ABSTRACT

WRENCH, NICOLA. Effect of Season on Sperm Membrane Protein 22 and Selected mRNAs in Fresh and Cryopreserved Stallion Sperm. (Under the direction of Drs. Charlotte E. Farin and Carlos R.F. Pinto).

The objective of this study was to determine if either season or semen cryopreservation would have an effect on the expression of fertility-related protein SP22 and selected mRNA transcripts in stallion sperm. Six stallions were collected in June 2005, September 2005, December 2005 and March 2006. Each ejaculate was partitioned for evaluation of sperm parameters in fresh and cryopreserved samples. The percentages of normal morphology, primary and secondary abnormalities, membrane integrity, viability, total motility, progressive motility and acrosome integrity were recorded. In addition, aliquots of fresh and cryopreserved ejaculates were analyzed for SP22 protein expression and expression of mRNA transcripts. For SP22 immunocytochemistry, samples were stained using a sheep anti-rat recombinant SP22 primary antibody and a FITC-conjugated secondary antibody. At least 200 stained sperm per sample were counted using a fluorescence microscope and categorized into one of two patterns: Pattern 1, staining overlying the equatorial region (ER) only; Pattern 2, staining overlying the acrosomal and equatorial region (AER), neck (N) and tail (T). For mRNA transcript expression, sperm were washed with a hypoosmotic solution to induce somatic cell lysis. RNA was then extracted from sperm samples using Tri-Reagent and cDNA was synthesized. PCR was performed using the cDNA to assess mRNA expression. Data were analyzed for all six stallions and for the subset of four stallions whose collections were repeated every season (subset stallions) using general linear model procedures (SAS Institute Inc., Cary, NC, USA).

The process of cryopreservation significantly ($P < 0.05$) affected all sperm parameters. In general, cryopreservation decreased the proportion of sperm exhibiting normal morphology and the proportions of viable and motile sperm. In contrast, cryopreservation was associated with an increased incidence of primary and secondary morphologic abnormalities. A significant ($P < 0.05$) effect of season was noted for normal morphology, primary abnormalities, total motility and progressive motility for all 6 stallions. In general, the same seasonal effects were present for the 4 subset stallions; however, there was no effect of season on total motility or progressive motility. Significant ($P < 0.05$) season x cryopreservation interactions were found for progressive motility and intact acrosomes for all 6 stallions. For the 4 subset stallions a significant ($P < 0.05$) season x cryopreservation interaction was found for total motility, progressive motility and secondary abnormalities. There were no significant stallion x cryopreservation interactions.

The proportion of sperm stained for SP22 was significantly ($P < 0.05$) affected by season, stallion and cryopreservation. A significant ($P < 0.05$) season x cryopreservation interaction was also present. A tendency ($P < 0.08$) for the intensity of staining for SP22 to differ among stallions was noted for the 4 subset stallions. For cryopreserved sperm, the proportion of sperm staining for SP22 on the equatorial segment was affected differently by stallion or season depending on the SP22 antibody used.

RNA yield from sperm was not affected by season, stallion, cryopreservation or their interactions. There was no effect of season or cryopreservation on the relative quantity of mRNAs for PGK2, TPX1, TIMP3 or ACTB. Also, no season x cryopreservation or stallion x cryopreservation interactions were found. However, differences between stallions ($n = 6$)

were apparent for PGK2 (P = 0.08) and ACTB (P = 0.01) content. For the 4 subset stallions there was a tendency (P = 0.1) for an effect of stallion on ACTB mRNA content.

Sperm parameters were affected by the process of cryopreservation and could be affected by season or stallion. The percentage of sperm staining for SP22 on the equatorial segment was affected by cryopreservation, season and stallion. Although the relative quantity of ACTB and PGK2 mRNAs was affected by stallion, no other effects on selected sperm RNAs were noted. Understanding differences that exist in sperm across seasons and stallions is beneficial when attempting to determine the best time to collect semen for cryopreservation. Based on data presented, we recommend collecting semen for cryopreservation between March and June.

Effect of Season on Sperm Membrane Protein 22 and Selected mRNAs in Fresh and Cryopreserved Stallion Sperm

By

Nicola Wrench

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APPROVED BY:

_____	_____
Chair of Advisory Committee Charlotte E. Farin	Co-chair of Advisory Committee Carlos R.F. Pinto
_____	_____
Gary R. Klinefelter	David J. Dix

_____

William L. Flowers

BIOGRAPHY

Nicola Wrench was born on May 12th, 1982 in Plymouth, England. She and her parents then moved to Germany when she was four years old. When Nicola was 12, she and her parents moved to Charlotte, NC. She attended middle school and high school in Charlotte and then decided to come to North Carolina State University for her bachelor's degree. Nicola graduated with her BS in Animal Science in 2004 and started graduate school at NCSU the next semester to pursue a Master of Science degree in Animal Science and Physiology.

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Introduction

Use of cryopreserved stallion semen has increased as a result of breed registries recently allowing the use of cryopreserved semen for artificial insemination (AI). However, AI with cryopreserved semen is not used to its full potential because of poor pregnancy rates. In addition to unsatisfactory pregnancy rates, other disadvantages of using cryopreserved semen include increased mare management, increased costs and the increased levels of expertise needed to perform AI procedures. However, there are several key advantages for using AI with cryopreserved semen. First, it is less expensive to ship semen in liquid nitrogen than to ship a horse; second, the stallion's breeding season can continue while he is in competition, ill, injured or even deceased; and third, there is less waste of an ejaculate since the entire ejaculate can be subdivided into individual insemination doses for cryopreservation. Post-thaw fertility of stallion sperm has been improved but techniques can vary tremendously from one laboratory to the next. Therefore, results are frequently difficult to compare between laboratories. It is commonly understood, however, that variation in sperm parameters such as motility, viability and membrane integrity exist between stallions and within stallion (3,6,9,16,24,26,28,32,33,50,65,79,92). Also consistent among studies is the observation that cryopreserved sperm are less motile, less viable and have fewer sperm cells with intact membranes compared to freshly ejaculated sperm (6,10,18,28,32,33).

Besides the stallion itself, age and breed are also sources of variation in semen quality. Stallions reach sexual maturity at about five years of age. Seminal characteristics such as ejaculate volume, concentration and viability are affected by age (20). Breed is also

a source of variation in semen characteristics. For example, Arabian stallions had the highest number of total sperm per ejaculate with the lowest number of dead sperm compared to all other breeds including Quarter Horse, Thoroughbred, Pony and Holsteiner (20). While it is possible that the characteristics of freshly ejaculated sperm influence the freezability of a stallion's semen, genetics most likely has a greater influence on freezability. As an example, the Mangalarga Marchador breed is considered to be poor for semen cryopreservation (25). This breed tended to have less than 30% total motility after the freeze-thaw process and had worse cryopreservation results when compared to Jumper breeds and Quarter horses (25). The process of spermatogenesis should not differ among breeds so breed differences are most likely explained by differences in genetics among breeds. Arabian horses do differ from other horses in their anatomy; but, whether other genetic differences are truly the reason for their improved live sperm output compared to other breeds has not been determined.

That some stallions' semen freezes better than others is often referred to as stallion semen freezability. This can be defined as the number of ejaculates selected to be used for artificial insemination compared to the total number of ejaculates collected (92). Selection of ejaculates is usually based on post-thaw motility, where less than 35% motile sperm after thawing is considered unacceptable (90). Based on this criteria, only 2 of 6 stallions had satisfactory post-thaw motility (16).

Sperm are expected to withstand many different stressors during the cryopreservation and thawing process. Stressors can include the addition of cryoprotectants, membrane stretching and shrinkage, dehydration, phase transitions of the membrane, elevated solute concentration and intracellular ice formation (71). These stressors can potentially damage

the sperm through a variety of actions including loss of plasma membrane, morphological alterations, membrane permeabilization and membrane destabilization (65).

The process of cryopreservation alters the sperm membrane and provides the most likely explanation for differences in sperm motility, viability and membrane integrity observed between fresh and cryopreserved sperm. The plasma membrane of sperm is composed of lipids and proteins. The lipids consist of phospholipids and cholesterol, the ratio of which is important for the rigidity of the membrane (63). More cholesterol appears to decrease the flexibility of the membrane (6). It is thought that the membrane damage caused by cryopreservation is due to reordering of membrane lipids (71) which may increase membrane permeability. During the cryopreservation process, as temperature is lowered, water moves out of the cell causing dehydration. At the same time, the ratio of intracellular potassium and sodium is also altered, allowing an influx of calcium (6). This influx eventually leads to a breakdown of phospholipids and an increase in the permeability of the membrane (6). If temperature changes are extremely rapid, then intracellular microcrystals (0.1- to 0.3 μm) form which greatly reduce the damage to the sperm compared with the damage incurred by large ice crystals (10 to 50 μm) which form with less rapid temperature changes (6). Membrane alterations occur during the cryopreservation process (71) but it is possible that rapid warming may rescue portions of the cell that were damaged by intracellular ice (6).

Seminal plasma appears to have detrimental (3,62) and beneficial (62) effects on sperm. For example, incubating sperm with 5% seminal plasma prior to cryopreservation increased post-thaw motility (62). In contrast, incubating with 20% seminal plasma prior to cryopreservation lead to decreased post-thaw motility (62). However, this was only seen 90

minutes post-thaw (62). If sperm were cryopreserved immediately after collection of the ejaculate, then the addition of seminal plasma had no effect (62). However, semen with poor post-thaw motility had improved progressive post-thaw motility when incubated with seminal plasma from stallions with good semen freezability (8). Because addition of seminal plasma can prevent premature capacitation, it may also be beneficial as an additive to cryopreserved semen prior to insemination to improve fertility.

Capacitation is a process necessary for fertilization and is one which sperm undergo while in the female's genital tract. During capacitation sperm undergo several changes including addition and removal of surface proteins, changes in the plasma membrane and loss of cholesterol (29). Sperm undergo capacitation in order to gain the potential for the acrosome reaction and hyperactivated motility (29). Cryocapacitation, also referred to as premature capacitation, is a process that occurs during the cryopreservation and thawing processes (86). Changes in osmotic condition influences the cell's ability to maintain proper signaling processes (53), which probably plays a role in cryocapacitation. However, these changes do not appear to be equivalent to those that occur in capacitated sperm (86). Most mammalian sperm undergo one membrane phase transition during cellular cooling (53). In contrast, stallion sperm membranes have a wide variety of lipid classes and therefore undergo three distinct phase transitions (53). Each of which is a possible source of damage to sperm as they are cryopreserved.

In summary, the process of cryopreservation causes many changes in the sperm membrane which may decrease the fertility of those sperm. Variability in sperm characteristics exists between stallions and within stallions because sperm characteristics have been shown to vary with age and breed of the stallion. While there are several

disadvantages to cryopreservation including unsatisfactory pregnancy rates and increased costs, the advantages of having a stallion's semen available, even after death, outweigh these disadvantages.

Extenders

Extenders are solutions that function to extend the viability of fresh and cryopreserved sperm and are used to dilute ejaculates for shipment (45). Extenders contain buffers, sugars, antibiotics and egg yolk or non-fat dried skim-milk (45). A variety of different extenders are available commercially for use with fresh semen for shipping purposes and also specifically for cryopreservation. An example of a commonly used extender is INRA82. It is composed of glucose, lactose, raffinose, sodium citrate, potassium citrate, HEPES, distilled water and ultra high temperature-treated skim milk (76). This extender can be used for fresh semen or for cryopreserved semen after addition of glycerol. Another example is Kenney's extender. This extender is superior to glucose-EDTA for preserving motility for fresh semen and also improves quality of cryopreserved semen when used for initial extension (3). Use of Modified Kenney's freezing medium decreased the percentage of acrosome reacted sperm after cryopreservation compared to lactose-EDTA freezing medium (79). Addition of Tyrode's medium, a modified phosphate buffer, to a typical skim-milk glucose extender has also been shown to be beneficial for improving sperm motility compared to the skim-milk glucose extender alone; however, only when seminal plasma has been removed from the semen (74).

Typically semen is collected and then transported to the laboratory where it is extended. However, it has been demonstrated that placing skim milk extender in the

ejaculate collection bottle improved the motility of cooled semen (3). The type of extender used with fresh and cryopreserved semen has an effect on semen quality. Differences between extenders are most likely due to the components of the extenders and determining which component has the greatest influence on sperm quality could be very beneficial. More research in this area would be beneficial to determine the best extender to decrease sperm damage and increase post-thaw quality.

Cryoprotectants

A cryoprotectant is added to the freezing extender in order to protect sperm during cryopreservation. An optimal cryoprotectant has a low molecular weight and high water solubility (52); rapidly permeabilizes the cell, decreases temperature dependence and decreases toxicity to cell (53). Glycerol (G) has been the most frequently used cryoprotectant; however, at high concentrations, glycerol can be toxic to sperm (5,84). Glycerol is typically used at concentrations of 2.5 to 6% (91). At these concentrations, glycerol (G) has been shown to depress fertility when added to fresh semen (19). By binding directly to phospholipid headgroups, glycerol causes a reduction in membrane fluidity (71). More recently, other cryoprotectants have been explored, including ethylene glycol (EG), dimethyl formamide (DMF), methyl formamide (MF), glutamine and dimethyl sulfoxide (DMSO). These alternative cryoprotectants have a lower molecular weight than glycerol which may enable them to penetrate the sperm plasma membrane more readily and decrease osmotic toxicity (84).

Original reports in the late 1980s led to the suggestion that G was superior to EG, for the use of cryopreservation (6). However, more recently EG has been shown to be

comparable to G for cryopreservation; thus, a possible replacement for G in freezing medium (51,83). Glutamine and G are synergistic, increasing post-thaw motility when both are included in basal medium (38). However, glutamine also shows osmotic toxicity at high concentrations (38,94). Dimethyl sulfoxide proved not to be comparable to G in that sperm cryopreserved with DMSO had lower total and progressive motility (4). Dimethyl formamide appears to be the most plausible replacement for G, because it showed either similar (28,84) or improved results when directly compared to G (4,5,25,91). Dimethyl formamide is also beneficial for stallions with poor semen freezability, in that use of DMF increased total and progressive motility of sperm in post-thaw samples. Progressive post-thaw motility was also improved for the Mangalarga Marchador breed, known to have poor post-thaw results (5,25). Unlike alternative cryoprotectants, it is unclear if DMF also displays osmotic toxicity at high concentrations.

Currently, dimethyl formamide appears to be the best option to be used in combination with or to replace glycerol, particularly for stallions classified as poor freezers. Not only does DMF improve the quality of semen post-thaw, but also DMF has low or no apparent osmotic toxicity. Decreasing the physiological stress that sperm experience during the cryopreservation process through the use of improved cryoprotectants may increase pregnancy rates when cryopreserved semen is used.

Cryopreservation Methodologies

The traditional method of cryopreservation involves diluting semen with extender, centrifuging, resuspending in freezing extender, filling straws, leaving straws in liquid nitrogen vapor for a specific period and then submerging them into liquid nitrogen (1). One

advancement in cryopreservation methodology has been the creation of automatic cryopreservation machines which allow for more control over cooling rates (1). Semen is commonly packaged into 0.5 ml straws but other options are available, including 0.25 ml and 5 ml straws, 0.1-0.2 ml pellets and also 3.6 ml vials (7). Observations from a survey on cryopreservation methods, involving approximately 10 different countries, underscored the conclusion that many differences in methodologies exist between farms and that comparisons of optimal procedures are difficult (78). Standardization of cryopreservation protocols across all farms will be needed to resolve this issue (78).

Centrifugation is necessary to concentrate the sperm cells for cryopreservation, however this step is also a potential source of damage. Adding an iso-osmotic cushion and layering the semen sample on top of this solution creates a cushioned centrifugation, allowing for an increase in revolution speed and duration of centrifugation. This improves recovery without significantly harming sperm cells (43). Speed and time of centrifugation are not the only possible sources of injury to sperm when centrifuging, temperature also has an effect. Adding glycerol and centrifuging at 22°C, instead of 4°C, resulted in improved recovery of sperm after centrifugation as well as improved post-thaw motility and fertility (94). The sperm membrane is still fluid at 22°C and is likely to be more flexible and better able to handle centrifugation stress (94).

Rapid removal of cryoprotectants results in relative hypo-osmotic shock which is associated with rapid loss of motility, membrane integrity and mitochondrial membrane potential (98). Relative hypo-osmotic shock also occurs when glycerol is rapidly added and removed from the extender (98). For fresh semen, use of a four step dilution process to remove cryoprotectant improved the maintenance of sperm motility and viability (98).

Unfortunately motility and viability were not improved when cryopreserved sperm were diluted in a similar manner (98).

Slow cooling is a process in which semen samples are cooled to 4°C prior to cryopreservation. Use of slow cooling resulted in increased post-thaw motility, preservation of sperm membrane and acrosome integrity, and mitochondrial membrane potential (44). Slow cooling allows for shipment of cooled semen to semen cryopreservation stations rather than requiring transport of the stallion to the laboratory contracted for semen cryopreservation (1,93,94). Because of the ability to transport semen and because slow cooling improved post-thaw motility (93,94) it is a beneficial tool for use prior to cryopreservation stallion semen.

Recently, a cryopreservation technique originally designed for foodstuffs (unique freezing technique; UFT), was tested on stallion semen. UFT is a high-speed freezing system that includes organic fluid with a heat capacity similar to water. The UFT achieved a freezing rate similar to that of liquid nitrogen and produced similar post-thaw motilities when compared to cryopreserving semen using traditional liquid nitrogen plunge methods (26). In that study, bovine and human semen extenders were used for equine semen (26). It would be beneficial to test this method using equine semen extenders instead.

Cholesterol is able to reduce the transition temperature of the plasma membrane and maintain the membrane in a fluid state (63). This reduces damage to the sperm cell (63). During the process of cyropreservation cholesterol is lost from the sperm plasma membrane and this is thought to be responsible for the reduced duration of sperm viability after thawing (63). Because cholesterol is easily incorporated into and extracted from the plasma membranes of cells, it has been suggested that adding cholesterol-loaded-cyclodextrins

(CLCs) to sperm cells may be beneficial (63). In fact, addition of CLCs appeared to be beneficial (83), in that CLC exposure increased the number of cryopreserved stallion sperm that bound to bovine zona pellucida (63). The addition of CLCs also increased longevity of sperm in the mare's genital tract (63). This would allow for a decrease in the intense management of mares that is currently necessary when using cryopreserved semen (63) because it would not be as critical to breed as close to the time of ovulation as possible.

Because the process of freezing appears to be the primary cause of damage to sperm cells, this process has been studied more extensively than the process of thawing. Traditional thawing methods involve submerging straws in a 37°C water bath for 30 seconds (1). Alternatively, straws can be thawed at 75°C for 7 seconds (12). This is a more difficult and time-sensitive procedure and did not improve progressive motility, normal morphology or acrosome defects compared to thawing at 37°C for 30 seconds (12). A third procedure is to thaw at 75°C for 7 seconds and then to immediately transfer the straws to 37°C for 5 seconds. This method did not improve post-thaw motility, normal morphology or acrosomal defects over thawing at 37°C for 30 seconds only (6).

During the process of cryopreservation necessary steps are taken to optimize post-thaw motility, even if some of those steps are a possible source of sperm damage. Continued advancements in technology, such as automatic cryopreservation machines, the UFT and CLCs may lead to improved post-thaw motility, viability and morphology. This in turn, could lead to improved pregnancy rates when using cryopreserved semen.

Effect of Season

Seasonality is much more evident in the mare than in the stallion. The mare experiences an anestrous period during the fall and winter, whereas spermatogenesis continues in stallions throughout the year, despite the fact that testicular concentration of testosterone and Leydig cells decrease during winter (35). It is unclear whether differences exist in semen collected during the breeding season compared to the non-breeding season. Therefore, studies have been done to determine effects of season on characteristics of fresh and cryopreserved stallion semen.

For fresh semen, ejaculate volume has been shown to be highest in the breeding season (spring and summer) compared to the non-breeding season (fall and winter; (32,33,50). Similarly, the number of sperm in an ejaculate is also highest in summer (6,32,33). Analyses of sperm concentration and motility have produced contradictory results. Both endpoints have been reported to be high and low in spring and summer (32,33,50). Differences in observations of sperm motility could be related to the differences in methods used for analysis in these studies. For example, Magistrini et. al. (50) used methods for assessing motility that were subjective whereas Janett et. al. (32,33) used a computer-based analysis. Other, more likely, causes for these discrepant results may include individual stallion or breed variation.

