THESIS

# OPTIMIZING STORAGE OF BOVINE SPERM BETWEEN SEMEN COLLECTION AND SEXING

Submitted by

Jennifer Lea Anema

Department of Biomedical Sciences

In partial fulfillment of the requirements

for the Degree of Master of Science

Colorado State University

Fort Collins, Colorado

Summer 2010

COLORADO STATE UNIVERSITY

July 7, 2010

WE HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER OUR SUPERVISION BY JENNIFER ANEMA ENTITLED OPTIMIZING STORAGE OF BOVINE SPERM BETWEEN SEMEN COLLECTION AND SEXING BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE.

Committee on Graduate Work

James Graham

Richard Kraig Peel

Advisor: George Seidel, Jr.

Department Head: Colin Clay

ABSTRACT OF THESIS

OPTIMIZING STORAGE OF BOVINE SPERM BETWEEN SEMEN COLLECTION AND SEXING

The primary objective of the experiments presented herein was to optimize procedures for storing bull sperm for 20 h between semen collection and staining sperm with Hoechst 33342 in preparation for sexing sperm prior to cryopreservation. Such storage would allow semen to be shipped overnight to a sorting facility, thereby minimizing the need to house bulls in close proximity to sorters. This would also allow sorters to run longer shifts with semen collected at earlier times.

The effect of storing sperm in a MOPS buffer solution or a MOPS + egg yolk solution prior to sex sorting was evaluated in Experiment 1. Two successive ejaculates were obtained for each bull. Semen was diluted 9:1 with a MOPS solution resulting in 24 mM MOPS (Treatment 1) or 24 mM MOPS + 2% egg yolk (Treatment 2). Nothing except standard antibiotics was added to the control samples until staining with Hoechst 33342 for sorting. Subsamples of each treatment and control were sorted by flow cytometry shortly after collection and then frozen. Other subsamples were stored at 15-18°C and sorted 20 h after collection followed by cryopreservation. Sperm were evaluated post-thaw for subjective progressive and total motility, by computer-assisted sperm analysis (CASA), and by flow cytometry for sperm viability. Second ejaculates were superior to first ejaculates. Treatment 1 was superior to the control, while Treatment 2

was similar to the control. For example, means for Treatment 1, Treatment 2, and the control for subjective progressive motility were 29.9, 23.1, and 22.0%, respectively ($P < 0.1$). Surprisingly, Treatment 1 also was superior to the control for the 0 h sort for several responses ($P < 0.1$).

Experiment 2 examined the effect of MOPS for storing bull sperm up to 20 h at 15-18°C prior to sex sorting. Treatments stored at 5°C in a MOPS + egg yolk solution were also evaluated. The effects of MOPS buffer were evaluated at a concentration of 25 mM added to three sperm concentrations ($9 \times 10^8$ spermatozoa/ml, $6 \times 10^8$ spermatozoa/ml, and $3 \times 10^8$ spermatozoa/ml) and at 100 mM MOPS added to two sperm concentrations ($6 \times 10^8$ spermatozoa/ml and $3 \times 10^8$ spermatozoa/ml), resulting in five treatments. Treatments were also prepared by diluting semen to three concentrations ($9 \times 10^8$ spermatozoa/ml, $6 \times 10^8$ spermatozoa/ml, and $3 \times 10^8$ spermatozoa/ml) with a medium resulting in 25 mM MOPS and 2% egg yolk in the samples. After storage at the indicated temperatures, samples were diluted and stained for sorting, but sperm processing during the sorting process was simulated by diluting the stained samples 20-fold with sheath fluid and storage at room temperature for one hour. Samples were then frozen following standard processing procedures and evaluated post-thaw by subjective motility and computer-assisted sperm analysis (CASA). Sperm diluted to $9 \times 10^8$ spermatozoa/ml and $6 \times 10^8$ spermatozoa/ml and stored at 15-18°C maintained the highest motility post-thaw. Additionally, MOPS buffer at a concentration of 25 mM performed better than at 100 mM concentration. Sperm stored at 5°C were markedly inferior to sperm stored at 15-18°C.

In Experiment 3, the effects of TEST buffer versus MOPS buffer on diluted semen samples after 20 h storage at 16°C were determined. Additionally, treatments stored at 5°C in Cornell University Extender (CUE) were evaluated. The effects of TEST buffer were evaluated at two sperm concentrations (9 x $10^8$ spermatozoa/ml and 6 x $10^8$ spermatozoa/ml) and three buffer concentrations (12.5, 25, and 50 mM), resulting in six treatments. MOPS buffer was evaluated at three buffer concentrations added to the 9 x $10^8$ spermatozoa/ml dilution (12.5, 25, and 50 mM) and at 25 mM MOPS added to the 6 x $10^8$ spermatozoa/ml dilution, resulting in four MOPS treatments. Two treatments were also prepared by diluting semen with CUE and egg yolk, resulting in 5% and 2.5% egg yolk in the samples. After storage at the indicated temperatures, samples were diluted and stained for sorting, but sperm processing during the sorting process was simulated by diluting the stained samples 20-fold with 20% Tris A fraction catch buffer and sheath fluid, and storage at room temperature for one hour. Samples were then frozen following standard processing procedures and evaluated post-thaw by computer-assisted sperm analysis (CASA). Samples stored at 16°C and diluted to 9 x $10^8$ spermatozoa/ml at buffer concentrations of 12.5 mM or 25 mM maintained the higher sperm parameters. No difference was found between TEST and MOPS. Sperm stored at 5°C were markedly inferior to sperm stored at 16°C.

Experiment 4 examined sperm stored in TEST buffer solution or TEST + egg yolk solution for up to 20 h between semen collection and staining for sex sorting. Two successive ejaculates were obtained for each bull. Semen was diluted 9:1 (semen: extender) with TEST resulting in 25 mM TEST (Treatment 1) or 25 mM TEST + 2% egg yolk (Treatment 2). Treatment 3 (applied to the first ejaculate) and Treatment 4 (applied

to the second ejaculate) both consisted of a 2:1 dilution of extender to neat semen, resulting in 25 mM TEST + 2% egg yolk. Nothing other than standard antibiotics was added to control samples until staining with Hoechst 33342 for sorting. A subsample of each treatment and control was sorted by flow cytometry shortly after collection and then frozen. The other subsample was stored at 16°C, sorted 20 h after collection, and then frozen. Samples were evaluated post-thaw by subjective progressive and total motility, by computer-assisted sperm analysis (CASA), and by flow cytometry for sperm viability. Second ejaculates were superior to first ejaculates. Treatment 2 was superior to the control and Treatment 1, resulting in higher percentages of live, properly oriented cells (48.5, 44.6, and 44.0%, respectively; $P < 0.1$) and decreased proportion of dead cells (28.4, 32.8, and 33.1%, respectively; $P < 0.05$) during sorting. Treatments 3 and 4 were inferior to the control.

The experiments described demonstrated that addition of 25 mM MOPS or 25 mM TEST buffer improved sperm viability after storage for 20 h. The 25 mM TEST + 2% egg yolk was the best treatment studied.

Jennifer Lea Anema
Department of Biomedical Sciences
Colorado State University
Fort Collins, Colorado 80523
Summer 2010

## ACKNOWLEDGEMENTS

Sincere appreciation is extended to the members of my graduate committee for their assistance, advice, and encouragement; Dr. George E. Seidel, Jr., advisor and Professor, Department of Biomedical Sciences; Dr. James K. Graham, Professor, Department of Biomedical Sciences; Dr. Richard K. Peel, Assistant Professor, Department of Animal Sciences.

Appreciation is also extended to Sexing Technologies for providing support and financial assistance. Special thanks are expressed to staff members of Sexing Technologies, including Dr. Rick Lenz, Cory Crissman, and the lab technicians.

Appreciation is extended to Thom Gilligan for his assistance.

I would also like to thank my family and friends for their continued love, support, and encouragement.

TABLE OF CONTENTS

## LIST OF TABLES AND FIGURES

Text Figures

# INTRODUCTION

Technology for sexing sperm has been adopted commercially in many countries, and thousands of calves have been born from the use of artificial insemination with sexed sperm (Seidel, 2007). Although many improvements have been made to increase the efficiency of this technology, limitations to sex sorting sperm still exist. Because a limited number of sperm are produced by flow cytometrically sexing the cells (Seidel and Garner, 2002), it is necessary to optimize every component of this technology for commercial application. This not only includes aspects of processing collected semen and mechanical issues, but also having bulls located near the sorting facility to collect semen so sperm deterioration during shipping is minimized.

The primary objective of these experiments was to develop optimum procedures for storing bull sperm for 20 h between semen collection and staining sperm with Hoechst 33342 in preparation for sexing sperm followed by cryopreservation. If efficacious, this would allow semen to be shipped by overnight carrier from almost anywhere in the country to a sorting facility, thereby minimizing the need to house bulls within close proximity of the sorters. This would also increase technology efficiency by allowing sorters to run longer shifts with semen collected at earlier times.

For these experiments, we not only had to design procedures that would preserve the viability of the fresh semen for an extended period of time, but they also had to enable

efficient sorting. This resulted in a number of constraints for sperm treatments, such as limiting the extent that semen was diluted.

Despite having a modicum of success in improving storage procedures, the best treatments for storing sperm for 20 h prior to processing for sexing still provided inferior sperm parameters after sorting and freezing compared to unstored sperm. However, results for sperm stored for 20 h prior to processing were still quite acceptable.

# CHAPTER I

## REVIEW OF LITERATURE

Artificial insemination, particularly in the cattle industry, has become the dominant assisted reproduction technology for genetic advancement in animal agriculture. Many obstacles have been overcome during development of this technology in order to make it an efficient and viable technique. One such problem addressed in numerous ways has been the issue of limited shelf life of fresh semen. To maintain the fertilizing capacity of sperm, factors such as storage medium, temperature and other conditions of storage must all be considered (Vishwanath and Shannon, 1997). Because of relevance to the research performed (as described in the subsequent chapters), methods for storing semen in a non-frozen state will be the focus of the first part this review.

### Semen storage at refrigerated temperatures

As the need grew to prolong the shelf life of collected semen so that it could be transported it for use in other locations, the initial storage conditions were at refrigerator temperatures (4-5$^{o}$C). The guiding principle behind the storage of semen at low temperatures is that the sperm would have a lower metabolic rate, thereby extending survival. Davis et al. (1940) concluded that the temperature of storage is directly related to the rate of decline in pH values during the storage period. He demonstrated that the least amount of decline in pH values was observed at the lowest storage temperatures (1.7$^{o}$ and 4.4$^{o}$C), thereby

maintaining superior motility and subsequent viability of the stored samples. Davis believed that it was not the actual pH value of the semen but the shift in pH values, indicative of particular catabolic events during storage, that are detrimental to the sperm and which are reduced by decreased temperature.

The discovery that egg yolk could be used as an additive with other media components to help extend the viability of semen had a significant impact on the diluents that were developed for use at refrigerated temperatures (Phillips, 1939). In relation to low temperatures, egg yolk supplies macromolecular products that provide thermal protection to sperm cells thereby preventing cold shock during cooling, freezing, and thawing. It was reported that the specific component of egg yolk providing this protection was the low density fraction of the lipoprotein complex (Pace and Graham, 1974; Foulkes, 1977).

Additionally, numerous buffering substances were found to enhance survival of sperm stored at refrigerated temperatures. A variety of zwitterionic buffers, first described by Good et al. (1966), are suitable for semen storage. It was confirmed that bull semen diluted in a TEST-yolk diluent (prepared by titrating TES against Tris and adding 20% vol/vol egg yolk) , cooled to 4°C and stored for up to 48 hours maintained motility, acrosome integrity and fertilizing ability (Ijaz and Hunter, 1989). Tris-based diluents, containing egg yolk and glycerol, have also been tested extensively and consistently maintained motile sperm after storage at 5°C (Davis et al., 1963; Foote, 1970; Foote, 1972; Tardif et al., 1997).

Many of these studies also included citrate salt in their extender solutions. In addition to providing buffering capacity, citrate also possesses chelating properties that improve the solubility of protein fractions in egg yolk (Vishwanath and Shannon, 2000).

Like egg yolk, several milk products and other biological fluids have also been used to preserve the fertility of bovine sperm. Homogenized whole milk, skim milk and coconut milk have all been used successfully for sperm storage in the liquid state (Foote et al., 2002; Bergeron and Manjunath, 2006; Norman et al., 1962). In a study utilizing skim milk as an extender for sperm stored at 4°C, Bergeron et al. (2007) showed that caseins – the actual protective component of milk – preserved sperm in the same manner as egg yolk; namely, casein micelles decreased the binding of deleterious bovine seminal proteins to sperm, thereby minimizing lipid loss from the sperm membranes during storage and maintaining sperm motility and viability.

Another diluent that can be used to effectively store semen for prolonged periods of time at refrigerated temperatures is the Cornell University extender (CUE). Extensive fertility tests performed by Foote and collaborators showed consistently higher non-return rates for semen stored at 5°C in CUE versus a standard control extender (Foote et al., 1960; Foote and Dunn, 1962). When compared to an egg yolk-glycerol-Tris extender, CUE maintained higher mean percentages of motile sperm (81% versus 69%, respectively; $P < 0.05$) over a three day storage period at 5° C (Tardif et al., 1997). Nonetheless, both of these extenders (CUE and egg yolk-glycerol-Tris) produced similar high fertility rates when equal numbers of sperm were inseminated (Foote, 1978).

Some diluents have been formulated to model the biochemical composition of bovine cauda epididymal plasma in an attempt to prolong the shelf life of fresh semen. CEP-2 is

one such diluent that was developed to have a similar ionic composition, pH and osmolality as cauda epididymal fluid. With the addition of 10% egg yolk and 1 mg/ml sorbitol, CEP-2 maintained greater than 40% progressively motile sperm up to 6 days of incubation at 5°C (Verberckmoes et al., 2004). A subsequent in vitro study showed similar fertilization and polyspermy rates for sperm stored in CEP-2 and Caprogen® diluent (Verberckmoes et al., 2005).

Semen storage at ambient temperatures

Depending on the situation and available resources, semen storage at refrigerated temperatures is not always convenient. Additionally, while storage at low temperatures effectively reduces metabolic activity, it can also be detrimental to sperm in various ways. For example, it has been shown that low temperature decreases $Na^+/K^+$ pump activity (Gonzalez and Santacana, 2001), reducing the capacity of the sperm to handle intracellular concentrations of $Na^+$ ions. In an attempt to resolve these problems, numerous methods to store semen at ambient temperatures, defined anywhere between 10-21°C, have been examined.

As with semen storage at refrigerated temperatures, the addition of egg yolk was also an important component for storing semen at ambient temperatures. As previously discussed, egg yolk provides thermal protection for sperm cells subject to cold shock. However, low-density lipoproteins present in egg yolk are also able to associate with detrimental factors of seminal plasma (Manjunath et al., 2002). Some proteins present in bull seminal plasma decrease the viability of sperm by inducing cholesterol and phospholipid removal from the sperm membrane, making the cells more susceptible to damage throughout the storage period (Bergeron and Manjunath, 2006). Protection is

provided to the cells through the interaction between egg yolk and seminal plasma proteins, thereby preventing these factors from binding to the sperm membrane (Al-Somai et al., 1994; Vishwanath, et al., 1992; Prendergast, et al., 1995).

Attempts have also been made to preserve sperm stored at ambient temperatures by reducing the metabolic activity of sperm. Norman et al. (1958) reported that a decrease in pH, caused by accumulating lactic acid in semen samples extended in coconut milk and stored for 150 h at room temperature (23-27$^{o}$C), effectively inhibited the sperm as measured by oxygen consumption, lactate production and motility. Metabolism was inhibited at a pH range of 5.5-5.8, however, no effect on the viability of the semen was seen when the pH was reduced to this level. Metabolic activity was successfully regained after six days of storage by resuspending the sperm samples in alkaline coconut milk diluents (pH 7.45). This suggested that in some respects the viability of sperm in vitro is inversely proportional to sperm activity.

Other methods of sperm storage at ambient temperatures have also been investigated. Similar to some of the diluents developed for use at refrigerated temperatures, conditions similar to those found in the epididymis have been used for storing semen for prolonged periods at ambient temperature. Specifically, one study examined the effects of low pH, high osmolality, high sperm concentration and low oxygen tension on semen stored for four days (De Pauw et al., 2003). The investigators concluded that optimal results were obtained when the Hepes-TALP base storage medium was adjusted to a pH of 6 and 300 mOsm/kg (isotonic). In contrast to the in vivo situation, better results were attained with a low concentration of $10x10^{6}$ spermatozoa/ml. Finally, no differences were seen in sperm stored under aerobic or nitrogen gassed conditions.

Probably one of the most significant contributions to storing semen at ambient temperatures has been the development of Caprogen. Caprogen evolved as a modification of CUE and has become a commercially available diluent used extensively for liquid semen storage in New Zealand (Shannon, 1964). Initially, this diluent was developed for use at 5°C but further testing showed that sperm survival and fertility were optimally maintained at a temperature of 16-21°C (Shannon and Curson, 1984).

A major problem with semen stored at ambient temperatures is an increase in peroxide production. Dead sperm release an aromatic L-amino acid oxidase. The degradation resulting from this oxidase produces hydrogen peroxide, which is toxic to sperm (Shannon and Curson, 1982). Temperature is a primary determinant of the activity of peroxide which is produced at much higher rates when semen is stored at ambient temperatures then at 5°C (Shannon and Curson, 1984). To counteract these detrimental effects on sperm stored at ambient temperatures, Caprogen is gassed with nitrogen to reduce dissolved oxygen levels in the medium. This considerably reduces the metabolic activity of the cells (Vishwanath and Shannon, 2000).

Additionally, further adjustments have been made to Caprogen over time to improve fertility and make it more practical for commercial use. First, addition of catalase, an enzyme used to reduce hydrogen peroxide concentrations, significantly increased non-return rates with semen stored at ambient temperatures of 15-23°C (Shannon and Curson, 1982). Peroxide concentrations were also reduced by decreasing the concentration of egg yolk, which is a substrate for aromatic-L-amino acid oxidase produced by the dead cells. Shannon and Curson (1983) found that reducing the amount of egg yolk from 20% to 5% in Caprogen diluent did not significantly affect fertility rates. Finally, by extending

semen with Caprogen, it was possible to reduce the dose used to inseminate cows. A decrease in sperm concentration from $5x10^6$ to $2x10^6$ total sperm/insemination did not compromise fertility (Shannon et al., 1984). Semen stored under these conditions can be used for three days without a decline in fertility (Verberckmoes et al., 2005).

Dilution

Another aspect of sperm storage to consider is the concentration to which sperm are diluted. Mann and Lutwak-Mann (1981) used the phrase "dilution effect" to describe the decreased viability of sperm observed with dilution. This was demonstrated by Garner et al. (1997) who showed that sperm viability significantly decreased with each decreasing increment of sperm concentration between 30 and 1 x $10^6$/spermatozoa/0.5 ml dose. This effect is attributed in part to the fact that dilution removes antioxidants and other components of seminal plasma that are necessary to preserve the cell membrane and maintain viability of sperm (Maxwell et al., 1997). Components present in seminal plasma are responsible for stabilizing the sperm cell membrane and inhibiting capacitation. Without these components, the cell membrane is weakened, and the acrosome reaction takes place, leading to premature cell death if fertilization does not occur (Prathalingam et al., 2006).

For the most part, the AI industry has not required that semen be diluted and reconcentrated (Schenk et al., 1999). However, with the advancement of sexing technology, this has become an issue since extreme dilution of sperm occurs during flow sorting (Maxwell and Johnson, 1999). Additionally, because of the time constraints and low throughput of sex sorting, considerably lower numbers of sperm are produced and

packaged into straws (Garner et al., 1997). Therefore, this is a relatively recent problem that has not been studied thoroughly.

