, and many protocols used for this technique are derived from conventional sperm cryopreservation protocols. While these procedures work, improvements might be made that cater to the needs of the highly stressed sexed sperm that may not be necessary or even desirable for conventional sperm processing for cryopreservation.

The objective of these experiments was to determine the effect of sperm concentration during storage between collection and staining for sex-sorting; sperm were stored in seminal plasma only, without seminal plasma or at controlled levels of seminal plasma. The effect of sperm concentrations, Hoechst 33342 concentrations, and seminal plasma content were analyzed to find an optimal combination for sorting sperm and for sperm survival.

METHODS AND MATERIALS

Ejaculate Collection and Initial Analysis

First ejaculates were collected with an artificial vagina from dairy bulls that were on a normal collection schedule, and housed at Sexing Technologies, Inc (Navasota, Texas, USA). Raw semen was processed by determining the initial sperm concentration (Nucleocounter® SP-100™, ChemoMetec, Allerod, Denmark), percent motile sperm, percent morphologically normal sperm, and pH. Antibiotics were added as recommended by Certified Semen Services. All ejaculates were evaluated by the same person, and only ejaculates with greater than 60% motile sperm and 70% morphologically normal sperm were accepted. All semen was centrifuged at 1000 x g for 15 min to remove seminal plasma by aspiration. The seminal plasma was clarified by an additional 15 min of centrifugation at 2000 x g. Fewer than one percent of sperm from the original ejaculate were removed and discarded with the seminal plasma.

Storage

In Experiment IV, four sperm concentrations: initial, 700, 1400 and $2100x10^6$ sperm per ml were created by addition or removal of seminal plasma. Samples were stored at 0.5ml in Eppendorf tubes at 16°C. At 0 and 4 h, subsamples were taken for staining and sorting.

In Experiment V, sperm concentrations of 700, 1400 and $2100x10^6$ sperm per ml were created by addition of staining TALP (pH 7.4; Schenk et al. 1999) and 0 or 10% seminal plasma. Sperm were stored for one hour before preparation for staining and sorting.

Staining

Hoechst 33342 (H33342; Molecular Probes, Eugene, Oregon, USA) was added to sperm that had been diluted to $160x10^6$ sperm per ml using staining TALP for a final H33342 concentration of 65µM. Sperm were incubated for 45 min in a 34.5° water bath to facilitate movement of H33342 into sperm cells. Sperm were diluted to $80x10^6$ sperm per ml by adding an equal amount of TALP (pH 5.5) + 0.002% food-coloring dye (red TALP; FD&C Red #40, Sensient Technologies Corporation, St. Louis, Missouri, USA) and allowed to sit for 5 min before sorting.

In Experiment VI, H33342 was added to create final concentrations of 49, 65 or 81µM in sperm diluted to 160 or $240x10^6$ sperm per ml with staining TALP and 0 or 10% seminal plasma. Sperm were incubated as above, and then diluted to $80x10^6$ sperm per ml by addition of an equal amount of red TALP to sperm stained at $160x10^6$ sperm per ml or by addition of an equal amount of TALP (3:1 red TALP, staining TALP) in order to maintain pH.

Sorting

All sperm 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 175mW. Event rates were held between 20,000 and 21,000 per sec. Responses were recorded based on sorting 50,000 sperm for % live-oriented cells, X sort rate, % membrane-impaired sperm, and % X-bearing sperm collected.

Freezing/Thawing

Freezing and thawing of sorted sperm was only done in Experiment V. Approximately 12 million bulk (both X-and Y-bearing sperm) sorted sperm 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 addition of an equal amount of TRIS buffer + 6% glycerol; added in two aliquots, 15 min apart. After addition of the second aliquot, tubes were centrifuged for 20 min at 850 x 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 per 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.

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. Sperm were analyzed by flow cytometry and computer-assisted sperm analysis (CASA). For flow cytometry analysis, sperm were stained with propidium iodide (1mg/ml) and FITC-PNA (1 mg/ml) and incubated for 5 min (Purdy and Graham, 2004). Sperm were diluted with 500µl of TALP, filtered through a 20µm mesh, and analyzed using a MoFlo™ High-Performance Cell Sorter (Dako, Fort Collins, CO, USA) for % live, acrosome-reacted (Hoechst 33342 and FITC-PNA positive), % live, non-acrosome reacted (only Hoechst 33342 positive), and % membrane-impaired (propidium iodide positive). For subjective and CASA sperm

motility, a second straw from each treatment was thawed. Subjective motility was evaluated by one evaluator for all samples. CASA was conducted with a minimum of 200 cells evaluated per treatment in eight fields of view. System parameters for these analyses were: 100 frames acquired at 60 frames per sec; minimum contrast = 70, minimum cell size = 4 pixels; lower VAP cut-off = 10 µm/sec; lower VSL cut-off = 40 µm/sec; VAP cut-off for progressive cells = 40 µm/sec and straightness = 75%.