The percent of freshly ejaculated sperm with normal morphology was low in summer (33) and then increased in fall (32). The best way to look at results is to see whether they make “physiological sense”. For instance, it does not seem logical for normal morphology to be higher during the non-breeding season than during the breeding season because the best sperm should be available during the breeding season. However, if that stallion is breeding

frequently then the increased spermatogenic demand may result in ejaculation of increased numbers of premature sperm, therefore resulting in decreased percentages of sperm with normal morphology.

Although there appears to be some seasonal variation among semen parameters, post-thaw quality of semen is comparable between the breeding and non-breeding seasons (10). Because there are few differences in semen parameters collected in different seasons, the current recommendation is to collect and freeze a stallion's semen during the non-breeding season. In addition, the demand on the stallion is reduced in the non-breeding season compared to that encountered during the breeding season. Because the concentration and volume for cryopreserved semen, and therefore, the total sperm number per straw, are controlled by the technician cryopreserving the semen, sperm numbers per straw should not vary from one season to another.

Because seasonal differences in semen parameters were observed in fresh semen, it would seem logical that those differences would also occur in cryopreserved semen. Analyses of post-thaw sperm viability have produced conflicting reports with viability being reported as either higher (50) or lower (33) during the breeding season. These two studies used different methods for viability assessments and this may explain their contradictory results. Janett et al (2003) used SYBR-14 and propidium iodide (PI) while Magistrini et al (1987) used eosin-nigrosin staining. Amann and Pickett (1987) also found that the viability of semen collected and cryopreserved during the breeding season was higher than that collected and cryopreserved during the non-breeding season. Possible explanations for discrepant observations in relation to sperm viability may include stallion or breed variability. Besides differences in sperm viability, it was also found that semen collected and

cryopreserved during the non-breeding season experienced greater sperm deterioration during storage than semen collected and cryopreserved during the breeding season (6). Other studies showed that post-thaw sperm motility, membrane damage, hyperactivation and acrosome-reacted sperm are greatest in the non-breeding season (10,32,33,50,96). Ejaculates collected from a single stallion exhibited greater variation when collected during the non-breeding season (3). Also, semen appears to survive the cryopreservation process better when collected during the breeding season (3). In order of importance, the main factors influencing post-thaw quality are cryopreservation, stallion and season (10).

In summary, a number of studies have analyzed the effect of season on fresh and cryopreserved semen. The concentration of sperm per ejaculate is highest in the breeding season and those sperm experience less deterioration. Within different studies, contradictory results for motility and viability occur more frequently than for other semen parameters. Discrepant results between studies may be due to lab technique, analytical methods used, individual stallion and breed variability. Because of these contradictions, more research is needed to determine when to collect and freeze stallion semen to optimize semen quality after thawing.

Fertility

If post-thaw sperm parameters could be correlated to fertility, they would become a useful tool for assessing the fertility of an ejaculate. Post-thaw sperm motility, ranging from 35% motile to greater than 60% motile, has been positively correlated to fertility (90); however, other investigators reported no correlation between motility and fertility. In cattle, similar discrepancies have been noted, one study correlated sperm motility to the fertility of

bulls (27) whereas another study saw no such correlation (34). In Europe, freezing and AI centers utilize standardized protocols. Assessments for fertility using semen cryopreserved at these centers are comparable to those obtained when fresh semen was used (90). For example, in 2003 foaling rates with cryopreserved semen packaged in France were 64% for the French national stud, 62% for a privately cryopreserved French breed and 57% for a privately cryopreserved foreign breed whose semen was shipped into France (90). During the same breeding season, foaling rate was 64% when hand mating was used (90). Apparently with protocol standardization, not only fertility can be improved but also post-thaw motility. For example, 80% of stallions in this study had a mean post-thaw motility higher than 40% (90).

Insemination protocol also has an effect on fertility of cryopreserved semen. Palmer and Magistrini (1992) saw improvement in fertility when two artificial inseminations (AI) were used instead of one (69). Multiple inseminations may be more effective because the number of sperm available, the stimulation of genital tract and the variability of sperm population are all increased. Together these factors may positively impact fertility (90). Another factor influencing fertility could be the semen extender. For example, INRA82 was shown to be superior to Kenney's FE based on per-cycle pregnancy rates (90). In contrast, Wilhelm et al (99) reported that the type of semen extender did not affect fertility. Age of the stallion or mare may also impact fertility. Because semen characteristics are affected by age, it is logical that age is also associated with a decrease in pregnancy rates (81). A mare's fertility may decrease with age due to cycle irregularity, development of endometrial cysts, fewer ovulations or compromised oocyte quality (2,15).

It has been suggested there is a critical number of sperm needed for inseminating a mare above which fertility will no longer increase with increasing sperm number (6). For example, foaling rate was higher when $80x10^6$ motile sperm were inseminated than when $40x10^6$ motile sperm were used (6). However, when mares were inseminated with $320x10^6$ or $800x10^6$ motile sperm, pregnancy rates were not affected (48). The critical number of sperm cells needed depends on the stallion and on post-thaw quality, glycerol concentration and on the interval between AI and ovulation (90).

One half ml straw was typically cryopreserved at a concentration of 200×10^6 sperm/straw and at least two 0.5ml straws were considered an insemination dose (400×10^6 sperm total). When inseminating with fresh semen, a breeding dose is considered to be at least 500×10^6 motile sperm. Because cryopreserved sperm are more damaged than freshly ejaculated sperm, to inseminate fewer sperm and expect similar pregnancy rates compared to those obtained with fresh semen inseminations is unreasonable. Unfortunately, the idea of inseminating with low doses of cryopreserved semen has spread across the industry, which may contribute to the reduced fertility observed with the use of cryopreserved semen (90). Interestingly, packaging sperm at a concentration of $1600x10^6$ sperm/ml did not result in decreased post-thaw motility or pregnancy rates compared to straws packaged at $400x10^6$ sperm/ml (48). Increasing the sperm concentration per straw would be beneficial because only one straw would be thawed for each insemination. Cryopreservation at high sperm concentrations also decreases the space required for straw storage.

Identification of correlations between semen characteristics and fertility would be beneficial for the prediction of fertility for fresh and cryopreserved semen. As an example, stallions with a high progressive motility after being stored at 5°C for 24 hours achieved

superior pregnancy rates (81). Although initial post-thaw motility does not appear to be correlated to fertility, motility assessments made at 90 minutes post-thaw were correlated to first cycle fertility (39).

Another possible factor influencing fertility is the procedure used for semen collection. A high frequency collection schedule with shortened interval between successive semen collections was associated with increased fertility of cryopreserved semen compared to the fertility of semen from stallions collected on a schedule characterized by a low collection frequency with an increased interval between successive collections (81).

When stallions were separated into high or low fertility groups based on fertility rate per cycle, the high fertility group exhibited a greater percentage of live cryopreserved sperm in their ejaculates (99). In addition, the fertility of stallions in the low group seemed to be more negatively affected by treatment with liposomes and various extenders compared to those in the high fertility group (99). If a stallion can be classified as a good semen freezer then visual motility analysis may be sufficient to determine possible fertility (39). However, if a stallion is considered a poor semen freezer, then motion and flow cytometry analyses may be necessary to adequately evaluate sperm quality (39) and estimate fertilizing potential.

Assessing fertility of cryopreserved stallion semen is difficult because pregnancy outcome is affected not only by the stallion but also by the mare artificially inseminated to that stallion. Relatively few studies have focused on fertility as an endpoint because they are expensive and time consuming. Extender composition, insemination protocol and the number of motile sperm inseminated have been shown to influence the fertility of cryopreserved semen. It would be beneficial to be able to correlate post-thaw sperm quality parameters such as motility, viability and morphology to fertility. Unfortunately, however,

this has yet to be consistently accomplished. If an optimized protocol for assessing cryopreserved sperm quality could be established and standardization of this protocol across labs was implemented, pregnancy rates with use of cryopreserved semen could be improved on an industry-wide basis.

Sperm Membrane Proteins Associated with Fertility

The sperm membrane is embedded with a variety of proteins including signaling molecules, receptors and transport molecules. Identifying and understanding the role of sperm membrane proteins is important to our understanding of sperm physiology and fertility.

Sperm Proteins in Other Species

A number of studies have been conducted to identify sperm proteins that may correlate with fertility. One such protein, SP-10, has been found to be associated with acrosomal membranes and was localized within the acrosomal matrix in human, baboon, pig, macaque, fox, bovine and mice sperm cells (23,73). Fertilization was shown to be inhibited by an antibody to SP-10 in mice, humans and pigs; furthermore, both sperm attachment to and penetration of the zona pellucida were also blocked (21). The SP-10 antibody also decreased fertilization of bovine oocytes by reducing the tight binding of sperm to the zona pellucida, rather than by disrupting sperm-zona primary binding (17).

Another sperm protein, P34H, appears on the acrosomal cap of human sperm. Similar to SP-10, antibody to P34H interferes with sperm binding to the zona pellucida (13). Over 50% of infertile men were shown to have low levels of P34H and their sperm were

unable to bind the zona pellucida (14). P34H has been shown to be antigenically and functionally similar to the hamster P26h protein (13). When a P26h antiserum was used against bovine sperm, two proteins were identified; P25b and P21b (70). Similar to P34H, P25b is localized to the acrosomal cap (70). P21b, on the other hand, is associated with the flagellum (70). A low P25b/P21b ratio was show to be indicative of subfertility in bulls (70), similar to results seen with the P34H acrosomal protein in humans.

SP-10, P34H, P26h and P21b all appear to have a similar acrosomal localization pattern on sperm. Similar effects on fertilization are also seen when antibodies are used against these proteins. Studying these and other fertility-associated sperm proteins may provide information helpful to determine potential causes of infertility.

Determining the function of proteins associated with sperm could explain reasons for infertility or possibly help with development of new contraceptives. One protein that has been studied is rat epididymal protein D/E. This protein is thought to be involved in sperm-egg fusion (22). When rats were immunized against D/E they became infertile (82) probably due to a decreased ability of the sperm to penetrate the eggs (22). Most likely there is not just one protein responsible for determining fertilizing ability. Identification of other proteins similar to D/E could be beneficial in helping to determine other possible causes of infertility.

Sperm Membrane Protein 22

In male rats, a powerful toxic insult is required to cause a notable decrease in fertility (41); however, in man minor toxic insults by specific chemicals can cause a decrease in fertility (37). Thus, to understand the high incidence of infertility in human males (36),

determining the effect of specific chemicals such as those found in drinking water (37), on sensitive indicators of sperm quality in sperm extracts has proven important in human health risk assessment.

Several toxicants have now been investigated for their effects on sperm proteins including ethane dimethanesulphonate (EDS), chloroethylmethanesulphonate (CEMS), epichlorohydrin (EPI) and hydroxyflutamide (HFLUT) which act in different ways on the epididymis, as well as dibromoacetic acid (DBA) and bromochloroacetic acid (BCA) which act on spermatogenesis (37,40).

When the effects of CEMS, EPI, EDS and HFLUT were tested on sperm protein expression, it was noted that expression of sperm membrane protein 22 (SP22) was decreased relative to the other 124 sperm membrane proteins identified (40). Epididymal toxicants decreased SP22 levels on epididymal sperm, presumably via loss of the pre-existing, testis-derived, sperm protein from the plasma membrane during epididymal transit (42).

Consistent with observations for the rat, DBA exposure in rabbits also diminished SP22 levels and reduced conception rates (89). The decrease in SP22 levels on sperm following exposure to testicular toxicants likely results from decreased expression of the protein in the post-meiotic germ cells.

SP22 is part of a 450kD protein complex and is expressed on sperm following meiosis (41). Sperm from the rete testis exhibit immunostaining for SP22 at the base of the head when using an affinity purified antibody (41). The signal for SP22 is translocated during epididymal transit and is exhibited at the base of the head and over the equatorial segment of sperm collected from the caput and corpus epididymis. A similar staining pattern was seen in bull and human sperm (41).

Because SP22 exhibits staining over the equatorial segment, which initiates sperm-egg adhesion, the role SP22 in sperm-egg binding has been studied. In rats, as discussed above, fertility can be experimentally assessed based on the number of fetal implants on day 9 of gestation relative to the total number of corpora lutea present on the ovaries (41). When female rats are inseminated with five million sperm, typical fertility is 75% (41). Incubation of sperm with affinity-purified SP22 antibody for five minutes prior to in utero insemination decreased fertility (41,42). Inhibition of fertilization was also seen in vitro and the block appeared to mainly be at the level of the zona pellucida (42) despite the protein's presence at the equatorial segment.

CAP1 has been shown to be identical to SP22 by Welch et al and Wagenfeld et al 1998 (42). In humans, SP22 has also been referred to as DJ-1 (64), RS (30) and PARK7 (11). CAP1 mRNA has been identified using RT-PCR from mouse, marmoset and human testicular and epididymal tissues (95). The nucleotide sequence for CAP1 has greater than 80% identity, and the protein sequence greater than 90% homology, to a protein identified as DJ-1 in mouse and human (82,95). In rats, a 1.0kb transcript for SP22 was identified in somatic and testicular tissue while a 1.5kb transcript for SP22 was identified in testicular tissue only (41,97). Consistent with the first wave of spermatogenesis in the rat, the 1.5kb testis-specific mRNA transcript was first detected on day 23 and increased through day 33 of spermatogenesis (41,97). The presence of this 1.5kb transcript appears to be related to the appearance of spermatogenic cells such as pachytene spermatocytes and round spermatids (41,97). Multiple SP22 transcripts may suggest that multiple SP22 forms may be expressed in or on sperm which may appear at different times and with different expression patterns (41).

SP22 has also been investigated in other species. Analysis of immunostaining for SP22 showed that bulls with higher breeding efficiencies tended to have higher levels of SP22 expression (41). In *Xenopus leavis,* SP22 (Xl-SP22) was identified using the same anti-rSP22 IgG as in the rat and was shown to be very similar to rat SP22 protein (61). Xl-SP22 protein expression was greatest in testicular tissue and Xl-SP22 mRNA expression was greatest around the time of fertilization (61), further implicating a role for SP22 in fertilization. In rabbits, ejaculated sperm exhibited staining for SP22 over the equatorial segment and this staining pattern persisted even after an induction of the acrosome reaction (42). These observations supported the suggestion that SP22 is likely involved in fertilization in the rabbit.

In stallions, localization of SP22 on sperm is altered after cryopreservation and the localization differed between freezing extenders used (58,59). These alterations in SP22 localization may be due to damage to the sperm membrane subsequent to the cryopreservation process. Thus, the observed alterations in localization may reflect differences in the ability of the extender to provide proper protection to the sperm during cryopreservation. SP22 mRNA transcripts in stallion testes and epididymides have also been identified (57). A 1.0kb transcript was identified in both testes and epididymides and a 1.5kb transcript was identified in the testis only (57). These authors also found that stallion cauda epididymis had more SP22 mRNA than the caput or the corpus (57).

Identification of SP22 in rat, bull, *Xenopus leavis*, hamster, rabbit, human (42) and stallion with consistent localization on sperm suggests that SP22 protein is highly conserved across species (97). The presence of SP22 in several species and its correlation with fertility confirm its probable importance in the physiology of fertilization.

Ribonucleic Acids in Sperm

Because it has been assumed that newly made sperm cells lose the majority of their cytoplasm during the final stages of spermiogenesis and that any residual cytoplast is absorbed by the Sertoli cell (56), it has been believed that the only contribution that sperm make to the oocyte at fertilization is the paternal genome. However, extraction of RNA from sperm has revealed that there are between 2,000 and 5,000 distinct mRNAs expressed in sperm cells, suggesting that sperm may contribute more than simply DNA during fertilization (56,66,87). The translation of mRNA has been verified in capacitating sperm indicating an active requirement for at least some retained mRNAs; however, the possible role of sperm RNAs in fertilization is still unclear (55). Ostermeier et al (2004) found some sperm mRNAs which encode proteins essential for early embryonic development. It is possible that sperm RNA-mediated chromatin repackaging may be important for maintaining epigenetic changes occurring during spermatogenesis and essential for normal syngamy (56). Currently the data suggest that sperm RNAs may play a role in the remodeling and imprinting of paternal chromatin (56).

More recently, Platts et al. (2007) discovered a difference in sperm RNA content between normal fertile men and men suffering from teratozoospermia. This study found that the mRNAs from genes in the ubiquitin-proteosome pathway (UPP) were diminished which is believed to be due to suppressed expression of proteosomal mRNAs (72). These data support the idea that differences in sperm RNAs may reflect disrupted spermatogenesis which leads to abnormal morphology and infertility.

Because most ejaculates contain somatic cells, it is necessary to treat ejaculates with some type of somatic cell lysis solution to ensure extraction of pure sperm RNA. Pure, fully processed, sperm mRNA is characterized by the absence of 28S and 18S rRNAs, absence of intronic sequences and the presence of 3'-poly-A tails (55). Sperm mRNA fingerprints would not only be useful for helping assess male fertility but could also be useful for determining possible contraceptives, and understanding possible generational consequences of environmental exposures (99).

RNA and Fertilization

Sperm mRNAs identified in humans correspond to about 3,000 genes, some of which have well-known roles in fertilization (88). Ostermeier et al (2004) identified six transcripts which were present in human sperm but absent in unfertilized human oocytes. This further implicates that sperm deliver necessary RNAs to the oocyte for fertilization to occur. Sperm RNAs may also participate in establishment of imprints in early embryos, pronuclear formation, early events leading to oocyte activation (68) and may also provide a new level of control to establish imprints during the transition from maternal to embryonic genome activation (67). It does appear that sperm mRNAs may play some role in fertilization, but the relative importance of these transcripts is still unclear.

RNA Correlated to Sperm Parameters and Infertility

Typically, when researchers discover that some sperm component correlates to fertility, the correlation between that component and sperm parameters are also investigated. It was discovered that sperm with poor motility have more RNA than sperm with good

motility (77). In contrast, morphologically normal sperm had a greater RNA content compared to morphologically abnormal sperm (77). It is believed that sperm with abnormal morphology may have impaired transcriptional and, or translational pathways and therefore cell function is negatively impacted (77). These studies focused on the general RNA content, but it is possible that specific transcripts correlate differently with sperm parameters. For example, there was a decrease in aromatase mRNA levels in sperm with low motility (46), but intense signals for eNOS and nNOS transcripts (47). Similar differences between sperm with high and low motility have also been observed in levels of Prm-1 compared to Prm-2 mRNA (47). Prm-1 transcript levels were higher in sperm with low motility (47). Thus, it may be beneficial to correlate transcript differences with differences in sperm parameters for development of potential diagnostic tool. This is what Platts et al. (2007) found by comparing sperm mRNA content between normal fertile men and men suffering from teratozoospermia.

Current assessments to determine male fertility include: physical exam, semen analysis, urologic assessment, genetic evaluation and testicular biopsy (60). These assessments are limited and probably not very pleasant for the male experiencing them. As a result, it would be beneficial to find another method for evaluating male infertility. Sperm RNA could be a valuable tool in helping evaluate male fertility, especially because most male factor infertilities result from oligogenic and/or polygenic effects on sperm production (31,49,54,80,85). It has been observed that expression of 19 genes varied significantly between testicular samples from fertile and infertile males (75). Looking at the sperm mRNA content of an infertile male and comparing it to that of a normal fertile man may be beneficial in diagnosing idiopathic infertilities. Genetic profiles created from RNA extracted

from human sperm show differences between fertile and infertile samples (72) and examination of gene pathways from these profiles has identified possible causes for male infertility. These data support the idea that differences in sperm RNAs may reflect disrupted spermatogenesis which leads to abnormal morphology and infertility.

Statement of the Problem

Causes of infertility of males in any species can often be difficult to determine. In the animal world, important decisions are often made about a male's life prior to the knowledge of his fertility. Sperm membrane protein 22 (SP22) has been correlated to fertility in several species including rats and rabbits (42,61,95,96). Very little information on the expression of SP22 on stallion sperm has been reported (57-59). Whether SP22 expression reflects seasonal changes and whether this protein is affected by the freeze-thaw process across seasons is unknown. mRNA profiles of ejaculated sperm also have been shown to differ between fertile and infertile males (72). To the best of our knowledge, there are no reports on the presence of mRNAs in ejaculated sperm of stallions. Therefore, the objectives described in this thesis were: first, to reconfirm the presence of SP22 and assess mRNA expression in equine sperm; second, to determine if variability exists in routine sperm characteristics such as sperm motility, morphology and viability, as well as expression of SP22 and sperm mRNAs across season; and third, to determine the effects of cryopreservation on sperm characteristics, and expression of SP22 and sperm mRNAs across season.