Considerable research has been done to investigate methods to minimize the effect of dilution. Mann and Lutwak-Mann (1981) proposed the use of isotonic media that include balanced salts, energy sources and buffering capacity to reduce the dilution effect. Another study compared the effect of different diluents on semen that was cryopreserved at high dilution rates (Vera-Munoz et al., 2009). They found that sperm motility and membrane integrity were best maintained when the semen was extended in egg yolk low-density lipoproteins (LDL; 8% dry matter) at concentrations of 15 and 5 million sperm per dose.

At low concentrations, sperm viability is significantly related to the volume and concentration of the undiluted, neat semen (Garner et al., 1997). Garner suggested a relationship between viability at decreased concentrations and the amount of seminal plasma present. Therefore, researchers have also examined the effect of the addition of seminal plasma on diluted semen samples. Maxwell et al. (1997) concluded that the addition of seminal plasma in the staining diluent (for boar and ram sperm) and the collection medium (for boar, bull and ram sperm) improved the motility and viability of highly diluted sperm samples prepared for flow-cytometric sorting. Another method investigated reduced sperm concentration while maintaining a constant level of seminal plasma (Garner et al., 2001). Reduced viability occurred as concentration of sperm cells was decreased. However, the results confirmed that the addition of seminal plasma was beneficial in maintaining sperm viability.

An important observation is the distinct bull variation in tolerance of sperm to dilution effects (Schenk et al., 1999). Post-thaw viability of cryopreserved sperm therefore decreases in a bull-dependent manner (Vera-Munoz et al., 2009). Differing responses among bulls were also discerned with addition of seminal plasma (Garner et al., 2001). This could be due to the fact that seminal plasma proteins associated with fertility differ among individual bulls (Killian et al., 1992, 1993), making the source of seminal plasma significant in contributing to dilution effects (Garner et al., 2001).

Sex sorting sperm

The ability to preselect sex has been an ambition of livestock producers for decades, and many methods to skew the sex ratio have been attempted. Guyer (1910) first reported microscopic detection of sex chromosomes and research on sex preselection was performed by many groups over the following years. Much of this work was summarized in a symposium held at the Pennsylvania State University in 1970 (Kiddy and Hafs, 1971). However, no effective method of sexing sperm could be gleaned from the papers presented in this symposium.

In the late 1960's, flow cytometry was introduced (Kamentsky and Melamed, 1967) and used for many applications to measure DNA in individual cells. Cell sorting instrumentation was added to flow cytometry and became commercially available in the 1970's; this technology was greatly enhanced in 1980 with the introduction of improved data acquisition capabilities (Johnson and Welch, 1999).

The idea to use DNA content of sperm as a marker to determine sex stems from work published by Moruzzi (1979). His data showed various differences in DNA content – measured by differences in chromosome length – between X and Y sperm for several

species. Related research is presented in a book edited by Amann and Seidel (1982), composed of the papers from a symposium held to update research accomplished in this area and provide future direction for the technology. The paper by Gledhill et al. (1982) in this symposium summarized use of flow cytometry to distinguish X- and Y-bearing sperm based on DNA content. However, the process killed the sperm.

A successful and repeatable method to separate X- and Y-chromosome-bearing sperm populations that maintained fertility was first reported by Johnson, et al. (1989). Since then, the technology has been improved with the capability of high-speed sorting (Johnson et al., 1999) and a new orienting nozzle system (Rens et al., 1998, 1999). Sorting speed is limited because each sperm cell must be assessed individually for DNA content. This technology can provide routine sorting rates of approximately 3000 live sperm of each sex per second, equivalent to $10 \times 10^6$ sperm per hour (Seidel, 2007; Seidel and Garner, 2002). Because only a limited number of sexed sperm are produced, it is necessary to optimize every component of this technology for commercial application. This not only includes aspects of processing the collected semen and mechanical issues, but also requiring bulls to be located near the sorting facility so little deterioration of the semen during shipping occurs.

The term "sortability" has been used to describe the "ability of a sperm sample prepared for sex-preselection to be sorted into resolvable X- and Y-chromosome bearing population" (Clulow et al., 2009). This, of course, depends on the capacity of the cell sorter to determine and separate these two populations accurately. Many factors influence this capacity, and investigations have been made to make the technology as efficient as possible.

The most important factor affecting sorting procedures is accurate measurement of DNA content of sperm. On average, there is 3.8% more DNA in X-bearing sperm than in Y-bearing bovine sperm. However, this difference can vary even between cattle breeds (Garner et al., 1983). To measure this difference, semen samples are incubated with Hoechst 33342, a fluorescent DNA-binding dye. The rate by which this dye is taken up by the DNA can vary between species (Garner, 2006) and individuals (Hollinshead et al., 2004), causing variability in the ability of sperm to be sorted.

The purity of sorted sperm can be increased by narrowing gate parameters, but efficiency of the process is decreased concomitantly. Technological efficiency also is lost as a result of prolonged sorting time because this negatively affects the quality of sorted semen (Clulow et al., 2009). To date, a reliable prediction method to determine sorting efficiency for an individual bull has not been established, and "test sorts" are the only measures available to determine this information (Rath et al., 2009). However, a "sortability index" has been suggested by Clulow and collaborators (2009) as a selection tool for sex-sorting stallion sperm. Based on the graphical representation of the sperm detected by the flow cytometer, this index uses differences in certain parameters of the computer-generated dot plot to rank the suitability of each stallion for sex-sorting.

Sorting is complicated by the fact that the sperm cells have a flat ovoid head shape and compact chromatin (Rens et al., 1996), and to some extent function as a lens. Consequently, to measure the fluoresce of the dye bound in each cell accurately, the sperm must be oriented correctly to a laser; that is, the sperm need to pass the laser beam with the flat side facing the forward fluorescence detector and the edge of the sperm towards the $90^{o}$ fluorescence detector (Pinkel et al., 1982; Johnson and Pinkel, 1986). As

mentioned previously, Rens and collaborators (1996) developed a new orienting nozzle system that greatly improved the proportion of correctly oriented sperm. Instead of the previously used beveled needle, they designed a novel nozzle equipped with an elliptical sample injection nozzle. This improved the correct cell orientation from 25% with the beveled needle to 70% using the new nozzle (Rens et al., 1999).

In an attempt to develop a method to sex-sort and then re-freeze previously frozen bull semen, the best sperm orientation during sorting was achieved for sperm stained with Hoechst 33342 in Androhep® diluent (Minitube, Sebastopol, Vic., Australia) compared to bovine sheath fluid or TALP buffer (Underwood et al., 2009). Additionally, Rath and collaborators (2009) reported a sperm sexing protocol that includes modifications to the processing and freezing of sex-sorted frozen semen with the goal of improving fertilizing capacity. One of the components of this procedure, summarized under the name Sexcess® (Masterrind GmbH, Verden, Germany), is the addition of fluoride to the sperm to temporarily inhibit sperm motility. In regards to the sortability of sperm processed under this method, immobilization improved orientation of the cells in front of the laser, and sorting rates increased slightly.

Other mechanical changes have been made that influence sorting parameters. The development of high speed sorters was partly accomplished by increasing pressure within the flow cytometer, and the industry standard became operating pressures of 50 psi (Schenk et al., 2009). However, high sorting pressures resulted in damage to the sperm cell membrane and reduced cell viability. Work by Suh et al. (2005) showed that decreasing the sorting pressure from 50 psi to 40 psi did not lower sort rate or purity, and sperm quality was greatly improved.

The laser power used also is important to obtain and maintain a stable bimodal histogram for sorting (Johnson and Welch, 1999). Schenk and collaborators (2009) reported use of a pulsed laser instead of the standard continuous wave laser. In addition to other benefits, the pulsed laser resulted in better excitation of Hoechst 33342 stain and improved the resolution between X- and Y-bearing sperm.

Obtaining adequate resolution between X- and Y-chromosome bearing sperm during sorting is essential to achieve sufficient purity of the sorted samples. This also affects sorting speed, so factors that affect resolution need to be considered. Decreased resolution is characterized by a broad fluorescence distribution lacking a distinct X- and Y-peak (Stap et al., 1998). Increased proportions of dead or damaged sperm in the sample causes resolution of the flow cytometer to deteriorate (Clulow et al., 2009). This also explains some of the reason why fresh semen samples sort better then those stored for longer periods of time (Seidel, 2007).

Several methods have been used to exclude dead sperm from being sorted via the flow cytometer. One procedure involved addition of propidium iodide to the sample (Johnson et al., 1994). Propidium iodide effectively quenches the Hoechst fluorescence of the dead cells, making it possible to discard unwanted dead sperm. By excluding the dead sperm, high proportions of viable sperm can be sorted. A disadvantage of this method, however, is that propidium iodide is an intercalating agent and toxic to the sperm cells (Stap et al., 1998). Therefore, semen contaminated with this dye should not be used in artificial insemination or in vitro fertilization procedures.

In an alternative procedure to propidium iodide, Stap and collaborators (1998) demonstrated that Percoll – in an isotonic solution not used as a gradient – has similar

capacity to quench the Hoechst fluorescence of dead cells thereby eliminating them from the sorting window. An advantage of using Percoll is that it does not alter the osmolarity when added to buffer solutions and is relatively nontoxic in low concentrations.

Currently, the method used to eliminate dead cells from being flow sorted is adding food coloring dye (e.g. FD&C #40) to the sample (Seidel and Garner, 2002). The dye penetrates the membrane of dead cells and quenches the Hoechst fluorescence in these sperm, allowing them to be removed from the sorted viable sperm (Schenk et al., 1999). Food coloring also has no mutagenic effects that are exhibited by propidium iodide (Johnson and Welch, 1999).

Attempts have also been made to sort semen that had been previously frozen. One of the major problems with this procedure is the presence of egg yolk in the original freezing extender. Egg yolk is opaque and causes poor resolution for semen samples containing it (Underwood et al., 2009). Therefore, it is best to remove the egg yolk by washing the sperm (Stap et al., 1998) or employing a gradient separation method before sorting frozen-thawed sperm (Underwood et al., 2009; Samardzija et al., 2006; Mousset-Simeon et al., 2004).

Commercial application of sexed sperm

The technology for sexing semen has become commercially adopted in many countries, and thousands of calves have been born from artificially inseminated sexed sperm. Over the years, the process of sexing has been refined to optimize sperm sorting parameters to achieve the highest quality sample in the least time. One of the primary areas of improvement has been the rate at which sperm are sexed (Seidel, 2009). However, current sorting speeds are still relatively slow; therefore, insemination doses of

sexed sperm contain few sperm compared to the numbers of sperm that are used conventionally for artificial insemination. It was therefore necessary to determine the minimum number of sexed sperm per insemination dose to account for this constraint, and 2 x $10^6$ sperm/dose has become the industry standard (Seidel, 2007).

An additional limitation to this technology is that normally only freshly ejaculated semen is sex-sorted (Underwood et al., 2009). The implication of this is that donor bulls need to be housed close to the sorting facility. However, this problem has been reduced by advancements made in the ability to sort frozen-thawed sperm (Underwood et al., 2009; DeGraaf et al., 2009).

Another constraint to the efficiency of sexing technology is damage to sperm as a result of the sorting process. There appears to be little genetic damage due to sorting (Garner, 2009), but there is a substantial effect on fertilizing ability of sorted sperm due to functional damage. A more thorough summary of issues affecting commercialization of this technology is presented by Amann (1999).

The majority of commercial sperm sorting has been for cattle with the main purpose of producing females for dairies (Seidel, 2009). Other applications include obtaining bull calves with superior genetics for use as breeding bulls (Seidel, 2007) and using sexed sperm for IVF (Seidel, 2009). Limitations to sex sorting remain, and there is considerable room for improvement through further research. As these issues are minimized, the application of this technology will become more widespread.

Evaluation of sperm

For this discussion, I will concentrate on methods used to evaluate cryopreserved semen. The ultimate test, obviously, would be the production of offspring. However, this is time consuming and extremely expensive, making such an evaluation impractical. Consequently, in vitro procedures are used to determine differences among treatments that might be correlated with fertility. While visual estimations of sperm for motility and morphology have been used for many years as a predictor of sperm health and viability, there can be considerable variability due to the observer with this assay. Therefore, more objective measurements of sperm parameters can be obtained using computer assisted sperm analysis (CASA) systems and flow cytometry.

Perhaps the most obvious sperm parameter that can be measured is sperm motility. As mentioned above, visual assessment using a microscope equipped with phase contrast optics is often employed to evaluate this, but substantial training is required to obtain reliable results. To remove some of the bias in visual estimations and to determine sperm velocity data, CASA was developed to evaluate sperm characteristics (Gravance and Davis, 1995; Holt and Palomo, 1996). These systems assess multiple motility characteristics including total sperm motility, progressive motility, track speed, progressive velocity, path velocity, linearity, amplitude of lateral head displacement, and beat cross frequency of sperm (Kathiravan et al., 2010).

The CASA system consists of a microscope, a video camera, a video frame grabber card and a computer (Kathiravan et al., 2010). Sperm are visualized using a microscope equipped with a choice of dark field, negative phase contrast or fluorescence optics. The microscopic image is captured by the camera and digitized by the computer based on the

pixel range covered by the sperm head. To distinguish sperm heads from debris and other cells, various instrument settings can be manipulated when analyzing the specimen.

CASA analysis requires proper user training, and the equipment must be appropriately calibrated and standardized for the species being evaluated in order to provide accurate data. However, if these aspects are properly addressed, CASA can be used as an efficient and reliable tool to evaluate sperm for multiple motility characteristics, some of which are correlated with fertility (Tardif et al., 1997; Farrell et al., 1998; Christensen et al., 1999; Kathiravan et al., 2010).

The second method that has proven reliable for sperm analysis is flow cytometry (Garner et al., 1994; Garner and Johnson, 1995). Sperm viability can be assessed using a combination of nucleic acid-specific fluorophores that have affinity for DNA to distinguish between living (plasma membrane intact) and dead (cells with disrupted plasma membranes) sperm in the sample (Garner et al., 1997). Specifically, the method utilizes SYBR-14 or Hoechst stains, which stains all sperm green (or blue), and propidium iodide (PI), which stains dead sperm red (the PI quenches the fluorescence of SYBR or Hoechst). It is only necessary to incubate fresh or cryopreserved sperm with these stains for only a short period of time (~ 15 min) before they are analyzed by the flow cytometer. This assay allows visualization of both the living and dead populations of a sperm sample (Garner et al., 1994). The population of the sperm exhibiting both red and green fluorescence is perceived as an orange color (Garner and Johnson, 1995). This occurs because these cells are moribund or damaged to some extent and therefore are incapable of excluding PI. While this might occur by coincidence if two oppositely dyed sperm are measured at the same time by the flow cytometer, this problem can be

minimized by modifying gating parameters. The combination of fluorescent staining with flow cytometry has made it possible to rapidly analyze thousands of sperm per sample, which achieves high precision (Christensen et al., 2005).

# CHAPTER II

## EXPERIMENT 1: EFFECT OF 24 mM MOPS ± 2% EGG YOLK ON BOVINE SPERM STORED FOR 20 HOURS BETWEEN COLLECTION AND SEXING

### ABSTRACT OF EXPERIMENT 1

Bull sperm were stored for 20 h in a MOPS buffered solution or a MOPS + egg yolk solution prior to sex sorting. Two successive ejaculates were obtained from mature bulls (Holstein, $n = 5$; Jersey, $n = 3$) via artificial vagina. Treatments were then applied to the neat semen to which antibiotics were added as recommended by Certified Semen Services. Nothing further was added to the control samples until staining with Hoechst 33342 for sorting. The semen was diluted 9:1 with a MOPS solution resulting in 24 mM MOPS (Treatment 1) or 24 mM MOPS + 2% egg yolk (Treatment 2). A subsample of each treatment and control was sorted by flow cytometry shortly after collection and then frozen following standard processing procedures. The other subsample was stored at 15-18°C and sorted 20 h after collection. pH measurements were made before staining samples for sorting. Samples were evaluated post-thaw for subjective progressive and total motility, by computer-assisted sperm analysis (CASA), and by flow cytometry for sperm viability using propidium iodide and SYBR-14. Diluting sperm in a MOPS diluent performed better than no dilution (control) while diluting sperm in a MOPS + egg yolk diluent was similar to the control. Second ejaculates were superior to first ejaculates. pH measurements showed that the addition of MOPS maintained the pH about 0.2 units higher than the control, but pH declined similarly over time for all treatments. While

results for sperm incubated for 20 h prior to sorting were lower than for sperm sorted immediately after collection, results were similar, and the majority of responses were acceptable for most, but not all bulls. In conclusion, storing sperm in 24 mM MOPS was beneficial. Surprisingly, 2% egg yolk negated the beneficial effect of MOPS in terms of post-thaw sperm survival, possibly due to increasing osmolarity by ~15 mOsM/kg due to pH adjustment. Addition of MOPS provided better results than the control for both the 0 h and 20 h sorts.

## INTRODUCTION

Storing sperm in a low oxygen environment for prolonged periods leads to an accumulation of lactic acid. This creates an unfavorable environment as the increasing lactic acid causes a decline in pH, which decreases sperm metabolic activity, motility and, eventually, viability (Norman et al., 1958; Carr et al., 1985).

To maintain a stable pH, zwitterionic buffers have often been added to cell media. One such buffer, Morpholinopropane sulfonic acid (MOPS), has been used successfully in sperm diluents (Graham et al., 1972; Parrish and Foote, 1980). A preliminary study showed that the pH of sperm samples decreased the least when stored for 20 h in a MOPS buffered solution compared to Hepes, Tris, or BES buffered solutions.

In addition, egg yolk in media also provides beneficial properties for preserving sperm. One of its protective contributions occurs as it can bind bovine seminal plasma components, reducing the capacity of detrimental components of seminal plasma to interact with the sperm (Manjunath et al., 2002; Al-Somai et al., 1994; Vishwanath, et al., 1992; Prendergast, et al., 1995). Another benefit of egg yolk is that its macromolecular

components provide thermal protection to sperm cells, reducing damage that can occur to sperm membranes during the cooling process (Pace and Graham, 1974; Foulkes, 1977).

The objective of this experiment was to develop a procedure for storing bull sperm in a MOPS buffered solution or a MOPS + egg yolk solution for up to 20 h, prior to sex sorting and cryopreservation.

## MATERIALS AND METHODS

### Semen Collection and Processing

Semen collection, treatment administration, sex sorting and cryopreservation were performed at Sexing Technologies (Navasota, TX). Two successive ejaculates were obtained from mature dairy bulls (Holstein, $n = 5$, Jersey, $n = 3$) via artificial vagina. Ejaculates were evaluated for acceptable viability, and only those with progressive sperm motility no less then 50% and with at least 70% normal morphology were used. Additionally, two second ejaculates were centrifuged (15 min at 1144 x g; model Centra CL2, Thermo Fisher Scientific Inc (Pittsburgh, PA)) to increase the sperm concentration of the sample because the initial concentrations were below $1 \times 10^9$ sperm/ml. Antibiotics, as recommended by Certified Semen Services (CSS, Inc, subsidiary of NAAB, Columbia, MO), were added to the samples and the samples were then split. Nothing further was added to one fraction (control) until staining with Hoechst 33342 (Schenk et al., 1999) for sorting. Treatments consisted of diluting the neat semen 9:1 with a 240 mM MOPS (Sigma M-5162) solution (in water) or 240 mM MOPS + 2% egg yolk solution (in water), resulting in a final concentration of 24 mM MOPS (Treatment 1) or 24 mM MOPS + 2% egg yolk (Treatment 2).

