EVALUATION OF STALLION FROZEN-THAWED SEMEN USING CONVENTIONAL AND FLOW CYTOMETRIC ASSAYS

by

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(ABSTRACT)

Field evaluation of frozen-thawed stallion semen has been limited to tests such as post-thaw motility and morphology that are not only subjective but also evaluate only a small population of cells. Flow cytometry has provided a quick, repeatable, objective method of evaluating a large number of cells, including spermatozoa. Two experiments were designed to first validate the use of several flow cytometric tests on frozen-thawed stallion semen and then determine a model that may best explain variation in fertility. Comparing samples that were live and freeze-killed validated the flow cytometric tests.

In experiment one, six ejaculates were collected from each of three stallions. The semen from each ejaculate was centrifuged and frozen in 0.5 ml polyvinyl chloride straws. Two straws from each ejaculate were thawed and evaluated. Semen was evaluated for post-thaw motility, morphology, mitochondrial activity using Rhodamine 123 (R123), plasma membrane integrity using propidium iodide (PI) and ethidium monoazide (EMA), and chromatin structure using the sperm chromatin structure assay (SCSA). Data was recorded as percentages for all but the SCSA for both experiment one and two. The extent of chromatin denaturation was calculated using the SCSA and the alpha-t population [αt = red/(red +green) fluorescence]. From the alpha-t population, statistics were calculated such mean ($X\alpha t$), standard deviation ($SD\alpha t$),

percentage of cells outside (COMPαt) the main alpha-t population and the mean green fluorescence (mean green) of the population.

Results from experiment one demonstrated that all flow cytometric tests except EMA were able to distinguish between live and freeze-killed samples ($p < 0.0001$). Also the stallion accounted for most of the variation in samples when compared to ejaculate and straw within an ejaculate. Therefore two straws could be chosen at random from a stallion and evaluated in experiment two.

In experiment two, twenty-nine stallions were evaluated using the same tests as experiment one excluding EMA. Fertility data was obtained from the 1998 or 1999 breeding season. Multiple linear regression was used to determine the best-fit model to predict overall pregnancy rate. SCSA and R123-PI assays accounted for the largest amount of variation in fertility ($R^2 = 0.65$, $p < 0.0004$). Within SCSA and the R123/PI assays Xαt and PI staining had the highest contribution to this variation in fertility ($R^2 = 0.11$, $R^2 = 0.47$) respectively. The best-fit model for predicting fertility included the assay combination listed above and the interactions between SDαt and mean green staining as well as R123 and mean green staining. Post-thaw motility and morphology did not account for significant variation in fertility ($p = 0.22$, $p = 0.46$) respectively.

Based on this project post-thaw motility and morphology are poor predictors of fertility in frozen-thawed stallion semen. However, through the addition of SCSA and R123-PI to the routine evaluation of frozen-thawed stallion semen time and money may be saved in advance by identifying those stallions with poor post-thaw fertility.

Keywords: equine spermatozoa, flow cytometry, motility, morphology, fertility

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Introduction

Equine frozen-thawed semen usage has increased over the past decades. Equine fertility has been less than optimal with the use of frozen-thawed semen as stallions are chosen for performance rather than fertility. Conventional methods of semen evaluation include motility and morphology. Through advancements in research it has become clear that these methods of semen evaluation do not adequately define the fertilizing characteristics of spermatozoa. Certain molecular events not evaluated by motility or morphologic examination must occur prior to and post zona pellucida penetration for successful zygote and embryo formation. Once morphologically normal spermatozoa reach the oviduct it becomes important for other compartments of spermatozoa to function correctly to complete capacitation, acrosome reaction, and fertilization of the oocyte.

Cryopreservation has enabled identification of flaws in current methods of semen evaluation, as decreased fertility has been noted in multiple species when using frozen-thawed semen versus fresh or cooled semen. Cryopreservation of spermatozoa, as with the freezing and thawing of any living cell, can irreversibly damage cellular organelles and the plasma membrane. Scientists need to determine new methods for evaluating the ultrastructural damage that might occur during cryopreservation, particularly to organelles not well visualized via light microscopy. Furthermore, a need exists for objective, repeatable tests for evaluation of frozen-thawed semen.

Flow cytometry has recently been identified as a quick, repeatable, and objective method of evaluating these other compartments of spermatozoa. The flow cytometer has been used in humans, mice, and cattle to evaluate the integrity of mitochondria, acrosomes, and

plasma membranes of spermatozoa.[1-4] By evaluating these compartments scientists have gained a broader understanding of ways to improve cryopreservation techniques improving sperm viability. This will prevent owners from wasting time and money on stallions that do not cryopreserve well. Although the flow cytometer is not currently available to all laboratories and practices, it is available at most universities as a viable new tool for semen evaluation.

This project will contain two experiments. The first objective of experiment one is to determine the ability for the flow cytometric stains to distinguish between cryopreserved semen and semen that has been freeze-killed. Next the components of variation (stallion, ejaculate, and straw) for the conventional and flow cytometric assays will be determined. The final objective of experiment one is the determination of the correlation between the flow cytometric assays and the conventional methods. Experiment two will examine which assays or combination of assays will provide the best prediction of fertility using frozen-thawed semen. The results will demonstrate that a battery of tests is needed to accurately evaluate or predict the fertility of stallion frozen-thawed semen.

Literature Review

Equine frozen-thawed semen popularity has risen over the years. It allows a stallion to be shown without interruption during the breeding season. It decreases the risk of injury with natural breeding to both mare and stallion. There are many other advantages to using frozen-thawed semen but there is one big disadvantage: lower fertility. Attempts are being made to adequately evaluate all components of spermatozoa in order to identify potential causes of reduced fertility and to find ways of improving cryopreservation techniques.

This section will examine the anatomy and physiology of the spermatozoon to serve as the basis of understanding changes that occur during cryopreservation that may result in dysfunction. Means of altering quality and quantity of spermatozoa will also be discussed. A review of conventional methods of semen evaluation (motility and morphology), as well as new methods using the flow cytometer, will be discussed.

Anatomy and physiology of the spermatozoon

The spermatozoon consists of 3 main parts: head, middle piece and tail. Each section has a specific function and organelles to enable a spermatozoon to achieve penetration and fertilization of an oocyte. The spermatozoon begins development at the base of the seminiferous tubule as an A spermatogonia. A long course of mitotic divisions, meiosis and differentiation follows before it is released as a mature spermatozoon. Cellular divisions undertaken by the spermatozoon have been defined as spermatocytogenesis, meiosis, and spermiogenesis.[5] Spermatocytogenesis is the mitotic divisions the A spermatogonia undergo

until it reaches the primary spermatocyte stage. Approximately 19.4 days are required for the equine spermatozoon to complete this phase of development.[6]

The next phase is meiosis, wherein the primary spermatocyte undergoes two meiotic divisions to become the haploid round spermatid. This phase is also completed in approximately 19.4 days.[6] During meiosis the spermatocyte moves from the adluminal to the luminal compartment of the seminiferous tubule via tight junctions between Sertoli cells. Tight junctions formed between Sertoli cells separate haploid cells from the blood and its elements, thus forming a blood-testis barrier.[5] The spermatocyte halves its original complement of chromosomes (haploid) during maturation and at that point is seen as foreign by the body. The blood-testis barrier prevents the immune system from contacting and destroying the spermatocyte and more mature stages of the spermatozoon.

Spermiogenesis is the final and one of the most important stages of spermatozoal maturation. During this phase the spermatozoon loses some of its organelles to limit the amount of unnecessary material transported to the oocyte. Endoplasmic reticulum and cytoplasmic lysosomes are deleted.[7] The Golgi apparatus begins to package the acrosomal enzymes in vesicles necessary for zona penetration. These vesicles will then move to the tip of the head and become the acrosome.[7]

The proximal centriole in the cytoplasm moves during elongation of the spermatid to the implantation fossa. The implantation fossa is the connecting point between the head and middle piece. The other centriole, the distal centriole, moves to the opposite pole of the acrosome to form the axoneme in the flagellum. The flagellum forms in a 9+9+2 pattern of microtubules and is similar in most animal species. Fawcett and Porter first described this microtubular pattern in 1954.[8] The outer 9 elements are dense fibers that aid in cytoskeletal structure for

tail bending and movement. The next layer is 9 paired (doublets) of microtubules that aid in motility as is the innermost layer, the 2 central microtubules.[7]

The flagellum is covered by a continuous plasma membrane but is divided into three sections: middle piece, principal piece, and end piece.[7] Under the plasma membrane of the middle piece a mitochondrial sheath surrounds the outer dense fibers. It consists of two parallel helices of mitochondria of about 60 coils.[9] Mitochondria are necessary for oxidative phosphorylation, which generates ATP and provides energy for the flagellum. The principal piece has a fibrous sheath and is the longest segment of the flagellum of the spermatozoon. The fibrous sheath is composed of two longitudinal columns connected by circumferential ribs [7], which provide additional support and aid in bending and movement of the flagellum.