DNA Fragmentation

During Experiment V, sperm samples for DNA fragmentation analysis were taken during the sex-sorting process: pre- and post-storage (1h at room temperature), pre-sort, post-sort, and post-thaw during Experiment V. Samples of 100µl were stored in 1.5ml 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, 5µl of sperm sample was mixed with 5µl 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 20x 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 sperm were analyzed per bull per treatment, and percent fragmented was calculated.

Statistical Analyses

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.

Experiment IV was a factorial design of 4 sperm concentrations by 2 time points (0 and 4h storage) with 10 bulls (Jersey, *n*=7; Holstein, *n*=2; Brown Swiss, *n*=1) for replication. Experiment V 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 24h) with 6 bulls (Jersey, *n*=3; Holstein, *n*=3) for replication. Experiment VI 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. Data collected 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 VI, second order interactions that included bull nested within breed were also considered. Least squares means are presented; linear contrasts were used for Experiment IV. Least squares means are presented with Tukey's HSD test used for multiple comparison tests

in Experiment V, VI and DNA fragmentation. Residual plots showed no obvious need for transformation.

RESULTS

Experiment IV

Effects of sperm concentration were 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), 700, 1400 and $2100x10^6$ sperm per ml. These concentrations were chosen as an incremental spread over the natural ejaculate range; initial ejaculate sperm concentration was a control. Sort responses for the initial ejaculates and samples reconstituted to $1400x10^6$ sperm per ml were similar. This was expected as $1400x10^6$ sperm per ml was similar to the average initial sperm concentration of the ejaculates ($1246x10^6$ sperm per 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 4h storage times and sperm concentration. Means are presented in Table 3.1.

For % live-oriented cells there was a positive linear effect of sperm concentration ($p<0.01$). With an increase in sperm concentration (seminal plasma more dilute during staining) there was an increase in % live-oriented cells. Sperm concentration also had a positive linear effect on sort rate, with samples containing higher sperm concentrations sorting better ($p<0.01$). Sperm concentration had a negative linear effect on % membrane-impaired sperm; therefore, there were lower percentages of membrane-impaired sperm at higher sperm concentrations ($p<0.01$). There were no linear effects of

sperm concentration on % X collected or on split, nor were there any quadratic effects on any response ($p>0.1$). Overall, this indicates an improved ability to sort sperm when sperm were stored at higher sperm concentrations.

Experiment V

Effects of sperm concentration in the presence of 0 or 10% seminal plasma and staining TALP during 1 h of storage were explored in this experiment (Table 3.2). There was no significant difference between any of the sorting or post-thaw responses between the three sperm concentrations ($p>0.1$). Samples incubated in 0% seminal plasma had higher % live-oriented cells (54% vs 50%) and sort rate (3.55 vs 3.20×10^3 sperm per sec) compared to samples incubated in 10% seminal plasma ($p<0.05$). The % membrane-impaired sperm was also lower for sperm incubated with 0% seminal plasma (19%) than with 10% seminal plasma (22%), indicating better sperm survival when seminal plasma is removed ($p<0.05$). Post-thaw motility, analyzed by CASA, was higher for sperm incubated with 0% seminal plasma (41%) than for sperm incubated with 10% seminal plasma (35%). There were no interactions ($p>0.1$) between sperm concentration and the percent of seminal plasma, for any response.

Storing and staining sperm with 0% seminal plasma was beneficial to both sorting and post-thaw responses. Sperm concentration did not affect sorting of sperm when the seminal plasma content was held constant across sperm concentrations, indicating that seminal plasma content, not sperm concentration, is affecting sort efficiency.

DNA Fragmentation

DNA fragmentation was analyzed for samples taken from six bulls during Experiment V. Interactions were significant ($p<0.001$) for sperm concentration by hour of storage and seminal plasma by hour of storage; therefore, two-way means are presented in Tables 3.3 and 3.4. 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 sperm incubated with 0% seminal plasma ($p<0.05$). Increasing sperm concentration also resulted in increasing rates of DNA fragmentation at 24h of incubation (Figure 3.1). All of these effects disappeared after sorting, which removed essentially all sperm that were positive for DNA fragmentation.