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Introduction

Assessments of stallion fertility are typically not possible until a stallion has reached reproductive maturity. However, determination of a young stallion's potential fertility prior to breeding would be more beneficial to equine breeders. While seasonal effects on the mare's reproductive cycle are considered to be more extreme, the stallion does undergo some changes during the nonbreeding season. These include a decrease in testosterone levels and a decrease in testicular size (3).

Potential markers of sperm fertility have been identified in rats, hamsters, rabbits and humans. One of these identified markers is sperm membrane protein 22 (SP22; 4). SP22 is thought to be involved in the process of fertilization, possibly at the stage of sperm-egg binding, based on its immunocytochemical localization to the equatorial segment of the sperm head (4). Differences in the localization pattern of SP22 have been recently reported for stallion sperm (8). Localization of SP22 varied between fresh and cryopreserved sperm and also varied with the type of semen extender used (9).

Previously, mRNAs were identified in mature sperm (7,11,12). Ostermeier et al. (2002) found that sperm mRNAs are correlated with testicular gene expression. Furthermore, expression of particular mRNAs in testicular tissue varied with fertility in mice and with azoospermia in men (13). More recently, Platts et al. (2007) discovered a difference in gene expression between normal fertile men and men suffering from teratozoospermia. This study found that the ubiquitin-proteosome pathway (UPP) is disrupted which is believed to be due to suppression of proteosomal RNAs associated with the UPP (12). These results may lead to development of a noninvasive diagnostic test to determine molecular genetics of

male infertility (12). These reports lend support to the proposal that sperm mRNAs may potentially be useful as markers of fertility.

To the best of our knowledge, there are no reports of the presence of mRNAs in ejaculated stallion sperm. Therefore, the objectives of the research described in this thesis were first, to reconfirm the presence and the effects of freezing on SP22 and examine the mRNA expression in equine sperm; second, to determine if variability exists in the expression of SP22 and selected sperm mRNAs across season; and third, to determine the effects of cryopreservation on expression of SP22 and selected sperm mRNAs across season.

Materials and Methods

Chemicals

Unless otherwise specified, all chemicals were obtained from Sigma Chemical Co.

Semen Collection

Semen was collected from six fertile stallions (mixed light-horse breeds, 7-18 years of age) with the aid of an artificial vagina (Colorado model; Animal Reproduction Systems, Chino, CA). Stallions that were not on a routine collection schedule were collected at least twice within the week prior to sample collection. Semen samples were obtained during 2005-2006 in the summer (June 2005), fall (September 2005), winter (December 2005) and spring (March 2006). Four of the six stallions were represented in all seasons. Stallions were located in Raleigh and Southern Pines, North Carolina, USA.

Semen Cryopreservation

Fresh semen was extended in Kenney's extender (Har-Vet, Spring Valley, WI) to a final concentration of 50 x 10^6/ml and was then centrifuged at 400 x *g* for 10 min (Eppendorf; Hamburg, Germany). Seminal plasma was removed and the sperm pellet resuspended in E-Z Freezin LE (Animal Reproduction Systems, Chino, CA) to a concentration of $400x10^6$ sperm/ml. A 20% loss was assumed for each sample when pellets were resuspended. Sperm were then packaged into 0.5 ml straws, placed on a Styrofoam float and held 2 cm above liquid nitrogen for 10 minutes. After 10 min, straws were plunged into the liquid nitrogen and stored.

Semen Thawing

In September 2006 straws were removed from the tank and thawed at 37°C in a water bath for 30 to 45 seconds. Straws were thawed by month collected, one month at a time.

One stallion's sample was thawed at a time and initial assessments were made prior to thawing the next stallion's sample. Thawed samples were kept in an incubator at 37°C until all stallion samples were processed. Stallions were thawed in the same order for each month. Ten control straws were thawed and processed with each thawing.

Semen Analysis

A basic flow diagram of semen processing is shown in Figure 1.

Motility: Total and progressive motility were visualized using phase contrast microscopy at 400x by at least two individuals and the average of the subjective assessments was recorded. For each assessment several fields were evaluated to estimate an overall percentage of motile sperm.

Membrane Integrity: Integrity of the sperm membrane was tested by adding 10 µl of fresh semen to 100 µl of a 100 mM sucrose solution (hypoosmotic swelling test, HOST). Response of the sperm was based on the degree of swelling of the plasma membrane, determined by coiling or curling of the sperm tail (10). Two hundred cells were classified as coiled or straight tail.

Morphology and Viability: Sperm were stained with eosin-nigrosin and 200 cells were classified into normal or abnormal (primary and secondary) morphology. An additional count of 200 sperm was performed and the number of nonviable (stained) sperm was recorded.

Acrosome Integrity: Semen smears were analyzed using Spermac stain (Conception Technologies, San Diego, CA). Two hundred sperm cells were counted for the presence or absence of an acrosome.

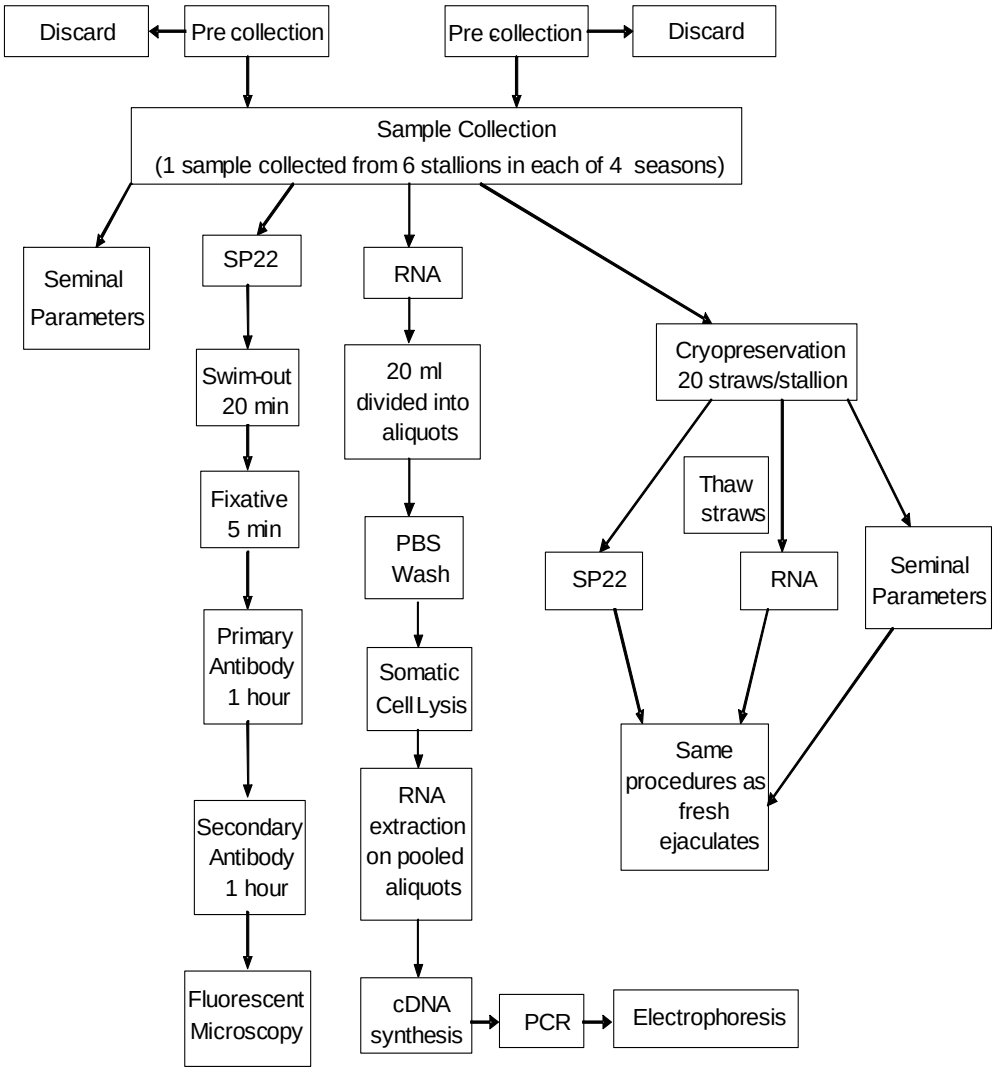

Figure 1. Basic schematic of methods for collecting, processing and analyzing semen samples for this study

SP22 Immunocytochemistry

This method was revised for use on stallion sperm from the method used on rat sperm to decrease handling and possible damage to the sperm. For each ejaculate, 2 ml of freshly ejaculated semen or 1 ml of cryopreserved semen was added to a 75µm mesh insert (CoStar Netwell, Corning, NY) that was resting on 1 ml of Dulbecco's phosphate buffered saline (DBPS) in one well of a 12-well culture plate. Sperm were allowed to swim through the mesh for at least 20 min at room temperature. The volume of semen above the insert was removed and sperm under the insert were transferred to a 15 ml conical centrifuge tube. The dispersed sperm were fixed by adding an equal volume of 4% paraformaldehyde in 0.1 M phosphate buffer containing 4% sucrose for 5 min. The volume was brought to 10 ml with DPBS and centrifuged (500 x *g*, 5 min). The pellet was resuspended in 1 ml of DPBS, transferred to a 1.5 ml microfuge tube and incubated for 60 min in affinity purified primary antibody (sheep anti-rat recombinant SP22-L; 1:200). In some cases antibodies recognizing different regions of the SP22 protein were compared (SP22-L, SP22-S or SP22-X; Figure 2). The sample was centrifuged (500 x *g*, 5 min), washed once in DPBS and incubated for 60 min in FITC-labeled second antibody (1:50, Pierce Biotechnology Inc.; Rockford, IL). The sample was centrifuged again, washed once in DPBS and Vector's fade retardant mounting medium (Vector laboratories; Burlingame, CA) was added. At least 200 stained sperm were counted using a fluorescent microscope. Sperm were categorized into one of two staining patterns: Pattern 1, stain overlying the equatorial region (ER); Pattern 2, stain overlying acrosomal and equatorial region (AER), neck (N) and tail (T).

For cryopreserved samples, intensity of the staining at the equatorial segment was measured using a Leica DM6000B (Leica Microsystems, Wetzlar, Germany).

1 M A S K R A L V I L A K G A E E M E T V I P V D 24

25 I M R R A G I K V T V A G L A G K D P V Q C S R 48

49 D V V I C P D T S L E E A K T Q G P Y D V V V L 72

73 P G G N L G A **Q N L S E S A L V K E I L K E Q E** 96

97 **N R K G L I** A A I C A G P T A L L A H E V G F G 120

121 C K V T S H P L A K D K M M N G S H Y S Y S E S 144

145 R V E K D G L I L T S R G P G T S F E F A L A I 168

169 V E A L S G K D M A N Q V K A P L V L K D 189

L-rSP22 Ig Amino Acid # 1-189
S-rSP22 Ig Amino Acid # 47-102 (underlined)
X-SP22 Ig Amino Acid # 80-102 (bold type and underlined)

Figure 2. Single letter amino acid designation of the sequences of SP22 that the L, S and X antibodies were targeted against.

RNA Preparation of fresh ejaculates

For each ejaculate, a volume of at least 20 ml was split evenly into four conical tubes which each were brought to a 10 ml volume with 1X phosphate buffer saline (PBS) and centrifuged (2,000 x *g*, 15 min). The pellets were resuspended by vortexing in 1 ml somatic cell lysis buffer (SCLB) and then brought to 10 ml with SCLB, vortexed and left at 4°C for at least 30 minutes. Samples were assessed for the absence of somatic cells following the lysis procedure and in all cases no somatic cells were found to be present. After verification of somatic cell lysis, samples were centrifuged (2,000 x *g*, 15 min) and the pellets were resuspended in 1 ml SCLB. Samples were transferred to a 1.5 ml microfuge tube and centrifuged again; pellets were then resuspended in 1 ml Tri-Reagent and stored at - 80°C until later RNA extraction.

RNA Preparation of cryopreserved ejaculates

About 7 ml of cryopreserved semen was diluted 1:1 with DPBS and split into two, 15 ml conical tubes. These were then brought to 10 ml with 1X phosphate buffer saline (PBS) and centrifuged (2,000 x *g*, 15 min). The pellets were resuspended by vortexing in 1 ml somatic cell lysis buffer (SCLB) and then brought to a 10 ml volume with SCLB, vortexed and left at 4°C for at least 30 minutes. Samples were verified for the absence of somatic cells following the lysis procedure. After verification, samples were centrifuged (2,000 x *g*, 15 min) and the pellets were resuspended in 1 ml SCLB. Samples were transferred to a 1.5 ml microfuge tube, centrifuged and the pellet was resuspended in 1 ml Tri-Reagent. Samples were stored at - 80°C until later RNA extraction.

Reverse Transcription

RNA was extracted from ejaculated sperm using TRI-Reagent as per manufacturer's instructions and the resulting wcRNA was dissolved in diethyl pyrocarbonate-treated water. RNA was also extracted from stallion testicular tissue to serve as test samples for the mRNA primer sequences. Concentrations of wcRNA were determined by absorbance at 260nm. The ratio of absorbance at 260 nm and 280 nm was calculated for each sample. The overall mean for all six stallions was 1.5 ± 0.04 based on 47 observations. The overall mean for the four subset stallions was 1.5 ± 0.04 based on 31 observations. For every stallion 2 µg of wcRNA were treated with 1.5 U DNase for 20 min at 37°C. Following DNase inactivation, wcRNA was reverse transcribed using random primers and SuperScript III reverse transcriptase. After cDNA synthesis, sample buffers were exchanged using the Qiagen PCR purification kit and the resulting clean cDNA was stored at 4°C. The TPX1 primer set was designed to span an intron so that genomic DNA could be evidenced by the presence of several other bands on the ethidium-bromide-stained gel if the DNase treatment did not work.

Specific primer sets used for RT-PCR and expected product lengths can be found in Table 1. Each PCR reaction consisted of 100 ng cDNA, 2 µl of appropriate forward and reverse primers, 3.2 µl dNTPs, 2 µl of 10 X PCR buffer (Roche) and 0.25 µl of Taq polymerase (Roche) to make a 20 µl reaction. A negative control lacking cDNA was included in each PCR assay. All PCR reactions were run in 96-well PCR plates. Plates were placed into a PTC-100 Thermal Cycler (MJ Research; Bio-Rad Laboratories, Hercules, CA). Each PCR program consisted of 2 min at 92°C, followed by appropriate number of cycles of 30 sec denaturation at 94°C and 30 sec annealing at appropriate temperature and 1 min extension at 72°C. After amplification cycles were completed, another extension for 5 min at

72°C was used. PCR products were visualized on ethidium bromide-stained 1.5% agarose gels. PCR amplification products representing beta actin (ACTB), tissue inhibitor of metalloproteinase 3 (TIMP3), testis specific protein 1 (TPX1) and phosphoglycerate kinase 2 (PGK2) were each verified by sequence analysis.

The mRNA transcript for Parkinson Disease 7 (Park7; SP22) was detected in testicular tissue extracts but could not be detected in ejaculated sperm extracts. The sequence was verified using the testicular tissue sample and can be found in the Appendix (Fig 13).

Linear Amplification

PCR was performed using cDNA from a control sample to determine the linear phase of amplification and the optimal conditions for each primer set. Reactions were set up for a total of 40 cycles, removing 2 tubes every 5 cycles starting at the 20^{th} cycle. Signal intensity of bands from ethidium bromide gels were determined using an AlphaImager system (Alpha Innotech, San Leandro, CA). A response curve was created for each primer set and the cycle number during which linear amplification occurred was determined (Appendix Figures 1-5).

Semi-quantitative RT-PCR analysis

Samples from June 2005, September 2005, December 2005 and March 2006 from all four stallions were used for assessment of gene expression of TPX1, TIMP3, GAPDH, ACTB and PGK2. Each assay was set up to assess one mRNA type and include all stallions' samples across seasons. PCR products were visualized on ethidium-bromide-stained gels and signal intensity was determined using an AlphaImager system.

The relative level of expression of specific mRNAs was calculated as a ratio of the signal intensity of the band of interest to that of GAPDH.

Statistical Analysis

Data were analyzed using general linear model procedures (SAS Institute Inc., Cary, NC, USA). The final model used for analyses of sperm characteristics, SP22 ICC and relative mRNA expression included the main effects of season and stallion, and their appropriate interactions. In addition, data for the four stallions that were collected in all seasons were run in a separate analysis. All percentage data were arcsin transformed prior to analysis. For all analyses, when a significant F-statistic was obtained, means were separated using Duncan's Multiple Range test. All data are reported as least squares means ± SEM.

Table 1. Primer sequences used for RT-PCR analysis of mRNA extracted from stallion sperm.

Primer		Sequence	Annealing Temp (ºC)	Cycle # (linear phase)	Fragment size (bp)
ACTB*	(Forward)	5'-GGCACCACACCTTCTACA			
	(Reverse)	5'-ACGCACGATTTCCCTCTC	60	37	376
GAPDH[a]	(Forward)	5'-GCCGTGAACCACGAGAAGTATAA			
	(Reverse)	5'-CCGTCCACGATGCCAAAGT	60	35	120
TPX1	(Forward)	5'-AAACCATACACCATCCTCT			
	(Reverse)	5'-ATCTCTCTTTGGACTTGGG	57	34	255
TIMP3	(Forward)	5'-CGGACAAGAGCATCATCA			
	(Reverse)	5'-GCGAGAAAACAGGCACTA	58	37	149
PGK2	(Forward)	5'-TGGAGGAAGAAGGGAAGG			
	(Reverse)	5'-CATCAAACAGGGAAGCAC	59	36	404

*ACTB = Beta Actin; GAPDH = Glyceraldehyde-3-phosphate dehydrogenase;
TPX1 = Testis specific protein 1; TIMP3 = Tissue inhibitor of metalloproteinase 3;
PGK2 = Phosphoglycerate kinase 2
[a]GAPDH; sequence obtained from Leutenegger et al (5)

Results

Sperm Parameters

All sperm parameters were significantly affected by cryopreservation (Table 2). In general, cryopreservation decreased the proportion of sperm exhibiting normal morphology and the proportions of viable and motile sperm. In addition, cryopreservation was associated with an increased incidence of primary and secondary abnormalities.

There was no significant effect of season on ejaculate volume, concentration, total sperm number, total motile sperm and total progressively motile sperm from collections of all six stallions (Table 3) and from the subset of four stallions whose collections were repeated every season (subset stallions; Table 4).

Significant season x cryopreservation interactions were found for progressive motility and intact acrosomes for the group of 6 stallions (Figures 3a and b) and for total motility, progressive motility and secondary abnormalities for the subset of 4 stallions (Figures 4a-c). For progressive motility (n = 6 stallions and n = 4 stallions) and total motility (n = 4 stallions) a significant difference between fresh sperm and cryopreserved sperm was present in June, September and December 2005 but not March 2006. Acrosome integrity (n = 4 stallions) was significantly different between fresh and cryopreserved sperm for all four seasons. However, the magnitude of difference was significantly greater in June 2005. A significant difference between fresh and cryopreserved sperm in regard to secondary abnormalities (n = 4 stallions) was found in March 2005 only. No significant stallion x cryopreservation interactions were present ($P > 0.1$). Table 5 represents a brief summary schematic of the responsiveness to the process of cryopreservation of specific endpoints including: normal morphology, primary abnormalities, progressive motility, intact

acrosomes, SP22 and mRNA. These endpoints were analyzed from sperm collected and cryopreserved during the breeding season and compared to sperm collected and cryopreserved during the non-breeding season. The schematic demonstrates that there may be some advantage to cryopreserving sperm during the breeding season compared to the non-breeding season.

In addition to collection of data across the four seasons starting in June 2005 and continuing until March 2006, an additional semen collection was performed during March 2005 (primarily for practice). An analysis of all endpoints incorporating data from all five collection periods (March 2005 through March 2006) was performed and are reported in Appendix B.