Spermatozoal movement occurs by shear forces between the microtubule doublets. Dynein arms remain attached until ATP causes shortening and disconnection of adjacent microtubule doublets. With the conversion of ATP to ADP and energy, the dynein arm tilts and lengthens to attach at a new site. The dynein arm then returns to its original length, which causes sliding of the two microtubule doublets past each other. This sliding action of the doublets along with the anchoring of the microtubules to the neck creates the characteristic beating pattern seen by the spermatozoon as it moves.[10]

During flagellar development the head of the spermatozoon is elongating and excess cytoplasm is shed as a cytoplasmic droplet. This droplet is lost as the sperm courses through the epididymis and is not present on the normal ejaculated equine spermatozoon. The nucleus of the spermatozoon now occupies the majority of the space in the spermatozoon head. Nuclear changes also occur during spermiogenesis. Histones, nuclear proteins of the diploid genome, are replaced by protamines made by the spermatids.[7] Protamines are small basic proteins rich

in cysteine and arginine. The positive charge of the protamine allows it to pack tightly into the minor grooves of the DNA and negate the negative charge on the phosphate backbone.[11, 12] This alteration in nuclear protein and disulfide bond formation between the protamines enhances compaction and stabilization of the DNA. [7]

As stated earlier, the acrosome arises from enzymatic vesicles formed by the Golgi apparatus. Acrosomal enzymes, including proacrosin, acrosin, hyaluronidase, and phospholipases digest the zona pellucida surrounding the oocyte.[7] The enzymatic release from the acrosome is called the acrosome reaction. It occurs when the outer acrosomal membrane fuses with the plasma membrane surrounding the spermatozoon and forms vesicles to release the hydrolytic enzymes. This process is an exocytotic reaction mediated by calcium. [13]

Another important compartment of the spermatozoon is the plasma membrane. It changes in composition throughout development and maturation. It covers the entire spermatozoon but has a junction between the head and tail at the posterior ring that prevents movement of proteins and lipids from the head section of the membrane to the tail.[7] The plasma membrane is a phospholipid bilayer containing a hydrophilic outer surface and hydrophobic interior.[14] Phospholipids contain a glycerol backbone and fatty acid side chains and have the ability to move laterally within one leaflet of the plasma membrane.[15] The composition and movement of phospholipids influence membrane fluidity.

Membrane fluidity is important in the area of cryopreservation. Membranes are fluid at room temperature (25°C) allowing movement of proteins and lipids but gel as the temperature decreases. The rate at which this occurs depends on which lipids are present within the plasma membrane.[14] Membranes containing short chain fatty acids and unsaturated fatty acids (at

least one double bond) remain fluid at reduced temperatures.[14] Cholesterol is also present on the plasma membrane and plays a role in membrane fluidity. The ratio of cholesterol to phospholipid varies with species and is 0.36 in the plasma membrane of the stallion spermatozoon.[16] Cholesterol intercalates into the membrane to separate fatty acid side chains.[14] It prevents lateral mobility of lipids and proteins within the membrane, thereby reducing its flexibility and increasing stabilization.[14] Membranes form a gel state at lower temperatures after the addition of cholesterol.[17-19]

Once spermatozoa mature they are released in a process of spermiation into the efferent ducts and course to the epididymis for further maturation, packaging and storage. The epididymis is divided into three segments: caput, corpus, and cauda.[20] The caput has the highest rate of spermatozoa protein synthesis and secretion of the three segments. Proteins and fluids synthesized in the testes that bathe the spermatozoa are resorbed in the caput to concentrate the sperm suspension. Changes in carbohydrate composition, membrane lipids and a decrease in intracellular pH of spermatozoa also occur in the caput. Most maturational changes to spermatozoa occur in the corpus and depend on dihydrotestosterone. During transit through the epididymis, the cytoplasmic droplet translocates down the flagellum.[20] Nuclear chromatin obtains more disulfide bonds for stabilization and plasma membrane permeability increases in the corpus. Spermatozoa gain motility in the cauda segment of the stallion epididymis. This is accomplished through the addition of a forward motility protein, alterations in the cAMP-protein kinase system, and reduction of intracellular calcium.[13] Spermatozoa also gain resistance to “cold-shock” in the cauda epididymis. Alterations in the plasma membrane with the addition of cholesterol, glycoproteins, and polypeptides protect the spermatozoa against

"cold-shock."[13] The cauda epididymis is the major site for spermatozoa storage until ejaculation.[20]

Alterations in the quality and quantity of spermatozoa

The entire process of spermatogenesis varies with the species but takes approximately 57 days[5] and daily sperm production averages five billion spermatozoa in the stallion.[21] However, directly measuring daily sperm production is difficult. One method of estimating daily sperm production is the measurement of daily sperm output.[21] Daily sperm output is best evaluated after stabilization of the extragonadal sperm reserves. The cauda epididymis may contain up to 61% of the total sperm output.[22] After the first ejaculation, spermatozoa concentration will decline with each consecutive ejaculate until the reserve population of spermatozoa is depleted. In one study collection of semen for seven consecutive days gave the best prediction of daily sperm output in stallions,[23] whereas another study demonstrated that only five successive daily ejaculations were needed to reach a predictable daily sperm output.[24] Season, ejaculation frequency, testicular size, and age of the stallion can also influence daily sperm output.

Stallions are seasonal breeders. Pickett and co-workers have demonstrated through alterations in daylight that stallions experience a decline in scrotal width, gel-free volume and sperm concentration with decreasing daylight.[25] In another experiment the effect of season on the quality of cryopreserved semen was evaluated.[26] In that experiment it was determined that there is no significant difference between post-thaw motility of semen collected in the non-breeding and breeding seasons.[26]

Ejaculation frequency also alters daily sperm output. The first ejaculate from a sexually rested stallion will have a sperm concentration twice that of a second ejaculate taken just one

hour later.[24] As ejaculation frequency increases past once every other day the sperm output per ejaculate decreases. Where stallion breeding frequency increases (approximately 4-6 x per week) fertility may decrease due to insufficient numbers of spermatozoa to impregnate all mares booked to the stallion.[25]

Daily sperm output has also been estimated through scrotal measurements.[5] Scrotal circumference is measured in bulls and rams and scrotal width is measured in stallions and boars. These measurements correlate positively with production of spermatozoa.[25, 27] Therefore stallions with larger scrotal width have the ability to produce more spermatozoa. However scrotal width can also vary with age, season and breed of the stallion.[25]

Age is a major determinant of sperm production. Puberty is defined as the capability for successful copulation and production of spermatozoa (50×10^6 spermatozoa with > 10% motility).[20] Stallions reach puberty at the average age of 2 years.[5] Nadan and colleagues designed an experiment using 15 colts to monitor reproductive development. They determined the average age to puberty was 83 weeks.[28] However this does not indicate sexual maturity. Sexual maturity is determined to be the state of maximum reproductive capacity. A stallion's testicles continue to grow and mature until sperm production reaches a plateau (20×10^6 spermatozoa/gram of testicular tissue) at approximately 6 years of age.[5] Testicular degeneration also affects sperm production but usually only occurs in the aged stallion (usually >20 years). However, neither age of the stallion, season, nor ejaculation frequency reduced the quality of cryopreserved semen.[15, 26]

Sertoli cell numbers in each seminiferous tubule influence daily sperm production. Sertoli cells are necessary for support of the developing spermatozoa. Each Sertoli cell can handle only a finite number of developing spermatozoa, thereby limiting the total daily

production of spermatozoa.[29] Where damage occurs to the Sertoli cells without affecting the spermatogonia pool the stallion will only be able to maintain spermatozoa production up to the capacity of the remaining Sertoli cells.[29]