Experiment VI

Multiple concentrations of both sperm and H33342 were explored in this experiment with 0 or 10% seminal plasma during staining. As seen in prior experiments, sperm incubated with 0% seminal plasma had higher % live oriented cells (56 vs. 53%) as well as greater sort rates (3.47 vs. $3.15x10^3$ sperm per sec) than 10% seminal plasma ($p<0.04$; Table 3.5). The % membrane-impaired sperm was lower for samples incubated with 0% seminal plasma (15 vs. 19%) than with 10% seminal plasma ($p<0.04$).

There was an interaction between H33342 dye concentration and sperm concentration for split and sort rate (Table 3.6). Sperm samples incubated at a sperm concentration of $160x10^6$ sperm per ml reached maxima for both split and sort rate when stained with 65µM H33342, while the samples stored at $240x10^6$ sperm per ml required 81 µM H33342 for optimum splits (Figure 3.2). Samples stained at 85µM

H33342 exhibited split and sort rates that were the same for both sperm concentrations, as were % live oriented cells and % X-bearing sperm collected. The % membrane-impaired sperm was the same 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. 7). Jersey bulls had higher % live oriented sperm at 49 and 65µM H33342 (56 and 58%) compared to Holsteins (46 and 53%), while samples stained with 81 µM H33342 exhibited % live oriented sperm that were similar for Jersey and Holstein bulls (58 vs. 55%). Sperm from Jersey and Holstein bulls had a similar % membrane-impaired sperm ($p>0.05$). For X sort rate, sperm from Jersey bulls sorted better at every dye concentration with $3.96x10^3$ sperm per sec compared to sperm from Holstein bull sperm at $3.28x10^3$ sperm per sec at 81µM H33342 ($p<0.05$). Split was also greater for Jersey bull sperm at all dye concentrations. Bull age did not account for any variation between bull breeds as Holstein bulls averaged 28 mo of age and Jersey bulls averaged 26 mo of age.

DISCUSSION

Initial sperm concentration of ejaculates has been a criterion for whether or not an ejaculate is used for sex-sorting due to belief that sperm concentrations $<10^9$ sperm per ml are less efficient to sort. In Experiment IV, sperm concentration did dictate how efficiently sperm sorted when seminal plasma was not manipulated. As seen in Table 3.1, higher concentrations of sperm resulted in more sperm sorted per sec and more membrane-intact sperm that were correctly oriented, which agrees with what has been

observed in industry. An ejaculate with $2100x10^6$ sperm per ml will contain about 7% seminal plasma after dilution for staining, compared to an ejaculate with $700x10^6$ sperm per ml that will contain about 23% seminal plasma during staining, leading to the question of whether it is sperm concentration, or amount of seminal plasma, that affects sorting efficiency.

In Experiment V, there was no difference in the sorting efficiency of sperm at several storage concentrations when seminal plasma content was controlled at either 0 or 10% (Table 3.2). However, sort rates were 11% higher for samples stored with 0% seminal plasma over those containing 10% seminal plasma. There was also a 14% decrease in sperm with compromised cell membranes in samples containing 0% seminal plasma. Not only did sperm sort more efficiently when seminal plasma was removed, but post-thaw motility also increased by 17%. An increase in post-thaw motility was also seen by Tibary et al. (1990) after sperm cryopreservation with seminal plasma removed. Therefore, seminal plasma impairs the ability of sperm to be sex-sorted and cryopreserved.

Some sperm DNA fragmentation is present in most ejaculates with variation among bulls, and high levels of this 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; 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. Sperm concentration may play a role in inaccuracy as higher sperm concentrations 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 700, 1400 and 2100x10^6 sperm per ml while pre-sort samples were at 80x10^6 sperm per ml, and post-sort samples had <1x10^6 sperm per ml.

It appears that sex-sorting of sperm removes DNA fragmented sperm from the population (Table 3.3). This agrees with conclusions made by Gosálvez et al. (2011) who found 63% of DNA fragmented sperm were removed by sex-sorting compared to neat semen. The percent of fragmented sorted sperm present post-thaw was negligible and probably does not affect overall sperm fertility.

During sex-sorting, sperm 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 sperm at concentrations of <10^6 sperm per ml for hours between sex-sorting and cryopreservation (Maxwell and Johnson, 1999). However, dilution may still be an issue. Determining whether sperm membranes are impaired just before adding glycerol, which would be after hours at a low sperm concentration, may give insight into how sperm 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 sperm were stored at 5°C, and then incubated at 37°C to determine livability, sperm with 0% seminal plasma lived 1.5 times longer after 2 days of storage and 3 times longer after 7 days of storage. Sperm only used for cryopreservation are typically exposed to seminal plasma for less than 1h before dilution, so effects of seminal plasma typically are not seen. But sperm 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 at times longer than the 5 hours studied.