A subsample from each treatment and the control was sorted by flow cytometry shortly after collection and then frozen following standard processing procedures (described below). Prior to sorting, the sperm concentration of each sample was adjusted to 160 x $10^6$ sperm/ml with Staining TALP (pH 7.4; Schenk et al., 1999), and 4 µl/ml of Hoechst 33342 (8.1 mM stock; Schenk et al., 1999) was added. The samples were incubated in a 34.5°C water bath for 45 min. Following staining, the sperm concentrations were adjusted to 80 x $10^6$ sperm/ml with TALP (pH 5.5) containing 4% egg yolk and 0.002% food-coloring dye (SortEnsure™ FD&C Red #40). The sperm samples were filtered (50 µm Cell Trics disposable filter #04-0042-2317, Partec GmbH, Munster, Germany) and sorted at 40 psi into 3.5 ml of 20% Tris A fraction catch buffer (SortEnsure™ Bovine XY® Tris A Working Solution, to which 20% egg yolk, antibiotics and water were added) to a total volume of 20 ml. Sorted sperm were cooled to 5°C over 90 min. Tris B fraction without egg yolk (SortEnsure™ Bovine XY® Tris medium with 12% glycerol) was added in two equal aliquots of 9.5 ml each, 15 min apart. Sperm were then centrifuged (20 min at 850 x g; Eppendorf model 5810R (Germany); 5°C) and the supernatant removed. The concentration of sperm in the pellet was measured using a Nucleocounter (ChemoMetec SP-100; Denmark) and Tris AB fraction, consisting of equal quantities of 20% egg-yolk Tris A fraction (SortEnsure™ Tris A Working Solution) and 20% egg-yolk Tris B fraction (SortEnsure™ Bovine Tris B Working Solution), was added to adjust the sperm concentration to 2.1 x $10^6$ sperm/ml with a final glycerol concentration of 6%. Sperm were packaged in 0.25 ml straws, sealed, and cryopreserved by holding the straws in liquid nitrogen vapor 15 min prior to immersion

in liquid nitrogen. A minimum of 3 h was allocated for cooling and glycerolization to "equilibrate" sperm before freezing.

The other subsample was stored at 15-18°C for 20 h after collection and then processed as described above. In addition, the pH of the sperm sample was determined prior to staining the samples for sorting with Hoechst 33342 for both sort times. Finally, sorting parameters were also recorded and analyzed. Splits were determined for each sort by the depth of the split as a percentage of the height of the distributions.

Evaluation

Frozen straws were shipped to Colorado State University (Fort Collins, CO) for analysis. The analysis consisted of two primary components. The first component evaluated was motility, both subjectively (visual) and objectively. Two straws for each treatment were thawed in a 37°C water bath for 30 sec. Straws were coded to prevent evaluators from knowing the treatment, and subjective motility was assessed by a single evaluator. CASA was done using the fluorescence capability of the machine and 10 fields of view; a minimum of 200 cells were counted for each treatment. Motility analyses were performed at the following two times for each sample: immediately after thawing of the straws (0 h) and again after 2 h of incubation at 37°C in 5% $CO_2$ in air.

The second analysis performed was the percent viable sperm, evaluated using flow cytometry as described by Purdy and Graham (2004). Briefly, a sample straw from each treatment was thawed and treated with 3 µl of propidium iodide (PI; 2.4 mM solution in water) and 5 µl of SYBR Green stain (20 µM solution in DMSO; Molecular Probes, Eugene OR) to obtain an accurate reading for the percent dead sperm cells. Following incubation, the samples were diluted with 0.80 ml of TALP, filtered through a 20 µm

nylon mesh, and analyzed. One straw from each treatment was processed according to this method.

Statistical Analysis

The semen collection, treatment administration and subsequent evaluation resulted in an 8 (bulls) x 2 (ejaculates) x 3 (treatments) x 2 (sort times) x 2 (evaluation times) factorial arrangement. Data collected from these evaluations were subjected to a mixed model ANOVA. Bulls were considered a random effect. When two straws were evaluated for each treatment, the values were averaged for each subclass. The statistical analysis included all main effects as well as first and second order interactions. Least squares means are presented, which adjusts for the very few missing values. Tukey's Honestly Significant Difference (HSD) test was used as a multiple comparison procedure to determine differences among means.

## RESULTS AND DISCUSSION

The incubation time between the motility observations served as a "stress test" for the sperm to provide information on how quickly the cells degrade during this period. The difference between the times evaluated after thaw (0 and 2 h) was significant ($P < 0.05$) for almost all of the motility and CASA parameters evaluated (see Appendix Table 3A). The values measured decreased after the 2 h incubation period for most of the parameters. For example, total motility recorded by the CASA analysis decreased from 40.6% initially to 30.8% at 2 h. Because there were no interactions between incubation period and any of the other factors, the averages of the two incubation times are presented for all responses.

The means for the most relevant responses for treated sperm sorted at 20 h are presented (Table 2.1; see Appendix Table 1A for complete data). For nearly all responses, Treatment 1 was either superior to the control or not significantly different. Surprisingly, Treatment 2 was nearly identical to the control for most responses. Therefore, adding 24 mM MOPS was beneficial to sperm stored 20 h prior to sorting, but adding 2% egg yolk in addition to MOPS negated the beneficial effect of MOPS. This was possibly due to increasing osmolality by ~15 mOsM/kg when adjusting the pH when making the treatment 2 solution. Means for sorting responses at 20 h are shown in Table 2.2. Neither treatment had a negative effect on sorting parameters, and Treatment 2 showed some improvement over the control and Treatment 1 for the percent of live oriented cells and the X sort rate. Thus, Treatment 2 appeared to be best for sperm at the time of sorting, but Treatment 1 was best when sperm were evaluated post-thaw.

The results also show that second ejaculates consistently performed better than first ejaculates, but there were no treatment by ejaculate interactions ($P > 0.05$) so only main effects are presented in Table 2.1. Additionally, while the results for the AM (second) sort were lower than the first sort, the differences were generally not significant and the majority of responses were acceptable for most, but not all bulls. Motility responses for individual bulls sorted at 20 h are shown in Appendix Table 2A. In general, Treatment 1 resulted in greater or not significantly different responses for most bulls compared to the control. Treatment 2 exhibited more variation in responses than Treatment 1 and had lower parameter values for some bulls compared to the control.

Table 2.1: Means of semen characteristics for 20 h sort – Experiment 1

| | Treatment | | | Ejaculate # | |
|---|---|---|---|---|---|
| Response | Control | MOPS | MOPS+ EY | Ejaculate 1 | Ejaculate 2 |
| Subjective Total Motility (%) | 28.4[a] | 36.6[b] | 29.8[ab] | 28.2[c] | 35.0[d] |
| Subjective Progressive Motility (%) | 22.0[a] | 29.9[b] | 23.1[ab] | 21.6[c] | 28.4[d] |
| CASA Total Motility (%) | 31.8 | 35.5 | 29.5 | 28.5[c] | 36.0[d] |
| CASA Progressive Motility (%) | 17.0 | 19.3 | 17.1 | 16.0 | 19.5 |
| pH | 5.9[e] | 6.1[f] | 6.1[f] | 5.9[g] | 6.1[h] |
| % Live | 43.1 | 44.8 | 40.2 | 40.2 | 45.2 |

[ab] Means without common superscripts within rows differ ($P < 0.1$)
[cd] Means without common superscripts within rows differ ($P < 0.05$)
[ef, gh] Means without common superscripts within rows differ ($P < 0.001$)

Table 2.2: Means of sorting responses for 20 h sort – Experiment 1

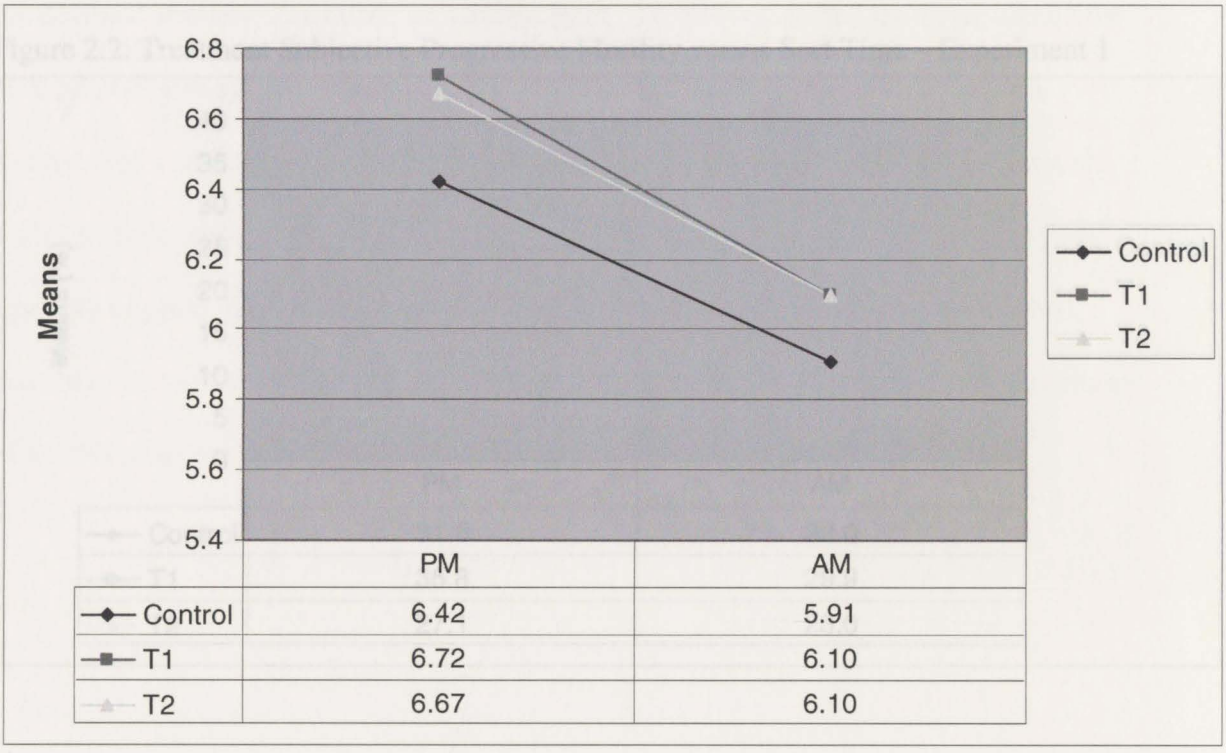
| | Treatment | | |
|---|---|---|---|
| Response | Control | MOPS | MOPS + EY |
| Event Rate (*$10^3$/sec*) | 33.2 | 34.2 | 33.3 |
| Live Oriented Cells (%) | 53.9[a, cd] | 53.1[ab, c] | 56.9[bd] |
| X Sort Rate (*$10^3$/sec*) | 4.8[a] | 4.8[a] | 5.1[b] |
| Coincidence Rate (*$10^3$/sec*) | 6.8 | 7.3 | 6.8 |
| Split (%) | 42.4 | 43.8 | 42.4 |

[ab] Means without common superscripts within rows differ ($P < 0.1$)
[cd] Means without common superscripts within rows differ ($P < 0.05$)

Figure 2.1 illustrates the pH changes with sort time by treatment. While the MOPS treatments maintained the pH about 0.2 units higher than the control, addition of the buffer was not sufficient to neutralize the acid produced by the sperm. We hypothesized that the pH would not have dropped as much with the MOPS treatments as for the control. However, although likely beneficial, the lack of any substantive buffering by the MOPS was disappointing. Perhaps some component in seminal plasma contributed to this lack of buffering. More likely, the severe constraint of being able to control pH was not being able to dilute the semen more than 10% in order to maintain the high sperm concentrations, as staining for sorting is much less effective when seminal plasma is more dilute. Therefore, it was not possible to add sufficient buffer molecules to the sperm samples to maintain a constant pH over the storage time without increasing the osmolality of the solution inappropriately.

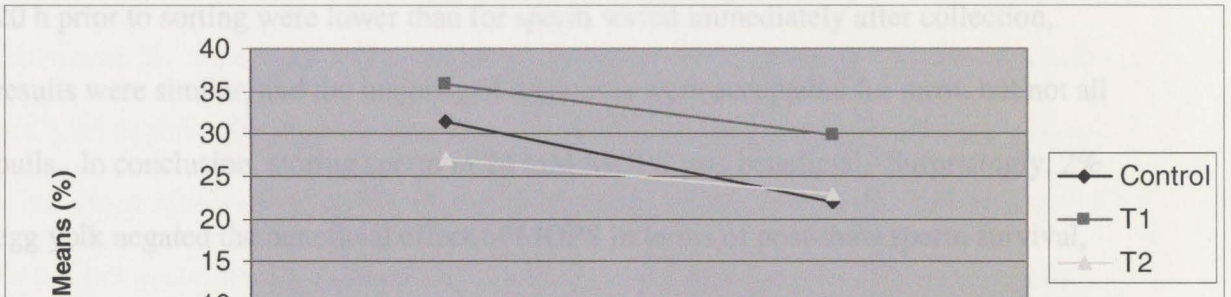
Interestingly, the added buffer provided benefits not only to sperm sorted after 20 h of storage but also to sperm sorted immediately (shown in Figure 2.2). While Treatment 1 maintained higher motility than the control after 20 h of incubation prior to sorting, it also resulted in higher motility when sperm were sorted immediately after treatment. The interaction between treatment and incubation time was not significant, however, though similar trends were noted for subjective total motility and CASA total and progressive motility (Appendix Table 1A). Additionally, neither Treatment 1 nor Treatment 2 was significantly different from the control for the PM sort, although Treatments 1 and 2 were significantly different from each other for subjective total and progressive motility ($P < 0.1$).

Figure 2.1: Treatment pH versus Sort Time – Experiment 1




| | PM | AM |
|---|---|---|
| Control | 6.42 | 5.91 |
| T1 | 6.72 | 6.10 |
| T2 | 6.67 | 6.10 |

Figure 2.2: Treatment Subjective Progressive Motility versus Sort Time – Experiment 1




| | PM | AM |
|---|---|---|
| Control | 31.5 | 22.0 |
| T1 | 35.8 | 29.9 |
| T2 | 27.1 | 23.0 |

## CONCLUSIONS

Bull sperm were stored for 20 h in a MOPS buffered solution or a MOPS + egg yolk solution prior to sex sorting. Diluting sperm in a MOPS diluent performed better than no dilution (control) while diluting sperm in a MOPS + egg yolk diluent was similar to the control. Second ejaculates were superior to first ejaculates. pH measurements showed that the addition of MOPS maintained the pH about 0.2 units higher than the control, but pH declined similarly over time for all treatments. While results for sperm incubated for 20 h prior to sorting were lower than for sperm sorted immediately after collection, results were similar, and the majority of responses were acceptable for most, but not all bulls. In conclusion, storing sperm in 24 mM MOPS was beneficial. Surprisingly, 2% egg yolk negated the beneficial effect of MOPS in terms of post-thaw sperm survival, possibly due to increasing osmolarity by ~15 mOsM/kg due to pH adjustment. Addition of MOPS provided better results than the control for both the 0 h and 20 h sorts.

# CHAPTER III

# EXPERIMENT 2: EVALUATION OF BOVINE SPERM CYROPRESERVED AFTER POST COLLECTION STORAGE FOR 20 HOURS AT 15-18°C OR 5°C WITH MOPS BUFFER

## ABSTRACT OF EXPERIMENT 2

The effect of MOPS for storing bull sperm up to 20 h prior to sex sorting was examined. Ejaculates were obtained from mature bulls (Holstein, $n = 8$, Jersey, $n = 2$) via artificial vagina. Treatments were then applied to the neat semen to which antibiotics were added as recommended by Certified Semen Services. Semen for the first set of treatments was initially standardized to either 1 x $10^9$ spermatozoa/ml (Treatments 1-3 and 6-8) or 9 x $10^8$ spermatozoa/ml (Treatments 4 and 5). The semen was then further diluted with the buffer solutions to result in the following treatments: Treatment 1 = 9 x $10^8$ spermatozoa/ml with 25 mM MOPS, Treatment 2 = 6 x $10^8$ spermatozoa/ml with 25 mM MOPS, Treatment 3 = 3 x $10^8$ spermatozoa/ml with 25 mM MOPS, Treatment 4 = 6 x $10^8$ spermatozoa/ml with 100 mM MOPS, Treatment 5 = 3 x $10^8$ spermatozoa/ml with 100 mM MOPS, Treatment 6 = 9 x $10^8$ spermatozoa/ml with 25 mM MOPS + 2% egg yolk, Treatment 7 = 6 x $10^8$ spermatozoa/ml with 25 mM MOPS + 2% egg yolk, Treatment 8 = 3 x $10^8$ spermatozoa/ml with 25 mM MOPS + 2% egg yolk. Samples were stored for 20 h at the following temperatures: 15-18° C (Treatment 1-5) and 5° C (Treatment 6-8). Samples were prepared for sorting by standard procedures, but the sperm processing for sorting was simulated by diluting the stained samples with sheath fluid and storage at room temperature for an hour. The samples were then frozen

following standard processing procedures. Samples were evaluated post-thaw by subjective motility and computer-assisted sperm analysis (CASA). Sperm diluted to 9 x $10^8$ spermatozoa/ml and 6 x $10^8$ spermatozoa/ml and stored at 15-18° C maintained the highest motility post-thaw. Additionally, MOPS buffer at a concentration of 25 mM performed better than at 100 mM concentration.

## INTRODUCTION

Since the ability of MOPS to maintain the pH in Experiment 1 was disappointing, it was decided to dilute the semen sample to various sperm concentrations to allow more buffering effect. An increased concentration of MOPS was also used for some treatments in an attempt to accomplish the same end. This decision was supported by a study that investigated the viability of diluted sperm following 24 h storage at room temperature (Prathalingam et al., 2006). They concluded that samples diluted to lower sperm concentrations (10 x $10^6$ spermatozoa/ml) had an increased proportion of sperm survive. Additionally, semen diluted at a higher concentration (60 x $10^6$ spermatozoa/ml) had a significantly lower pH then the more dilute semen sample following storage (6.9 versus 7.1, respectively; $P < 0.01$)

In addition to the above treatments, some cooled storage treatments were included. The main principle behind the storage of semen at 5°C is to lower the sperm metabolic rate, thereby extending survival. Many diluents have been examined for use at 5°C, utilizing a variety of additives to preserve the fertility of bovine sperm, the most common being egg yolk (Vishwanath and Shannon, 2000). Therefore, samples diluted similarly to treatments at 15-18° C were stored in a MOPS plus egg yolk solution at 5°C.

Egg yolk was included in the buffer solution to provide thermal protection to the sperm cells, as previously discussed in Experiment 1.

Therefore, the objective of this experiment was to determine the effect of MOPS buffer on semen samples diluted to different concentrations after a 20 h storage period followed by cryopreservation. Additionally, some treatments stored at 5°C were also evaluated.

## MATERIALS AND METHODS

### Semen Collection and Processing

Semen collection, treatment administration and cryopreservation were performed at Sexing Technologies (Navasota, TX). Ejaculates were obtained from mature dairy bulls (Holstein, $n = 8$; Jersey, $n = 2$) via artificial vagina. Only those ejaculates with sperm motility $\geq 50\%$ and at least 65% normal sperm morphology were used. Antibiotics were added to the neat semen as recommended by Certified Semen Services (CSS, Inc, subsidiary of NAAB, Columbia, MO). The neat semen was then split into two main fractions and further sub-divided into individual treatments, as described below.