Table 2.Effect of Cryopreservation on Stallion Sperm Characteristics from Sperm Collected in Four Separate Seasons (Least squares means ± SEM).

	n = 6 Stallions		n = 4 Stallions	
	Fresh	Cryopreserved	Fresh	Cryopreserved
Normal Morphology (%)	77 ± 1[a]	43 ± 1[b]	78 ± 2[c]	47 ± 2[d]
Primary Abnormalities (%)	18 ± 1[a]	43 ± 1[b]	17 ± 1[c]	44 ± 1[d]
Secondary Abnormalities (%)	5 ± 1[a]	14 ± 1[b]	5 ± 1[c]	9 ± 1[d]
Intact Plasma Membrane (HOST;%)	70 ± 2[a]	50 ± 2[b]	70 ± 3[c]	51 ± 3[d]
Viability (%)	76 ± 2[a]	37 ± 2[b]	82 ± 3[c]	37 ± 3[d]
Total Motility (%)	71 ± 2[a]	45 ± 2[b]	74 ± 2[c]	53 ± 2[d]
Progressive Motility (%)	64 ± 2[a]	38 ± 2[b]	67 ± 2[c]	46 ± 2[d]
Intact Acrosome (%)	93 ± 1[a]	68 ± 1[b]	93 ± 2[c]	70 ± 2[d]

[ab]$P < 0.0001$ between treatments within data from six stallions
[cd]$P < 0.0001$ between treatments within data from four stallions

Table 3.Effect of Season on Stallion Sperm Characteristics from Sperm Collected from 6 Stallions in Four Separate Seasons (Least squares means ± SEM).

	Jun 2005	Sep 2005	Dec 2005	Mar 2006	P-value
Volume (ml)	63 ± 14	65 ± 11	50 ± 12	74 ± 11	NS
Concentration (10^6)	308 ± 68	243 ± 53	348 ± 59	221 ± 53	NS
Total Sperm (10^9)	18 ± 3	12 ± 3	14 ± 3	14 ± 3	NS
Total Motile Sperm (10^9)	13 ± 2	9 ± 2	9 ± 2	9 ± 2	NS
Total Progressive Sperm (10^9)	12 ± 2	8 ± 2	8 ± 2	9 ± 2	NS

Table 4.Effect of Season on Stallion Sperm Characteristics from Sperm Collected from Four Subset Stallions in Four Separate Seasons (Least squares means ± SEM).

	Jun 2005	Sep 2005	Dec 2005	Mar 2006	P-value
Volume (ml)	68 ± 10	84 ± 10	40 ± 11	75 ± 10	NS
Concentration (10^6)	300 ± 49	186 ± 49	381 ± 58	235 ± 49	NS
Total Sperm (10^9)	18 ± 3	13 ± 3	11 ± 4	15 ± 3	NS
Total Motile Sperm (10^9)	14 ± 2	10 ± 2	8 ± 3	10 ± 2	NS
Total Progressive Sperm (10^9)	12 ± 2	9 ± 2	8 ± 3	9 ± 2	NS

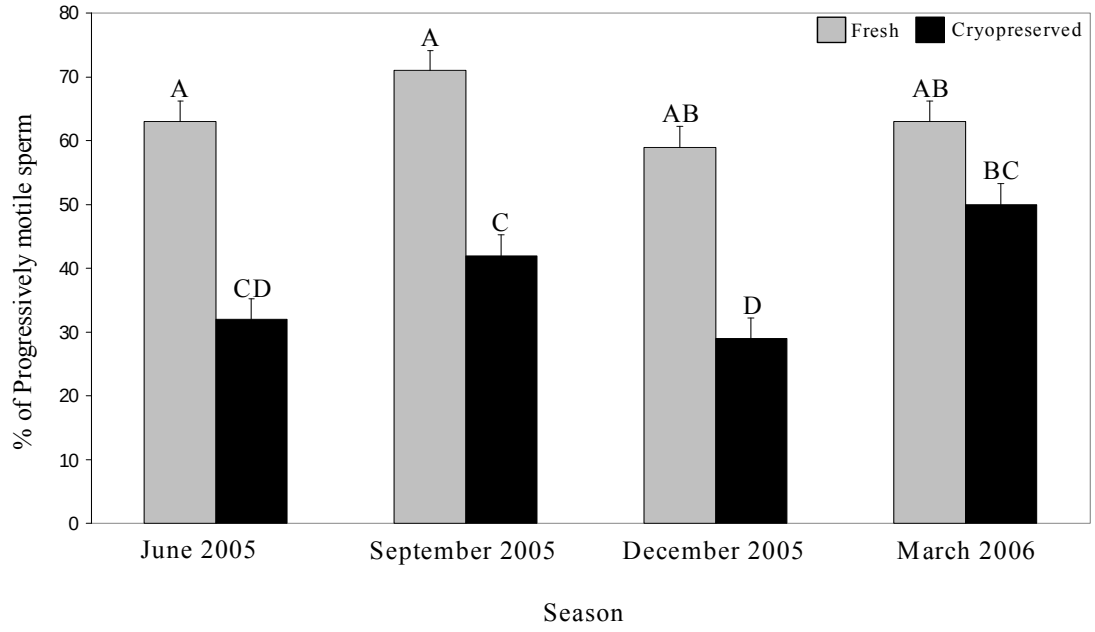

Figure 3a. Percentage of progressively motile sperm across season and across cryopreservation. n = 6 stallions. Significant season x cryopreservation interaction, means with different superscripts are significantly different; $P < 0.05$.

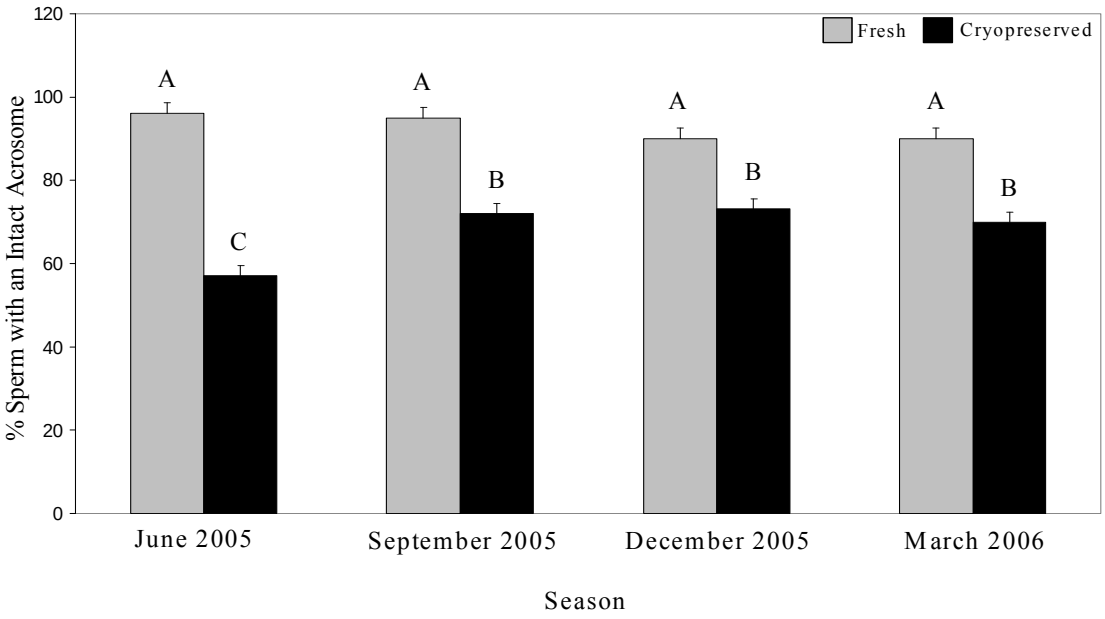

Figure 3b. Percentage of sperm with intact acrosome across season and across cryopreservation. n = 6 stallions. Significant season x cryopreservation interaction, means with different superscripts are significantly different; $P < 0.05$.

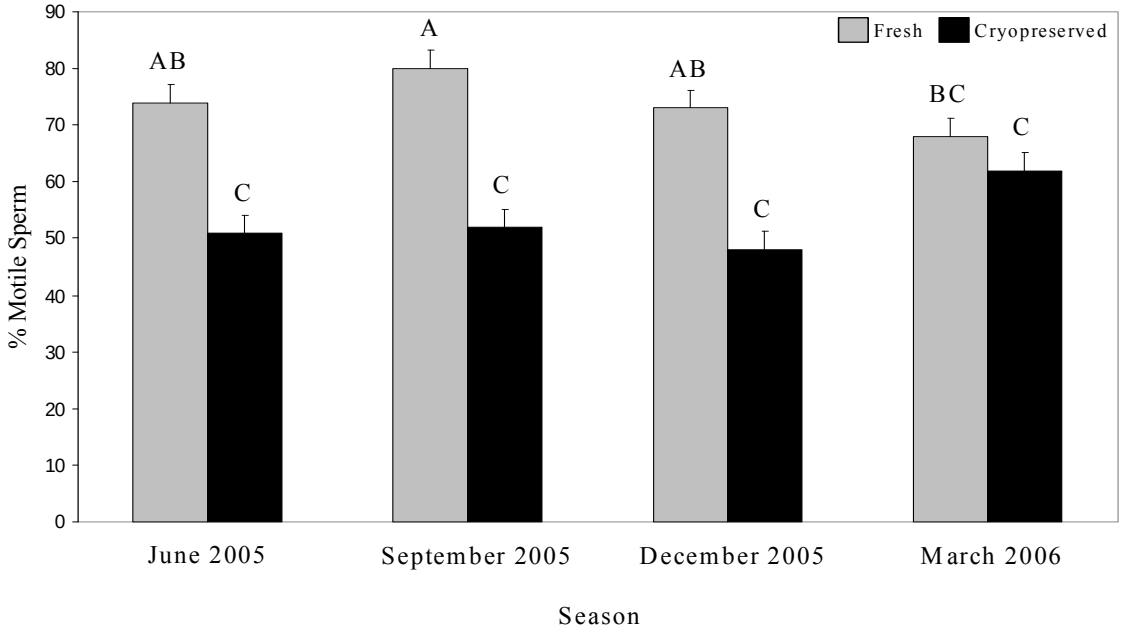

Figure 4a. Percentage of motile sperm across season and across cryopreservation. n = 4 stallions. Significant season x cryopreservation interaction, means with different superscripts are significantly different; $P < 0.05$.

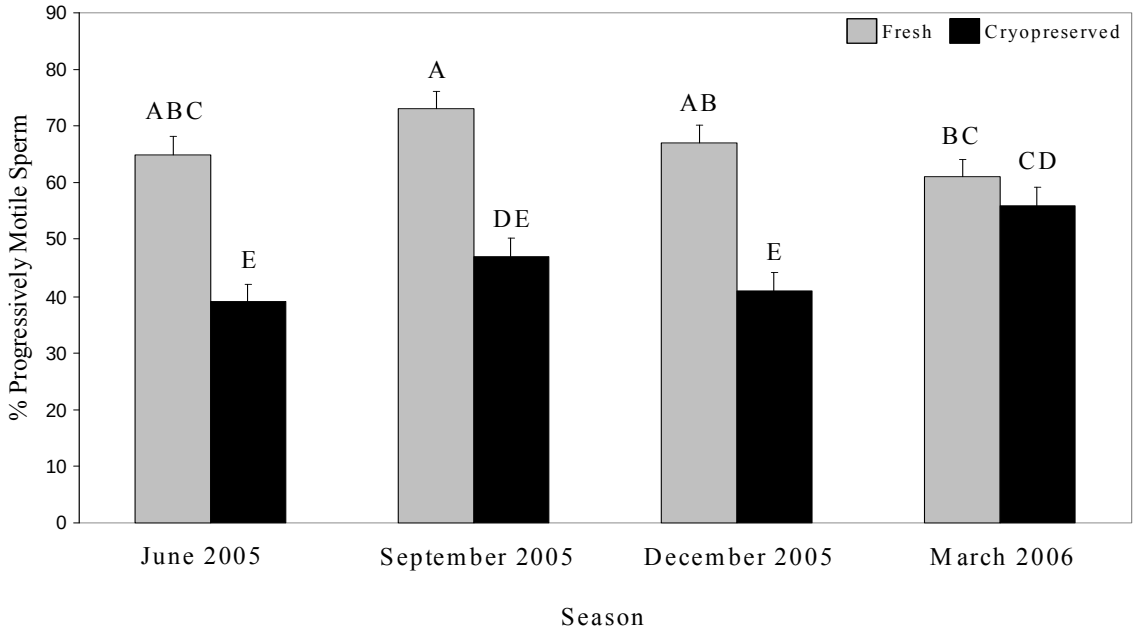

Figure 4b. Percentage of progressively motile sperm across season and across cryopreservation. n = 4 stallions. Significant season x cryopreservation interaction, means with different superscripts are significantly different; $P < 0.05$.

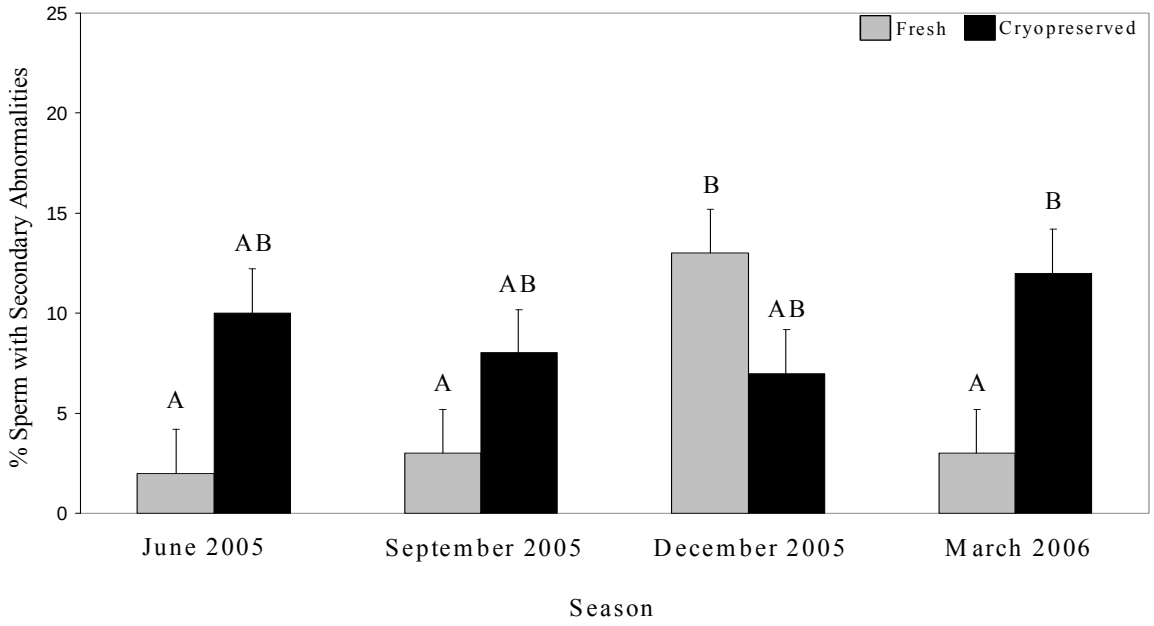

Figure 4c. Percentage of sperm exhibiting secondary abnormalities across season and across cryopreservation. n = 4 stallions. Significant season x cryopreservation interaction, means with different superscripts are significantly different; $P < 0.05$.

Table 5. A Brief Summary Schematic of Specific Endpoints and their Responsiveness to the Cryopreservation Process when Semen was Collected during the Breeding Season (March and June) Compared to Semen Collected during the Non-breeding Season (September and December)

	Responsiveness to Cryopreservation	
	Breeding Season	Non-breeding Season
Normal Morphology	Better	Worse
Primary Abnormalities	Better	Worse
Progressive Motility	Same	Same
Intact Acrosomes	Worse	Better
SP22	Same	Same
mRNA	Same	Same

Breeding Season: ↑ ↑ — ↓ — —

Non-breeding Season: ↓ ↓ — ↑ — —

Sperm Protein 22 (SP22)

An example of a sperm staining for SP22 on the equatorial segment is seen in Figure 5. The percentage of sperm staining for SP22 on the equatorial segment was highest in the fall (Fig 6). There was a significant effect of cryopreservation (Fig 7) and a significant season x cryopreservation interaction (Figures 8a and b) on the proportion of sperm staining for SP22 on the equatorial segment. There was a significant difference between fresh and cryopreserved sperm staining for SP22 on the equatorial segment in September 2005 for the four subset stallions. Interestingly, March was the only month in which the percentage of sperm staining over the equatorial segment was not decreased by cryopreservation. The proportion of sperm staining for SP22 varied significantly between stallion for all 6 stallions ($P = 0.03$; Fig 9) and for the subset stallions ($P = 0.01$; Fig 10). In addition, the intensity of staining for SP22 tended ($P = 0.08$) to vary for subset stallions. When SP22 staining percent and intensity data were calculated, it appeared that those stallions with the higher percentage staining also had increased staining intensities. This is shown by groups 1 and 2 (Fig 9 and 10) for all 6 stallions and the 4 subset stallions.

For cryopreserved sperm, three different antibodies were used (L, S and X). When data from $n = 6$ stallions were analyzed, there was a significant effect of season on antibody S ($P = 0.02$) and X ($P = 0.001$) staining (Appendix Fig 8). The percentage of sperm staining for SP22 was lowest in June 2005 for the S and X antibody and greatest in March 2006 for the X antibody. A significant stallion effect was present for antibody S ($P = 0.03$) and antibody X ($P = 0.005$) staining (Appendix Fig 11). Stallions 2 and 4 exhibited the lowest percentage of staining for SP22 and Stallion 5 exhibited a greater percentage of staining for SP22. When data from the subset of stallions were analyzed there was a significant ($P =$

0.01) effect of season with the X antibody (Appendix Fig 9). The percentage of sperm staining for SP22 was lowest in June 2005. A significant (P = 0.01) effect of stallion (n = 4 stallions) for antibody X was noted. The percentage of sperm staining for SP22 was decreased in stallions 2 and 4.

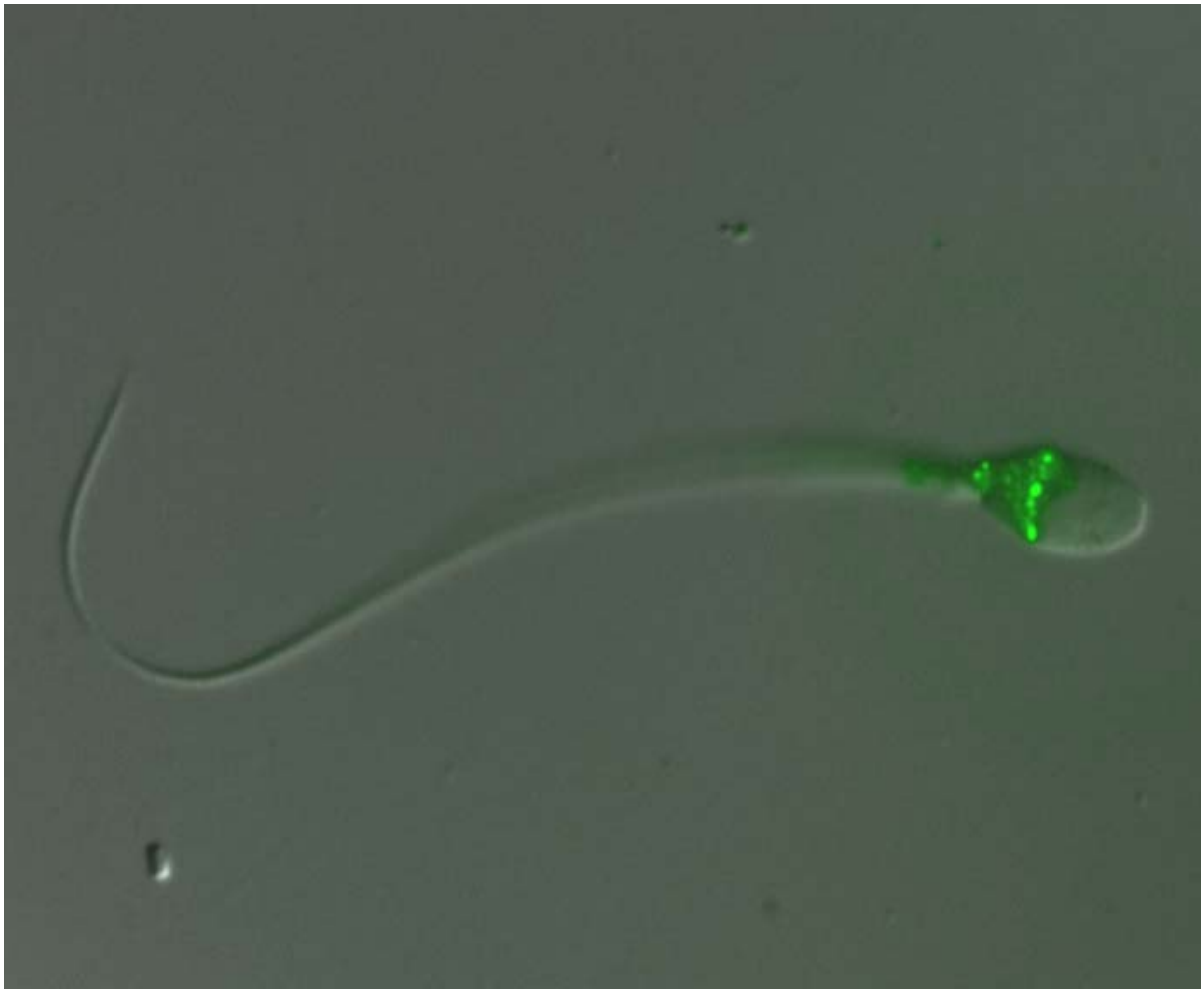

Figure 5. A stallion sperm exhibiting staining for SP22 on the equatorial segment.