The increase in sperm survival seen with seminal plasma removal could be due to removal of acrosomal enzymes that were released into seminal plasma upon sperm death. Acrosomal enzymes such as hyaluronidase are present in seminal plasma, and at increasing concentrations over time (Foulkes and Watson, 1975). High levels of glutamic-oxalacetic transaminase, another acrosomal enzyme, have been shown to correlate with decreased fertility (Breeuwsma, 1972; Pace and Graham, 1970). 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 percent of membrane-intact sperm seen in these experiments.

Movement of Hoechst 33342 (H33342) into the nucleus is one of the most important parts of the sex-sorting process. Many factors impact efficiency of H33342

movement, including: temperature, pH, dye concentration, sperm membrane permeability, diluent components, and sperm concentration (Garner, 2009). Study of all these factors simultaneously would be nearly impossible. Therefore, dye concentration and sperm concentration were chosen for study with removal of seminal plasma to determine whether the seminal plasma affects H33342 movement. Having 0% seminal plasma 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 65µM H33342, while the higher sperm concentrations required 81µM H33342. The greatest example of this was with the split response, which could indicate when sperm are reaching the saturation point for dye (Figure 3.1).

Breed differences between Jersey and Holstein bulls were seen in Experiment VI. There has been reported to be a difference in H33342 fluorescence between X- and Y-bearing sperm in Holstein sperm of 3.98%, while the difference in Jersey sperm is 4.24% (Garner and Seidel, 2008). From this information, Jerseys should sort more efficiently. The data herein support this as Jersey sperm sorted more optimally than Holstein sperm. The difference fluorescence between the two breeds could be accounted for because H33342 binds to adenine-thymine base pairs, which may be more prevalent on the Jersey X chromosome or the Holstein Y chromosome. 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.

Implementation of seminal plasma removal into sex-sorting procedures not only improved sort rates and number of live sperm collected per ejaculate, but sperm also were of higher quality post-thaw. An added bonus is that ejaculates of low sperm concentration 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 motility post-thaw. While it could be hypothesized that some of the highest quality sperm are being removed with seminal plasma, data indicate 14% fewer membrane-impaired sperm at sorting. Furthermore, less than 1% of sperm were lost in the discarded seminal plasma.

Table 3.1. Sex-sorting responses with varying sperm concentrations and storage time before staining- Experiment IV (10 bulls)

Sort Response	Sperm Concentration (10^6 sperm/ml)	0 hour	4 hour	Average
% Live Oriented Cells SEM±1.0	*Initial (1246)*	67	61	64
	700	66	60	63[a]
	1400	67	62	64
	2100	69	64	66
	Average	67	62[b]	64
X Sort Rate (10^3 sperm/sec) SEM±0.19	*Initial (1246)*	3.97	3.63	3.80
	700	3.73	3.17	3.45[a]
	1400	4.05	3.64	3.85
	2100	4.10	3.77	3.94
	Average	3.96	3.55[b]	3.76
% Membrane-Impaired Sperm SEM±1.3	*Initial (1246)*	14	20	17
	700	15	21	18[a]
	1400	14	20	17
	2100	13	17	15
	Average	14	20[b]	17
% X Collected SEM±1.5	*Initial (1246)*	38	38	38
	700	38	36	37
	1400	38	38	38
	2100	39	37	38
	Average	38	37	38
Split SEM±5.6	*Initial (1246)*	35	34	34.5
	700	30	28	29
	1400	37	38	37.5
	2100	38	32	35
	Average	35	33	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 0h storage ($p<0.01$).

Table 3.2. Main effect means for sex-sorting responses of 0 and 10% seminal plasma concentrations and sperm concentrations- Experiment V (10 bulls)

	Sorting Responses					Post-Thaw Responses			
Treatment	**% Live-Oriented Cells**	**X Sort Rate**	**% Membrane-Impaired Sperm**	**% X Collected**	**Split (%)**	**Live Non-Reacted Sperm**	**Live Reacted Sperm**	**% Membrane-Impaired Sperm**	**% Motile Sperm**
0% Seminal Plasma	54[a]	3.55[a]	19[a]	42[a]	38[a]	39[a]	3[a]	61[a]	41[a]
10% Seminal Plasma	50[b]	3.20[b]	22[b]	41[a]	36[a]	37[a]	2[a]	62[a]	35[b]
SEM	±2.8	±0.25	±3.8	±1.4	±6.9	±2.9	±0.4	±3.5	±2.8
700x10^6 sperm/ml	53[a]	3.47[a]	20[a]	42[a]	40[a]	39[a]	3[a]	59[a]	38[a]
1400x10^6 sperm/ml	53[a]	3.37[a]	21[a]	41[a]	37[a]	37[a]	2[a]	62[a]	38[a]
2100x10^6 sperm/ml	51[a]	3.30[a]	21[a]	41[a]	35[a]	38[a]	3[a]	62[a]	38[a]
SEM	±2.8	±0.25	±3.8	±1.4	±7.1	±3.2	±0.5	±3.6	±3.4