Fraction 1

The semen aliquotted into this fraction was stored at 15-18°C. The following treatments were prepared:

- Treatment 1: 9 x $10^8$ spermatozoa/ml with 25 mM MOPS
- Treatment 2: 6 x $10^8$ spermatozoa/ml with 25 mM MOPS
- Treatment 3: 3 x $10^8$ spermatozoa/ml with 25 mM MOPS
- Treatment 4: 6 x $10^8$ spermatozoa/ml with 100 mM MOPS
- Treatment 5: 3 x $10^8$ spermatozoa/ml with 100 mM MOPS

Neat semen was first standardized to either 1 x $10^9$ spermatozoa/ml (Treatments 1 through 3) or 9 x $10^8$ spermatozoa/ml (Treatments 4 and 5) by diluting it with TALP (Table 3.1). Semen was then further diluted with the buffer solutions for each treatment according to the following procedures:

- Treatment 1: 1 part 250 mM MOPS (Sigma M-5162)+25 mM NaCl (Sigma S-9625) solution plus 9 parts 1 x $10^9$ semen
- Treatment 2: 2 parts of Treatment 1 plus 1 part TALP/MOPS solution (9 parts TALP and 1 part 250 mM MOPS+25 mM NaCl solution)
- Treatment 3: 1 part of Treatment 1 and 2 parts of TALP/MOPS solution (as described for Treatment 2)
- Treatment 4: 1 part 300 mM MOPS solution and 2 parts of 9 x $10^8$ semen
- Treatment 5: 1 part 300 mM MOPS solution and 1 part TALP and 1 part of 9 x $10^8$ semen.

Fraction 2

Semen for this fraction was cooled gradually over a period of 2-2.5 h to the storage temperature of 5°C by placing each tube containing the semen in a 150 ml beaker filled with 100 ml of water equilibrated to room temperature (~20-22°C) and placed in a 5°C refrigerator. Prior to cooling, semen was diluted to the following concentrations in a medium resulting in 2% egg yolk and 25 mM MOPS:

- Treatment 6: 9 x $10^8$ spermatozoa/ml
- Treatment 7: 6 x $10^8$ spermatozoa/ml
- Treatment 8: 3 x $10^8$ spermatozoa/ml

Table 3.1: Composition of TALP[b] – Experiment 2

| Ingredients | mM | Amount/100 ml |
|---|---|---|
| NaCl | 117.0 | 0.684 g |
| KCl | 3.2 | 0.023 g |
| $Na_2HPO_4$ (Dibasic, anhydrous) | 0.3 | 0.0043 g |
| $MgCl_2 \cdot 6\ H_2O$ | 0.5 | 0.011 g |
| $NaHCO_3$ | 25.0 | 0.21 g |
| Na-pyruvate | 0.2 | 0.0022 g |
| Glucose[a] | 5.0 | 0.09 g |
| Nanopure $H_2O$ to make | | 100 ml |
| Na-lactate 60% syrup | 10.0 | 0.09 ml |
| BSA[a] | 0.3% (w/v) | 0.3 g |
| Gentamycin sulfate stock solution | 25 µg/ml | 25.6 µl |

[a]Dissolve separately in about 50% of total volume of water, prior to mixing other ingredients

[b]pH of final solution adjusted to 7.3 with HCl

To prepare the above treatments, the neat semen was first standardized to 1 x $10^9$ spermatozoa/ml by diluting it with TALP. The semen was then further diluted with the buffer solution for each treatment according to the following procedures:

- Treatment 6: 1 part 312.5 mM MOPS+20% egg yolk solution and 9 parts 1 x $10^9$ semen
- Treatment 7: 2 parts of Treatment 6 and 1 part of TALP/MOPS solution (9 parts TALP and 1 part 312.5 mM MOPS+20% egg yolk solution
- Treatment 8: 1 part of Treatment 6 and 2 parts of TALP/MOPS solution (as described for Treatment 7)

General

All samples were stored at the indicated temperatures for 20 h after collection and then prepared for sorting following standard processing procedures. Additionally, pH measurements were obtained for each sample after treatment administration and before dilution of semen for staining. Sperm concentrations were adjusted to 160 x $10^6$ spermatozoa/ml with Staining TALP (pH 7.4) (Schenk et al., 1999). 4 µl/ml of Hoechst 33342 (8.1 mM stock) (Schenk et al., 1999) was added, and samples were incubated in a 34.5°C water bath for 45 min. Following staining, sperm concentrations were adjusted to 80 x $10^6$ spermatozoa/ml with TALP (pH 5.5) to which 4% egg yolk and 0.002% food-coloring dye (SortEnsure™ FD&C Red #40) was added; the samples were then filtered (50 µm Cell Trics disposable filter #04-0042-2317, Partec GmbH, Munster, Germany). However, the samples were not sorted by flow cytometry. The sorting process was simulated instead by diluting 2 ml of the stained sample with 18 ml of sheath fluid (Schenk et al., 1999) and storing the samples at room temperature for an hour. Cooling,

centrifugation, and glycerolization were then done (as described previously for Experiment 1). Sperm were packaged in 0.25 ml straws at of 2.1 x $10^6$ sperm/dose. The straws were sealed and held in liquid nitrogen vapor 25 min prior to immersion in liquid nitrogen. Sperm were kept a minimum of 3 h for cooling and glycerolization before freezing.

Evaluation

Frozen straws were shipped to Colorado State University (Fort Collins, CO) for analysis. Motility was determined both subjectively and objectively (computer assisted sperm analysis (CASA), Hamilton-Thorne IVOS; Hamilton Thorne, Inc., Beverly, MA). Two straws for each treatment were thawed by immersion in a 37°C water bath for 30 sec. Straws were thawed randomly and placed into numbered tubes to provide a "blind" analysis. Subjective motility was assessed by visual estimation by one person. CASA imaging was enabled using the fluorescence objective and 10 fields of view or a minimum of 200 cells were counted.

Statistical Analysis

Data were subjected to a mixed model ANOVA. Bulls were considered a random effect and the average value of the two straws evaluated for each treatment was used. The statistical analysis included all main effects. Least squares means are presented. Tukey's Honestly Significant Difference (HSD) test was used as a multiple comparison procedure to determine differences among means.

One of the bulls had consistently very poor post-thaw motility for all treatments. Therefore, he was not included in the ANOVA, so only 9 bulls (Holstein, $n = 7$; Jersey, $n = 2$) were analyzed. This resulted in a 9 (bulls) x 8 (treatments) factorial arrangement.

## RESULTS AND DISCUSSION

Treatments of sperm held at 5°C were inferior ($P < 0.05$) to the best treatments of sperm held at 15-18°C for all responses (except straightness and linearity, which are of minor importance) (Appendix Table 7A), and therefore will not be considered further. Dilution of sperm to $9 \times 10^8$ and $6 \times 10^8$ /ml supported higher post-thaw motility than dilution to $3 \times 10^8$ ($P < 0.05$). Others have shown that sperm viability decreases with increasing dilution (Garner et al., 1997; Maxwell et al., 1997; Prathalingam et al., 2006). This "dilution effect" may be partly due to decreases of components in seminal plasma that are essential for maintaining the integrity of the sperm membrane. Without these components, the cell membrane is weakened and premature cell death can occur.

Additionally, 25 mM MOPS buffer resulted in higher motility then the 100 mM buffer concentration for both the subjective and CASA motility measurements ($P < 0.001$). This could be because of some toxic effect of MOPS, or more likely, that the increased MOPS molecules effectively diluted other important ions (e.g. sodium) in the solution due to keeping osmolarity constant. Ionic strength also decreased with increased MOPS.

## CONCLUSIONS

The effect of MOPS for storing bull sperm up to 20 h prior to sex sorting was examined. Sperm diluted to $9 \times 10^8$ spermatozoa/ml and $6 \times 10^8$ spermatozoa/ml and stored at 15-18° C maintained the highest motility post-thaw. Additionally, MOPS buffer at a concentration of 25 mM performed better than at 100 mM concentration. These treatments therefore were chosen to be included in Experiment 3.

Table 3.2: Treatment means for all responses – Experiment 2

| | **Treatment** | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | *Stored at 15-18°C* | | | | | *Stored at 5°C*[g] | | |
| Sperm conc ($10^8$) /mM MOPS: | Trt 1: 9/25 | Trt 2: 6/25 | Trt 3: 3/25 | Trt 4: 6/100 | Trt 5: 3/100 | Trt 6: 9/25 | Trt 7: 6/25 | Trt 8: 3/25 |
| **Response** | | | | | | | | |
| Subjective Total Motility (%) | 35.8 | 34.7 | 25.0[b] | 21.1[c] | 11.7[c] | 13.9 | 14.7 | 15.3 |
| Subjective Progressive Motility (%) | 30.3 | 28.9 | 19.7[b] | 16.4[c] | 7.2[c] | 8.9 | 9.4 | 10.3 |
| CASA Total Motility (%) | 35.6 | 32.1 | 25.7[b] | 23.1[c] | 13.7[c] | 13.6 | 18.3 | 19.4[d] |
| CASA Progressive Motility (%) | 16.9 | 14.7 | 10.7[b] | 9.3[b] | 5.2[c] | 5.8 | 7.8 | 8.6 |
| VAP (μm/s) | 92.3 | 96.6[a] | 94.4 | 91.6 | 88.9 | 78.2 | 82.1 | 78.2 |
| VSL (μm/s) | 66.4 | 68.7 | 67.8 | 63.6 | 61.6[b] | 58.0 | 61.1 | 60.1 |
| VCL (μm/s) | 183.3 | 189.6 | 185.2 | 179.3 | 176.2 | 152.4 | 157.5 | 151.8 |
| ALH (μm) | 7.1 | 6.9 | 7.0 | 6.6 | 6.4[a] | 6.0 | 6.1 | 6.3 |
| BCF (beats/s) | 24.6 | 23.6 | 22.8 | 23.6 | 23.8 | 22.3 | 21.7 | 20.8 |
| STR (%) | 74.6 | 74.2 | 74.9 | 73.6 | 73.9 | 77.3 | 77.8 | 79.4 |
| LIN (%) | 40.0 | 40.2 | 40.8 | 40.1 | 40.5 | 42.8 | 44.0 | 44.1 |
| VD: Rapid (%) | 33.2 | 29.7 | 23.2[b] | 20.5[c] | 12.2[c] | 11.5 | 15.7[b] | 16.1[b] |
| VD: Medium (%) | 2.5 | 2.5 | 2.4 | 2.7 | 1.7[a] | 2.2 | 2.7 | 3.2[d] |
| VD: Static (%) | 45.3 | 38.8 | 47.7 | 41.6 | 59.0[b] | 64.2 | 57.4 | 50.1[e] |

[a] Means without common superscripts within rows differ from Treatment 1 ($P < 0.1$); [d] Means without common superscripts within rows differ from Treatment 6 ($P < 0.1$)

[b] Means without common superscripts within rows differ from Treatment 1 ($P < 0.05$); [e] Means without common superscripts within rows differ from Treatment 6 ($P < 0.05$)

[c] Means without common superscripts within rows differ from Treatment 1 ($P < 0.001$); [f] Means without common superscripts within rows differ from Treatment 6 ($P < 0.001$)

[g] Treatments stored at 5°C were inferior to Treatment 1 for all responses (except straightness and linearity)

# CHAPTER IV

## EXPERIMENT 3: EVALUATION OF BOVINE SPERM STORED FOR 20 HOURS AFTER COLLECTION AT 16°C IN EITHER TEST OR MOPS BUFFERED DILUENTS AT DIFFERENT SPERM CONCENTRATIONS OR STORED AT 5°C IN CUE EXTENDER WITH EGG YOLK

## ABSTRACT OF EXPERIMENT 3

Effects of MOPS, TEST or CUE + egg yolk for storing bull sperm up to 20 h prior to sex sorting were examined. Ejaculates were obtained from mature bulls (Holstein, $n = 6$, Gyr, $n = 1$) via artificial vagina. Treatments were then applied to the neat semen to which antibiotics were added as recommended by Certified Semen Services. Semen for the first set of treatments was initially standardized to 1 x $10^9$ spermatozoa/ml, and then further diluted with buffer solutions to result in the following treatments: Treatment 1 = 9 x $10^8$ spermatozoa/ml with 12.5 mM TEST, Treatment 2 = 6 x $10^8$ spermatozoa/ml, Treatment 3 = 9 x $10^8$ spermatozoa/ml with 25 mM TEST, Treatment 4 = 6 x $10^8$ spermatozoa/ml with 25 mM TEST, Treatment 5 = 9 x $10^8$ spermatozoa/ml with 50 mM TEST, Treatment 6 = 6 x $10^8$ spermatozoa/ml with 50 mM TEST, Treatment 7 = 9 x $10^8$ spermatozoa/ml with 12.5 mM MOPS, Treatment 8 = 9 x $10^8$ spermatozoa/ml with 25 mM MOPS, Treatment 9 = 6 x $10^8$ spermatozoa/ml with 25 mM MOPS, Treatment 10 = 9 x $10^8$ spermatozoa/ml with 50 mM MOPS. Semen for the second set of treatments was diluted with CUE + egg yolk solution to result in the following treatments: Treatment 11 = CUE + 5% egg yolk, Treatment 12 = CUE + 2.5% egg yolk. The samples were stored for 20 h at the following temperatures: 16° C

(Treatment 1-10) and 5° C (Treatment 11-12). Samples were prepared for sorting by standard procedures, but the sperm processing for sorting was simulated by diluting the stained samples with 20% Tris A fraction catch buffer and sheath fluid and storage at room temperature for an hour. The samples were then frozen following standard processing procedures. pH measurements were made before staining samples for sorting. Samples were evaluated post-thaw by computer-assisted sperm analysis (CASA). Treatments at 5°C storage of sperm were inferior to those concerning storage at 16°C. Sperm diluted to 9 x $10^8$ spermatozoa/ml and stored at 16°C maintained the highest motility post-thaw. TEST buffer at concentrations of 12.5 mM and 25 mM performed better than at 50 mM concentration. There was no difference between TEST and MOPS for post-thaw sperm survival.

## INTRODUCTION

Results from Experiment 2 showed that semen diluted to 6 x $10^8$ and 9 x $10^8$ spermatozoa/ml had better post-thaw motility than semen diluted to 3 x $10^8$ spermatozoa/ml. This experiment was designed to screen treatments diluted to 6 or 9 x $10^8$ spermatozoa/ml and to determine if TEST buffer solution would provide similar benefit to sperm stored for 20 h when compared to MOPS.

TEST is a double zwitterionic buffer solution that has frequently been used for storage of bull sperm (Underwood et al., 2009; Ijaz and Hunter, 1989; Graham et al., 1972). It is normally prepared by titrating a N-tris (hydroxymethyl) methyl-2-amino ethane sulfonic acid (TES) buffer solution against a Tris (hydroxymethyl) amino methane (Tris) solution, each at 300 mOsm, until a desired pH is reached. This procedure has the major advantage that the osmolality of the solution remains constant during pH adjustment.

Furthermore, it was decided to store these treatments at a constant temperature of 16°C. The hypothesis was that maintaining a consistent storage temperature would be less harmful to sperm cells then one that fluctuates 3-4 degrees throughout the storage period.

Although the samples held at 5°C in the preliminary experiment had significantly lower post-thaw results when compared to samples stored at 15-18°C, it was decided to again include cooled treatments; Cornell University Extender (CUE) plus egg yolk diluted simply 1:1 or 1:2 with neat semen (v/v CUE: semen) was the storage solution examined in this study. CUE is a liquid semen diluent that has been used at both 5°C and ambient temperatures for storing bovine semen (Shannon, 1964; Foote, 1978).

Consequently, the objective of this experiment was to determine the effect of TEST buffer versus MOPS on diluted semen samples after a 20 h storage period. In addition, treatments stored at 5°C in CUE extender were also evaluated. The results of this study were then used to design Experiment 4.

## MATERIALS AND METHODS

### Semen Collection and Processing

Semen collection, treatment administration and cryopreservation were performed at Sexing Technologies (Navasota, TX). Ejaculates were obtained from mature bulls (Holstein, $n = 6$, Gyr, $n = 1$) via artificial vagina. Ejaculates were evaluated for acceptable viability and only those with progressive sperm motility no less then 50% and morphology at least 65% normal were used (all ejaculates had 70% or greater normal morphology except that one bull had 35% abnormalities.) One ejaculate was centrifuged (15 min at 1144 x g; model Centra CL2, Thermo Fisher Scientific Inc (Pittsburgh, PA))

to increase the sperm concentration because the initial concentration was below 1 x $10^9$ sperm/ml. Antibiotics were added to the neat semen as recommended by Certified Semen Services (CSS, Inc, subsidiary of NAAB, Columbia, MO) and the semen was then split into two main fractions, which were further sub-divided into individual treatments as described below.

Fraction 1

Semen divided into this fraction was stored at 16°C (Minitube incubator, model DCR082W). The following is a list of the treatments that were prepared for this component:

- Treatment 1: 9 x $10^8$ spermatozoa/ml with 12.5 mM TEST
- Treatment 2: 6 x $10^8$ spermatozoa/ml with 12.5 mM TEST
- Treatment 3: 9 x $10^8$ spermatozoa/ml with 25 mM TEST
- Treatment 4: 6 x $10^8$ spermatozoa/ml with 25 mM TEST
- Treatment 5: 9 x $10^8$ spermatozoa/ml with 50 mM TEST
- Treatment 6: 6 x $10^8$ spermatozoa/ml with 50 mM TEST
- Treatment 7: 9 x $10^8$ spermatozoa/ml with 12.5 mM MOPS
- Treatment 8: 9 x $10^8$ spermatozoa/ml with 25 mM MOPS
- Treatment 9: 6 x $10^8$ spermatozoa/ml with 25 mM MOPS
- Treatment 10: 9 x $10^8$ spermatozoa/ml with 50 mM MOPS

To prepare the above treatments, the neat semen was first standardized to 1 x $10^9$ spermatozoa/ml by diluting it with TALP (Table 4.1). The semen was then further

Table 4.1: Composition of TALP[b] – Experiment 3

| Ingredients | mM | Amount/100 ml |
|---|---|---|
| NaCl | 117.0 | 0.684 g |
| KCl | 3.2 | 0.023 g |
| $Na_2HPO_4$ (Dibasic, anhydrous) | 0.3 | 0.0043 g |
| $MgCl_2 \cdot 6\ H_2O$ | 0.5 | 0.011 g |
| $NaHCO_3$ | 25.0 | 0.21 g |
| Na-pyruvate | 0.2 | 0.0022 g |
| Glucose[a] | 5.0 | 0.09 g |
| Nanopure $H_2O$ to make | | 100 ml |
| Na-lactate 60% syrup | 10.0 | 0.09 ml |
| BSA[a] | 0.3% (w/v) | 0.3 g |
| Gentamycin sulfate stock solution | 25 µg/ml | 25.6 µl |

[a]Dissolve separately in about 50% of total volume of water, prior to mixing other ingredients
[b]pH of final solution adjusted to 7.3 with HCl

diluted with the buffer solutions for each treatment according to the following procedures:

- Treatment 1: 1 part 125 mM TEST (TES, Sigma #T6022, Trizma®, Sigma #T1503)+87 mM NaCl (Sigma S-9625) solution and 9 parts 1 x $10^9$ semen
- Treatment 2: 2 parts of Treatment 1 and 1 part TALP/TEST solution (9 parts TALP diluted with 1 part 125 mM TEST solution)
- Treatment 3: 1 part 250 mM TEST solution and 9 parts 1 x $10^9$ semen
- Treatment 4: 2 parts of Treatment 3 and 1 part TALP/TEST solution (9 parts TALP diluted with 1 part 250 mM TEST solution)
- Treatment 5: 2 parts 250 mM TEST solution and 8 parts 1 x $10^9$ semen
- Treatment 6: 2 parts of Treatment 5 and 1 part TALP/TEST solution (8 parts TALP diluted with 2 parts 250 mM TEST solution)
- Treatment 7: 1 part 125 mM MOPS (Sigma M5162)+56.25 mM NaCl solution and 9 parts 1 x $10^9$ semen
- Treatment 8: 1 part 250 mM MOPS solution and 9 parts 1 x $10^9$ semen
- Treatment 9: 2 parts of Treatment 8 and 1 part TALP/MOPS solution (9 parts TALP diluted with 1 part 250 mM MOPS solution)
- Treatment 10: 2 parts 250 mM MOPS solution and 8 parts 1 x $10^9$ semen

Fraction 2

Diluted semen for this fraction was cooled gradually over 2-2.5 h to 5°C for storage by placing each tube containing the semen in a 150 ml beaker filled with 100 ml of water equilibrated to room temperature (~20-22°C) that was placed into a 5°C refrigerator.