Factors also exist that alter the quality of spermatozoa. Thermal stress to the testicle alters morphology of spermatozoa and may result from elevated body temperature, high environmental temperature, trauma, or localized inflammation. Experiments have been designed using scrotal insulation to mimic thermal stress. Thermal stress alters spermatogenesis at predictable points in time.[30, 31] Damage to spermatozoa may not be evident on the day of injury but by 6 days post injury, alterations such as detached heads may be found in the ejaculate.[31, 32] Cytoplasmic droplets are first seen by day 9, head abnormalities by day 12 (pyriform heads, crater defects), and abnormal tails by day 18.[31] Stages of spermatozoal development that are most susceptible to thermal stress are pachytene primary spermatocytes, B spermatogonia, and round spermatids.[20, 33] Karabinus and colleagues evaluated chromatin structure and identified ultrastructural abnormalities to spermatozoa as early as day 3 post-scrotal insulation.[34] These abnormalities result from alterations to the epididymis that occurs with thermal stress. The most severe changes in the stallion are decreased spermatozoa concentration and morphologic changes noted about 40 days post thermal stress. Resolution of the abnormalities will not occur for at least 60 days after removal of the insult because of the length of the spermatogenic cycle.[20]

Another insult that may alter the quality and quantity of spermatozoa is testicular trauma. Integrity of the blood-testis barrier may be compromised, leading to the development of anti-sperm antibodies and destruction of developing spermatozoa. .[35] Severe trauma may cause pressure necrosis due to edema formation or blood occupying space in the scrotum.[35]

Toxins/drugs may selectively damage certain stages of developing spermatozoa or cause a generalized destruction and reduction in spermatogenesis.[5] Administration of anabolic steroids, testosterone or estrogen can decrease spermatozoa production and testicular size.[36] Nutritional deficiencies, radiation and other factors can also alter quality of spermatozoa.[5]

Cryopreservation of spermatozoa

All alterations in the quality of spermatozoa discussed above occur in the stallion. Other iatrogenic (man-made) changes in spermatozoa may also occur after ejaculation. Many of these changes can occur during attempts to cryopreserve semen. The discovery of glycerol enabled Polge and co-workers in 1949 to revive spermatozoa after storage at low temperatures.[37] However, the first equine pregnancy was not established with cryopreserved semen until 1957 by Barker and Gandier.[38]

Many advantages exist with the use of frozen-thawed semen. One advantage is the reduced risk of injury to the stallion and/or mare that can occur during natural breeding. Genetic material from valuable breeding animals can also be saved in case of an untimely death. In the cattle industry, sperm cryopreservation has enabled semen transport to other countries that are in need of new genetic material to improve the quality of their stock. Quarantine procedures make overseas animal transport much more difficult than shipping a tank of semen. Quality control procedures have improved with the use of cryopreserved semen. Quality control procedures may potentially decrease transmission of diseases such as equine contagious metritis, equine influenza, equine infectious anemia, equine viral arteritis, and coital exanthema.

Although semen cryopreservation has many advantages, the big disadvantage is lower fertility. There is also an increased need to understand cryopreservation techniques and develop expertise in insemination timing in the mare. Since a decrease in fertility using frozen-thawed

stallion semen has been observed, research has focused on determining what occurs during the cryopreservation process to damage spermatozoa.[15] Important ultrastructural compartments of the spermatozoon that can not be visualized using light microscopy are at risk of being damaged during the freeze-thaw process. Therefore techniques for evaluating these compartments need to be developed and used during semen evaluation.

Spermatozoa from a thawed sample must retain certain properties. Energy production, motility, and functional acrosomal enzymes and proteins on the plasma membrane are all required by the spermatozoon for proper oocyte attachment and penetration.[15] Damage to any of these properties reduces fertility. To understand what structures may be damaged during cryopreservation it is important to first understand what happens to spermatozoa as they freeze and thaw. As spermatozoa cool from room temperature (25 °C) to the freezing point of water (0 °C) they may undergo damage known as "cold-shock." "Cold-shock" is the term for the collective damage to spermatozoa after cooling stress. This is characterized by abnormal swimming patterns, loss of motility, damage to the plasma and acrosomal membranes, and loss of intracellular components.[10] Stallion spermatozoa are particularly susceptible to "cold-shock", which can cause loss of some or all of the properties listed above.[39] Stallion spermatozoa are most susceptible to "cold-shock" between the temperatures of 19 and 8 °C.[40]

The plasma membrane plays a crucial role in the resistance to "cold-shock." A membrane's fluidity depends on its degree of saturation. Larger numbers of unsaturated bonds in the fatty acid side chains allow the membrane to stay fluid at lower temperatures. Also as the surrounding temperature decreases fatty acid chains elongate and straighten to become more compact. [41] Fluidity is crucial during the cooling phase because as the membrane becomes rigid, water cannot diffuse out of the cell. As more water remains in the cell

more subcellular damage can occur due to intracellular ice crystal formation.[42] A reduction in ATP pump efficiency also occurs as the temperature decreases. This leads to an increase in intracellular calcium concentration that activates phospholipases.[43] These phospholipases hydrolyze phospholipids, causing membrane damage.[15]

Several experiments in other species have attempted to alter plasma membrane composition of spermatozoa to improve stability and protect spermatozoa from "cold-shock." Phillips and Lardy evaluated the effect egg yolk had in minimizing "cold-shock" to bull spermatozoa.[44] They determined the low-density lipoprotein fraction of egg yolk had the greatest stabilizing effect on the plasma membrane. This fraction was believed to actually fuse to the membrane of spermatozoa, however, other studies with bull semen demonstrated this does not occur.[45-47]

Liposomes (lipid bilayer vesicles) have also been investigated as a means of protecting plasma membranes of spermatozoa against "cold-shock."[45] Liposomes containing phosphatidylserine, or phosphatidylserine with cholesterol were used in the experiment. The researchers concluded that liposomes generally enhanced post-thaw motility and viability of stallion spermatozoa. This experiment also evaluated the ability for spermatozoa to undergo a normal capacitation and acrosome reaction after the addition of the liposomes. It was determined that spermatozoa incubated with liposomes were able to undergo a normal acrosome reaction.

When spermatozoa are placed in a solution and cooled, extracellular ice crystals start to form as the pure water freezes. However, if water cools below its freezing point without ice crystal formation it is referred to as supercooling.[42] This normally occurs in salt solutions such as those that spermatozoa are suspended in during the freezing process. Ice crystals form

in the extracellular space at random. The pure water freezes and the remaining solute and cells in the solution concentrate and reside in the unfrozen channels of water.[42]

Damage resulting from extracellular ice crystal formation may occur through exposure of spermatozoa to high (toxic) solute concentrations. This is termed “solution effects” and was described by Meryman in 1966.[48] It is now thought that damage may not result from a high solute concentration but rather a reduction in the fraction of unfrozen water in which spermatozoa must reside.[49] More extracellular ice crystals form as a sample cools, decreasing the unfrozen channel volume remaining surrounding the cell. This reduces the amount of space for spermatozoa, causing structural damage.[49]

Methods to reduce the solute concentration or increase the amount of unfrozen channels have been investigated. Experiments were designed that added substances to “protect” spermatozoa. Polge first added glycerol to fowl spermatozoa in 1949 for cellular protection during temperature reduction.[37] Even though glycerol is a permeating cryoprotectant, extracellular activity was thought to dilute the solute concentration and increase the volume of unfrozen channels.[49] This decreases the time of exposure of spermatozoa to high solute concentrations as the solution freezes.