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

Table 3.3. Two-way means for DNA fragmentation with 0 and 10% seminal plasma by 2 incubation times for samples taken during the sex-sorting process- Experiment V (6 bulls)

Seminal Plasma	Incubation (hour)	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] Means without common superscripts differ ($p<0.05$) within columns.

Table 3.4. Two-way means for DNA fragmentation with 3 sperm concentrations by 2 incubation times for samples taken during the sex-sorting process- Experiment V (6 bulls)

Sperm Concentration (10^6 sperm/ml)	Incubation (hour)	Pre-Storage (%)	Post-Storage (%)	Pre-Sort (%)	Post-Sort (%)	Post-Thaw (%)
700	0	6.25[a]	5.22[a]	4.28[a]	0.00[a]	0.98[a]
1400	0	6.52[a]	5.55[a]	4.72[a]	0.05[a]	0.48[a]
2100	0	6.18[a]	5.33[a]	5.01[ab]	0.05[a]	0.74[a]
700	24	10.18[a]	14.37[b]	8.98[b]	0.13[a]	1.11[a]
1400	24	19.78[ab]	18.19[c]	16.27[c]	0.13[a]	0.72[a]
2100	24	30.00[b]	19.94[c]	17.36[c]	0.43[b]	1.18[a]
SEM		±4.47	±1.29	±1.26	±0.07	±0.18

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

Table 3.5. Main effect means for sex-sorting responses with 0 or 10% seminal plasma averaged across dye and sperm concentrations- Experiment VI (22 ejaculates)

Treatment	% Live-Oriented Cells	X Sort Rate	% Membrane-Impaired Sperm	% X Collected	Split
0% Seminal Plasma	56[a]	3.47[a]	15[a]	39[a]	27[a]
10% Seminal Plasma	53[b]	3.15[b]	19[b]	37[a]	24[a]
SEM	±2.6	±0.21	±2.5	±1.1	±3.0

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

Table 3.6. Two-way means for sex-sorting responses for dye by sperm concentrations during staining averaged over 0 and 10% seminal plasma- Experiment VI (22 ejaculates)

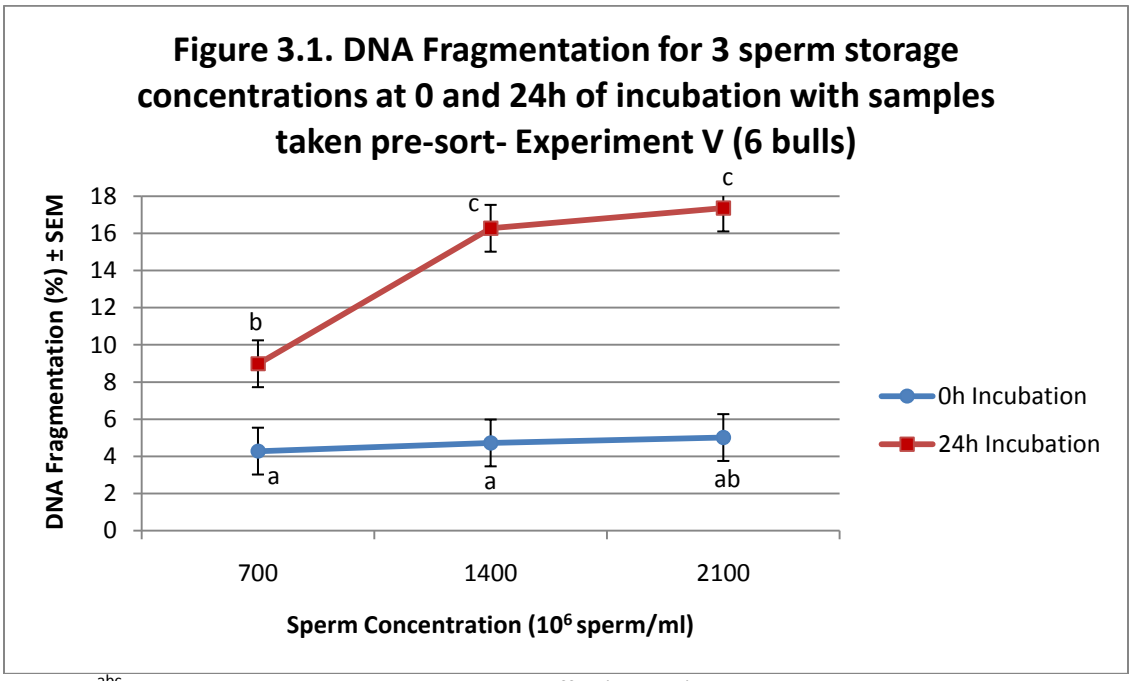
Dye	Response	160x10^6 sperm/ml	240x10^6 sperm/ml	Average
49µM H33342	% Live-Oriented	54^{b}	48^{a}	51^{a}
	X Sort Rate	3.27^{b}	2.38^{a}	2.83^{a}
	Split	27^{bc}	3^{a}	15^{a}
65µM H33342	% Live-Oriented	57^{b}	55^{b}	56^{b}
	X Sort Rate	3.47^{c}	3.22^{b}	3.48^{b}
	Split	37^{c}	15^{ab}	26^{b}
81µM H33342	% Live-Oriented	56^{b}	56^{b}	56^{b}
	X Sort Rate	3.68^{c}	3.56^{bc}	3.62^{b}
	Split	35^{c}	35^{c}	35^{c}