Semen was diluted for each treatment in the following manners in CUE extender (with indicated egg yolk added; see Appendix Table 4A for composition):

- Treatment 11: 1 part extender (10% egg yolk solution) and 1 part neat semen, resulting in 5% egg yolk in sample
- Treatment 12: 1 part extender (7.5% egg yolk solution) and 2 parts neat semen, resulting in 2.5% egg yolk in sample

General

All samples were stored at the indicated temperatures for 20 h after collection and then processed. Samples were prepared for sorting following standard processing procedures. Additionally, pH measurements were made for each sample after treatment administration (0 h) and before dilution of semen for staining (20 h). After storage, sperm concentrations were adjusted to 160 x $10^6$ spermatozoa/ml with Staining TALP (pH 7.4; Schenk et al., 1999). Next, 4 µl/ml of Hoechst 33342 (8.1 mM stock; Schenk et al., 1999) was added and the samples were incubated in a 34.5°C water bath for 45 min. Following staining, sperm concentrations were adjusted to 80 x $10^6$ spermatozoa/ml with TALP (pH 5.5) containing 4% egg yolk and 0.002% food-coloring dye (SortEnsure™ FD&C Red #40) and the samples were filtered (50 µm Cell Trics disposable filter #04-0042-2317, Partec GmbH, Munster, Germany). However, the samples were not sorted by flow cytometry. The sperm processing steps for sorting were simulated by diluting 0.5 ml of the stained sample with 3.5 ml of 20% Tris A fraction catch buffer (SortEnsure™ Bovine XY® Tris A Working Solution, to which 20% egg yolk and antibiotics were added) plus 16 ml of sheath fluid (Schenk, et al., 1999) and storing the samples at room temperature for an hour. Cooling, centrifugation, and glycerolization were then

performed, as described previously for Experiment 1. Sperm were packaged in 0.25 ml straws at a concentration of 2.1 x $10^6$ sperm/straw. The straws were sealed and held in vapor 25 min prior to immersion in liquid nitrogen for storage. A minimum of 3 h was allowed for cooling and glycerolization before freezing.

Evaluation

Frozen straws were shipped to Colorado State University (Fort Collins, CO) for analysis. Motility was determined using a computer assisted sperm analyzer (computer assisted sperm analysis (CASA), Hamilton-Thorne IVOS; Hamilton Thorne, Inc., Beverly, MA). Two straws/treatment were thawed by immersion in a 37°C water bath for 30 sec. Straws were thawed randomly and placed into numbered tubes to provide a "blind" analysis. Imaging was conducted using the fluorescence objective and 10 fields of view or a minimum of 200 cells were counted.

Statistical Analysis

Data collected from these evaluations were arranged into two factorial structures. The first analysis consisted of all six TEST treatments, resulting in a 2 (semen concentration) x 3 (buffer concentration) x 6 (bulls) factorial arrangement. The second analysis consisted of all six treatments at the 9 x $10^8$ spermatozoa/ml dilution, resulting in a 2 (buffer solution) x 3 (buffer concentration) x 6 (bulls) factorial arrangement. These data were subjected to a mixed model ANOVA. Bulls were considered a random effect and the average value of the two straws evaluated for each treatment was used. The statistical analysis included all main effects and first order interactions. Least squares means are presented.

One of the bulls had consistently very poor post-thaw motility for all treatments. Therefore, he was not included in the ANOVA and only 6 bulls (Holstein, $n = 5$; Gyr, $n = 1$) were analyzed.

## RESULTS AND DISCUSSION

In general, the samples that were cooled and stored at 5°C (Treatments 11 and 12) were inferior to the treatments kept at 16°C (Appendix Table 5A). This could be due to the fact that CUE was designed for storing sperm around $10 \times 10^7$ sperm/ml while we stored the sperm at 50-100 times that concentration in this experiment.

Individual treatment means for all of the responses are presented in Appendix Table 4A. The treatments in which the sperm were diluted to $9 \times 10^8$ spermatozoa/ml were superior for most of the responses evaluated compared to the sperm stored at $6 \times 10^8$ spermatozoa/ml (Table 4.2). Of most importance, the percentages of total and progressive motility for sperm stored at $9 \times 10^8$ spermatozoa/ml was greater then those for sperm stored at $6 \times 10^8$ spermatozoa/ml ($P < 0.001$ and $P < 0.01$, respectively). This could be because dilution reduced the concentrations of components in seminal plasma that are needed for maintaining the integrity of the sperm membrane (Maxwell et al., 1997). Without these components, the membrane is weakened and premature cell death can occur (Prathalingam et al., 2006). Put another way, the TALP-TEST solutions used for dilution were inferior to the seminal plasma for maintaining the sperm. Diluting the sperm contributes to a decrease in sperm viability that is proportional to increasing dilution (Garner et al., 1997). As one would expect, the pH was lower in samples that had the higher sperm concentration. This was probably because less acid was

Table 4.2: Main effect means and significances of treatment effects for all responses – Experiment 3

| | **Treatment Effects** | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | TEST Treatments | | | | | | | | $9 \times 10^8$ spermatozoa/ml Treatments[c] | | |
| | *Spermatozoa/ml* | | | *Buffer Concentration (mM)* | | | | | *Buffer Type* | | |
| | **$9 \times 10^8$** | **$6 \times 10^8$** | | **12.5** | **25** | **50** | | | **MOPS** | **TEST** | |
| **Response** | | | Pr > F | | | | Pr > F | | | | Pr > F |
| *Total Motility (%)* | 34.4 | 26.7 | < 0.001 | 32.7 | 31.6 | 27.3 | 0.064 | | 33.7 | 34.4 | 0.679 |
| *Progressive Motility (%)* | 16.5 | 13.0 | 0.01 | 15.2 | 15.2 | 13.9 | 0.63 | | 15.3 | 16.5 | 0.308 |
| *VAP (μm/s)* | 95.4 | 85.2 | < 0.001 | 93.4 | 90.4 | 87.1 | 0.074 | | 96.5 | 95.4 | 0.562 |
| *VSL (μm/s)* | 71.9 | 66.4 | 0.002 | 70.4 | 68.9 | 68.1 | 0.494 | | 71.0 | 71.9 | 0.566 |
| *VCL (μm/s)* | 189.6 | 168.3 | < 0.001 | $186.6^{a}$ | $179.0^{ab}$ | $171.2^{b}$ | 0.025 | | 190.9 | 189.6 | 0.721 |
| *ALH (μm)* | 7.4 | 6.6 | < 0.001 | 7.1 | 7.1 | 6.8 | 0.356 | | 7.2 | 7.4 | 0.366 |
| *BCF (beats/s)* | 22.5 | 22.5 | 1.000 | 22.7 | 22.4 | 22.5 | 0.853 | | 22.9 | 22.5 | 0.330 |
| *STR (%)* | 77.3 | 79.8 | 0.001 | $77.4^{a}$ | $78.3^{a}$ | $80.0^{b}$ | 0.014 | | 76.4 | 77.3 | 0.289 |
| *LIN (%)* | 41.3 | 42.9 | 0.032 | 41.4 | 41.7 | 43.1 | 0.139 | | 41.0 | 41.3 | 0.807 |
| *VD: Rapid (%)* | 32.1 | 23.9 | < 0.001 | $30.1^{a}$ | $29.2^{a}$ | $24.7^{b}$ | 0.047 | | 31.1 | 32.1 | 0.517 |
| *VD: Medium (%)* | 2.3 | 2.8 | 0.104 | 2.5 | 2.4 | 2.7 | 0.785 | | 2.6 | 2.3 | 0.395 |
| *VD: Slow (%)* | 15.1 | 15.1 | 0.987 | 17.9 | 16.6 | 10.9 | 0.236 | | 20.0 | 15.1 | 0.159 |
| *VD: Static (%)* | 50.4 | 58.2 | 0.036 | $49.5^{a}$ | $51.8^{a}$ | $61.8^{b}$ | 0.020 | | 46.2 | 50.4 | 0.254 |
| *Start pH* | 6.89 | 7.16 | < 0.001 | $7.00^{a}$ | $7.00^{a}$ | $7.07^{b}$ | < 0.001 | | 6.93 | 6.89 | 0.009 |
| *End pH* | 6.26 | 6.90 | < 0.001 | $6.50^{a}$ | $6.50^{a}$ | $6.74^{b}$ | < 0.001 | | 6.51 | 6.26 | < 0.001 |
| *pH Change* | 0.63 | 0.26 | < 0.001 | $0.50^{a}$ | $0.50^{a}$ | $0.33^{b}$ | < 0.001 | | 0.42 | 0.63 | < 0.001 |

[ab] Means without common superscripts within rows differ ($P < 0.05$)

[c] Main effect means for buffer concentrations for the MOPS/TEST analysis (12.5, 25, and 50 mM) are not presented because there were no significant differences ($P > 0.1$) except for pH responses ($P < 0.001$)

produced by the sperm cells in the more dilute samples. However, the final pH values were still within an acceptable range for the more concentrated samples (6.2-6.8).

In regard to level of buffer used, the highest buffer concentration resulted in a smaller decline in pH over the storage period (pH change of 0.33 versus 0.50 for both of the lower concentrations). However, the 12.5 mM and 25 mM buffer concentrations performed better than the 50 mM concentration. This could be because the 50 mM concentration had more buffer molecules present in the sample then the 12.5 mM and 25 mM concentrations. The increased number of buffer molecules required that other ions be reduced to maintain the proper osmolality. Therefore, the reduction of other important ions (e.g. sodium) in the solution may explain why the highest buffer concentration failed to be more beneficial. Additionally, no statistical difference was found for the CASA responses when comparing MOPS versus TEST at the 9 x $10^8$ spermatozoa/ml dilution. However, the MOPS buffer was slightly but clearly superior ($P < 0.001$) to TEST in maintaining pH (Table 4.2).

## CONCLUSIONS

Effects of MOPS, TEST or CUE + egg yolk for storing bull sperm up to 20 h prior to sex sorting were examined. The 5°C storage treatments were inferior to those at 16°C. Sperm diluted to 9 x $10^8$ spermatozoa/ml and stored at 16°C maintained the highest motility post-thaw. TEST buffer at 12.5 mM and 25 mM performed better than at 50 mM. Finally, although no difference was found between TEST and MOPS for post-thaw sperm survival, TEST was chosen to be examined more extensively in Experiment 4 because of ease of adjusting pH without altering the osmolality of the buffer solution.

# CHAPTER V

## EXPERIMENT 4: EFFECT OF 25 mM TEST ± 2% EGG YOLK ON DILUTED BOVINE SPERM STORED FOR 20 HOURS FROM COLLECTION TO SEXING

### ABSTRACT OF EXPERIMENT 4

Incubating bull sperm in a TEST buffer solution or a TEST + egg yolk solution for up to 20 h prior to sex sorting was evaluated. Two successive ejaculates were obtained from mature bulls (Brahman, $n = 4$, Holstein, $n = 3$, Simmental, $n = 2$, Jersey, $n = 1$, Angus, $n = 1$, Red Angus, $n = 1$) via artificial vagina. Treatments were then applied to the neat semen to which antibiotics were added as recommended by Certified Semen Services. Nothing further was added to the control samples until staining with Hoechst 33342 for sorting. The semen was diluted 9:1 with a TEST solution resulting in 25 mM TEST (Treatment 1) or 25 mM TEST + 2% egg yolk (Treatment 2). Treatment 3 (applied to the first ejaculate) and Treatment 4 (applied to the second ejaculate) both consisted of a 2:1 dilution of neat semen to buffer, resulting in 25 mM TEST + 2% egg yolk. Subsamples of each treatment and control were sorted by flow cytometry shortly after collection and then frozen following standard processing procedures. The other subsample was stored at 16°C and sorted 20 h after collection. pH measurements were made before staining samples for sorting. Samples were evaluated post-thaw for subjective progressive and total motility, by computer-assisted sperm analysis (CASA), and by flow cytometry for sperm viability using propidium iodide and SYBR-14. Treatment 2 maintained higher responses then the control or Treatment 1. It improved the percentage of live oriented

cells ($P < 0.1$) and decreased the proportion of dead cells during sorting ($P < 0.05$). pH remained highest in Treatment 1, but a decline in pH occurred in all groups over time. Second ejaculates performed better then first ejaculates, as demonstrated by slightly higher results with Treatment 4 compared to Treatment 3. However, Treatment 3 was comparable to the Control, Treatment 1, and Treatment 2. In conclusion, Treatment 2 was the most advantageous treatment for this experiment. It improved the sperm sort rates about 10% because of fewer dead sperm when sorting occurred 20 h after sperm collection. Additionally, survival post-thaw was improved, though not significantly.

## INTRODUCTION

The results of the first experiment showed that addition of a pH buffer to a stored sperm sample was beneficial, as documented by motility and live/dead parameters post-thaw. Based upon Experiment 3, evaluating various concentrations of buffer with different sperm concentrations, it was decided to examine storing sperm in TEST buffer solutions. TEST, like MOPS, is a zwitterionic buffer that has been successfully used in storage solutions for bovine sperm.

For this experiment, semen was diluted to a standardized concentration of $9 \times 10^8$ spermatozoa/ml for storage. Results from Experiment 2 showed that this dilution supported higher post-thaw motility than sperm samples diluted to lower concentrations.

In addition, a simple dilution treatment was incorporated into this experiment. The thought was that a straight-forward application of one part buffer solution to two parts neat semen would be more convenient for technicians preparing a collected ejaculate at a satellite location than a sample requiring standardization of sperm concentration.

Finally, all samples were stored in an incubator set at 16°C. This step was performed to eliminate the variation in temperature during the storage period, thereby minimizing additional stress that might affect the sperm. Preliminary results showed that samples stored at this temperature maintained higher post-thaw motility than those stored at 5°C. The main objective of this experiment was to compare storing bull sperm in a TEST buffer solution and a TEST + egg yolk solution for 20 h prior to sex sorting followed by cryopreservation.

## MATERIALS AND METHODS

### Semen Collection and Processing

Semen collection, treatment administration, sex sorting and cryopreservation were performed at Sexing Technologies (Navasota, TX). Two successive ejaculates were obtained from mature bulls (Brahman, $n = 4$, Holstein, $n = 3$, Simmental, $n = 2$, Jersey, $n = 1$, Angus, $n = 1$, Red Angus, $n = 1$) via artificial vagina. Semen had been collected from each bull at least once during the previous week. Only ejaculates containing $\geq 50\%$ motility and with $\geq 65\%$ morphology were used (all ejaculates had 70% or greater normal morphology, except one bull had 35% abnormalities). Ejaculates were centrifuged gently (15 min at 1144 x g; model Centra CL2, Thermo Fisher Scientific Inc (Pittsburgh, PA)) if the initial concentration was between $7 \times 10^8$ and $1 \times 10^9$ spermatozoa/ml to increase the concentration to $1 \times 10^9$ spermatozoa/ml or greater. Ejaculates were discarded if the concentration was below $7 \times 10^8$ spermatozoa/ml. Therefore, one first ejaculate was centrifuged (12 total collected and treated) and four second ejaculates were centrifuged (9 total collected and treated). Treatments were then applied to the neat semen to which antibiotics were added as recommended by Certified Semen Services (CSS, Inc,

subsidiary of NAAB, Columbia, MO). For consistency, samples were diluted to 9 x $10^8$ spermatozoa/ml by diluting the ejaculates with staining TALP (Table 5.1). Nothing further was added to the control samples until staining with Hoechst 33342 for sorting. Treatment 1 consisted of diluting the semen 9:1 with a TEST (TES, Sigma # T6022 and Trizma®, Sigma # T1503)+ 24 mM NaCl (Sigma S-9625) solution resulting in 25 mM TEST. Treatment 2, resulting in 25 mM TEST + 2% egg yolk in the sample, was diluted in the same manner. Treatments 3 and 4 were applying the simplified procedure of using 2 parts undiluted, neat semen and 1 part TEST + egg yolk solution (plus 107 mM NaCl), resulting in 25 mM TEST + 2% egg yolk. Treatment 3 referred to applying this solution to first ejaculates while treatment 4 was applying it to second ejaculates.

A subsample from each treatment was sorted by flow cytometry shortly after collection and frozen using standard processing procedures. Briefly, sperm concentrations were adjusted to 160 x $10^6$ spermatozoa/ml with Staining TALP (pH 7.4; Schenk et al., 1999); 4 µl/ml (for Holstein and Jersey bulls) or 4.5 µl/ml (for other breeds) of Hoechst 33342 (8.1 mM stock; Schenk et al., 1999) was added, and the samples were incubated in a 34.5°C water bath for 45 min. Following staining, sperm concentrations were adjusted to 80 x $10^6$ spermatozoa/ml with TALP (pH 5.5) containing 4% egg yolk and 0.002% food-coloring dye (SortEnsure™ FD&C Red #40). Sperm samples were filtered (50 µm Cell Trics disposable filter #04-0042-2317, Partec GmbH, Munster, Germany) and sorted at 40 psi into 3.5 ml of 20% Tris A fraction catch buffer (SortEnsure™ Bovine XY® Tris A Working Solution, to which 20% egg yolk, antibiotics and water were added) until 1 x $10^7$ cells were collected. Sorted sperm were cooled to 5°C over 90 min. Tris B fraction without egg yolk (SortEnsure™ Bovine XY® Tris

Table 5.1: Composition of TALP[b] – Experiment 4

| Ingredients | mM | Amount |
|---|---|---|
| NaCl | 117.0 | 0.684 g |
| KCl | 3.2 | 0.023 g |
| $Na_2HPO_4$ (Dibasic, anhydrous) | 0.3 | 0.0043 g |
| $MgCl_2 \cdot 6\ H_2O$ | 0.5 | 0.011 g |
| $NaHCO_3$ | 5.0 | 0.042 g |
| Na-pyruvate | 0.2 | 0.0022 g |
| HEPES (Hemi-sodium salt)[a] | 20.0 | 0.5 g |
| Glucose[a] | 5.0 | 0.09 g |
| Nanopure $H_2O$ to make | | 100 ml |
| Na-lactate 60% syrup | 10.0 | 0.09 ml |
| BSA (Fraction V)[a] | 0.3% (w/v) | 0.3 g |
| Gentamycin sulfate stock solution | 25 µg/ml | 0.25ml |

[a]Dissolve HEPES separately in about 50% of total volume of water. Dissolve glucose and BSA next, prior to mixing other ingredients

[b]pH of final solution adjusted to 7.3 with HCl

medium with 12% glycerol) was added in two equal aliquots of 5 ml each, 15 min apart. The sperm were then centrifuged (20 min at 850 x g; Eppendorf model 5810R (Germany); 5°C) and the supernatant removed. The concentration of sperm in the pellet was determined using a Nucleocounter (ChemoMetec SP-100; Denmark). Tris AB fraction, consisting of equal volumes of 20% egg-yolk Tris A fraction (SortEnsure™ Tris A Working Solution) and 20% egg-yolk Tris B fraction (SortEnsure™ Bovine Tris B Working Solution), was added to adjust the sperm concentration to 2.1 x $10^6$ spermatozoa/ml with a final glycerol concentration of 6%. The sperm were packaged in 0.25 ml straws, sealed, and frozen in liquid nitrogen vapor 25 min prior to immersion in liquid nitrogen for storage. A minimum of 3 h was required to elapse for cooling and glycerolization before freezing.