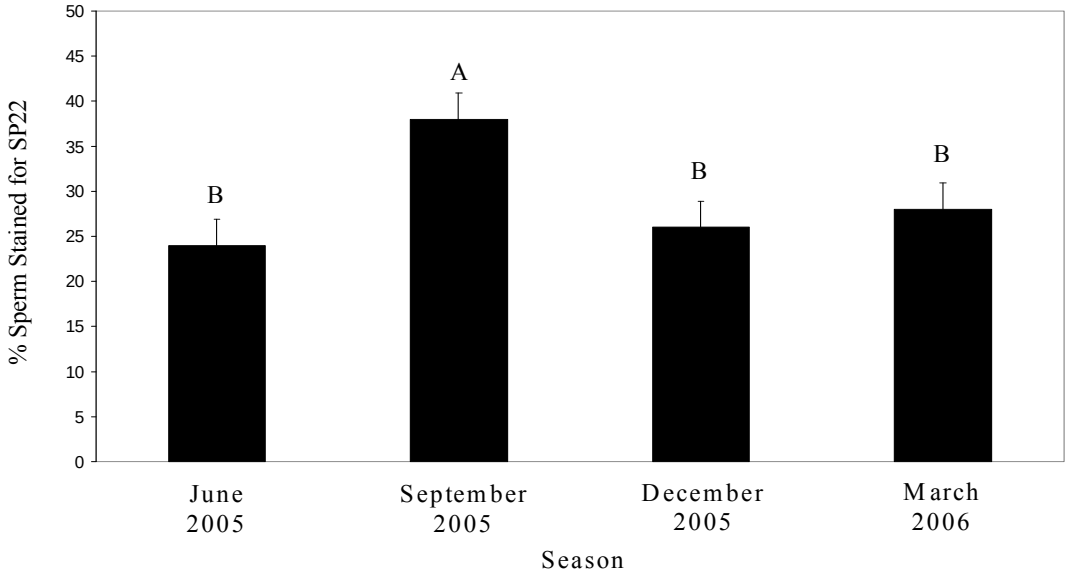

Figure 6. Effect of season on SP22 staining on the equatorial segment of sperm collected from the 4 subset stallions. Detected using SP22 antibody L. Means with different superscripts are significantly different; $P < 0.05$.

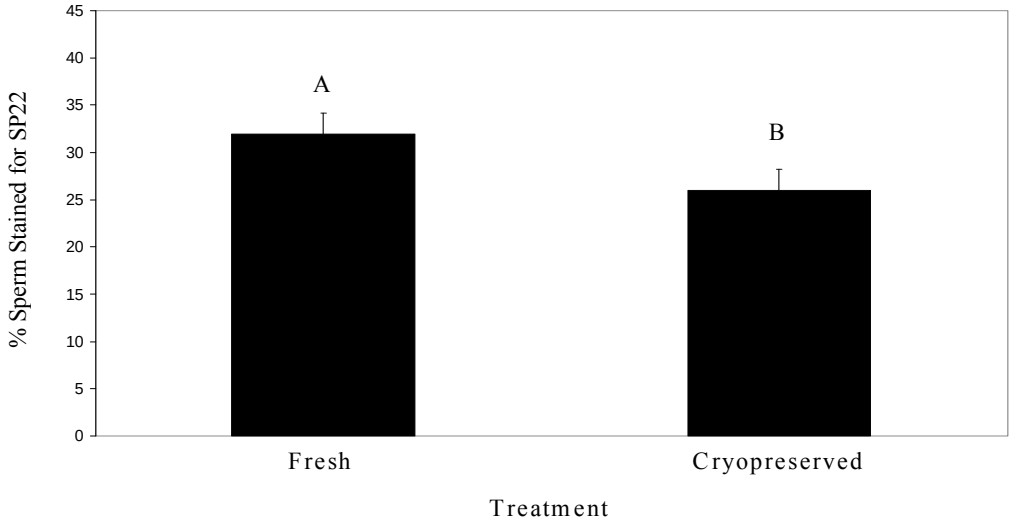

Figure 7. Effect of cryopreservation on SP22 staining on the equatorial segment of sperm collected from the 4 subset stallions. Detected using SP22 antibody L. Means with different superscripts are significantly different; $P = 0.05$.

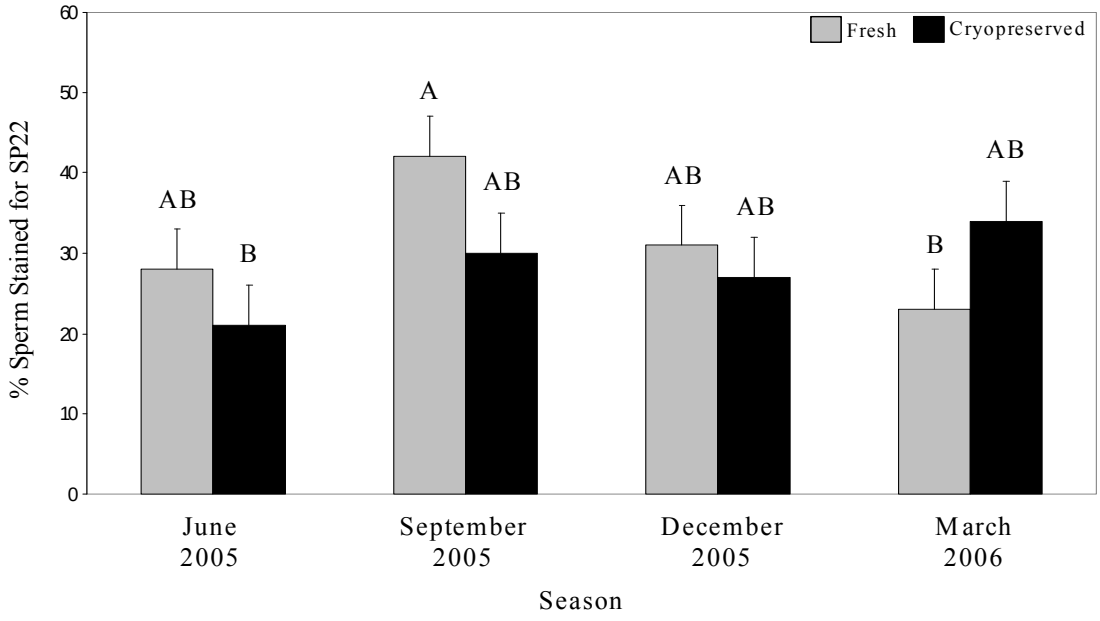

Figure 8a. Percentage of sperm exhibiting staining for SP22 on the equatorial segment across season and across cryopreservation. n = 6 stallions. Detected using SP22 antibody L. Significant season x cryopreservation interaction, means with different superscripts are significantly different; $P < 0.05$.

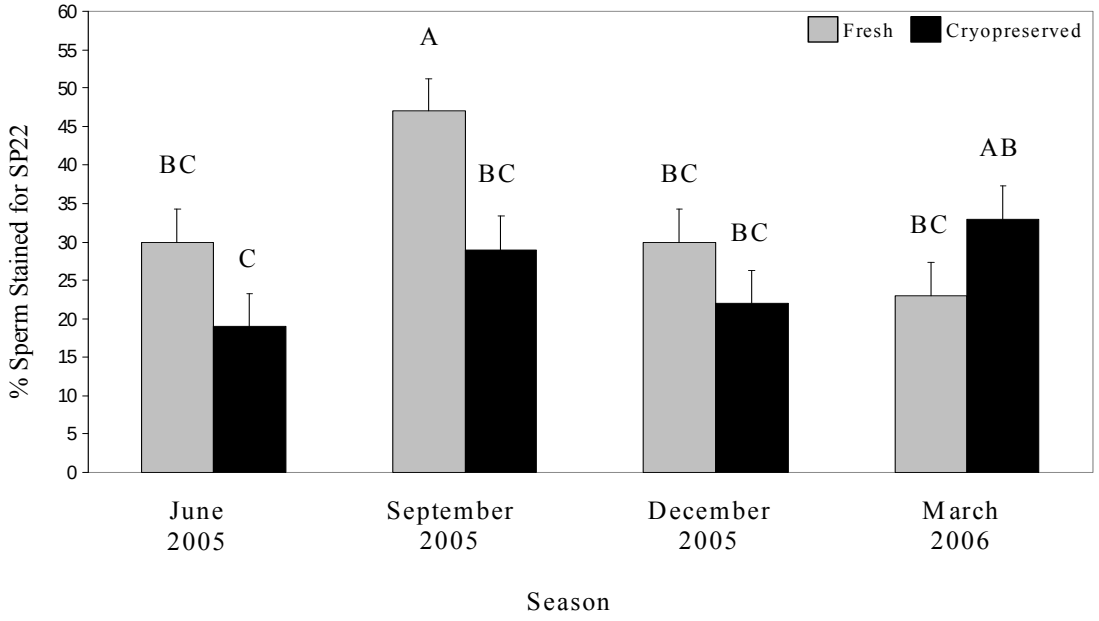

Figure 8b. Percentage of sperm exhibiting staining for SP22 on the equatorial segment across season and across cryopreservation. n = 4 stallions. Detected using SP22 antibody L. Significant season x cryopreservation interaction, means with different superscripts are significantly different; $P < 0.05$.

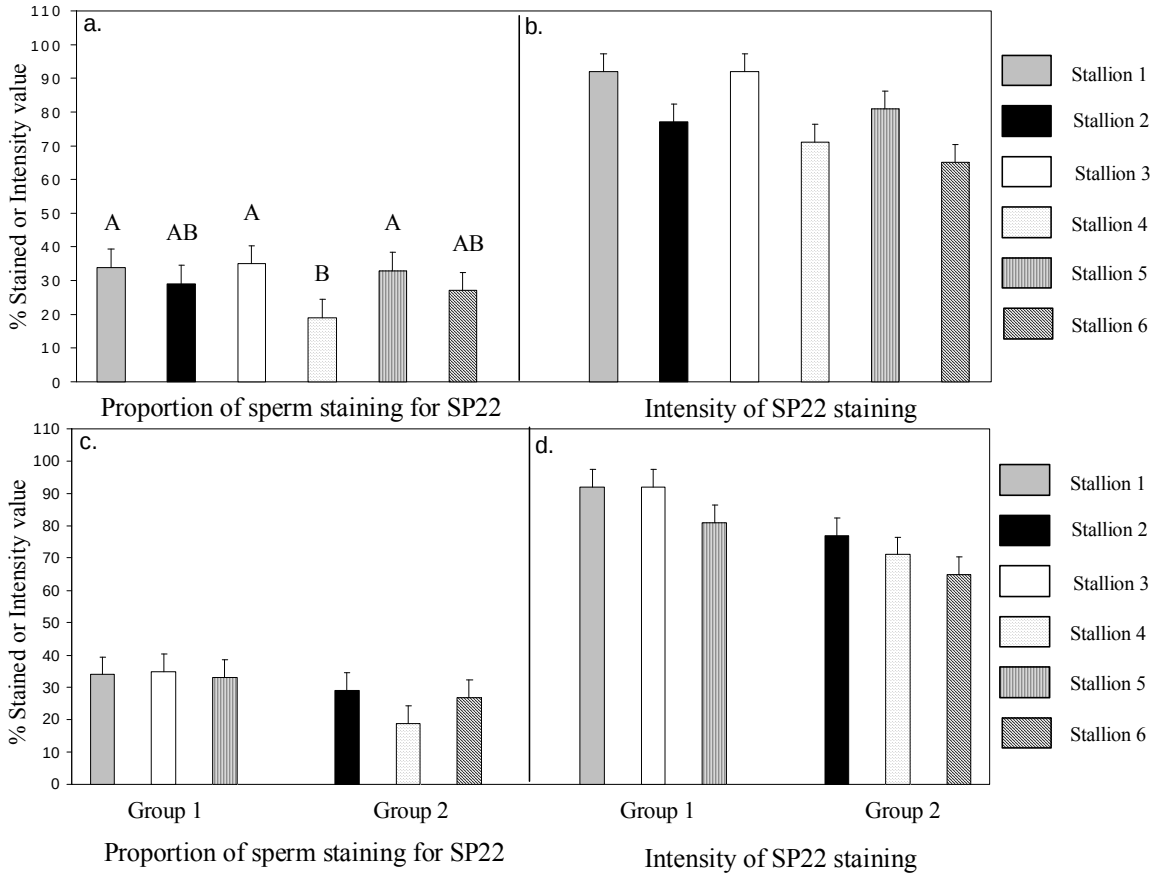

Figure 9. Panel a shows the effect of stallion for the 6 stallions on the proportion of sperm staining for SP22 on the equatorial segment. Panel b displays the intensity of SP22 staining across stallions. In panels c and d stallions 1, 3 and 5 had the highest percentage of SP22 staining and intensity of SP22 signal, designated as Group 1. Stallions 2, 4 and 6 had a lower percentage of SP22 staining and intensity of SP22 signal, designated as Group 2. Detected using SP22 antibody L. Means with different superscripts are significantly different; $^{A,B}P < 0.05$.

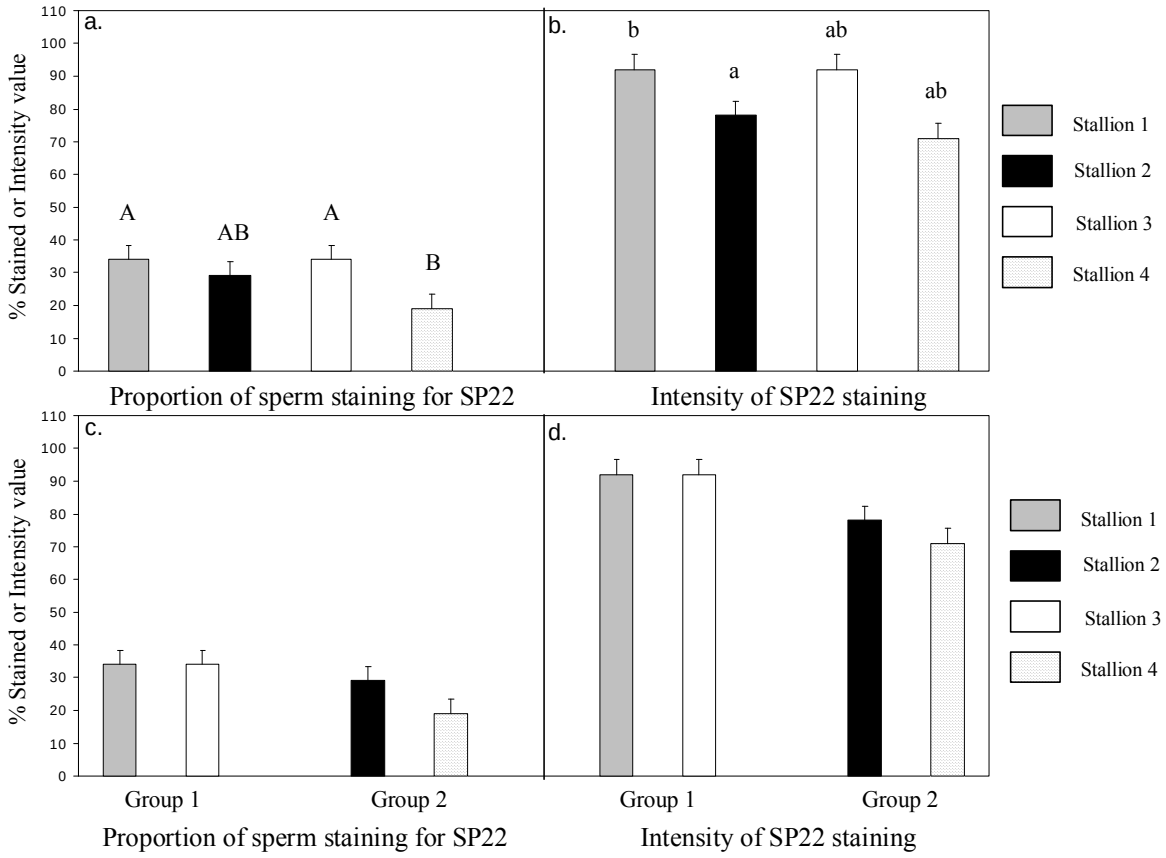

Figure 10. Panel a shows the effect of stallion for the 4 subset stallions on the proportion of sperm staining for SP22 on the equatorial segment. Panel b displays the intensity of SP22 staining across stallions. In panels c and d stallions 1 and 3 had the highest percentage of SP22 staining and intensity of SP22 signal, designated as Group 1. Stallions 2 and 4 had a lower percentage of SP22 staining and intensity of SP22 signal, designated as Group 2. Detected using SP22 antibody L. Means with different superscripts are significantly different; $^{A,B}P < 0.05$ and $^{a,b}P = 0.08$.

Messenger Ribonucleic Acid

The yield of RNA extracted was calculated as nanograms of RNA per million sperm. The overall mean for RNA yield was 15±2 ng per million sperm. There was no significant effect of season, stallion, cryopreservation or their interaction on RNA yield.

In Figure 11 is displayed an example of RT-PCR analysis of RNA extracted from sperm and testicular tissue. Note that all data for content of mRNA expression were calculated as a ratio to the content of GAPDH mRNA. Data for the non-housekeeper adjusted mRNA content can be found in the Appendix (Tables 3 and 4).

Data for the effect of stallion, season and cryopreservation on expression of selected mRNAs are presented in Table 6 (six stallions) and Table 7 (subset stallions). There was no effect of season or cryopreservation on relative expression of mRNAs for PGK2, TPX1, TIMP3 or ACTB. Also, no season by cryopreservation or stallion x cryopreservation interactions were found. However, differences between stallions (n = 6 stallions) were apparent for PGK2 ($P = 0.08$) and ACTB ($P = 0.01$). For the subset stallions there was a tendency ($P = 0.10$) for a stallion effect on ACTB mRNA.