^{abcd} Means without common superscripts differ ($p<0.05$) within responses.
For % Live-Oriented Sperm, SEM±2.7; for X Sort Rate, SEM±0.22; for Split, SEM±4.0.

Table 3.7. Two-way means for sex-sorting responses for breed by dye concentration from 7 Jersey bulls and 4 Holstein bulls-
Experiment VI (22 ejaculates)

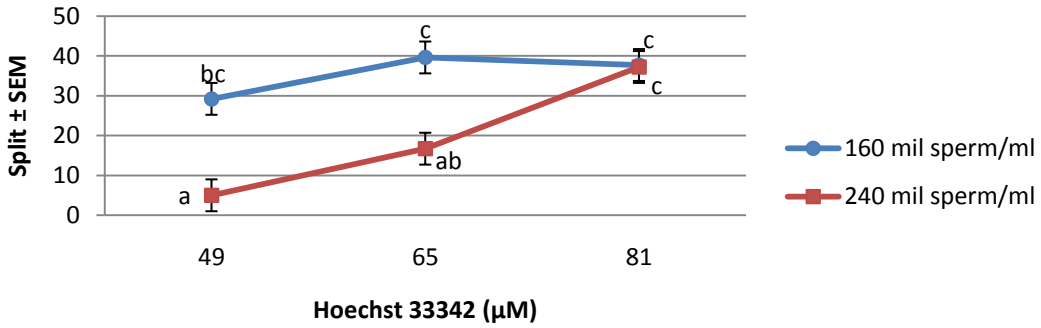
Response	Breed	49µM H33342	65µM H33342	81µM H33342
% Live Oriented Sperm	**Jersey** *SEM±3.1*	56^{b}	58^{b}	58^{b}
	Holstein *SEM±4.2*	46^{a}	53^{b}	55^{b}
X Sort Rate (10^3sperm/sec)	**Jersey** *SEM±0.26*	3.37^{b}	3.81^{c}	3.96^{c}
	Holstein *SEM±0.34*	2.29^{a}	3.15^{b}	3.28^{b}
% X Collected	**Jersey** *SEM±1.4*	37^{b}	42^{b}	43^{b}
	Holstein *SEM±1.9*	31^{a}	37^{b}	38^{b}
Split (%)	**Jersey** *SEM±3.9*	22^{ab}	34^{bc}	44^{c}
	Holstein *SEM±5.2*	9^{a}	18^{ab}	26^{bc}

^{abcd} Means without common superscripts differ ($p<0.05$) within responses.



[abc] Means without common superscripts differ ($p<0.05$) averaged over seminal plasma.

Figure 3.2. Effect of interaction between sperm and dye concentrations during staining on split - Experiment VI (22 ejaculates)



[abcd] Means without common superscripts differ ($p<0.05$) averaged over seminal plasma content.

CHAPTER IV

CONCLUSIONS

The purpose of these experiments was to evaluate how characteristics of semen influence sex-sorting efficiency of bovine sperm. This project arose from the current industry dogma that ejaculates with an initial sperm concentration $<10^9$ sperm per ml were inefficient for sex-sorting. Whether this inefficiency was due to an actual ejaculate characteristic, initial sperm concentration, or the seminal plasma content of semen was explored.