Other cryoprotective agents such as dimethyl sulfoxide, ethylene glycol, and propylene glycol have been used with variable success.[15] Most of these agents penetrate to protect against intracellular damage but also act to decrease solute concentration in the extracellular compartment. Sugars like lactose, mannose and trehalose act solely in the extracellular compartment.[15] These reduce the salt concentration in the unfrozen channels to increase unfrozen water availability at a given temperature.[50]

Since cryoprotective agents can be toxic at high levels, optimal concentrations need to be determined.[15] Optimal concentrations of cryoprotectant vary between species. Boar semen is very sensitive to cryoprotectants such as glycerol and can only tolerate concentrations of 3-4%.[51] Bull and stallion semen can handle 7-9% and 5-6% glycerol, respectively.[52, 53]

Cryoprotectant concentration tolerated by a semen sample also depends on the temperature and step-wise rate at which it is added, as well as how long it is allowed to equilibrate before freezing. For ram and bull semen glycerol is added in a step-wise procedure at 5ºC. The semen is then allowed to equilibrate at that temperature for up to 24 hours.[54] However, other studies have shown that a step-wise addition of glycerol is not necessary and equilibration time can be as short as 2-3 hours.[54] Another study demonstrated that glycerol has the ability to penetrate the cell in seconds and therefore equilibration may not be necessary.[15] Boar semen must be stored at 15ºC and the glycerol added at that temperature.[54] Glycerol can be added to stallion semen at room temperature and frozen without any equilibration time.[53]

As the solute concentration starts to rise in the remaining unfrozen channels, cells with a permeable membrane like spermatozoa try to maintain osmotic balance through passive diffusion of water out of the cell. This causes cellular dehydration. However dehydration is advantageous, because as water leaves the cell there is less present in the intracellular space to form ice crystals as the temperature decreases. Intracellular ice crystal formation is damaging to subcellular components of spermatozoa.[42]

The cooling rate influences the degree of intracellular ice crystal formation. Where the sample is frozen at a fast rate water movement out of the cell is not rapid enough for the cell to equilibrate, thereby forming intracellular ice crystals. However Amann and Pickett discuss

cooling at a very fast rate to decrease intracellular damage.[15] They discussed the ability of rapid cooling rates to cause only microcrystal formation intracellularly which may not be as damaging. A slow cooling rate allows the cell to dehydrate, thereby decreasing intracellular ice crystal formation. Sperm solutions that are cooled too slowly are also damaging by virtue of excessive dehydration and subsequent membrane deformations.[42] The optimum range in freezing rate to minimize cellular damage and optimize sperm viability post-thaw differs based on freezing extenders and packaging systems.

Numerous extenders have been used to freeze stallion semen with varying success.[15] Most extenders contain glucose, egg yolk and glycerol to provide energy, membrane stabilization and cryoprotection, respectively.[55, 56] Varying concentrations of these components are used based on experience of the freezing center, cooling rates and packaging system utilized.

Different semen packaging systems have also been evaluated and used. Glass ampules were one of the original packaging systems used to freeze semen. Ampule volume has ranged from 1 to 10 milliliters in stallions.[15] Pelleted semen has also been used in various species and continues to be used in rams, boars and dogs.[54] Pellets range in volume from 0.1 to 1 milliliter and are cooled on a block of dry ice.[15] This allows very rapid cooling of semen but poor identification of individual pellets and an increase risk of contamination.[15, 57]

Polyvinyl chloride plastic straws have now become more popular for packaging stallion semen. Straw volume ranges from 0.5 to 5 milliliters.[15] Numerous experiments have compared straws to other packaging methods.[15] Significant differences were found between semen quality and fertility based on packaging with straws being superior to the other methods.

Due to the ease of labeling, storage, and reduced contamination risks, straws have taken over as the preferred packaging system for equine semen.

Thawing is the final stage of cryopreservation where damage to spermatozoa can occur. To prevent recrystallization damage, sample thawing should occur at the same rate at which it was frozen.[42] For example, a sample that was cooled at a fast rate may experience intracellular microcrystal formation. If this sample is thawed at a slow rate these microcrystals may enlarge and irreversibly damage spermatozoa.[42]

Many experiments have evaluated thawing rates. Most have concluded the same results, that a fast cool should have a fast thaw in the stallion.[52, 55] Thawing temperature and duration depends on the packaging system. Pellets are usually thawed at a temperature of 40°C.[15] Four milliliter straws should be thawed at 50°C for 40 seconds.[15] One-half milliliter straws are used most frequently and are best thawed at 75°C for 7 seconds.[55] When fast thawing, leaving the straw in the water bath longer than recommended is strongly discouraged. Prolonged exposure to high temperatures can irreversibly damage spermatozoa.

Stallion semen cryopreservation has improved over the years but has yet to be perfected. Reliable methods to evaluate thawed semen for ultrastructural damage need to be developed. Once these methods have been identified, cryopreservation techniques can be modified in an attempt to reduce damage incurred during the freezing and thawing process.

Conventional methods of semen evaluation

Due to differences in fertility between animals in the same species, methods of evaluating semen and their correlation with fertility have been developed. Magistrini defined a "good sperm" as one that can reach the oocyte, circumventing female barriers.[58] The spermatozoon must have an intact acrosome, plasma membrane, chromatin, mitochondria and

flagellum prior to, at the site of, and after fertilization.[58] The Society for Theriogenology has established standards to predict a stallion's ability to cover a book of mares.[59] To pass a breeding soundness examination at least 1 x 10^9 motile, morphologically normal spermatozoa must be present in the second of two ejaculates collected one hour apart after one week of sexual rest.[60]

These standards factor in several variables such as gel-free ejaculate volume, semen concentration (sperm/ml), motility and morphology of spermatozoa. As was discussed earlier these variables can change with season, age, and frequency of ejaculation. Rousset and colleagues found that the best samples to evaluate are those that are representative of the daily sperm output (ejaculates after extragonadal sperm reserve depletion).[61] Ejaculate volume is usually measured in a pre-warmed graduated cylinder. Concentration can be measured with a hemacytometer or by spectrophotometric methods.[60] The latter evaluates changes in optical density and therefore raw semen must be used to prevent false readings resulting from particles that may exist in extenders.[60] Ejaculate color is also important. Semen should be an opaque-white color and any deviation from this may indicate contaminants. Both urine (yellow color) and blood (red color) will damage spermatozoa if left in contact with these cells.[62] Seminal pH is also measured since acidity may alter motility and viability.[63]

Motility and morphology are considered qualitative tests of semen analysis and are the two most widely used parameters to evaluate semen and predict fertility. The pitfall of both motility and morphologic assessment is their relative subjectivity. Motility is routinely analyzed using a drop of raw or diluted semen on a pre-warmed (37ºC) slide.[62] Many factors can alter the results of this evaluation method. As discussed above, pH can alter motility and temperature fluctuations may lead to "cold-shock" and lowered motility values. Also, Van

Duijn and Hendriske found that the more concentrated the sample the higher the subjective assessment of motility.[63]

Even though its use is widespread, motility may not be the best method for evaluating semen quality. Past experiments have yielded conflicting correlations between motility and fertility. A few experiments using equine semen have demonstrated a weak relationship between motility and fertility.[64, 65] Also one study found a low correlation[58] and another no correlation between motility and fertility in stallions.[66] One explanation may be that low motility in a sample can be overcome by increasing the sperm dose inseminated.[67] Subsequent studies have examined whether the initial motility analysis was a better predictor of fertility than motility after a 24-48 hour incubation at 4ºC, but this point remains in dispute.[58]

Even though motility is known to be a subjective measurement, nevertheless it is still an important parameter to evaluate. Alternative methods of semen evaluation have been established. Computerized systems known as computer assisted sperm analysis (CASA) are now used to obtain more objective results. Companies market machines that use computer algorithms based on spermatozoal head movement and dimensions for each species.[68] Several parameters are analyzed, including motility, velocity, flagellar beat frequency and lateral head displacement.[68] Experiments utilizing CASA have been performed on spermatozoa of humans[69] bulls[70] and stallions.[64, 71] Varner and colleagues found the computerized system was more repeatable than visual methods or videomicrography.[71] One short-coming was discovered when using sperm concentrations greater than $25x10^6$ spermatozoa/ml. Erroneous readings resulted because of a high number of intersecting sperm tracks.[71] Even with the use of these computerized motility analyzers only a small correlation with fertility has been noted in stallions.[64]

Morphology has also been extensively used to assess semen quality. Percent of morphologically normal spermatozoa indicates fertility in the bull[72, 73] and stallion.[72, 74] Morphologic abnormalities are good indicators of biologic stress, trauma, toxin exposure or cryodamage.[18, 75-77] However, some breeding trials indicate that while the morphologic examination may meet certain minimum criteria, fertility may still be poor.[78] This is particularly true with frozen-thawed semen where morphologic examination may be acceptable but no pregnancies are achieved.[66, 78]

Morphologic classification schemes have been established for the stallion.[74, 79] These place spermatozoa abnormalities into two categories: primary and secondary.[79] Primary abnormalities arise within the testicle and include head and middle piece defects and proximal droplets along with others.[62] Secondary abnormalities occur during transport in the excurrent duct system and include defects such as detached heads, bent tails, and distal droplets.[62] Some scientists are beginning to use a different classification scheme, major versus minor defects.[74] Head and middle piece abnormalities are classified as major defects and have been associated with impaired fertility in the bull[80] and stallion[74]. Minor defects such as bent tails and distal droplets are not thought to significantly affect fertility.[74]