Figure 11. RT-PCR gene expression analysis: testis vs. sperm

FIG 11a. Lanes 1 and 11: 100 bp ladder; Lane 2: PGK2 TT*; Lane 3: PGK2 SP**; Lane 4: PGK2 WB*** Lanes 5-7: Blank; Lane 8: TPX1 TT; Lane 9: TPX1 SP; Lane 10: TPX1 WB

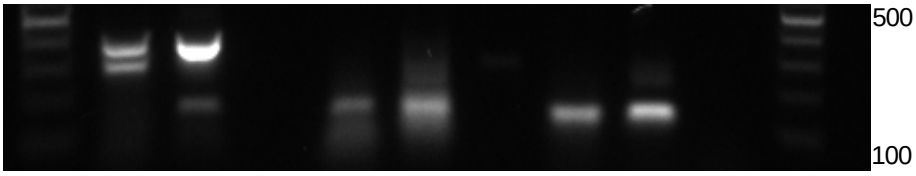

FIG 11b. Lanes 1 and 11: 100 bp ladder; Lane 2: ACTB TT; Lane 3: ACTB SP; Lane 4: ACTB WB; Lane 5: TIMP3 TT; Lane 6: TIMP3 SP; Lane 7: TIMP3 WB; Lane 8: GAPDH TT; Lane 9: GAPDH SP; Lane 10: GAPDH WB

* Testicular tissue, ** Sperm, *** Water Blank
Etbr stained 1.5% agarose gel in TBE

Table 6. Effect of stallion, season and cryopreservation on relative mRNA content in sperm from six stallions (Least squares means ± SEM).

mRNA	Stallion (1-6)	P-value	Season (J,S,D,M)	P-value	Fresh, Cryopreserved	P-value
ACTB*	0.41 ± 0.16^{bc}	0.0086	0.63 ± 0.20	0.1321	0.54 ± 0.10	0.4514
	0.46 ± 0.19^{bc}		0.87 ± 0.14		0.66 ± 0.12	
	0.37 ± 0.18^{bc}		0.50 ± 0.14			
	0.23 ± 0.16^{c}		0.40 ± 0.13			
	0.90 ± 0.18^{ab}					
	1.24 ± 0.20^{a}					
PGK2	0.45 ± 0.08^{a}	0.0845	0.49 ± 0.10	0.8366	0.44 ± 0.05	0.9736
	0.53 ± 0.10^{a}		0.46 ± 0.07		0.44 ± 0.06	
	0.39 ± 0.09^{ab}		0.40 ± 0.07			
	0.59 ± 0.08^{a}		0.40 ± 0.06			
	0.47 ± 0.09^{a}					
	0.20 ± 0.10^{b}					
TPX1	1.48 ± 1.65	0.1841	3.23 ± 2.05	0.6750	2.23 ± 0.97	0.7969
	0.49 ± 1.95		1.38 ± 1.44		2.64 ± 1.23	
	0.67 ± 1.79		1.70 ± 1.39			
	1.04 ± 1.68		3.41 ± 1.30			
	6.06 ± 1.82					
	4.86 ± 2.07					
TIMP3	1.56 ± 0.47	0.4316	1.61 ± 0.59	0.1985	1.38 ± 0.28	0.9603
	1.31 ± 0.56		1.95 ± 0.41		1.36 ± 0.35	
	0.83 ± 0.51		0.66 ± 0.40			
	1.48 ± 0.48		1.25 ± 0.37			
	2.21 ± 0.52					
	0.82 ± 0.59					

*Beta Actin (ACTB), Tissue Inhibitor of Metalloproteinase 3 (TIMP3), Testis Specific Protein 1 (TPX1) and Phosphoglycerate Kinase 2 (PGK2)

Table 7. Effect of stallion, season and cryopreservation on relative mRNA content in sperm from four subset stallions (Least squares means ± SEM).

mRNA	Stallion (1-4)	P-value	Season (J,S,D,M)	P-value	Fresh, Cryopreserved	P-value
ACTB*	0.44 ± 0.06[a]	0.1006	0.37 ± 0.07	0.8971	0.34 ± 0.04	0.5797
	0.39 ± 0.07[ab]		0.39 ± 0.06		0.38 ± 0.05	
	0.40 ± 0.06[ab]		0.35 ± 0.06			
	0.22 ± 0.06[b]		0.33 ± 0.06			
PGK2	0.43 ± 0.09	0.4960	0.54 ± 0.10	0.8413	0.47 ± 0.06	0.6876
	0.51 ± 0.10		0.49 ± 0.09		0.51 ± 0.07	
	0.41 ± 0.09		0.50 ± 0.09			
	0.59 ± 0.09		0.42 ± 0.09			
TPX1	1.56 ± 0.64	0.8816	1.76 ± 0.76	0.7356	1.38 ± 0.45	0.4341
	0.96 ± 0.76		0.83 ± 0.65		0.83 ± 0.51	
	0.90 ± 0.70		1.09 ± 0.70			
	1.00 ± 0.65		0.74 ± 0.64			
TIMP3	1.60 ± 0.40	0.5975	1.43 ± 0.47	0.5567	1.46 ± 0.28	0.3273
	1.06 ± 0.47		1.49 ± 0.40		1.03 ± 0.32	
	0.88 ± 0.43		0.69 ± 0.43			
	1.45 ± 0.40		1.38 ± 0.40			

*Beta Actin (ACTB), Tissue Inhibitor of Metalloproteinase 3 (TIMP3), Testis Specific Protein 1 (TPX1) and Phosphoglycerate Kinase 2 (PGK2)

Discussion

In the present study the percentage of sperm exhibiting normal morphology was greatest in June. Morphologically normal sperm are important for obtaining high fertilization rates; therefore, it is expected that a greater percentage of morphologically normal sperm would be present during the natural breeding season. These observations are in contrast to previously reported data where the proportion of morphologically normal sperm were found to be low in the summer (2) and then increased in the fall (1). These two studies included Warmblood stallions (2) or Franches-Montagnes (1) only and were conducted in Switzerland where seasonal variation may be more extreme compared to North Carolina where the present study was conducted.

In addition to sperm having the highest percentage of morphologically normal cells, the percentage of viable sperm and those with intact membranes was also highest in June. As noted above, it would be expected that the best sperm would be available during the natural breeding season rather than during the non-breeding season.

The process of cryopreservation had a significant effect on sperm parameters, with all parameters showing a decrease following the cryopreservation procedure. Differences between sperm characteristics of fresh and cryopreserved sperm appeared to be least in June, particularly with respect to sperm morphology. These data support the suggestion that cryopreserving semen in June may be better compared to cryopreserving during the non-breeding season.

In the present study, the percentage of sperm staining for SP22, specifically on the equatorial segment, was significantly reduced after cryopreservation of sperm when only data from the 4 stallions collected across all seasons was included in the analysis. The observed

effect of cryopreservation on SP22 staining is consistent with previous reports (8,9). More sperm stained for SP22 on the equatorial segment in September compared to other seasons. Why the percentage of sperm stained with SP22 antibody L was highest in September is unclear. However, when data from all 6 stallions including the 4 subset stallions were included in the analysis, there was a significant season x cryopreservation interaction where the difference between fresh and cryopreserved sperm staining for SP22 on the equatorial segment was greatest in September. Thus, when the percentage of freshly ejaculated sperm staining for SP22 is greatest, those sperm appear more susceptible to the cryopreservation process compared to sperm collected in the other seasons. Presumably, the sperm membrane is destabilized after the breeding season. Interestingly, the percentage of cryopreserved sperm staining for SP22 was actually greater than the percentage of freshly ejaculated sperm staining for SP22 in the March 2006 collection. Because the same antibody and the same technique were used throughout the study it is unclear as to why this would occur. However, it may suggest that SP22 is most stabilized in March in preparation for the breeding season.

Both the proportion of sperm stained for SP22 and the intensity of SP22 staining was affected by stallion. When stallions were grouped based on the percentage of sperm staining for SP22, those stallions with the highest percentage were also the stallions with the greatest staining intensity. Stallion 4 had the lowest percentage of sperm stained and staining intensity in both data sets analyzed. Although all six stallions were of proven fertility, it is possible that stallion 4 may exhibit decreased fertility and this may be reflected in the decreased staining for SP22 over the equatorial segment of sperm from this stallion. In a separate study that included this particular stallion, the effects of different types of semen extenders on basic semen parameters over a period of time was evaluated. Interestingly, it

was found that the motility of this particular stallion's sperm decreased to ≤ 35% after only 24 hours and to ≤ 20% after 48 hours with all extenders used (Pinto, unpublished data). Such a decrease in motility is undesirable for insemination of mares with fresh cooled semen as it may lead to decreased fertilization rates.

Observations based on the percentage of sperm stained from the different SP22 antibodies used showed antibody X was likely the most sensitive, at least when attempting to determine differences between stallions. Because the three different SP22 antibodies were only used on cryopreserved sperm in this study, it would be interesting to see if any differences exist in the percentage of sperm staining for SP22 between the SP22 antibodies when freshly ejaculated semen is used.

To the best of our knowledge this is the first report on the presence of mRNA in equine sperm. mRNA translation has been verified in capacitating sperm indicating an active requirement for at least some retained mRNAs; however, the possible role of sperm RNAs in fertilization is still unclear (6). Ostermeier et al (2004) found some sperm mRNAs which encode proteins essential for early embryonic development. It is possible that sperm RNA-mediated chromatin repackaging may be important for maintaining epigenetic changes occurring during spermatogenesis and essential for normal syngamy (7). Currently the data suggest that sperm RNAs play a role in the remodeling and imprinting of paternal chromatin (7).

RNA was extracted from stallion testicular tissue to ensure that the primer sets were detecting the expected product. The Park7 (SP22) mRNA transcript was detected in testicular tissue extract but was not detected in RNA extracted from ejaculated sperm. That

Park7 was not detected in sperm is likely because the mRNA is not present in ejaculated stallion sperm or because it is present at such low concentrations that it cannot be detected.

The RNA yield from human sperm extraction has been found to be around 1 µg per million sperm. In the current study, the most RNA extracted was 39 ng (0.039 µg) per million sperm using the same extraction method used for human sperm RNA extraction. RNA extraction of bull sperm appears to be more difficult than extraction of human sperm RNA (Dr. David Dix, personal communication). Therefore, it may be necessary to alter the RNA extraction method used for stallion sperm to increase RNA yield from this species.

Differences between stallions were noted for relative expression of ACTB and PGK2 mRNAs. These transcripts are possibly more abundant than TIMP3 and TPX1, which were not significantly affected by stallion, allowing for a difference between stallions to be noted. A stallion effect on mRNAs could possibly help detect differences between the fertility of stallions; however, stallions in this study had no known differences in fertility. There was no effect of season or cryopreservation on expression of any of the mRNA transcripts. Perhaps the mRNA transcripts are competent enough to not be affected by seasonal changes or the process of cryopreservation.

Sperm parameters were affected by the process of cryopreservation and could be affected by season or stallion. The percentage of sperm staining for SP22 on the equatorial segment was affected by cryopreservation, season and stallion. The relative quantity of ACTB and PGK2 mRNAs were affected by stallion, no other effects on selected sperm RNAs were noted. Understanding differences that exist in sperm across seasons and stallions is beneficial when attempting to determine the best time to collect semen for

cryopreservation. Based on data presented, we recommend collecting semen for cryopreservation between March and June.

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Appendices

APPENDICES

Appendix A. Figures 1-5. Determination of the cycle numbers within the linear amplification range for assessment of GAPDH, ACTB, PGK2, TIMP3 and TPX1 mRNAs by semiquantitative RT-PCR.

Appendix B. Tables 1-3; Figures 6a-e and 7a-e. Sperm parameters data for sperm collected from March 2005 continuing to March 2006.

Appendix C. Figures 8-12. Effects of season and stallion on SP22 staining using SP22 antibodies L, S and X on cryopreserved sperm.

Appendix D. Figure 13. Park7 (SP22) nucleotide sequence.

Appendix E. Tables 3 and 4. Effect of stallion, season and cryopreservation on relative non-housekeeper adjusted mRNA content.

Appendix A

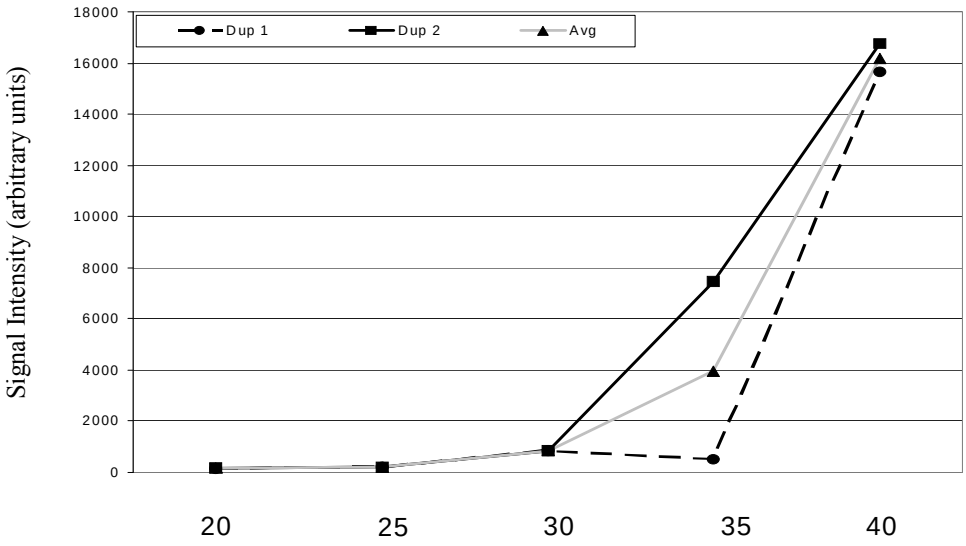

Figure 1. Response curve for GAPDH mRNA transcript.

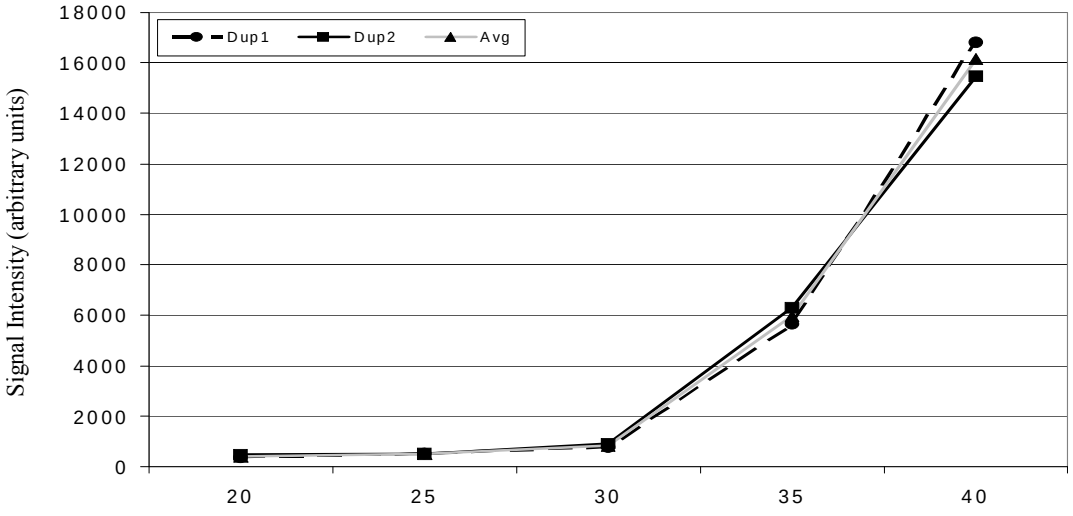

Figure 2. Response curve for ACTB mRNA transcript.

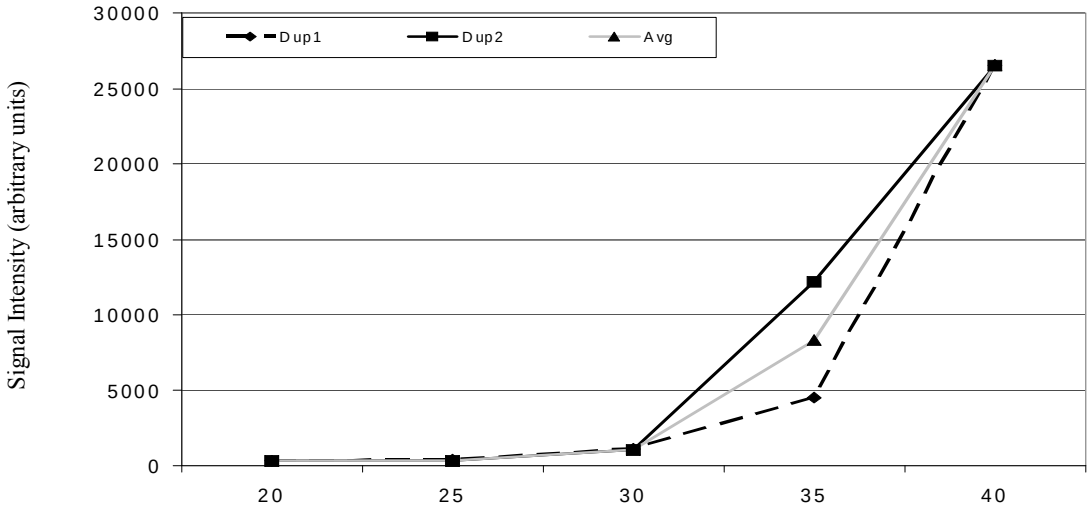

Figure 3. Response curve for PGK2 mRNA transcript.

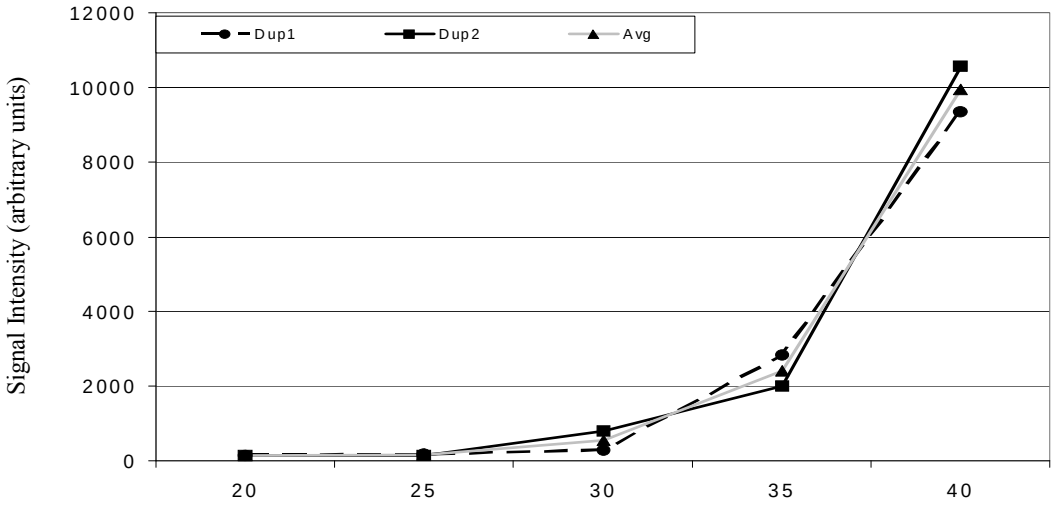

Figure 4. Response curve for TIMP3 mRNA transcript.

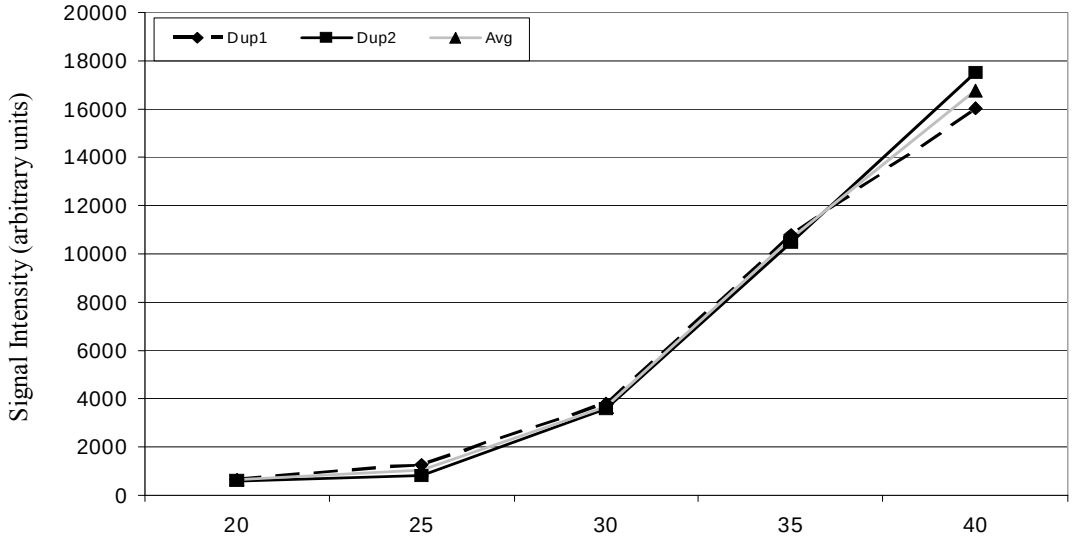

Figure 5. Response curve for TPX1 mRNA transcript.