Several conclusions resulted from this series of experiments, although the most important is that removing seminal plasma improves sex-sorting of bovine sperm. Sort rates were increased by 11% in multiple experiments with the bonus that there were 14% fewer membrane impaired sperm. When sperm were evaluated for post-thaw motility, there was a 17% increase in progressive motility, an additional benefit.

Sorting second ejaculates was more efficacious than sorting first ejaculates due to higher numbers of living sperm. There does not appear to be any beneficial effect of seminal plasma from other bulls, nor does there appear to be any effect of BSA when added at 0, 0.3 or 0.9% to medium already containing 0.3% BSA.

When seminal plasma was not manipulated, staining sperm at higher concentrations had a positive effect on sorting, but when seminal plasma was either

removed or held at a constant 10% across sperm concentrations, this effect was removed. Therefore, seminal plasma, not initial sperm concentration is impairing sorting efficiency.

With the knowledge that removing seminal plasma is beneficial, optimal efficacy for staining and sorting sperm, under the conditions studied, was the combination of a sperm concentration of $160x10^6$ sperm per ml, a final Hoechst 33342 concentration of 65µM, and no seminal plasma. Therefore, the only change the industry needs to make to optimize the current sperm sex-sorting protocol is addition of a 15 min centrifugation step to remove seminal plasma.

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APPENDIX A

Correlations among ejaculate parameters

MATERIALS AND METHODS

Data were collected on ejaculates used in all experiments. Bull age and days since last collection were from Sexing Technologies, Inc records. Ejaculate volume, sperm concentration, pH, seminal plasma pH, % motile sperm and % abnormal sperm were evaluated at collection. Ejaculates were culled on total % abnormalities, sperm concentration and total number of sperm for quality control and practicality of running experiments; therefore, ejaculates used were not representative of the entire population. Seminal plasma was frozen and stored until evaluation for protein content with a refractometer. Statistical analyses were performed in SAS using Proc Corr on 59 ejaculates from 28 bulls collected over seven months.

RESULTS AND DISCUSSION

Age of bull and volume of ejaculate had a positive correlation coefficient of 0.683 ($p<0.0001$); therefore, older bulls had larger ejaculate volumes. Age of bull also correlated highly with seminal plasma protein with a coefficient of 0.496 ($p<0.0001$). In addition, ejaculate volume and seminal plasma protein were highly correlated with a coefficient of 0.507 ($p<0.0001$). Therefore, one source of volume (likely seminal vesicles) had a high protein concentration. Ejaculate volume was also correlated with days since

last collection, as would be expected, with a coefficient of 0.388 ($p<0.01$). Sperm motility correlated negatively with days since last collection (-0.305), which indicates a decrease in overall sperm quality with more time between collections ($p<0.04$). Total number of sperm correlated with bull age (0.386) and days since last collection (0.358; $p<0.01$). Seminal plasma pH was negatively correlated with total motile sperm (-0.600) and total morphologically normal sperm (-0.581), indicating that a lower seminal plasma pH was found with higher quality sperm. Other correlation coefficients were not significant ($p>0.05$).

Mean values for ejaculate parameters are presented in Table 1A and are similar to those in the literature (Mann, 1964).

Table 1A. Mean values for ejaculate parameters for 59 ejaculates

Parameter	Mean	Standard Deviation	Minimum	Maximum
Bull Age (mo)	33	21.2	13	92
Days since last collection	6	8.8	1	54
Motile Sperm (%)	70	4.4	60	80
Semen pH	6.55	0.21	6.10	7.00
Seminal Plasma pH	6.40	0.28	5.77	7.00
Seminal Plasma Protein (%)	7.0	1.95	3.0	13.0
Sperm Concentration (10^6 sperm/ml)	1417	491	533	3111
Total Abnormalities (%)	22.0	6.8	7.0	33.0
Total Morphologically Normal Sperm (10^6 sperm/ml)	6777	3691	1128	18031
Total Progressively Motile Sperm (10^6 sperm/ml)	6107	3383	1128	19599
Total Sperm (10^6 sperm/ml)	8730	4838	1612	26132
Volume (ml)	6.2	2.9	2.0	14.5

APPENDIX B

Correlations among ejaculate and sorting parameters

MATERIALS AND METHODS

Data were collected on ejaculates used in all experiments. Bull age and days since last collection were from Sexing Technologies, Inc records. Ejaculate volume, sperm concentration, pH, seminal plasma pH, % motile sperm and % abnormal sperm were evaluated at collection. Ejaculates were culled on total % abnormalities, sperm concentration, and total number of sperm for quality control and practicality of running experiments; therefore, ejaculates used were not representative of the entire population. Seminal plasma was frozen and stored until evaluation for protein content with a refractometer. Parameters were collected during sorting and include: % live oriented cells, X sort rate, coincidence rate, % membrane-impaired sperm, % X-bearing sperm collected, and split.