Saacke has discussed classifying spermatozoa traits as compensable versus uncompensable.[67] Compensable traits can be overcome by increasing the dose of semen. These are factors that are not capable of reaching the site of fertilization or stimulate the block to polyspermy.[67] Included in this group are motility, plasma membrane integrity, and tail abnormalities.[67] Uncompensable traits cannot be overcome by semen dose. Zona penetration occurs and polyspermy is blocked but fertilization cannot be completed or an increased incidence of embryo mortality.[67] Head abnormalities such as nuclear vacuoles and alterations

in chromatin packaging are included in this group.[67] Bielanski discussed differences in spermatozoa structure that are considered normal in the stallion but abnormal in other species. These include an asymmetrical head, abaxial tail position, and small acrosomal volume.[9]

Methods of sperm morphologic evaluation include phase-contrast microscopy, electron microscopy, differential interference contrast microscopy (DIC) and computerized morphometry. The best method of sample preparation for phase-contrast and DIC microscopy uses a buffered formol-saline mixture because different stains have been shown to cause morphologic changes, especially alterations in head and tail shape.[81, 82] However, even morphology is thought to be subjective.[83, 84] Most reports of poor correlation between percent normal morphology and fertility in stallions have evaluated only small groups of animals and limited breedings.[85] Jasko and colleagues reported a weak positive correlation ($r = 0.34$, $p < 0.01$) between the percent normal spermatozoa and per cycle fertility estimate in 66 stallions over a two-year period.[85]

Since morphology is considered a subjective measurement, computerized systems (morphometry) have been developed to provide an objective means of semen evaluation. Moruzzi and colleagues in 1988 found that by measuring length, width, area, and perimeter of a spermatozoon, 95% of fertile human spermatozoa were correctly classified.[86] Katz in 1986 determined that the ratio of spermatozoa length to width had the greatest significance when comparing fertile versus infertile men.[87] In 1996 Gravance and coworkers determined that sperm head width, area and length were larger in subfertile stallions versus fertile stallions.[88]

Through the use of cryopreserved semen, it has been determined that motility and morphologic evaluation may not accurately assess all of the damage that can occur to the

spermatozoa. Researchers are starting to realize that there are other parts of the spermatozoa to assess and tests needed to be performed in order to better evaluate semen quality.

Other methods of measuring quality of spermatozoa

Cervical mucus penetration is thought to be a more accurate assessment of spermatozoa motility.[89] In the cow and woman, cervical mucus hinders spermatozoa penetration. In the cervical mucus penetration test a capillary tube is loaded with cervical mucus from the estral bovine and one end is placed in the semen sample.[89] A formula was established to evaluate the amount of penetration by spermatozoa.[90] Motion characteristics correlated strongly with mucus penetration.[91] Feneux and coworkers found a positive correlation between cervical mucus penetration and CASA-derived lateral head displacement in humans.[92] They concluded that the spermatozoa must have significant lateral head deviation in order to move through the thick mucus. Mortimer and colleagues reported similar results in humans in 1986.[93]

Proper acrosome function is necessary for zona penetration and can be evaluated in bull and boar semen with a differential interference contrast microscope.[94] However it is not as easily seen in the stallion.[66] Spermac stain (Minitube, Verona, WI) has been used to assess acrosomal integrity with variable success.[58, 95] This stain causes a green coloration of the intact acrosome enabling better visualization with a phase-contrast microscope. Varner and co-workers reported on the use of a fluorescent stain containing chlortetracycline (CTC) that had five characteristic staining patterns to differentiate between true acrosome-reacted spermatozoa and those undergoing the acrosome reaction.[96] The zona-free hamster oocyte penetration test assesses the ability to complete the acrosome reaction.[89] Some question the validity of the penetration test since all barriers up to the oocyte plasma membrane are removed. This test and

a comparison of the test results with fertility are currently under investigation in our laboratory using stallion semen.

Plasma membrane integrity of spermatozoa has been assessed using the hypoosmotic swelling test (HOS).[58] The basis of this test is to place the cells in a hypoosmotic solution so water will enter spermatozoa through passive diffusion. If the membrane is intact then the flagellum will swell and curl.[97] This test is repeatable, accurate and compatible with light microscopy. However, the HOS test correlates poorly with morphology and motility and does not correlate with the zona-free hamster oocyte penetration test.[98] Results have conflicted as to correlation with stallion fertility.[58]

Other biochemical stains and solutions are being developed to evaluate sperm quality. Many of these tests use light microscopy, which is laborious and evaluates only a limited number of cells. The flow cytometer is considered a more accurate, quick, objective tool currently being investigated for multicompartment assessment of spermatozoa.[99]

Basics of flow cytometry

Flow cytometry measures the physical and chemical properties of a cell as it passes through a fluid stream.[100] In 1965, the first flow cytometer was developed using spectrophotometry and light scatter.[99] Most flow cytometers are designed for sample insertion into an injector. The injector draws cells into the capillary tube and the cells mix with sheath fluid. The pressure difference between the sheath fluid and cells force the cells into a single file line so the light beam, usually a laser, hits each cell individually (Appendix A).[99]

Light is emitted from the cell in two forms: scattered and fluorescent.[99] The cell will undergo laser excitation to release fluorescent light. Probes used in cell staining emit light at different wavelengths corresponding to colors in the visible spectrum.[99] The color scheme is

as follows: ultraviolet <390nm, violet 400-450nm, blue 450-500nm, green 500-570nm, yellow 570-590nm, orange 590-620nm, and red 620-750nm. The cells are excited at certain wavelengths and then emit fluorescence at a different wavelength. These wavelengths are usually sufficiently separated from each other in order to trap scatter with filters. The most common excitation wavelength is 488nm.[99] After laser excitation, light is collected and focused by a lens to a photomultiplier detector tube (PMT) (Appendix A). These PMTs change the light wavelength to an electrical signal and transfer the information to a computer that records it on a histogram.

Absorbance and interference(dichroic) filters are used in the flow cytometer. These filters are located in the path of emitted light to either select for or reflect certain wavelengths away from the PMTs.[99] Absorbance filters allow passage of long wavelengths and block short. Interference filters can function as either short or long wavelength pass filters and block the remaining wavelengths with the use of mirrors. Multiparameter systems, flow cytometers that analyze more than one parameter at a time, use these filters to shift light to different PMTs.[99] An example of a filter setup can be shown in Appendix A using propidium iodide (red fluorescence) and rhodamine 123 (green fluorescence). This setup consists of four filters. Filter one block wavelengths <488nm. Filter two is a 590nm short pass which reflects >590nm and allows passage of wavelengths <590nm. Filter three is a 610nm long pass filter which receives the >590nm light that was reflected by the last filter. The 610nm long pass filter only allows passage of wavelengths >610nm which is the red fluorescent range (propidium iodide) to a PMT. Filter four is a 530nm short pass filter that receives the wavelengths that passed through the 590nm short pass filter. A wavelength range of 488-530nm (green range) passes to the other PMT.[99] The PMTs then pass this information to the computer for analysis.

Advantages of flow cytometry are objectivity, speed of evaluation, and the ability to evaluate large numbers of cells, thereby improving experimental design.[99] Flow cytometry also has the ability to concurrently evaluate multiple parameters. Disadvantages include cost of the machine and probes, inability to determine which cells have a specific characteristic, and the technical expertise needed to operate and maintain the equipment.[99] Many probes have been designed to measure various compartments of cells and the following will discuss several compartments of interest in spermatozoa.