Appendix B

Table 1. Effect of Cryopreservation on Stallion Semen Characteristics (Least squares means ± SEM).

	n = 6 Stallions		n = 4 Stallions	
	Fresh	Cryopreserved	Fresh	Cryopreserved
Normal Morphology (%)	76 ± 1[a]	45 ± 1[b]	77 ± 1[c]	49 ± 1[d]
Primary Abnormalities (%)	18 ± 1[b]	41 ± 1[a]	18 ± 1[d]	42 ± 1[c]
Secondary Abnormalities (%)	6 ± 1[b]	13 ± 1[a]	6 ± 1[d]	8 ± 1[c]
Intact Plasma Membrane (HOST;%)	68 ± 2[a]	47 ± 2[b]	68 ± 3[c]	48 ± 3[d]
Viability (%)	75 ± 3[a]	37 ± 3[b]	81 ± 4[c]	38 ± 4[d]
Total Motility (%)	71 ± 2[a]	41 ± 2[b]	74 ± 2[c]	49 ± 2[d]
Progressive Motility (%)	64 ± 2[a]	34 ± 2[b]	67 ± 2[c]	42 ± 2[d]
Intact Acrosome (%)	93 ± 1[a]	63 ± 1[b]	94 ± 1[c]	65 ± 2[d]

[ab]$P < 0.0001$ between treatments within data from six stallions
[cd]$P < 0.01$ between treatments within data from four stallions

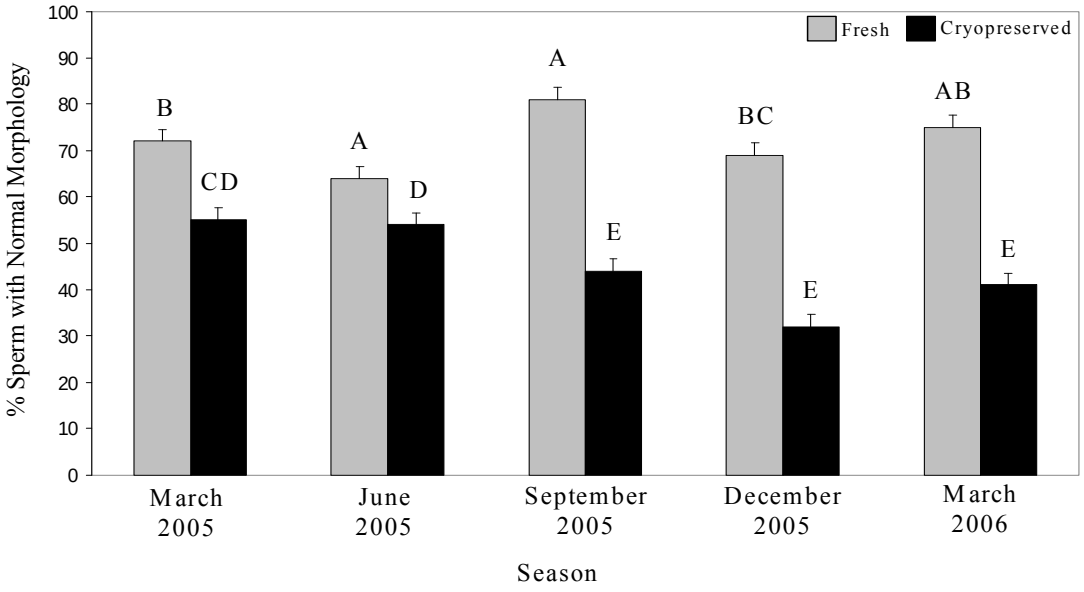

Figure 6a. Percentage of sperm exhibiting normal morphology across season and across cryopreservation. n = 6 stallions. Significant season x cryopreservation interaction, means with different superscripts are significantly different; $P < 0.05$.

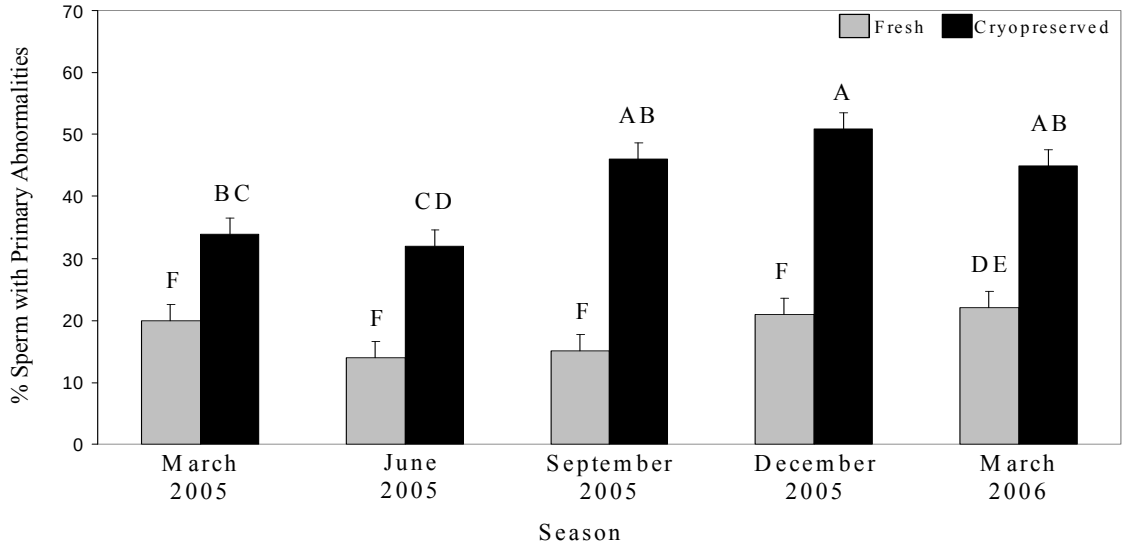

Figure 6b. Percentage of sperm exhibiting primary abnormalities across season and across cryopreservation. n = 6 stallions. Significant season x cryopreservation interaction, means with different superscripts are significantly different; $P < 0.05$.

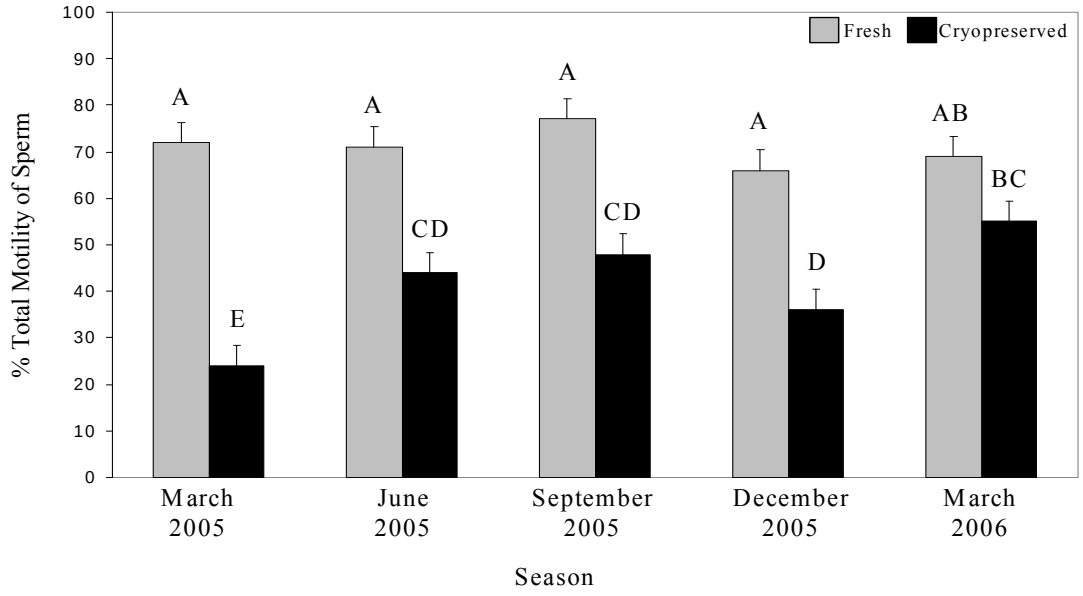

Figure 6c. Total motility of sperm across season and across cryopreservation. n = 6 stallions. Significant season x cryopreservation interaction, means with different superscripts are significantly different; $P < 0.05$.

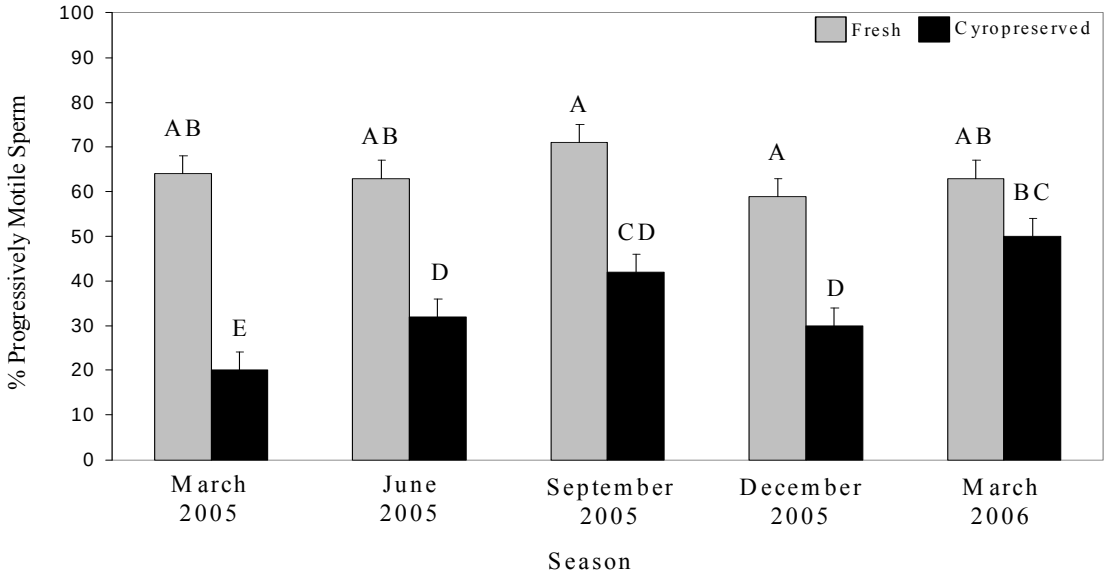

Figure 6d. Progressive motility of sperm across season and across cryopreservation. n = 6 stallions. Significant season x cryopreservation interaction, means with different superscripts are significantly different; $P < 0.05$.

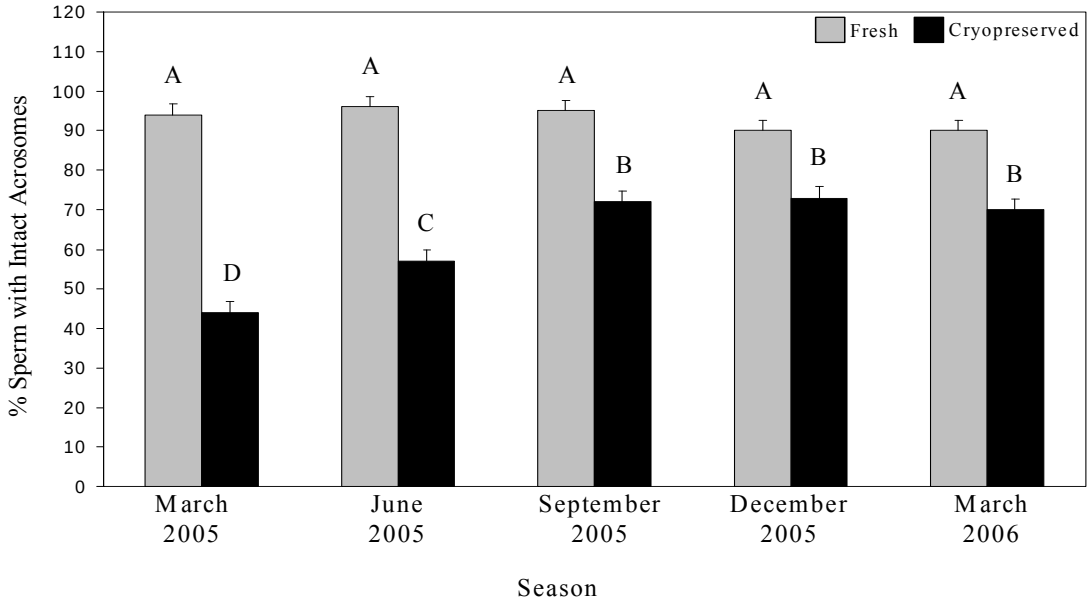

Figure 6e. Percentage of sperm with an intact acrosome across season and across cryopreservation. n = 6 stallions. Significant season x cryopreservation interaction, means with different superscripts are significantly different; $P < 0.05$.

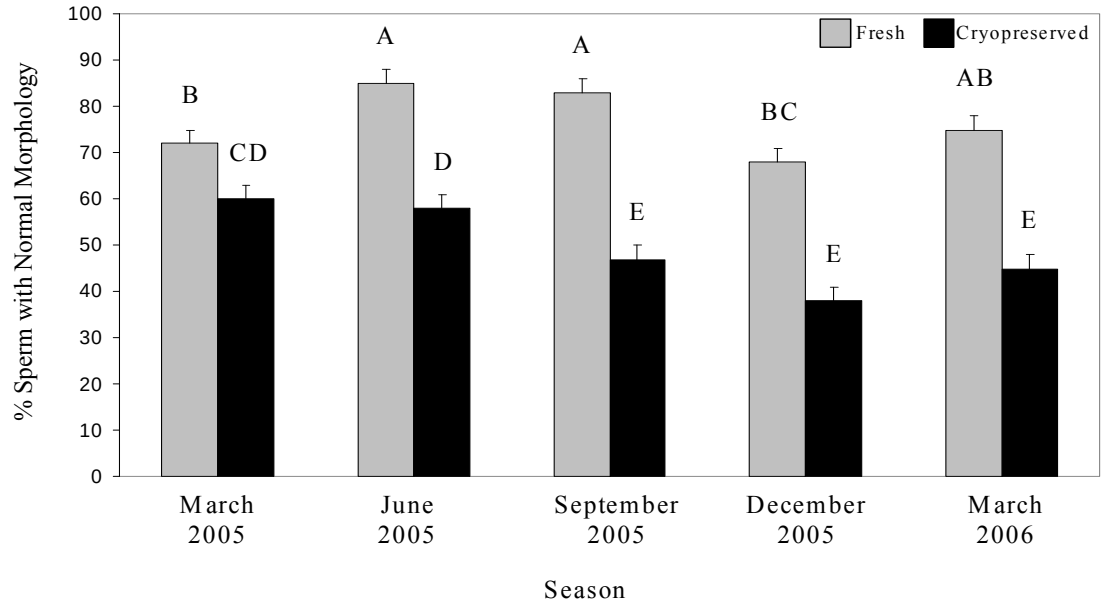

Figure 7a. Percentage of sperm exhibiting normal morphology across season and across cryopreservation. n = 4 stallions. Significant season x cryopreservation interaction, means with different superscripts are significantly different; $P < 0.05$.

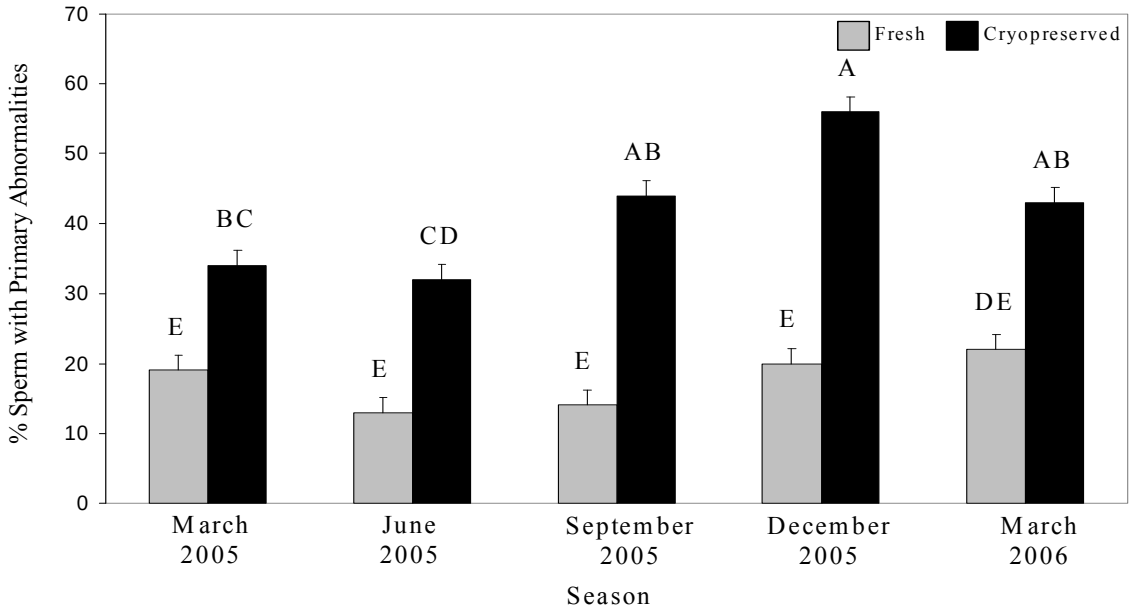

Figure 7b. Percentage of sperm exhibiting primary abnormalities across season and across cryopreservation. n = 4 stallions. Significant season x cryopreservation interaction, means with different superscripts are significantly different; $P < 0.05$.

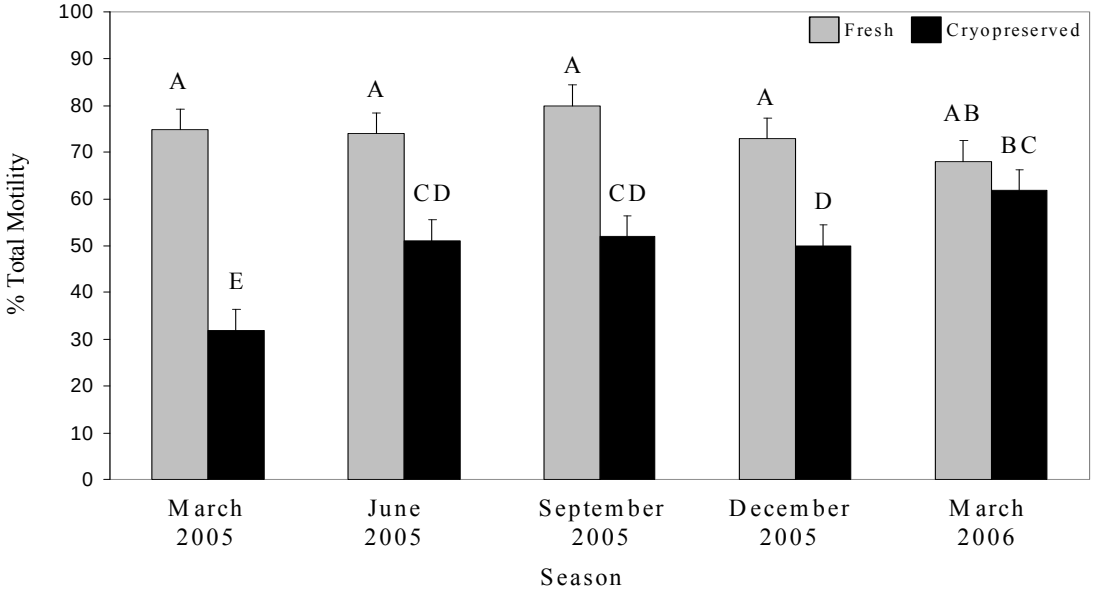

Figure 7c. Total motility of sperm across season and across cryopreservation. n = 4 stallions. Significant season x cryopreservation interaction, means with different superscripts are significantly different; $P < 0.05$.

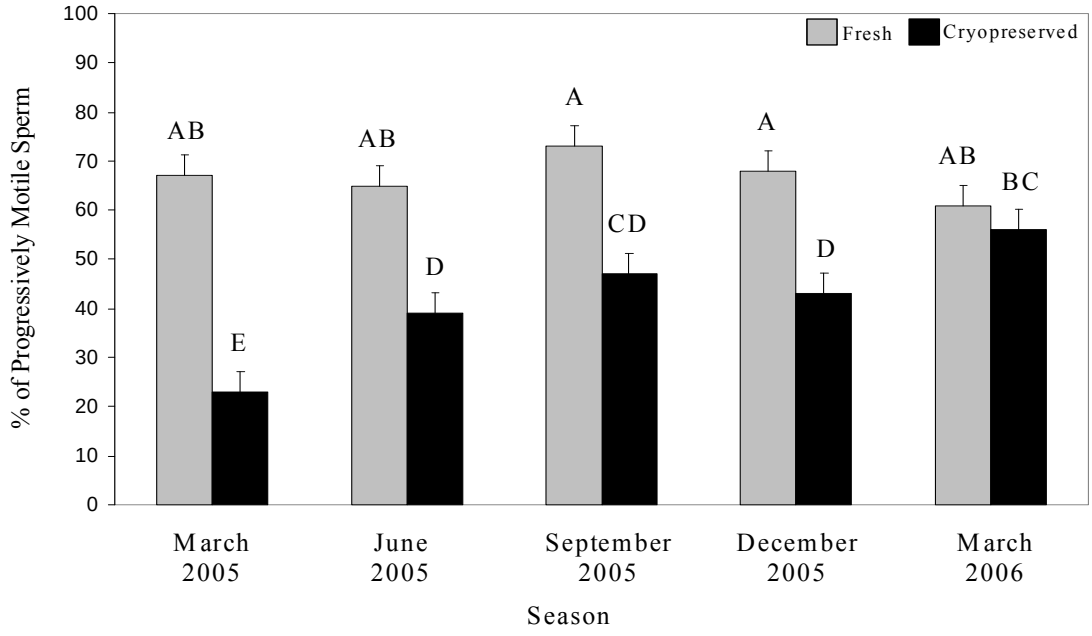

Figure 7d. Progressive motility of sperm across season and across cryopreservation. n = 4 stallions. Significant season x cryopreservation interaction, means with different superscripts are significantly different; $P < 0.05$.