The other subsample was stored at 16°C (Minitube incubator, model DCR082W) for 20 h and then processed as previously described. (For 4 bulls, it was necessary to increase the stain level of some of the treatments by adding approximately 2-6 µl to increase the resolution for sorting.) Additionally, pH measurements were made just before staining the samples for sorting with Hoechst 33342. Finally, sorting parameters (event rate, live oriented cells, X sort rate, coincidence rate and splits) were also recorded and analyzed. Splits were evaluated for each sort by the depth of the split as a percentage of the height of the distributions.

Evaluation

Frozen straws were shipped to Colorado State University (Fort Collins, CO) for analysis. The analysis consisted of two primary components. The first component evaluated was motility, both subjectively (visual) and objectively. Two straws for each

treatment were thawed in a 37°C water bath for 30 sec. Motility, both total and progressive, was then observed and recorded approximately 15 min after thawing. Straws were coded to prevent evaluators from knowing the treatment and subjective motility was assessed by a single evaluator. CASA was done using the fluorescence capability of the machine and 10 fields of view and a minimum of 200 cells were counted for each treatment.

The second analysis performed was the percent viable sperm, evaluated using flow cytometry as described by Purdy and Graham (2004). Briefly, 0.25 ml samples were incubated with 3.5 µl of propidium iodide (PI; 2.4 mM solution in water; Molecular Probes, Eugene OR). Sorting parameters were gated off of the Hoechst 33342 stain in the sample and PI. Following the 15 min incubation, the samples were diluted with 0.20 ml TALP, filtered through a 20 µm nylon mesh, and analyzed. One straw from each treatment was processed according to this method.

For both the motility and the live/dead analyses, straws were thawed randomly and placed into numbered tubes to provide a "blind" analysis.

Statistical Analysis

Data were subjected to a mixed model ANOVA. Bulls were considered a random effect and treatments and sort times were fixed effects. Where two straws were evaluated for each treatment, the values were averaged. The statistical analysis included all main effects as well as all first order interactions. Least squares means are presented. Dunnett's method was used as a multiple comparison procedure to compare differences of means to the control.

## RESULTS AND DISCUSSION

The addition of the buffer to the semen samples reduced the drop in pH observed in the control samples. The pH in buffered samples did not drop below 6.08 whereas that in the control sample dropped to 5.96 ($P < 0.01$; Table 5.2). However, the pH still decreased more then desired. Adding sufficient buffer to control the pH would require diluting the samples to a greater extent and that could compromise sortability.

Table 5.2 shows treatment means for both sort times for selected responses (10 bulls included in statistical analysis). Adding TEST to the sperm resulted in an improvement compared to the Control samples in pH only ($P < 0.001$). However adding egg yolk with TEST resulted in slightly superior responses compared to the Control for many parameters, and this treatment significantly improved several important sorting parameters, specifically the proportion of live oriented cells ($P < 0.1$). It also resulted in fewer dead cells after storage ($P < 0.05$). Improving these parameters is important because they both affect the speed and accuracy of the sorting process.

Treatment 3 – diluting 1 part buffer + egg yolk to 2 parts neat semen (first ejaculate) – was not beneficial for sperm health after sorting and freezing compared to the Control, Treatment 1 or Treatment 2 (Table 5.3). It did show some improvement at the 20 h sort for the sort rate of X-chromosome bearing cells ($P < 0.05$). However, Treatment 4 – same media solution as Treatment 3 but applied to the second ejaculate of the same bull – was somewhat better than Treatment 3 in terms of sperm health after sorting and freezing. Other research not concerning sperm sexing has shown similar results (Seidel and Foote, 1969; Nel-Themaat et al., 2006). Finally, both Treatments 3 and 4 resulted in

Table 5.2: Treatment means for selected responses for the indicated sort times. 10 bulls and 3 treatments were included in the statistical analysis – Experiment 4

| | | Treatment | | | | | |
|---|---|---|---|---|---|---|---|
| | | *Control*[A] | | *Treatment 1*[B] | | *Treatment 2*[C] | |
| | | 0 h | 20 h | 0 h | 20 h | 0 h | 20 h |
| Analysis | Response | | | | | | |
| **Motility** | *Subjective Total Motility (%)* | 54.8 | 46.8 | 57.0[b] | 50.0 | 56.8[b] | 46.0 |
| | *Subjective Progressive Motility (%)* | 48.2 | 37.0 | 50.0[b] | 45.0 | 50.2[b] | 40.8 |
| | *CASA Total Motility (%)* | 58.0 | 52.2 | 56.6 | 52.7 | 55.8 | 53.1 |
| | *CASA Progressive Motility (%)* | 33.0 | 27.6 | 30.8 | 26.2 | 30.0 | 30.0 |
| **Sorting Parameters** | *Event Rate ($10^3$/sec)* | 30.0 | 28.1 | 30.6 | 27.6 | 31.5[b] | 27.2 |
| | *Live Oriented Cells (%)* | 55.7[c] | 44.6 | 52.7[c] | 44.0 | 55.4[c] | 48.5[a] |
| | *X Sort Rate ($10^3$/sec)* | 4.8[c] | 3.7 | 4.9[c] | 3.5 | 5.0[c] | 3.9 |
| | *Coincidence Rate ($10^3$/sec)* | 5.5 | 4.9 | 5.9[b] | 4.9 | 6.6 | 4.8 |
| | *Death Rate (%)* | 19.7[c] | 32.8 | 20.4[c] | 33.1 | 16.3[c] | 28.4[b] |
| | *Split (%)* | 37.3 | 36.6 | 36.7 | 34.5 | 37.9 | 31.5 |
| | *pH* | 6.59[c] | 5.96 | 6.97[c] | 6.23[c] | 6.87[c] | 6.08 |
| **Live/Dead** | *% Live* | 52.3 | 48.4 | 53.5 | 49.8 | 55.1 | 48.9 |

[A] Nothing added; [B] 25 mM TEST; [C] 25 mM TEST + 2% egg yolk

Differs from 20 h sort control: [a] ($P < 0.1$); [b] ($P < 0.05$); [c] ($P < 0.001$)

Table 5.3: Treatment means for selected responses for the indicated sort times. 8 bulls and 5 treatments were included in the statistical analysis – Experiment 4

| | | Treatment | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | *Control*[A] | | *Treatment 1*[B] | | *Treatment 2*[C] | | *Treatment 3*[D] | | *Treatment 4*[E] | |
| | | 0 h | 20 h | 0 h | 20 h | 0 h | 20 h | 0 h | 20 h | 0 h | 20 h |
| Analysis | Response | | | | | | | | | | |
| **Post-Thaw Motility** | *Subjective Total Motility (%)* | 51.3 | 44.2 | 54.7 | 48.4 | 53.1 | 42.2 | 55.3 | 50.6 | 55.6[a] | 51.3 |
| | *Subjective Progressive Motility (%)* | 44.7 | 37.9 | 49.1 | 43.4 | 46.9 | 36.9 | 50.3 | 45.3 | 50.6 | 42.5 |
| | *CASA Total Motility (%)* | 57.2 | 53.5 | 54.5 | 50.4 | 53.0 | 50.8 | 57.2 | 44.5 | 61.0 | 54.9 |
| | *CASA Progressive Motility (%)* | 33.2 | 29.5 | 31.8 | 26.5 | 29.0 | 29.8 | 33.3 | 24.9 | 33.9 | 32.6 |
| **Sorting Parameters** | *Event Rate ($10^3$/sec)* | 30.0 | 27.9 | 30.0 | 27.3 | 31.0 | 26.5 | 26.8 | 28.1 | 27.4 | 26.9 |
| | *Live Oriented Cells (%)* | 54.6[c] | 43.0 | 50.6[b] | 41.6 | 54.0[c] | 46.4 | 54.0[c] | 47.1 | 54.8[c] | 47.3 |
| | *X Sort Rate ($10^3$/sec)* | 4.8[b] | 3.5 | 4.7[b] | 3.3 | 4.8[b] | 3.7 | 4.4[a] | 4.0[b] | 4.7[b] | 3.8 |
| | *Coincidence Rate ($10^3$/sec)* | 5.5 | 4.9 | 5.8 | 5.0 | 6.6[a] | 4.7 | 5.0 | 5.2 | 5.0 | 4.7 |
| | *Death Rate (%)* | 21.8[c] | 34.3 | 23.1[c] | 36.0 | 18.2[c] | 31.0 | 18.4[c] | 28.3[b] | 14.0[c] | 24.8[c] |
| | *Split (%)* | 36.4 | 35.6 | 33.4 | 34.5 | 32.5 | 28.6 | 35.1 | 35.1 | 29.3 | 32.3 |
| | *pH* | 6.62[c] | 6.02 | 6.99[c] | 6.29[b] | 6.89[c] | 6.13 | 6.83[c] | 5.99 | 6.84[c] | 6.12 |
| **Live/Dead** | *% Live* | 52.5 | 46.6 | 50.6 | 47.0 | 53.9 | 47.3 | 53.0 | 46.3 | 57.0[b] | 51.2 |

[A] Nothing added; [B] 25 mM TEST; [C] 25 mM TEST + 2% egg yolk; [D] 25 mM TEST + 2% egg yolk applied to first ejaculate; [E] 25 mM TEST + 2% egg yolk applied to second ejaculate

Differs from 20 h sort control: [a] ($P < 0.1$); [b] ($P < 0.05$); [c] ($P < 0.001$)

significantly lower proportions of dead cells ($P < 0.05$ and $P < 0.001$, respectively) compared to the other treatments and Control.

As noted above, TEST was not different from the appropriate control for any response other than pH. On the other hand, TEST + egg yolk improved several sperm responses; in particular, it maintained more live, oriented sperm and resulted in a lower percentage of dead cells during sorting. Although not statistically significant, the post-thaw progressive motility (whether measured subjectively or by CASA) was higher for this treatment than for control sperm. Note that there was no significant advantage or disadvantage of Treatment 2 for other measurements of sperm health or sortability (see data for all responses in Appendix Table 6A).

## CONCLUSIONS

Incubating bull sperm in a TEST buffer solution or a TEST + egg yolk solution for up to 20 h prior to sex sorting was evaluated. TEST + egg yolk maintained higher responses then the Control or TEST. It improved the percentage of live oriented cells ($P < 0.1$) and decreased the proportion of dead cells during sorting ($P < 0.05$). pH was kept the highest in samples incubated in TEST, but a decline in pH was seen in all groups over time. Second ejaculates performed better then first ejaculates, as demonstrated by slightly higher results with Treatment 4 (second ejaculates) compared to Treatment 3 (first ejaculates). However, Treatment 3 was comparable to the Control, TEST, and TEST + egg yolk. In conclusion, TEST + egg yolk was the most advantageous treatment for this experiment. It improved the sperm sort rates about 10% because of fewer dead sperm when sorting occurred 20 h after sperm collection. Additionally, survival post-thaw was improved, though not significantly.

## LITERATURE CITED


Al-Somai N, Vishwanath R, Molan PC, Shannon P. 1994 Anionic and cationic components from aggregates in bovine seminal plasma and their effects on sperm motility. Mol Reprod Dev 39: 328-336.

Amann RP, Seidel GE, Jr (eds). 1982. Prospects for sexing mammalian sperm. Boulder, CO: Colorado University Press. 288 pp.

Amann RP. 1999. Issues affecting commercialization of sexed sperm. Theriogenology 52: 1441-1457.

Bergeron A, Brindle Y, Blondin P, Manjunath P. 2007. Milk caseins decrease the binding of the major bovine seminal plasma proteins to sperm and prevent lipid loss from the sperm membrane during sperm storage. Biol Reprod 77: 120-126.

Bergeron A, Manjunath P. 2006. New insights towards understanding the mechanisms of sperm protection by egg yolk and milk. Mol Reprod Dev 73: 1338-1344.

Christensen P, Boelling D, Pedersen KM, Korsgaard IR, Jensen J. 2005. Relationship between sperm viability as determined by flow cytometry and nonreturn rate of dairy bulls. J Androl 26: 98-106.

Christensen P, Brockhoff PB, Lehn-Jensen H. 1999. The relationship between semen quality and the non-return rate of bulls. Reprod Dom Anim 34: 503-507.

Clulow JR, Evans G, Morris LHA, Maxwell WMC. 2009. Factors influencing the "sortability" of stallion spermatozoa into X- and Y-chromosome bearing populations. Anim Reprod Sci 113: 220-228.

Davis HP, Underbjerg GKL, Williams NK. 1940. The effect of storage temperatures upon certain characteristics of bovine semen. J Dairy Sci 23: 1057-1068.

Davis IS, Bratton RW, Foote RH. 1963. Livability of bovine spermatozoa at 5°C in Tris-buffered and citrate-buffered yolk-glycerol extenders. J Dairy Sci 46: 57-60.

De Graaf SP, Beilby KH, Underwood SL, Evans G, Maxwell WMC. 2009. Theriogenology 71: 89-97.

De Pauw IMC, Van Soom A, Mintiens K, Verberckmoes S, de Kruif A. 2003. In vitro survival of bovine spermatozoa stored at room temperature under epididymal conditions. Theriogenology 59: 1093-1107.

Farrell PB, Presicce GA, Brockett CC, Foote RH. 1998. Quantification of bull sperm characteristics measured by computer assisted sperm analysis (CASA) and their relationship to fertility. Theriogenology 49: 871-879.

Foote RH, Brockett CC, Kaproth MT. 2002. Motility and fertility of bull sperm in whole milk extender containing antioxidants. Anim Reprod Sci 71: 13-23.

Foote RH, Dunn HO. 1962. Motility and fertility of bull semen extended at high rates in yolk extender containing catalase. J Dairy Sci 45: 1237-1241.

Foote RH, Gray LC, Young DC, Dunn HO. 1960. Fertility of bull semen stored up to four days at 5°C in 20% egg yolk extenders. J Dairy Sci 43: 1330-1334.

Foote RH. 1970. Fertility of bull semen at high extension rates in Tris-buffered extenders. J Dairy Sci 53: 1475-1479.

Foote RH. 1972. Tris and other organic buffers for the conservation of semen of various species. Animal reproduction and artificial insemination. Edizioni Agricole, Bologna p 99-105.

Foote RH. 1978. Extenders and extension of unfrozen semen. In: Salisbury GW, VanDemark ML, Lodge JR (eds). Physiology of reproduction and artificial insemination of cattle. San Francisco: Freeman. p 442-477.

Foulkes JA. 1977. The separation of lipoproteins from egg yolk and their effect on the motility and integrity of bovine spermatozoa. J Reprod Fertil 49: 277-284.

Garner DL, Gledhill BL, Pinkel D, Lake S, Stephenson D, Van Dilla MA, Johnson LA. 1983. Quantification of the X-and Y-chromosome bearing spermatozoa of domestic animals by flow cytometry. Biol Reprod 28: 312-321.

Garner DL, Johnson LA, Yue ST, Roth BL, Haugland RP. 1994. Dual DNA staining assessment of bovine sperm viability. J Androl 15: 620-629.

Garner DL, Johnson LA. 1995. Viability assessment of mammalian sperm using SYBR-14 and propidium iodide. Biol Reprod 53: 276-284.

Garner DL, Thomas CA, Allen CH. 1997. Effect of semen dilution on bovine sperm viability as determined by dual-DNA staining and flow cytometry. J Androl 18: 324-331.

Garner DL, Thomas CA, Gravance CG, Marshall CE, DeJarnette JM, Allen CH. 2001. Seminal plasma addition attenuates the dilution effect in bovine sperm. Theriogenology 56: 31-40.

Garner DL. 2006. Flow cytometric sexing of mammalian sperm. Theriogenology 65: 943-957.

Garner DL. 2009. Hoechst 33342: The dye that enabled differentiation of living X-and Y-chromosome bearing mammalian sperm. Theriogenology 71: 11-21.

Gledhill BL, Pinkel D, Garner DL, Van Dilla MA. 1982. Identifying X- and Y-chromosome-bearing sperm by DNA content: Retrospective perspectives and prospective opinions. In: Amann RP, Seidel GE, Jr (eds). Prospects for sexing mammalian sperm. Boulder, CO: Colorado University Press. p 177-191.

Gonzalez O, Santacana GE. 2001. Effect of low temperature on tracheal smooth muscle contractile and relaxing responses evoked by electrical field stimulation. P R Health Sci J 20: 237-244.

Good NE, Wingest GD, Winter W, Connolly TN, Izawa S, Singh RMM. 1966. Hydrogen ion buffers for biological research. Biochem 5: 467-477.

Graham EF, Crabo BG, Brown KI. 1972. Effect of some zwitter ion buffers on the freezing and storage of spermatozoa 1. Bull. J Dairy Sci 55: 372-378.

Gravance CG, Davis RO. 1995. Automated sperm morphometry analysis (ASMA) in the rabbits. J Androl 16: 88-93.

Guyer MF. 1910. Accessory chromosomes in man. Biol Bull Marine Biol Lab (Woods Hole) 19: 219-221.

Hollinshead FK, Evans G, Evans KM, Catt SL, Maxwell WMC, O'Brien JK. 2004. Birth of lambs of a pre-determined sex after in vitro production of embryos using frozen-thawed sex-sorted and re-frozen-thawed ram spermatozoa. Reproduction 127: 557-568.

Holt WV, Palomo MJ. 1996. Optimization of a continuous real time computerized semen analysis system for ram sperm motility assessment, and evaluation of four methods of semen preparation. Reprod Fertil Dev 8: 219-230.

Ijaz A, Hunter AG. 1989. Induction of bovine sperm capacitation by TEST-yolk semen extender. J Dairy Sci 72: 2683-2690.

Johnson LA, Flook JP, Hawk HW. 1989. Sex preselection in rabbits: live births from X and Y sperm separated by DNA and cell sorting. Biol Reprod 41: 199-203.

Johnson LA, Pinkel D. 1986. Modification of a laser-based flow cytometer for high resolution DNA analysis of mammalian spermatozoa. Cytometry 7: 268-273.

Johnson LA, Welch GR, Garner DL. 1994. Improved flow sorting resolution of X and Y chromosomes bearing viable sperm separation using dual staining and dead cell gating. Cytometry 17: 83. (Abstract).

Johnson LA, Welch GR, Rens W. 1999. The Beltsville Sperm Sexing Technology: High-speed sperm sorting gives improved sperm output for in vitro fertilization and AI. J Anim Sci 77: 213-220.

Johnson LA, Welch GR. 1999. Sex preselection: High-speed flow cytometric sorting of X and Y sperm for maximum efficiency. Theriogenology 52: 1323-1341.

Kamentsky LA, Melamed RR. 1967. Spectrophotometric cell sorter. Science 156: 1364-1365.

Kathiravan P, Kalatharan J, Karthikeya G, Rengarajan K, Kadirvel G. 2010. Objective sperm motion analysis to assess dairy bull fertility using computer-aided system – A review. Reprod Dom Anim (in press; PM I.D. 20403134).