Flow cytometric probes

Many probes have been designed to evaluate the plasma membrane of spermatozoa. The plasma membrane must be intact in order to bind to the zona pellucida and initiate the acrosome reaction. Propidium iodide (PI) is a nuclear probe that intercalates into the DNA of spermatozoa with damaged membranes and emits fluorescence in the red range (>610 nm).[99] Propidium iodide is usually combined with other probes such as carboxy fluorescein diacetate (CFDA) and SYBR-14 (Molecular Probes, Inc., Eugene, OR) and are used to evaluate plasma membrane integrity.[99]

The use of propidium iodide in combination with other probes such as SYBR-14 has enabled scientists to distinguish multiple populations of cells. Garner and Johnson identified three populations of spermatozoa when using SYBR-14 and PI.[2] One population of spermatozoa emitted only green fluorescence and was considered to be the viable population. Another population contained cells emitting both red and green fluorescence and was considered moribund. The last population contained cells that fluoresced red only and was considered dead.[2] Using fluorescent microscopy it was determined that as a spermatozoon

expires the red fluorescence is demonstrated first at the posterior segment of the head, leading to the red and green dual staining seen in the flow cytometer.[2]

Experiments have been designed in multiple species such as fowl [101], rat [102], stallion [103], and bull [4] to evaluate PI's ability to distinguish between live and dead samples. Use of propidium iodide increased once two populations could be identified (viable and non-viable). Later PI was used to evaluate alterations in spermatozoal cryopreservation techniques.[3, 4, 101, 103] However a disadvantage was determined when using PI; it is not a reliable probe in fixed samples.[104] Cells that have damaged membranes and are then fixed in paraformaldehyde, allow PI to leach out.[104] This consideration is important in samples such as human bone marrow where there are health risks to the technical personnel if the sample is not fixed.[105]

It was thought that another nuclear probe with similar properties to PI might be more stable in fixed samples. Ethidium bromide is a nuclear probe that only gains access to DNA if the plasma membrane is damaged[99] However it also is incapable of remaining stable in fixed samples. In 1980 Cantrell and Yielding discovered ethidium monoazide (EMA) another probe in the same family as ethidium bromide.[106]

Ethidium monoazide is a positively charged molecule excluded from cells with an intact membrane.[104] It attaches to nucleic acids by intercalating between alternating purine and pyrimidine bases in the DNA. Through photoactivation the probe changes to a reactive nitrene binding covalently with DNA.[104] Photoactivation enables EMA to become resistant to washing and fixing. Ethidium monoazide excites and emits fluorescence at the same wavelength as propidium iodide, possibly making it a good alternative probe.[105] Henley and colleagues in 1994 used EMA in human spermatozoa to determine membrane integrity and

validated this probe as a viable alternative to propidium iodide.[105] However EMA has not gained the same popularity as propidium iodide. This may be due to the technique involved. Ethidium monoazide must be photoactivated prior to analysis, by placing a 60-watt fluorescent light 18-cm from the samples. The samples are then washed with polyvinyl pyrrolidone 40 to remove unbound EMA.[105]

Motility as discussed earlier is an important test for evaluating semen. The flagellum gains its propulsive energy from ATP produced in the mitochondria. Improper handling of the semen during evaluation may alter motility assessment and misrepresent the sample in vivo. The mitochondrial activity of a cell may be a better predictor of cell viability than motility. Auger and colleagues in 1989 found Rhodamine 123 (R123) a better predictor of fertility than motility in human semen samples.[107] Karabinus and colleagues determined that R123 did not correlate with post-thaw motility but attributed the result to lack of variation in motility scores.[4]

A membrane-permeable, cationic, fluorescent, dye, R123 is taken up and bound to mitochondria.[99] Mitochondrial binding requires energy and depends on the mitochondrial membrane potential and mass.[99] Due to this potential gradient as a cell dies R123 will not be lost immediately but slowly dissipates with time.[108, 109] Windsor and White in 1993 evaluated incubation time of ram spermatozoa with R123 and the stain's ability to differentiate between live and dead samples.[110] Maximum uptake time for R123 was 30 minutes and R123 was capable of differentiation between live and dead samples.[110]

Since R123 fluoresces green and PI red, the two probes were combined in an experiment to simultaneously measure spermatozoal mitochondrial activity and plasma membrane integrity.[4] This experiment evaluated cryopreserved bull semen after alteration of freezing

extender to identify an extender component that may be superior to others.[4] Karabinus and colleagues determined that egg yolk extender had the lowest percentage of membrane damage (lowest PI staining). In 1997 Papaioannou and colleagues validated one technique using R123 + PI in stallion semen.[111]

However, since Ahmadi and Ng suspected and confirmed the lack of direct contribution of spermatozoal mitochondria to fertilization, [112] researchers began looking at other compartments that may penetrate the oocyte but prevent embryo formation.[7] Nuclear DNA is one such compartment of the spermatozoon that has been evaluated and gains stability through transfer of the nuclear proteins, protamines for histones, and the formation of disulfide bonds.[99] Darzynkiewicz and colleagues studied a metachromatic nuclear probe called acridine orange(AO).[113] This probe fluoresces red when bound to single-stranded DNA and green when bound to double-stranded DNA. Darzynkiewicz determined that applying heat and acid to a cell will denature susceptible DNA and the shift from double stranded to single stranded DNA can be measured.[113]

Evenson and colleagues later used this technology and applied it to the spermatozoon.[114] The main goal of this research project was to establish another test for semen evaluation that correlates well with fertility and identifies subtle developmental changes resulting from toxicant exposure.[114] The new test was defined as the sperm chromatin structure assay (SCSA) because of the difference in composition of mature DNA of spermatozoa and the ability of acidic conditions to denature spermatozoa in the absence of thermally-induced denaturation.[114] Sperm chromatin structure assay has been used with both flow cytometry and fluorescent microscopy. Spermatozoa have been stained with AO to determine at what stages of spermatogenesis they are most susceptible to denaturation.[115,

116] Through acid denaturation, spermatozoa in the testicle stain red due to the DNA organization with histones but stain green in the proximal cauda of the epididymis.[116] Elongating spermatids had the most variable staining because of the transition from histones to protamines during this stage of spermatogenesis.[115]

Sperm chromatin structure assay measures a green fluorescence shift to red with increased denaturation of DNA. Data is collected and analyzed as a ratio of red fluorescence to the total fluorescence (red/red +green) and is defined as alpha-t.[113] This ratio is measured on a scale from 0 (native DNA) to 1 (denatured DNA).[113] Another parameter measured is the ($COMP_{\alpha t}$), cells outside the main population, which measures the percentage of abnormal cells, those with high alpha-t relative to the normal population.[113] This is the most repeatable SCSA parameter.[117] The standard deviation of alpha-t ($SD_{\propto t}$) and mean of alpha-t ($X_{\propto t}$) are other important parameters measured.[113] The variation in DNA abnormalities is measured with $SD_{\propto t}$.[117] Of the SCSA parameters measured, $SD_{\propto t}$ has been found to have the highest correlation with fertility.[118]

Sperm chromatin structure assay correlates with fertility as defined by non-return rates in bulls.[119] A negative correlation was found between bovine fertility, $SD_{\propto t}$ and $COMP_{\propto t}$, meaning fertility decreased as more cells became denatured under acidic conditions. Sperm chromatin structure assay is a good test for evaluating semen samples of poor quality and low fertility bulls.[119] Ballachey and colleagues in 1988 used SCSA and other conventional methods of semen evaluation to compare heterospermic performance of bulls.[118] Sperm chromatin structure assay inversely correlates with conventional semen tests and has a low error rate since a large number of cells are measured.[118]

Experiments have evaluated SCSA in other species,[114, 120, 121] in cryopreserved samples,[4, 120] and alterations that affect spermatogenesis with damage to the testicle either through heat or toxicant exposure.[33, 34, 122] Sperm chromatin structure assay has become an important tool for predicting fertility in young animals and measuring fertility in mature animals.[117, 123-127] These experiments reveal that high $SD_{\propto t}$ and $COMP_{\propto t}$ values are indicative of decreased fertility. Sperm chromatin structure assay has been used in raw and preserved stallion semen [121] but has not been used in cryopreserved semen and correlated with fertility.

Other fluorescent probes exist that can be used to measure additional compartments of the spermatozoon. The probes described above will be used in the following experiments with frozen-thawed semen from stallions. Once the tests are validated, fertility will be correlated with the various assays.

Materials and Methods

Experiment 1: The validation of several flow cytometric tests to be used in the assessment of stallion frozen-thawed semen.

Three stallions were chosen between the ages of 3 and 20 years for experiment 1. One stallion was housed at Select Breeders Service, Inc., in Colora, Maryland and was collected at that location. The other two stallions were housed in Blacksburg, Virginia: one privately owned and the other owned by Virginia Tech. All three stallions passed a breeding soundness examination as set by the standards of the Society for Theriogenology.[59] Stallions were collected using one of two artificial vaginas, either the Colorado State University model (Animal Reproduction Systems, Chino, CA) or the Missouri model (NASCO,Fort Atkensen, WI).