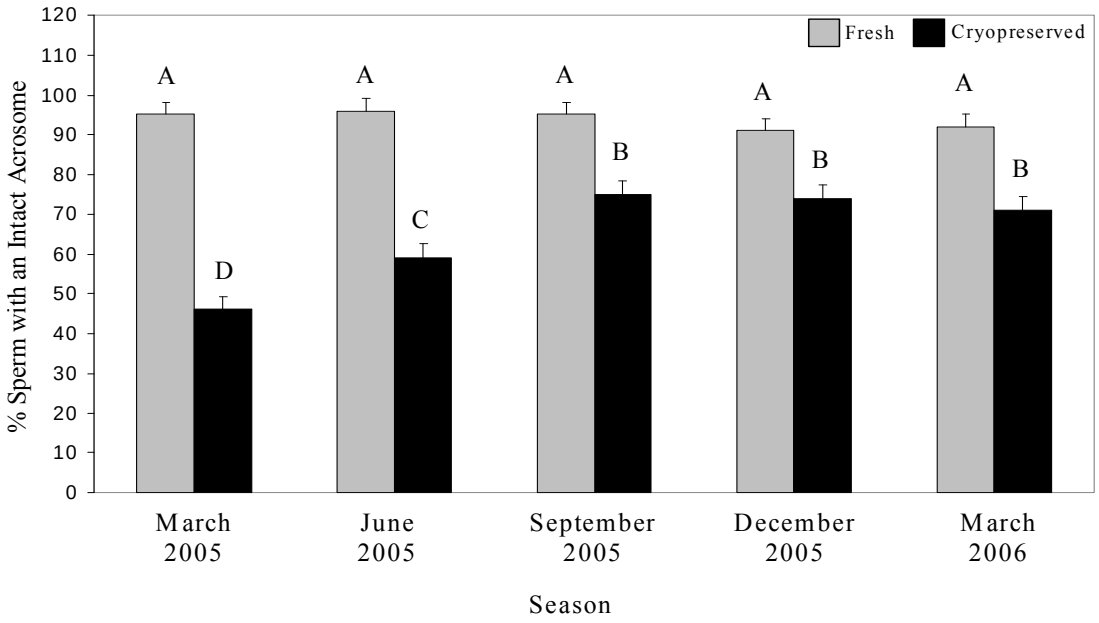

Figure 7e. Percentage of sperm with an intact acrosome across season and across cryopreservation. n = 4 stallions. Significant season x cryopreservation interaction, means with different superscripts are significantly different; $P < 0.05$.

Appendix C

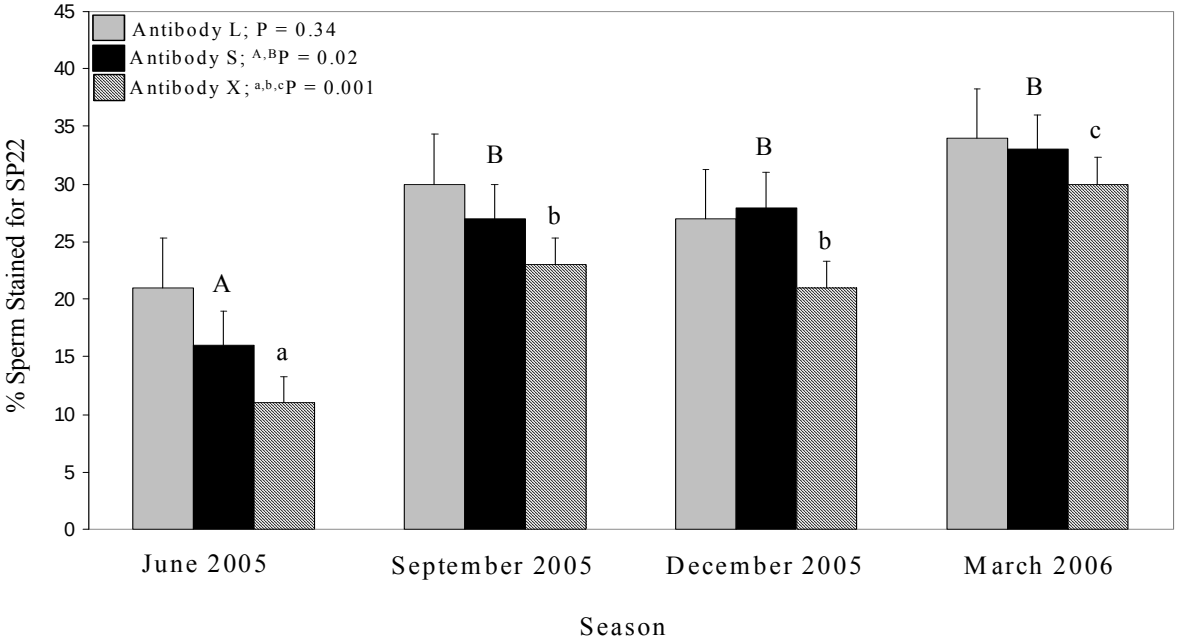

Figure 8. Effect of season (n = 4) on SP22 staining on the equatorial segment of sperm collected from all six stallions using the three different SP22 antibodies. Means with different superscripts are significantly different; $P < 0.05$.

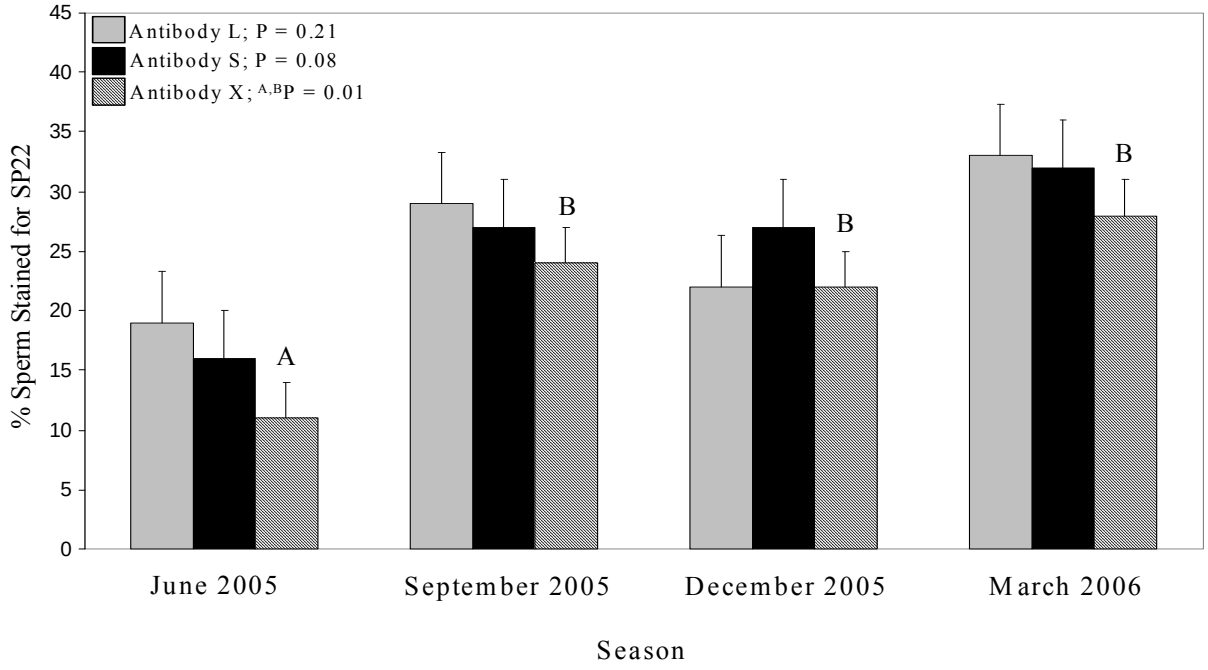

Figure 9. Effect of season (n = 4) on SP22 staining on the equatorial segment of sperm collected from the four subset stallions using the three different SP22 antibodies. Means with different superscripts are significantly different; $P < 0.05$.

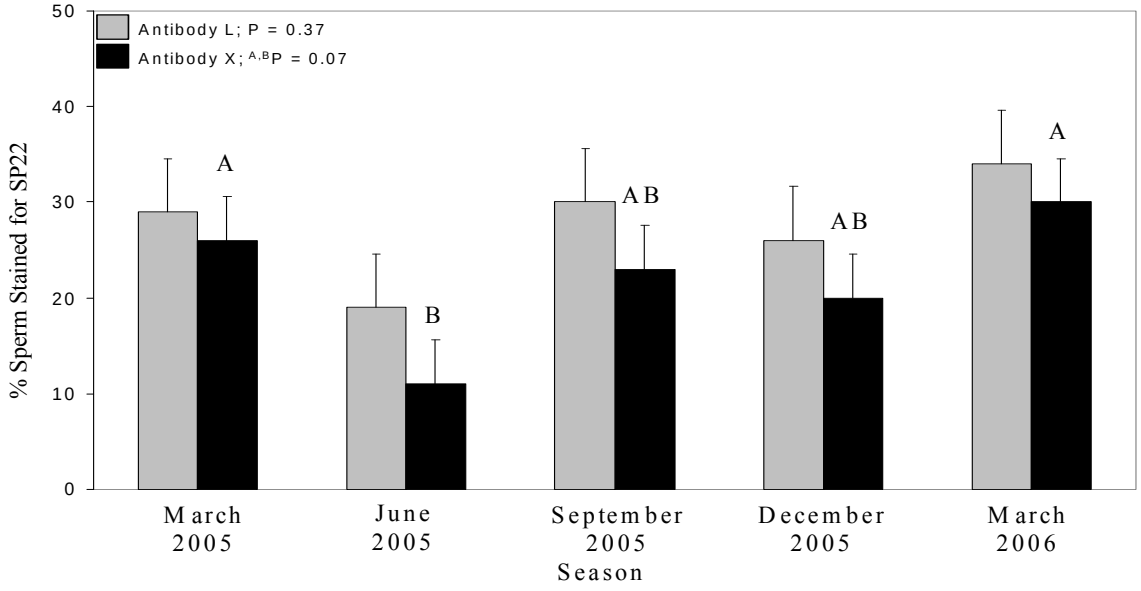

Figure 10. Effect of season (n = 5) on SP22 staining on the equatorial segment of sperm collected from all six stallions.

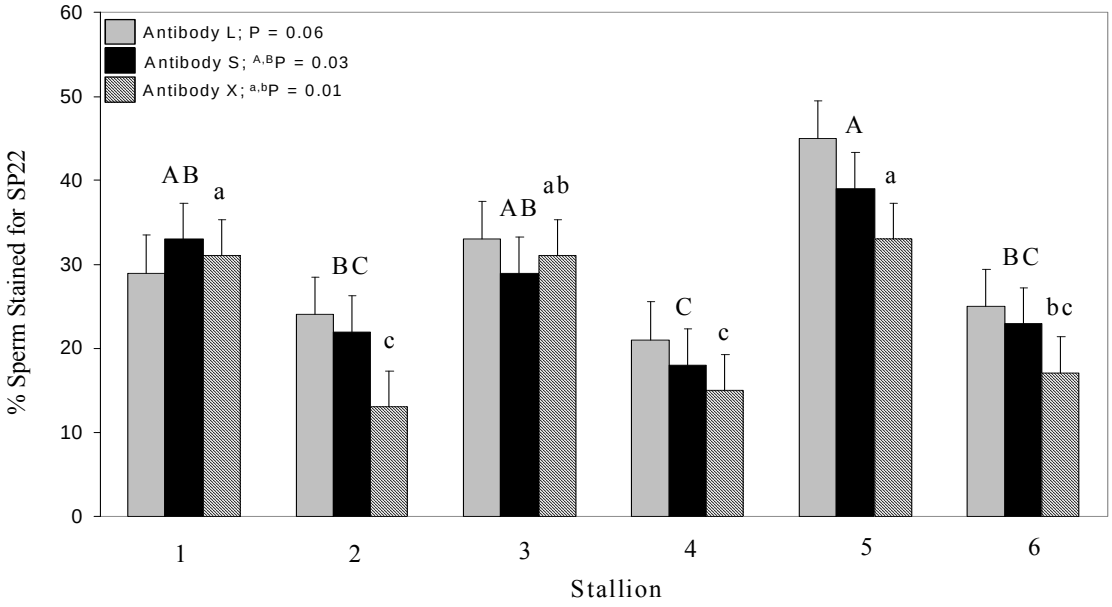

Figure 11. Effect of stallion on SP22 staining on the equatorial segment of sperm collected from all 6 stallions. Means with different superscripts are significantly different; $P < 0.05$.

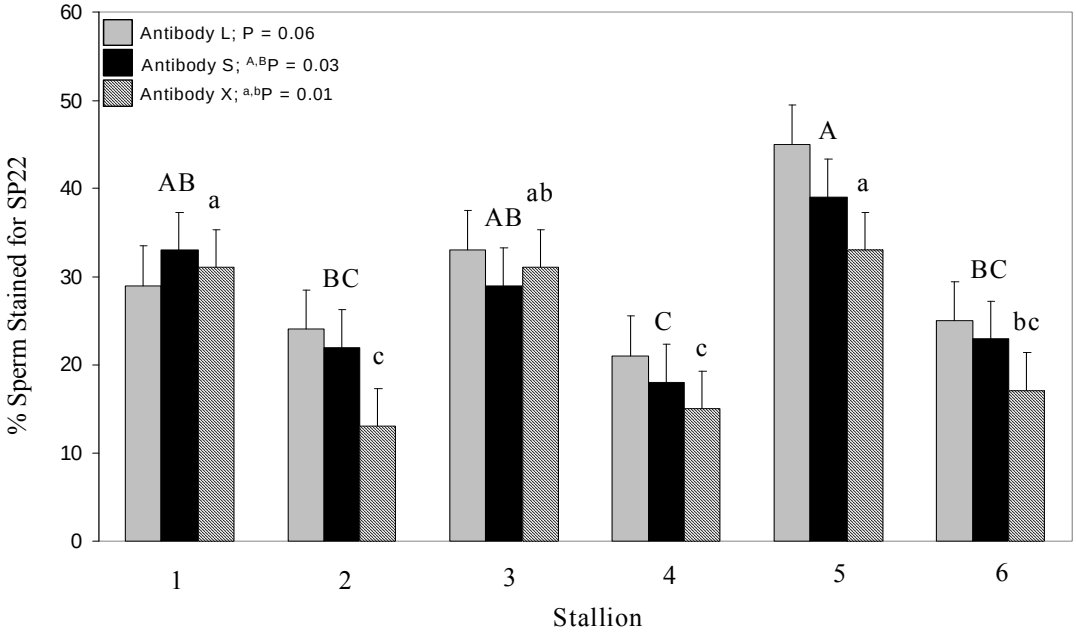

Figure 12. Effect of stallion on SP22 staining on the equatorial segment of sperm collected from the 4 subset stallions.

Appendix D

gtt atc cct gta gat gtc atg aga cga gct gga att aag gtc acc atc gcg ggt ctg gct gga aaa gac cca gta cag tgt agt cga gat gtt gtc att tgt cct gat gcc agt ctg gaa gat gca aag aaa cag gga cct tat gac gta gtg gtt cct cca gga ggt aat ctg ggt gcg cag aat tta tct gag tct gct gct gtt aaa gag ata ctg aag gaa caa gag aag

Figure 13. Equine Park7 PCR amplicon. Determined using Park7 primers: 5'-CGGTTATCCCTGTAGATG (forward) and 5'-CCTCTTCTCTTGTTCCTT (reverse).

Appendix E

Table 2. Effect of stallion, season and cryopreservation on relative non-housekeeper adjusted mRNA content in sperm from the four subset stallions (Least squares means ± SEM).

	Stallion (1-4)	P-value	Season (J,S,D,M)	P-value	Fresh, Cryopreserved	P-value
ACTB	3462 ± 577	0.3638	2397 ± 647	0.2916	3991 ± 480[a]	0.0118
	3564 ± 750		4064 ± 647		2126 ± 430[b]	
	2140 ± 577		2619 ± 695			
	3067 ± 695		3153 ± 577			
PGK2	3753 ± 654	0.4136	3705 ± 733	0.2577	5359 ± 544[a]	0.0010
	5029 ± 851		5081 ± 733		2330 ± 488[b]	
	3584 ± 654		2835 ± 789			
	3013 ± 789		3759 ± 654			
TPX1	5822 ± 1031	0.2346	6769 ± 1155	0.5288	6915 ± 858	0.0670
	7016 ± 1341		4443 ± 1155		4629 ± 768	
	3791 ± 1031		5785 ± 1242			
	6460 ± 1242		6092 ± 1031			
TIMP3	10322 ± 1413	0.4505	7366 ± 1584	0.1564	10392 ± 1176[a]	0.0310
	8965 ± 1839		10538 ± 1584		6605 ± 1054[b]	
	7407 ± 1413		5873 ± 1704			
	7300 ± 1704		10216 ± 1413			
GAPDH	9045 ± 1448	0.7287	6357 ± 1721	0.3839	10339 ± 1162[a]	0.0153
	8841 ± 1721		9907 ± 1466		5984 ± 1013[b]	
	6930 ± 1466		7336 ± 1581			
	7831 ± 1581		9047 ± 1448			

Table 3. Effect of stallion, season and cryopreservation on relative non-housekeeper adjusted mRNA content in sperm from all six stallions (Least squares means ± SEM).

	Stallion (1-6)	P-value	Season (J,S,D,M)	P-value	Fresh, Cryopreserved	P-value
ACTB	3462 ± 517	0.0942	1970 ± 602[ab]	0.0473	3067 ± 364	0.0906
	3665 ± 661		3840 ± 453[a]		2199 ± 329	
	2140 ± 517		2228 ± 476[b]			
	3079 ± 613		2494 ± 422[ab]			
	1604 ± 619					
	1848 ± 619					
PGK2	3753 ± 641[ab]	0.0201	2941 ± 747	0.0814	3920 ± 451[a]	0.0123
	5162 ± 820[a]		4368 ± 562		2262 ± 329[b]	
	3584 ± 641[abc]		2208 ± 590			
	3056 ± 761[abc]		2848 ± 523			
	1645 ± 768[bc]					
	1349 ± 768[c]					
TPX1	5822 ± 1402	0.5143	7364 ± 1635	0.3267	6494 ± 987	0.7357
	6717 ± 1792		4960 ± 1228		6039 ± 892	
	3791 ± 1402		5160 ± 1291			
	6087 ± 1664		7582 ± 1145			
	7908 ± 1679					
	7274 ± 1679					
TIMP3	10322 ± 1341[a]	0.0097	5716 ± 1564	0.0559	7619 ± 944	0.2564
	9350 ± 1715[ab]		9403 ± 1175		6136 ± 853	
	7407 ± 1341[ab]		4724 ± 1235			
	7457 ± 1592[ab]		7668 ± 1095			
	4894 ± 1607[bc]					
	1837 ± 1607[c]					
GAPDH	9047 ± 1209[a]	0.0022	4238 ± 1504	0.2761	6888 ± 900	0.1905
	8756 ± 1433[a]		7539 ± 1059		5329 ± 713	
	6914 ± 1235[ab]		5779 ± 1017			
	7996 ± 1312[a]		6878 ± 959			
	2887 ± 1335[bc]					
	1051 ± 1520[c]					