Kiddy CA, Hafs HD (eds). 1971. Sex ratio at birth – Prospects for control. J Anim Sci Symp-Suppl: 104 pp.

Killian GJ, Chapman DA, Rogowski LA. 1993. Fertility-association proteins in Holstein bull seminal plasma. Biol Reprod 49: 1202-1207.

Killian GJ. 1992. Fertility factors in seminal plasma. Proc 14th NAAB Tech Conf Artif Insem Reprod. pp. 33-38.

Manjunath P, Nauc V, Bergeron A, Menard M. 2002. Major proteins of bovine seminal plasma bind to the low-density lipoprotein fraction of hen's egg yolk. Biol Reprod 67: 1250-1258.

Mann T, Lutwak-Mann C. 1981. Male reproductive function and semen: Themes and trends in physiology, biochemistry and investigative andrology. Berlin; New York: Springer-Verlag. 495 pp.

Maxwell WMC, Johnson LA. 1999. Physiology of spermatozoa at high dilution rates: The influence of seminal plasma. Theriogenology 52: 1353-1362.

Maxwell WMC, Welch GR, Johnson LA. 1997. Viability and membrane integrity of spermatozoa after dilution and flow cytometric sorting in the presence or absence of seminal plasma. Reprod Fertil Dev 8: 1165-1178.

Moruzzi JF. 1979. Selecting a mammalian species for the separation of X- and Y-chromosome-bearing spermatozoa. J Reprod Fertil 57: 319-323.

Mousset-Simeon N, Rives N, Masse L, Chevallier F, Mace B. 2004. Comparison of six density gradient media for selection of cryopreserved donor spermatozoa. J Androl 25: 881-884.

Nel-Themaat L, Harding GD, Chandler JE, Chenevert JF, Damiani P, Fernandez JM, Humes PE, Pope CE, Godke RA. 2006. Quality and freezing qualities of first and second ejaculates collected from endangered Gulf Coast Native rams. Anim Reprod Sci 95: 251-261.

Norman C, Johnson CE, Porterfield ID, Dunbar RS. 1958. Effect of pH on the life-span and metabolism of bovine sperm kept at room temperatures. J Dairy Sci 41: 1803-1812.

Norman C, Johnson CE, Porterfield ID, Goldberg E, Dunbar RS, Min HS, Dunn HO. 1962. Survival of bovine sperm left at variable temperature in coconut milk extender. J Agric Sci (Camb) 59: 33-39.

Pace MM, Graham EF. 1974. Components in egg yolk which protect bovine spermatozoa during freezing. J Anim Sci 39: 1144-1149.

Parrish JJ, Foote RH. 1980. Organic buffer for freezing bovine spermatozoa. J Anim Sci 1(Suppl.): 314-315. (Abstract).

Phillips PH. 1939. Preservation of bull semen. J Biol Chem 130: 415. (Letter to the Editors)

Pinkel D, Gledhill BI, Van Dilla MA, Stephenson D, Watchmaker G. 1982. High resolution DNA measurements of mammalian sperm. Cytometry 3: 1-9.

Prathalingam NS, Holt WV, Revell SG, Jones S, Watson PF. 2006. Dilution of spermatozoa results in improved viability following a 24 h storage period but decreased acrosome integrity following cryopreservation. Anim Reprod Sci 91: 11-22.

Prendergast E, Vishwanath R, Shannon P. 1995. Protection of bovine sperm from the deleterious effects of seminal plasma by charged lipoprotein fractions of egg yolk. Proc 27th Ann Conf Austr Soc Reprod Biol Melbourne 27: 50. (Abstract).

Purdy PH, Graham JK. 2004. Effect of cholesterol-loaded cyclodextrin on the cryosurvival of bull sperm. Cryobiology 48: 36-45.

Rath D, Moench-Tegeder G, Taylor U, Johnson LA. 2009. Improved quality of sex-sorted sperm: A prerequisite for wider commercial application. Theriogenology 71: 22-29.

Rens W, Welch GR, Houck DW, van Oven CH, Johnson LA. 1996. Slit-scan flow cytometry for consistent high resolution DNA analysis of X- and Y-chromosome bearing sperm. Cytometry 25: 191-199.

Rens W, Welch GR, Johnson LA. 1998. A novel nozzle for more efficient sperm orientation to improve sorting efficiency of X and Y chromosome bearing sperm. Cytometry 33: 476-481.

Rens W, Welch GR, Johnson LA. 1999. Improved flow cytometric sorting of X- and Y-chromosome bearing sperm: Substantial increase in yield of sexed semen. Molec Reprod Dev 52: 50-56.

Samardzija M, Karadjole M, Matkovic M, Cergolj M, Getz I, Dobranic T, Tomaskovic A, Petric J, Surina J, Grizelj J, Karadjole T. 2006. A comparison of BoviPure® and Percoll® on bull sperm separation protocols for IVF. Anim Reprod Sci 91: 237-247.

Schenk JL, Cran DG, Everett RW, Seidel GE, Jr. 2009. Pregnancy rates in heifers and cows with cryopreserved sexed sperm: Effects of sperm numbers per inseminate, sorting pressure and sperm storage before sorting. Theriogenology 71: 717-728.

Schenk JL, Suh TK, Cran DG, Seidel GE, Jr. 1999. Cryopreservation of flow-sorted bovine spermatozoa. Theriogenology 52: 1375-1391.

Seidel GE, Jr, Foote RH. 1969. Influence of semen collection interval and tactile stimuli on semen quality and sperm output in bulls. J Dairy Sci 52: 1074-1079.

Seidel GE, Jr, Garner DL. 2002. Current status of sexing mammalian spermatozoa. Reproduction 124: 733-743.

Seidel GE, Jr. 2007. Overview of sexing sperm. Theriogenology 68: 443-446.

Seidel GE, Jr. 2009. Sperm sexing technology – The transition to commercial application: An introduction to the symposium "Update on sexing mammalian sperm". Theriogenology 71: 1-3.

Shannon P, Curson B, Rhodes AP. 1984. Relationship between total spermatozoa per insemination and fertility of bovine semen stored in Caprogen at ambient temperature. N Z J of Agric Res 27: 35-41.

Shannon P, Curson B. 1982. Kinetics of the aromatic L-amino acid oxidase from dead bovine spermatozoa and the effect of catalase on fertility of diluted bovine semen stored at 5°C and ambient temperatures. J Reprod Fert 64: 463-467.

Shannon P, Curson B. 1983. Effect of egg yolk levels on the fertility of diluted bovine sperm stored at ambient temperatures. N Z J of Agric Res 26: 187-189.

Shannon P, Curson B. 1984. Effect of storage temperature on the viability and fertility of bovine sperm diluted and stored in Caprogen. N Z J Agric Res 27: 173-177.

Shannon P. 1964. The effect of diluents containing glycine, and glycine and glycerol, on the fertility of diluted bovine semen. N Z J Agric Res 7: 357-363.

Stap J, Hoebe RA, Merton JS, Haring RM, Bakker PJM, Aten, JA. 1998. Improving the resolution of cryopreserved X- and Y-sperm during DNA flow cytometric analysis with the addition of Percoll to quench the fluorescence of dead sperm. J Anim Sci 76: 1896-1902.

Suh TK, Schenk JL, Seidel GE, Jr. 2005. High pressure flow cytometric sorting damages sperm. Theriogenology 64: 1035-1048.

Tardif AL, Farrell PB, Trouern-Trend V, Foote RH. 1997. Computer-assisted sperm analysis for assessing initial semen quality and changes during storage at 5°C. J Dairy Sci 80: 1606-1612.

Underwood SL, Bathgate R, Maxwell WMC, Evans G. 2009. Development of procedures for sex-sorting frozen-thawed bovine spermatozoa. Reprod Dom Anim 44: 460-466.

Vera-Munoz O, Amirat-Briand L, Diaz T, Vasquez L, Schmidt E, Desherces S, Anton M, Bencharif D, Tainturier D. 2009. Effect of semen dilution to low-sperm number per dose on motility and functionality of cryopreserved bovine spermatozoa using low-density lipoproteins (LDL) extender: Comparison to Triladyl® and Bioxcell®. Theriogenology 71: 895-900.

Verberckmoes S, Van Soom A, Dewulf J, de Kruif A. 2005. Comparison of three diluents for the storage of fresh bovine semen. Theriogenology 63: 912-922.

Verberckmoes S, Van Soom A, Dewulf J, De Pauw I, de Kruif A. 2004. Storage of fresh bovine semen in a diluent based on the ionic composition of cauda epididymal plasma. Reprod Dom Anim 39: 410-416.

Vishwanath R, Shannon P, Curson B. 1992. Cationic extracts of egg yolk and their effects on motility, survival and fertilising ability of bull sperm. Anim Reprod Sci 29: 185-194.

Vishwanath R, Shannon P. 1997. Do sperm cells age? A review of the physiological changes in sperm during storage at ambient temperature. Reprod Fertil Dev 9: 321-331.

Vishwanath R, Shannon P. 2000. Storage of bovine semen in liquid and frozen state. Anim Reprod Sci 62: 23-53.

# APPENDIX I

# CHAPTER II, III, AND IV EXTENDED TABLES

Table 1A: Means and significances of main effects for 0 and 20 h data for all responses – Experiment 1

| | | Treatment | | | | | | Ejaculate # | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | *Control* | | *MOPS* | | *MOPS + EY* | | *Ejaculate 1* | | *Ejaculate 2* | |
| | | 0 hr | 20 hr | 0 hr | 20 hr | 0 hr | 20 hr | 0 hr | 20 hr | 0 hr | 20 hr |
| Analysis | Response | | | | | | | | | | |
| **Subjective Motility** | *Total Motility (%)* | $38^{ij}$ | $28^{a}$ | $42^{i}$ | $37^{b}$ | $34^{j}$ | $30^{ab}$ | $35^{q}$ | $28^{c}$ | $41^{r}$ | $35^{d}$ |
| | *Progressive Motility (%)* | $32^{ij}$ | $22^{a}$ | $36^{i}$ | $30^{b}$ | $27^{j}$ | $23^{ab}$ | $29^{q}$ | $22^{c}$ | $34^{r}$ | $28^{d}$ |
| **CASA Parameters** | *Total Motility (%)* | 38 | 32 | 42 | 36 | 37 | 30 | $35^{q}$ | $29^{c}$ | $44^{r}$ | $36^{d}$ |
| | *Progressive Motility (%)* | 20 | 17 | 21 | 20 | 18 | 17 | $17^{o}$ | 16 | $22^{p}$ | 20 |
| | *VAP (μm/s)* | $80.0^{k}$ | $79.3^{s}$ | $78.7^{kl}$ | $75.6^{t}$ | $76.6^{l}$ | $75.1^{t}$ | 77.2 | 75.4 | 79.6 | 77.9 |
| | *VSL (μm/s)* | 63.3 | 63.7 | 62.2 | 61.1 | 61.0 | 61.2 | 61.1 | 61.3 | 63.2 | 62.7 |
| | *VCL (μm/s)* | $148.8^{k, ij}$ | $146.6^{as}$ | $148.2^{kl, i}$ | $142.0^{b, st}$ | $143.5^{lj}$ | $140.1^{ab, t}$ | 145.7 | 141.0 | 148.0 | 144.8 |
| | *ALH (μm)* | 6.0 | 6.0 | 6.1 | 6.0 | 5.8 | 5.8 | 6.0 | $6.1^{u}$ | 6.0 | $5.9^{v}$ |
| | *BCF (beats/s)* | $21.4^{i}$ | 21.9 | $22.5^{j}$ | 22.4 | $22.0^{ij}$ | 22.2 | 21.7 | 21.8 | 22.3 | 22.6 |
| | *STR (%)* | 81.0 | 81.9 | 81.0 | 82.6 | 81.6 | 83.0 | 81.2 | 82.7 | 81.3 | 82.2 |
| | *LIN (%)* | 46.1 | 47.3 | 45.6 | 46.8 | 46.8 | 47.3 | 45.8 | 47.1 | 46.5 | 47.2 |
| | *VD: Rapid (%)* | 33.8 | 26.9 | 35.9 | 29.8 | 31.7 | 24.7 | $29.9^{q}$ | $23.9^{c}$ | $37.7^{r}$ | $30.4^{d}$ |
| | *VD: Medium (%)* | $4.6^{k}$ | 4.9 | $6.4^{l}$ | 5.7 | $5.5^{kl}$ | 4.9 | $4.7^{q}$ | $4.6^{u}$ | $6.3^{r}$ | $5.7^{v}$ |
| | *VD: Slow (%)* | 22.3 | 25.6 | 22.7 | 24.0 | 23.0 | 22.8 | 21.9 | 24.6 | 23.5 | 23.6 |
| | *VD: Static (%)* | 39.3 | 42.6 | 35.0 | 40.6 | 39.8 | 47.6 | $43.6^{q}$ | $46.9^{c}$ | $32.5^{r}$ | $40.3^{d}$ |
| **Sort Information** | *Event Rate ($10^3$/sec)* | $34.4^{i}$ | 33.2 | $33.1^{j}$ | 34.2 | $33.1^{j}$ | 33.3 | 33.2 | $34.3^{c}$ | 33.8 | $32.8^{d}$ |
| | *Live Oriented Cells (%)* | 56.2 | $53.9^{a, st}$ | 55.7 | $53.1^{ab, s}$ | 57.5 | $56.9^{bt}$ | 55.9 | 55.2 | 56.9 | 54.2 |
| | *X Sort Rate ($10^3$/sec)* | 5.1 | $4.8^{a}$ | 5.0 | $4.8^{a}$ | 5.2 | $5.1^{b}$ | 5.0 | 5.0 | 5.1 | 4.8 |
| | *Coincidence Rate ($10^3$/sec)* | $7.4^{ik}$ | 6.8 | $6.8^{j, kl}$ | 7.3 | $6.7^{ij, l}$ | 6.8 | 6.9 | $7.3^{u}$ | 7.1 | $6.7^{v}$ |
| | *Split (%)* | 47.9 | 42.4 | 45.9 | 43.8 | 47.3 | 42.4 | 45.0 | $47.7^{u}$ | 49.1 | $38.0^{v}$ |
| | *pH* | $6.4^{m}$ | $5.9^{e}$ | $6.7^{n}$ | $6.1^{f}$ | $6.7^{m}$ | $6.1^{f}$ | $6.5^{q}$ | $5.9^{g}$ | $6.7^{r}$ | $6.1^{h}$ |
| **Live/Dead** | *% Live* | 43.2 | 43.1 | 50.5 | 44.8 | 46.0 | 40.2 | 44.7 | 40.2 | 48.4 | 45.2 |

$^{ab, uv}$ Means without common superscripts within rows differ for 20 h sort (P<0.1); $^{ij, op}$ Means without common superscripts within rows differ for 0 h sort (P<0.1)
$^{st, cd}$ Means without common superscripts within rows differ for 20 h sort (P<0.05); $^{kl, qr}$ Means without common superscripts within rows differ for 0 h sort (P<0.05)
$^{ef, gh}$ Means without common superscripts within rows differ for 20 h sort (P<0.001); $^{mn}$ Means without common superscripts within rows differ for 0 h sort (P<0.001)

Table 2A: Motility responses by treatment for individual bulls for 20 h sort[a] – Experiment 1

| | Subjective Total Motility (%) | | | Subjective Progressive Motility (%) | | | CASA Total Motility (%) | | | CASA Progressive Motility (%) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Bull I.D. | Control | MOPS | MOPS + EY | Control | MOPS | MOPS + EY | Control | MOPS | MOPS + EY | Control | MOPS | MOPS + EY |
| HO406 | 11.3 | 37.5 | 41.3 | 6.3 | 25.6 | 35.6 | 13.3 | 31.6 | 45.3 | 6.4 | 19.0 | 29.4 |
| HO416 | 36.3 | 34.4 | 34.4 | 30.6 | 29.4 | 28.8 | 44.8 | 29.0 | 27.9 | 26.6 | 12.0 | 14.8 |
| HO420 | 19.4 | 28.8 | 24.4 | 16.3 | 23.1 | 17.5 | 24.4 | 25.3 | 25.4 | 13.3 | 13.1 | 13.1 |
| HO445 | 38.1 | 51.3 | 50.6 | 28.8 | 43.8 | 41.9 | 34.9 | 54.4 | 45.3 | 21.1 | 33.5 | 29.9 |
| HO5705 | 16.3 | 30.0 | 16.3 | 10.0 | 24.4 | 10.0 | 26.0 | 27.5 | 18.1 | 11.4 | 12.3 | 8.5 |
| JE599 | 35.6 | 42.5 | 26.9 | 26.3 | 36.3 | 18.8 | 38.9 | 34.8 | 33.5 | 21.5 | 21.6 | 19.8 |
| JE607 | 48.8 | 46.3 | 21.3 | 40.6 | 38.8 | 12.5 | 44.8 | 56.9 | 17.0 | 23.0 | 31.3 | 7.1 |
| JE717 | 21.9 | 22.5 | 23.1 | 16.9 | 18.1 | 19.4 | 27.1 | 24.8 | 23.5 | 12.6 | 11.6 | 14.1 |
| Mean | 28.5 | 36.7 | 29.8 | 22.0 | 29.9 | 23.1 | 31.8 | 35.5 | 29.5 | 17.0 | 19.3 | 17.1 |

[a]Values are averages of 1st and 2nd ejaculates and the 2 evaluation times (after thawing)

Table 3A: Means of 0 and 2 h evaluation times for motility responses – Experiment 1

| | | **Treatment** | | | | | |
|---|---|---|---|---|---|---|---|
| | | *Control* | | *MOPS* | | *MOPS + EY* | |
| | | 0 h | 2 h | 0 h | 2 h | 0 h | 2 h |
| Analysis | Response | | | | | | |
| **Subjective Motility** | *Total Motility (%)* | 37.0 | 29.2[b] | 43.0 | 35.9[b] | 36.9 | 26.9[c] |
| | *Progressive Motility (%)* | 31.0 | 22.4[b] | 37.2 | 28.5[b] | 30.5 | 19.7[c] |
| **CASA Parameters** | *Total Motility (%)* | 39.1 | 30.8[c] | 44.3 | 33.5[c] | 38.3 | 28.3[c] |
| | *Progressive Motility (%)* | 22.9 | 14.0[c] | 26.0 | 14.5[c] | 22.8 | 12.7[c] |
| | *VAP (μm/s)* | 92.6 | 66.6[c] | 91.4 | 62.9[c] | 90.4 | 61.3[c] |
| | *VSL (μm/s)* | 72.6 | 54.3[c] | 72.0 | 51.3[c] | 72.0 | 50.1[c] |
| | *VCL (μm/s)* | 178.9 | 116.6[c] | 176.8 | 113.4[c] | 174.1 | 109.5[c] |
| | *ALH (μm)* | 6.8 | 5.3[c] | 6.8 | 5.2[c] | 6.7 | 5.1[c] |
| | *BCF (beats/s)* | 24.2 | 19.1[c] | 24.2 | 20.7[c] | 24.3 | 20.0[c] |
| | *STR (%)* | 79.9 | 83.0[b] | 80.3 | 83.3[b] | 81.0 | 83.7[b] |
| | *LIN (%)* | 44.1 | 49.3[b] | 44.4 | 48.0[b] | 45.1 | 49.0[b] |
| | *VD: Rapid (%)* | 37.3 | 23.4[c] | 41.7 | 23.9[c] | 36.1 | 20.3[c] |
| | *VD: Medium (%)* | 2.1 | 7.4[c] | 2.6 | 9.5[c] | 2.2 | 8.1[c] |
| | *VD: Slow (%)* | 24.3 | 23.6 | 24.0 | 22.6 | 22.8 | 23.1 |
| | *VD: Static (%)* | 36.3 | 45.7[b] | 31.6 | 44.0[c] | 39.0 | 48.5[b] |