The stallions were collected once a day, every day for five collections to stabilize the extragonadal reserve and stabilize daily sperm output. The stallions were then rested for two days and collected every other day for six collections. The semen was collected into a plastic bottle (NASCO) that contained a plastic baby bottle liner (Gerber Products Co., Fremont, MI) and in-line milk filter (2 1/4" x 8", Schwartz Manufacturing Co., Two Rivers, WI). The sperm rich portion of the ejaculate was transported to the laboratory for further analysis.

Motility of the fresh semen was evaluated on prewarmed (37°C) 25x75x1mm slides (Fisher Scientific, Pittsburgh, PA) with 22 mmsq coverslips (Corning Inc., Corning, NY) using a phase contrast microscope (Image Systems, Columbia, MD) at 400x magnification. Both the total motility and percent progressively motile were estimated for each ejaculate. Sperm

concentration was determined using a densimeter purchased from Animal Reproduction Systems as described by Pickett in 1993.[16] The remaining sperm-rich fraction was diluted in a 1:1 ratio using a skim milk-glucose extender (EZ Mixin BF, Animal Reproduction Systems). The diluted sample was placed in 50ml polypropylene centrifuge tubes (Corning) and centrifuged at 400 x g for 10 minutes (Marathon 22RBR, Fisher Scientific). This was to remove the seminal plasma and concentrate the sperm. The supernatant was extracted and the remaining pellet resuspended in the freezing extender to a concentration of 100-125 x 10^6 cells/ml. (Appendix A)

The sample was packaged in eight (0.5 ml) polyvinyl chloride plastic straws (IMV, Minneapolis, MN). The semen was frozen in a Kryo 10 controlled rate cell freezer (Planer Products Ltd., Middlesex, UK) at a rate of –10°C/min from 20°C to a temperature of –15°C was reached and then the rate was increased to –25°C/min until reaching –120°C. Once this temperature was reached the straws were plunged into liquid nitrogen (-196°C). The semen from one stallion was obtained from Maryland through shipment in a MVE SC 4/2v liquid nitrogen vapor shipper (MVE Inc., Bloomington, MN) provided by the CREATE (Center for Reproductive Excellence using Assisted Technology and Endocrinology) lab, VA-MD Regional College of Veterinary Medicine, Virginia Tech, Blacksburg, VA. All semen was stored in a MVE XLC 511 liquid nitrogen holding tank until use.

One straw of semen from each ejaculate of a stallion was removed from the liquid nitrogen tank and placed in a 37°C water bath for 30 seconds. After thawing, the straw was dried completely, one end of the straw was cut with scissors placed over a labeled 15 ml polypropylene test tube (Corning) then the other end was cut open and the contents dispersed into the test tube. The semen was diluted with one milliliter of skim milk-glucose extender (EZ

Mixin BF, Animal Reproduction Systems) and thoroughly mixed. A 100 µl aliquot of semen was removed and placed in a 1.5 ml polyethylene microcentrifuge tube (Sigma Chemical Co., St. Louis, MO) and set aside for motility and morphologic examination, while the remainder of the sample was centrifuged at 400 x g for 10 minutes. A 10 µl aliquot of semen was placed on a pre-warmed slide with a coverslip. Motility was evaluated on a prewarmed slide (37°C) in triplicate at 400x magnification using a phase contrast microscope and the percent motile spermatozoa recorded to the nearest 5%.

Morphology was evaluated by adding 10 µl of semen and 10 µl of Hancock stain (Society for Theriogenology, Nashville, TN) to one end of a slide, mixing the two, making a smear, and letting it air dry. The slide was later examined using bright field microscopy at 1000x magnification. A total of 200 cells were counted randomly and the average percentage of normal spermatozoa, primary abnormalities, and secondary abnormalities were calculated and recorded. Abnormalities counted as primary included abnormal head shape, midpiece defects, knobbed acrosomes, nuclear vacuoles, and proximal droplets. Secondary abnormalities included distal cytoplasmic droplets, bent tails, and detached heads. Two slides were evaluated per straw. The remainder of the semen from the straw was washed twice in Modified Tyrode's Media (MTM, Sigma, Appendix B) by centrifugation at 400 x g for 10 minutes and then diluted to 2 x 10^6 spermatozoa/ml with MTM. The semen sample was divided using pipetting and one half kept as the live sample. The other half was plunged into liquid nitrogen and then thawed at 120°F. This was repeated three times to provide the killed sample. These samples were then transported to the flow cytometry lab for evaluation of plasma membrane integrity, mitochondrial function, and chromatin structure.

Determination of the mitochondrial function and plasma membrane integrity was performed using a technique adapted from Karabinus and colleagues[4]. Rhodamine 123 (R123, Sigma) was diluted in distilled water to form a 1 mg/ml aqueous stock solution. One milliliter (2 $x10^6$ spermatozoa) of semen was placed in a polypropylene test tube (12x75mm, Elkay Products Inc., Shrewsbury, MA) and 10 µl R123 was added at 20°C. The sample was covered to protect from light and incubated for 10 minutes. The sample was then centrifuged for 8 minutes at 400 x g and the supernatant discarded. The pellet was resuspended using one milliliter of MTM. Propidium iodide (PI, Sigma) was diluted similar to R123 to form a 1 mg/ml aqueous stock solution. A 10 µl aliquot of PI was added to the sample. The sample was covered and incubated for 13 minutes. The sample was then read immediately.

Fluorescent intensities were determined using a Coulter EPICS XL flow cytometer (Coulter Electronics, Hialeah, FL,). The EPICS XL was equipped with an air cooled argon laser that excited cells at 488 nm. The filter setup contained a 620 nm and 525 nm band pass filter, to measure PI and R123, respectively. The peak fluorescence channels were determined using the EPICS XL (Coulter) software and expressed on a logarithmic scale. Ten thousand cells were analyzed per sample. The results of the R123 - PI dual staining were expressed as a percentage of live (R123 only), moribund (R123 + PI), and dead (PI only) cells. Each straw had live and killed samples run and each sample was run in duplicate. This gives a total of four tests evaluated per straw. A sample was also set aside and not stained with R123 or PI to determine any autofluorescence.

Plasma membrane integrity was assessed using ethidium monoazide (EMA) described by Henley and colleagues in 1994.[105] To one milliliter of the semen in MTM (2 $x10^6$ spermatozoa) was added 10 µl of EMA (Molecular Probes, Eugene, OR) dissolved in ethanol

(final concentration of 10 µl/ml). The sample was incubated in a light box (cardboard box, 60cm x 20cm x 40cm) containing a 42 cm long fluorescent white light source (60watt) that was placed 18 cm away from the sample at 20°C for 20 minutes. Once the light source was removed the samples were washed (centrifugation at 300 x g for 8 minutes) in 2 ml of 2 % (w/v) polyvinyl pyrolidine-40 in phosphate buffered saline (Sigma) to remove unbound EMA. The supernatant was removed and the pellet was resuspended in 2 ml of phosphate buffered saline (Sigma) and evaluated immediately. The sample was analyzed using an Epics XL (Coulter) flow cytometer in the same manner as previously described, utilizing the 620 nm band pass filter to measure the ethidium monoazide. Ten thousand cells were analyzed per sample and the percentage of fluorescence emitted measured. Both live and killed samples were analyzed for each straw and each sample was run in duplicate. A sample was analyzed that did not have EMA added to assess autofluorescence.

Sperm chromatin structure assay (SCSA) was evaluated similar to an earlier experiment by Ley and colleagues in 1991.[121] Briefly, 200 µl of the diluted sample (2 x 10^6 spermatozoa/ml) was mixed with 400 µl of acid buffer (0.1 % v/v Triton-X-100, 0.08 N HCl and 0.15 M NaCl, pH 1.2, Sigma) and placed in polypropylene 12 x 75 mm tubes (Elkay). After thirty seconds, 1.2 ml of acridine orange staining solution (AO, Polysciences, Warrington, PA, Appendix C) was added to the solution. The sample was covered to protect from light and after two minutes the samples were analyzed. The EPICS XL (Coulter) was used, and only red (>600 nm) and green (530 nm) fluorescence were recorded. Alpha-t (αt) is defined as the ratio of red to total (red/red+green) fluorescence and was computed for each sample. This and the wavelength that the cells fluoresce known here on out as mean green fluorescence were computed over 1024 channels (wavelengths) of fluorescence. Ten thousand cells were

measured for each sample. Standard deviation of αt (SDαt), mean of αt (Xαt), and cells outside the main peak population of alpha-t (COMPαt) were computed using the WinList program for the EPICS XL (Verity Software House, Inc., Topsham, MA). All samples were run in duplicate.