Statistical analysis was performed in SAS using Proc Corr on 49 ejaculates from 21 bulls collected over seven months.

RESULTS AND DISCUSSION

Correlation coefficients are given in Tables 1B and 2B for 52 ejaculates. The % live oriented cells correlated with both % membrane impaired sperm and X sort rate ($p<0.0001$). Correlations between these parameters are to be expected as more live

sperm will result in more X-bearing sperm being collected faster, and more live sperm logically means there will be fewer membrane-impaired sperm.

The % live oriented cells also correlated negatively with days since last collection (-0.334), initial ejaculate volume (-0.300), initial ejaculate concentration (-0.390) and seminal plasma protein (-0.424). Although these coefficients were all significant ($p<0.04$), they are small enough that there may be little practical value since the percent variance accounted for is the correlation coefficient squared.

Both X sort rate and % membrane impaired sperm were correlated with initial sperm concentration (-0.395 and 0.340) and seminal plasma protein (-0.316 and 0.505). Coincidence rate also correlated with seminal plasma protein (0.443; $p<0.003$). Therefore, high initial sperm concentration and high seminal plasma protein were associated with less efficient sorting.

As in Appendix A, initial ejaculate volume correlated with bull age (0.670), days since last collection (0.440) and seminal plasma protein (0.398; $p<0.01$). Bull age also correlated with seminal plasma protein (0.364; $p<0.02$). From these data, older bulls have higher initial volumes with more seminal plasma protein.

Total number of sperm, total number of motile sperm, and total number of normal sperm were all negatively correlated with % live-oriented cells and X sort rate, while being positively correlated with % membrane-impaired sperm and days since last collection (Table 2B). When there are more days since last collection, the total number of abnormal sperm increases and causes inefficient sorting. Ejaculate volume and sperm concentration contribute to the total number of sperm; volume has been shown to

negatively impact sperm when high because of increased seminal plasma content, while sperm concentration is correlated with % membrane-impaired sperm (0.322). Therefore, a high total number of sperm negatively impacts sort efficiency.

Overall, high seminal plasma protein appears to be associated with decreased sorting efficiency, and older bulls, or high volume ejaculates, contain high concentrations of seminal plasma protein. Collecting bulls more often will help improve sort efficiency by decreasing overall sperm numbers as well as abnormal sperm and immotile sperm in most bulls.

Table 1B. Correlation coefficients among ejaculate and sort responses for 52 ejaculates

	% Live Oriented Cells	X Sort Rate	% Membrane Impaired Sperm	Bull Age	Days since last collection	Initial Volume	Initial Sperm Concentration	Total Sperm	Total Motile Sperm	Total Normal Sperm	Seminal Plasma Protein
% Live Oriented Cells	1.000	0.796	-0.862	-0.021	-0.334	-0.300	-0.390	-0.511	-0.448	-0.493	-0.424
X Sort Rate		1.000	-0.630	0.224	-0.249	-0.252	-0.395	-0.458	-0.409	-0.436	-0.316
% Membrane Impaired Sperm			1.000	0.050	0.048	0.171	0.340	0.362	0.310	0.378	0.505
Bull Age				1.000	0.183	0.670	-0.271	0.359	0.342	0.377	0.364
Days Since Last Collection					1.000	0.440	0.025	0.488	0.430	0.421	-0.029
Initial Volume						1.000	-0.085	0.788	0.755	0.780	0.398
Initial Sperm Concentration							1.000	0.530	0.559	0.512	0.062
Total Sperm								1.000	0.980	0.969	0.310
Total Motile Sperm									1.000	0.970	-0.010
Total Normal Sperm										1.000	0.303
Seminal Plasma Protein											1.000

Correlation coefficients >0.273 are significant ($p<0.05$), and >0.354 ($p<0.01$).

Table 2B. Continued correlation coefficients among ejaculate and sorting responses for 52 ejaculates

	% Motile Sperm	% Abnormal Sperm	% Live Oriented Cells	X Sort Rate	% Membrane Impaired Sperm	Days since last collection
% Motile Sperm	1.000	-0.027	0.322	0.248	-0.310	-0.209
% Abnormal Sperm		1.000	-0.050	-0.075	-0.049	0.292
% Live Oriented Cells			1.000	0.796	-0.862	-0.334
X Sort Rate				1.000	-0.630	-0.249
% Membrane Impaired Sperm					1.000	0.048
Days since last collection						1.000

Correlation coefficients >0.273 are significant ($p<0.05$), and >0.354 ($p<0.01$).