Differs from respective 0 h: [a] ($P < 0.1$); [b] ($P < 0.05$); [c] ($P < 0.001$)

Table 4A: Composition of CUE (with 10% and 7.5% egg yolk) – Experiment 3

| **Ingredients** | **Amount/100 ml** | **Amount/100 ml** |
|---|---|---|
| Buffers: | | |
| Sodium citrate dehydrate (g.) | 1.45 | 1.45 |
| Sodium bicarbonate (g.) | 0.21 | 0.21 |
| Potassium chloride (g.) | 0.04 | 0.04 |
| Glucose (g.) | 0.3 | 0.3 |
| Glycine (g.) | 0.937 | 0.937 |
| Citric acid (g.) | 0.087 | 0.087 |
| Nanopure water (final volume) (ml.) | Add up to 100 ml | Add up to 100 ml |
| | | |
| Extenders: | | |
| Buffer (% by volume) | 90 | 92.5 |
| Egg yolk (% by volume)[a] | 10 | 7.5 |
| Gentamycin Sulfate stock solution (ml.) | 0.0256 | 0.0256 |

[a] Centrifuge egg yolk and use supernatant to add percent egg yolk

Table 5A: Treatment means and P values for all responses – Experiment 3

| | | **Treatment** | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | *Stored at 16°C* | | | | | | | | | | *Stored at 5°C* | |
| | | Trt 1: 9/12.5T | Trt 2: 6/12.5T | Trt 3: 9/25T | Trt 4: 6/25T | Trt 5: 9/50T | Trt 6: 6/50T | Trt 7: 9/12.5M | Trt 8: 9/25M | Trt 9: 6/25M | Trt 10: 9/50M | Trt 11: 5% EY | Trt 12: 2.5% EY |
| **Response** | **Pr > F** | | | | | | | | | | | | |
| *Total Motility (%)* | 0.0170 | 36.5 | 28.8 | 35.8 | 27.5 | 31.0 | 23.7 | 32.1 | 35.1 | 30.7 | 33.8 | 28.7 | 25.2 |
| *Progressive Motility (%)* | 0.1126 | 16.4 | 13.9 | 17.3 | 13.1 | 15.8 | 11.9 | 14.5 | 16.2 | 15.9 | 15.3 | 14.8 | 10.5 |
| *VAP (μm/s)* | <0.0001 | 97.6 | 89.3 | 97.3 | 83.4 | 91.3 | 82.8 | 93.0 | 99.9 | 92.7 | 96.5 | 93.8 | 88.9 |
| *VSL (μm/s)* | 0.0155 | 72.3 | 68.6 | 73.0 | 64.8 | 70.5 | 65.8 | 68.9 | 73.9 | 70.0 | 70.3 | 72.0 | 66.2 |
| *VCL (μm/s)* | 0.0001 | 195.3 | 177.8 | 194.0 | 164.0 | 179.4 | 162.9 | 186.5 | 195.1 | 182.3 | 191.2 | 184.4 | 175.3 |
| *ALH (μm)* | 0.0135 | 7.5 | 6.8 | 7.5 | 6.7 | 7.2 | 6.5 | 7.2 | 7.2 | 7.3 | 7.2 | 7.3 | 6.8 |
| *BCF (beats/s)* | 0.2840 | 22.8 | 22.5 | 22.4 | 22.4 | 22.3 | 22.7 | 22.5 | 23.8 | 22.6 | 22.4 | 23.8 | 23.5 |
| *STR (%)* | 0.0311 | 76.1 | 78.8 | 76.8 | 79.7 | 79.1 | 80.9 | 76.5 | 76.3 | 77.9 | 76.3 | 79.0 | 77.6 |
| *LIN (%)* | 0.5827 | 40.6 | 42.3 | 40.6 | 42.8 | 42.6 | 43.6 | 41.0 | 41.4 | 41.4 | 40.6 | 42.7 | 42.6 |
| *VD: Rapid (%)* | 0.0019 | 34.0 | 26.3 | 33.8 | 24.7 | 28.7 | 20.8 | 29.3 | 32.5 | 28.8 | 31.6 | 26.0 | 21.8 |
| *VD: Medium (%)* | 0.7633 | 2.4 | 2.5 | 2.1 | 2.8 | 2.3 | 3.1 | 2.8 | 2.6 | 2.8 | 2.3 | 2.8 | 3.3 |
| *VD: Slow (%)* | 0.1701 | 16.5 | 19.3 | 15.5 | 17.7 | 13.4 | 8.3 | 20.2 | 24.4 | 17.8 | 15.5 | 29.2 | 26.3 |
| *VD: Static (%)* | 0.0372 | 47.1 | 51.8 | 48.6 | 54.9 | 55.6 | 67.9 | 47.8 | 40.8 | 50.8 | 50.0 | 42.3 | 48.6 |
| *Start pH* | <0.0001 | 6.8 | 7.2 | 6.9 | 7.1 | 7.0 | 7.2 | 6.9 | 6.9 | 7.1 | 7.0 | 7.1 | 6.9 |
| *End pH* | <0.0001 | 6.2 | 6.8 | 6.2 | 6.8 | 6.5 | 7.0 | 6.4 | 6.4 | 6.9 | 6.8 | 6.9 | 6.7 |
| *pH Change* | <0.0001 | 0.69 | 0.32 | 0.67 | 0.33 | 0.53 | 0.13 | 0.52 | 0.54 | 0.15 | 0.19 | 0.24 | 0.18 |

Table 6A: Means and significances of main effects for all responses. 8 bulls and 5 treatments were included in the statistical analysis (P values were determined by Dunnett's method by comparison to the 20 h Control) – Experiment 4

| | | Treatment | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | *Control* | | *TEST* | | *TEST + EY* | | *Ejaculate 1* | | *Ejaculate 2* | |
| | | 0 h | 20 h | 0 h | 20 h | 0 h | 20 h | 0 h | 20 h | 0 h | 20 h |
| Analysis | Response | | | | | | | | | | |
| **Subjective Motility** | *Total Motility (%)* | 51.3 | 44.2 | 54.7 | 48.4 | 53.1 | 42.2 | 55.3 | 50.6 | 55.6[a] | 51.3 |
| | *Progressive Motility (%)* | 44.7 | 37.9 | 49.1 | 43.4 | 46.9 | 36.9 | 50.3 | 45.3 | 50.6 | 42.5 |
| **CASA Parameters** | *Total Motility (%)* | 57.2 | 53.5 | 54.5 | 50.4 | 53.0 | 50.8 | 57.2 | 44.5 | 61.0 | 54.9 |
| | *Progressive Motility (%)* | 33.2 | 29.5 | 31.8 | 26.5 | 29.0 | 29.8 | 33.3 | 24.9 | 33.9 | 32.6 |
| | *VAP (μm/s)* | 94.5 | 88.5 | 93.3 | 85.3 | 88.0 | 89.2 | 87.7 | 85.4 | 89.4 | 88.6 |
| | *VSL (μm/s)* | 73.5 | 69.1 | 72.4 | 65.4 | 67.4 | 70.8 | 68.9 | 66.9 | 69.4 | 71.0 |
| | *VCL (μm/s)* | 174.5 | 168.7 | 173.5 | 163.6 | 169.8 | 167.2 | 161.9 | 161.3 | 166.9 | 160.7 |
| | *ALH (μm)* | 6.5 | 6.6 | 6.5 | 6.6 | 6.3 | 6.7 | 6.2 | 6.8 | 6.3 | 6.5 |
| | *BCF (beats/s)* | 24.9 | 23.8 | 24.4 | 23.8 | 24.1 | 23.7 | 23.6 | 22.3 | 24.0 | 24.4 |
| | *STR (%)* | 79.9 | 79.7 | 79.4 | 78.9 | 78.7 | 80.8 | 80.4 | 80.1 | 79.6 | 81.6 |
| | *LIN (%)* | 46.0 | 44.4 | 45.0 | 43.4 | 43.2 | 45.8 | 46.3 | 45.3 | 45.4 | 48.1[b] |
| | *VD: Rapid (%)* | 53.8 | 48.8 | 51.4 | 45.8 | 48.9 | 46.9 | 52.7 | 40.4 | 56.1 | 50.1 |
| | *VD: Medium (%)* | 3.5 | 4.2 | 3.1 | 4.7 | 4.0 | 4.0 | 4.5 | 4.1 | 5.1 | 5.0 |
| | *VD: Slow (%)* | 21.8 | 18.7 | 20.9 | 20.8 | 21.3 | 18.4 | 19.5 | 24.1 | 22.6 | 24.3 |
| | *VD: Static (%)* | 21.1 | 28.0 | 24.8 | 28.8 | 25.8 | 30.9 | 23.4 | 31.4 | 16.3 | 20.6 |
| **Sort Information** | *Event Rate ($10^3$/sec )* | 30.0 | 27.9 | 30.0 | 27.3 | 31.0 | 26.5 | 26.8 | 28.1 | 27.4 | 26.9 |
| | *Live Oriented Cells (%)* | 54.6[c] | 43.0 | 50.6[b] | 41.6 | 54.0[c] | 46.4 | 54.0[c] | 47.1 | 54.8[c] | 47.3 |
| | *X Sort Rate ($10^3$/sec)* | 4.8[b] | 3.5 | 4.7[b] | 3.3 | 4.8[b] | 3.7 | 4.4[a] | 4.0[b] | 4.7[b] | 3.8 |
| | *Coincidence Rate ($10^3$/sec)* | 5.5 | 4.9 | 5.8 | 5.0 | 6.6[a] | 4.7 | 5.0 | 5.2 | 5.0 | 4.7 |
| | *Death Rate (%)* | 21.8[c] | 34.3 | 23.1[c] | 36.0 | 18.2[c] | 31.0 | 18.4[c] | 28.3[b] | 14.0[c] | 24.8[c] |
| | *Split (%)* | 36.4 | 35.6 | 33.4 | 34.5 | 32.5 | 28.6 | 35.1 | 35.1 | 29.3 | 32.3 |
| | *pH* | 6.62[c] | 6.02 | 6.99[c] | 6.29[b] | 6.89[c] | 6.13 | 6.83[c] | 5.99 | 6.84[c] | 6.12 |
| **Live/Dead** | *% Live* | 52.5 | 46.6 | 50.6 | 47.0 | 53.9 | 47.3 | 53.0 | 46.3 | 57.0[b] | 51.2 |

[a] Means without common superscripts within rows differ from 20 h control ($P < 0.1$); [b] Means without common superscripts within rows differ from 20 h control ($P < 0.05$); [c] Means without common superscripts within rows differ from 20 h control ($P < 0.001$).

Table 7A: Means and significances of main effects for all responses. 10 bulls and 3 treatments were included in the statistical analysis (P values were determined by Dunnett's method by comparison to the 20 h Control) – Experiment 4

| | | Treatment | | | | | |
|---|---|---|---|---|---|---|---|
| | | *Control* | | *TEST* | | *TEST + EY* | |
| | | 0 h | 20 h | 0 h | 20 h | 0 h | 20 h |
| Analysis | Response | | | | | | |
| **Subjective Motility** | *Total Motility (%)* | 54.8 | 46.8 | 57.0[b] | 50.0 | 56.8[b] | 46.0 |
| | *Progressive Motility (%)* | 48.3 | 39.2 | 50.0[b] | 45.0 | 50.3[b] | 40.8 |
| **CASA Parameters** | *Total Motility (%)* | 58.0 | 52.2 | 56.6 | 52.7 | 55.8 | 53.1 |
| | *Progressive Motility (%)* | 33.1 | 27.1 | 30.8 | 26.2 | 30.0 | 30.0 |
| | *VAP (μm/s)* | 96.0[a] | 90.1 | 93.4 | 87.7 | 90.1 | 89.7 |
| | *VSL (μm/s)* | 74.3[a] | 69.0 | 71.3 | 66.5 | 68.8 | 70.8 |
| | *VCL (μm/s)* | 179.0 | 174.3 | 176.5 | 170.9 | 174.2 | 170.6 |
| | *ALH (μm)* | 6.7 | 6.8 | 6.6 | 6.9 | 6.4 | 6.9 |
| | *BCF (beats/s)* | 24.7 | 23.0 | 23.8 | 22.9 | 23.9 | 23.4 |
| | *STR (%)* | 79.5 | 78.4 | 78.3 | 77.9 | 78.3 | 80.4 |
| | *LIN (%)* | 45.3 | 43.0 | 43.7 | 42.2 | 42.8 | 45.1 |
| | *VD: Rapid (%)* | 54.4 | 47.9 | 53.4 | 48.5 | 52.0 | 49.0 |
| | *VD: Medium (%)* | 3.6 | 4.0 | 3.3 | 4.2 | 3.7 | 4.2 |
| | *VD: Slow (%)* | 22.6 | 19.3 | 20.4 | 20.3 | 20.3 | 17.7 |
| | *VD: Static (%)* | 19.6 | 28.7 | 23.3 | 27.2 | 24.1 | 29.3 |
| **Sort Information** | *Event Rate ($10^3$/sec)* | 30.0 | 28.1 | 30.6 | 27.6 | 31.5[b] | 27.2 |
| | *Live Oriented Cells (%)* | 55.7[c] | 44.6 | 52.7[c] | 44.0 | 55.4[c] | 48.5[a] |
| | *X Sort Rate ($10^3$/sec)* | 4.8[c] | 3.7 | 4.9[c] | 3.5 | 5.0[c] | 3.9 |
| | *Coincidence Rate ($10^3$/sec)* | 5.5 | 4.9 | 5.9[b] | 4.9 | 6.6 | 4.8 |
| | *Death Rate (%)* | 19.7[c] | 32.8 | 20.4[c] | 33.1 | 16.3[c] | 28.4[b] |
| | *Split (%)* | 37.3 | 36.6 | 36.7 | 34.5 | 37.9 | 31.5 |
| | *pH* | 6.59[c] | 5.96 | 6.97[c] | 6.23[c] | 6.87[c] | 6.08 |
| **Live/Dead** | *% Live* | 52.3 | 48.4 | 53.5 | 49.8 | 55.1 | 48.9 |

[a] Means without common superscripts within rows differ from 20 h control ($P < 0.1$); [b] Means without common superscripts within rows differ from 20 h control ($P < 0.05$); [c] Means without common superscripts within rows differ from 20 h control ($P < 0.001$)

# APPENDIX II

# EGG YOLK SORTABILITY TRIAL: EVALUATION OF THE ABILITY TO SORT BOVINE SPERM WITH THE ADDITION OF EGG YOLK

## INTRODUCTION

Egg yolk present in a semen sample (e.g. from the freezing extender if semen was previously cryopreserved) before staining for sorting may cause decreased resolution of the flow cytometer. However, egg yolk provides many protective properties to sperm and could prove to be a beneficial additive to storage solutions. This trial was performed to determine how different concentrations of egg yolk added to the semen would affect the ability to sex sort.

The objective of this preliminary trial was to determine if there was a difference in sortability with various concentrations of egg yolk added to the semen sample before staining with Hoechst 33342.

## MATERIALS AND METHODS

Semen collection, treatment administration and sorting were performed at Sexing Technologies (Navasota, TX). Ejaculates were obtained from 3 mature bulls. The following treatments were applied to a subsample of each ejaculate at the start of incubating of sperm with Hoechst 33342; egg yolk was added as a percent of TALP:

- Control = 0% egg yolk
- Treatment 1 = 0.2% egg yolk
- Treatment 2 = 0.4% egg yolk
- Treatment 3 = 0.8% egg yolk

Additionally, each treatment was further divided and stained with Hoechst 33342 (8.1 mM stock) at two different levels: 16 (4 µl/ml) and 18 (4.5 µl/ml). Therefore, there were a total of 8 samples for each bull. Samples were then incubated and sorted following standard processing procedures. However, no sorted sperm were collected. Instead, the

sort time was only long enough to sufficiently determine how well the samples split. This was accomplished by saving a photograph of the sorter screen showing the height distribution of the split for each of the samples. Splits were determined for each sort by how deep the split was as a percentage of the height of the distributions.

## RESULTS

The means of the percent split for the Control, Treatment 1, Treatment 2, and Treatment 3 stained at the 16 level were 55.7, 57.5, 58.1 and 55.4, respectively. The means of the percent split for each of these same groups at the 18 stain level were 55.0, 57.1, 57.2 and 57.9, respectively. Therefore, there was very little difference among the groups, and the addition of egg yolk at any of the concentrations tested did not adversely affect the ability of the semen samples to be sorted.

# APPENDIX III

# SORTABILITY TRIAL: EVALUATION OF THE ABILITY TO SORT BOVINE SPERM STORED IN EITHER MOPS OR TEST FOR 20 HOURS AFTER COLLECTION

## INTRODUCTION

Based on the results of Experiment 3, semen diluted with TEST buffer was chosen to be examined more extensively in Experiment 4. However, since the samples were not actually sorted by flow cytometry, it was decided to perform a preliminary trial to confirm that samples diluted with TEST would be able to be sorted satisfactorily.

The objective of this preliminary trial was to determine if there was a difference in sortability between storage treatments using both TEST and MOPS buffers versus an untreated control after 20 h of storage.

## MATERIALS AND METHODS

### Semen Collection and Processing

Semen collection, treatment administration and sorting were performed at Sexing Technologies (Navasota, TX). Ejaculates were obtained from mature bulls (Brahman, $n$ = 8; Holstein, $n$ = 6; Angus, $n$ = 3; Red Brahman, $n$ = 3; Red Angus, $n$ = 2; Simmental, $n$ = 1; Jersey, $n$ = 1) via artificial vagina. Ejaculates were then evaluated for viability. Motility was 50% or greater for all ejaculates, and all but one ejaculate ($8.1 \times 10^8$ spermatozoa/ml) had a concentration greater then $1 \times 10^9$ spermatozoa/ml. Eight bulls had greater than 30% abnormal morphology; ejaculates from 15 bulls had less than or equal to 30% abnormal morphology; and morphology of one ejaculate was not determined. Treatments were then applied to the neat semen to which antibiotics were added as recommended by Certified Semen Services (CSS, Inc, subsidiary of NAAB, Columbia, MO). Nothing further was added to the control samples until staining with Hoechst 33342 for sorting. Treatment 1 consisted of diluting the semen 9:1 with a TEST (TES, Sigma # T6022 and Trizma®, Sigma # T1503) solution, resulting in 25 mM TEST.

Treatment 2, resulting in 25 mM MOPS in the sample, was diluted in the semen in the same manner.

All samples were stored at 16°C (Minitube incubator, model DCR082W) for approximately 20 h (storage time ranged from 18.3 h to 25 h) and then processed. Samples were stained, incubated and sorted following standard processing procedures, as described in the previous experiments. However, no sorted sperm were collected. Instead, the sort time was only long enough to determine how well the samples split. This was accomplished by saving a photograph of the sorter screen showing the height distribution of the split for each of the samples.

Evaluation

Splits were determined for each sort by how deep the split was as a percentage of the height of the distributions. The split percents were then analyzed by ANOVA and Fisher's LSD was performed to determine differences between the treatment group means.

## RESULTS AND DISCUSSION

The means of the percent split for the Control, TEST and MOPS groups were 53.4, 54.4 and 53.5, respectively. No difference in split percent was found among the three groups ($p = 0.96$). Therefore, the addition of the buffer solutions did not affect the ability of the samples to sort when compared to an untreated control after a storage period of approximately 20 h after collection. Finally, no difference in the split percent was found between the bulls with greater than 30% abnormal morphology and the bulls with less than 30% abnormal morphology when averaged over all three treatments (52.3 and 53.7, respectively).