Statistical analysis included paired-t test to determine the ability for the flow cytometric stains to distinguish between frozen-thawed semen and frozen-thawed semen that was freeze killed. Next the components of variation were evaluated using nested random effects analysis of variance. The model included differences between stallion (df = 2), ejaculate within a stallion (df = 5), and straw within an ejaculate (df = 1). Correlations between all of the components of each assay were evaluated using Pearson correlation coefficients.

Experiment 2: The selection of assays that provide the best prediction of fertility

Once experiment one was completed, 33 stallions were randomly picked from three equine semen freezing centers. Paul Loomis (Select Breeders Service, Colora, MD), Dr. Lisa Metcalf (Honalee, Eugene, OR), and Dr. Juan Samper (Vancouver, BC, Canada) are in charge of the three participating freezing centers. These stallions were between the ages of 3 and 20 years and have passed a breeding soundness examination as set by the standards of the Society for Theriogenology.[59] These stallions have been collected and the semen used in the 1998-1999 breeding season in the United States, Canada, and Europe. The stallions were collected using either the Colorado (Animal Reproduction Systems) or Missouri (NASCO) artificial vagina. The stallions were ejaculated every other day for three to five collections prior to collection for freezing. Stallions were then collected two to three times per week. Stallions underwent 2 to 40 collections during the freezing period depending on how many doses of

semen were needed. The semen examined for motility, morphology, and concentration as described earlier in Experiment 1.

The straws used were either 5 ml, 4 ml, or 0.5 ml polyvinyl chloride (IMV or Minitube) and each insemination dose contained 750-1000 x 10^6 total spermatozoa. This equated to either one 5 ml straw, one 4 ml straw, or eight 0.5 ml straws. The freezing extender used varied with the stallion and freezing center. The general formula was similar to the freezing extender described in Experiment 1 (Appendix A) but the cryoprotectant and type of sugar may have varied. The method of freezing the semen also varied with the freezing center. These differences were not a concern and were expected to better represent what will be seen in the field. Two straws were chosen at random from each stallion and shipped to the CREATE lab in a MVE SC 4/2v liquid nitrogen vapor shipper. The semen was then transferred to the MVE XLC 511 liquid nitrogen tank until further evaluation.

One straw of semen (0.5 ml) was removed from the liquid nitrogen tank and placed in a 37°C water bath for 30 seconds. Straws containing a volume of 5 or 4 ml were thawed at 50°C for 45 seconds. After thawing, each straw was dried completely, opened, and the contents placed into an appropriately labeled 15 ml polypropylene test tube (Corning). The semen was diluted in one milliliter of skim milk-glucose extender (EZ Mixin) and thoroughly mixed. A 100 µl aliquot of semen was removed and placed in a 1.5 ml polyethylene microcentrifuge tube (Sigma) and set aside while the remainder of the sample was centrifuged at 400 x g for 10 minutes. A 10 µl aliquot of semen was placed on a pre-warmed slide with a coverslip. Motility was evaluated in triplicate at 400x magnification using a phase contrast microscope and the percent motile spermatozoa recorded.

Morphology was evaluated by adding 10 µl of semen and 10 µl of Hancock stain (Society for Theriogenology) to one end of a slide, mixing the two, making a smear, and letting it air dry. The slide was later examined using bright field microscopy at 1000x magnification. A total of 200 cells were counted randomly and the percentage of normal spermatozoa, primary abnormalities, and secondary abnormalities were calculated and recorded. Two slides were evaluated per straw. The remaining semen from the straw was washed twice in Modified Tyrode's Media (MTM ,Sigma) by centrifugation at 400 x g for 10 minutes and then diluted to 2 x 10^6 spermatozoa/ml with MTM. These samples were then transported to the flow cytometry lab for evaluation of plasma membrane integrity, mitochondrial function, and chromatin structure.

Determination of the mitochondrial function, plasma membrane integrity, and sperm chromatin structure were performed using similar techniques as Experiment 1. EMA was not used in Experiment 2 due to inconsistent results seen in Experiment 1.

During the 1998 or 1999 breeding season at least 10 mares were artificially inseminated per stallion. The mares were housed at farms located throughout the United States, Canada, and Europe. These mares varied in their age and reproductive status (maiden, post-partum, barren). The stage of the estrous cycle was determined through palpation per rectum, ultrasound, and/or teasing with a stallion. Once the mare was ready for insemination through palpation per rectum of a large, fluctuant follicle and a relaxed cervix; the mare's perineal area was cleaned, thoroughly rinsed with water, and dried. Frozen semen from the stallion was thawed in a water bath either at 37°C for 30 seconds (0.5 ml straws) or 50°C for 45 seconds (5 or 4 ml straws). The straw was then dried and loaded into a sterile artificial insemination gun or pipette. Once this was completed, one insemination dose of 800-1000 x 10^6 spermatozoa was deposited

directly into the mare's uterus. The mare was later examined through palpation per rectum and ultrasonography by the field veterinarian, for pregnancy status. Information regarding subsequent pregnancy status was sent to the CREATE lab with details of the breeding techniques and pregnancy determination.

A postcard was sent with each batch of frozen semen with information about the breeding: number of times inseminated per cycle, number of straws of frozen semen used per insemination, estimated post thaw motility, time of each insemination, time of ovulation, and any drugs administered to enhance ovulation and/or uterine clearance. The CREATE lab attempted to obtain age of the mare, reproductive status (maiden, post-partum, barren), and any uterine fluid present either prior to or after breeding. The postcards were filed until the end of the breeding season when all of the breeding and pregnancy information was returned.

Multiple linear regression was used to determine the best model for prediction of fertility. The data was standardized for each of the components of the assays. The different assays use different units, therefore it was important to standardize the units so the magnitude of any effect would be real. The results were weighted based on number of mares bred. The mean, standard deviation, and range were calculated for each assay component. Other diagnostics were performed on the model including pair wise correlations and variation inflation factors to determine any collinearity and high influence observations that may make it difficult to formulate an accurate model. Also interactions between each of the assay components were performed to determine if any interaction prevents individual assay components from entering the model. Residual plots were also analyzed to check for model adequacy looking for the amount of unexplained variation.

Results

Experiment 1

Definition of the population

Three stallions between the ages of 6 and 20 years were chosen for this experiment. We attempted to choose stallions that were not undergoing puberty or testicular degeneration. All three stallions had to pass a breeding soundness exam taking into consideration volume, concentration, motility, and morphology. These stallions therefore had been pre-selected based on adequate ejaculated fresh semen motility and morphology. All three stallions in this experiment were various breeds of warmbloods. Table 1 outlines the values for each of the stallions. The stallions ranged in post-thaw motility from 1 – 47 % with a mean of 21.94 ± 14.69 %. Morphology ranged from 62 – 94 % with a mean of 78.03 ± 6.65 % normal spermatozoa. Most of the abnormalities seen on morphology were primary abnormalities including proximal cytoplasmic droplets and middle piece defects.

Comparison of a sample to a freeze-killed sample

A straw of frozen stallion semen from each ejaculate was thawed and divided into two equal samples. Each sample was again divided in half giving four samples from each straw. The first two of the four were evaluated as the live sample and the second two were freeze-killed in liquid nitrogen to represent the killed sample. Each sample was evaluated using R123, PI, and EMA. Each sample was also analyzed for autofluorescence. The results were evaluated using a paired t-test. Significance was determined as $p < 0.05$. It was determined that neither the extender nor semen contained autofluorescence. The mean percentage of cells ± standard

error exhibiting R123 only staining was 21.88±1.96 and 1.43±0.10 for the live and dead sample respectively. The mean percentage of cells exhibiting only PI staining was 6.41±0.77 and 14.81±1.32 for live and dead samples respectively. The rest of the cells stained both with R123 and PI and were not used in the determination of the model in Experiment 1 or 2. Both R123 and PI were able to differentiate between the live and killed samples ($p < 0.0001$) (Figure 1). A killed sample should have a lower percentage of R123 only staining and higher PI only staining indicating semen that has reduced mitochondrial activity and damaged plasma membranes, respectively.

Ethidium monoazide was also evaluated for the mean percentage of cells with staining for both the live and dead sample, 31.29±1.61 and 35±1.85 respectively. EMA was unable to differentiate between the live and killed samples ($p = 0.1207$)(Figure 1). Data values for EMA were inconsistent. Some of the killed samples had lower values than the live which is the opposite of what is expected.

Determination of the components of variation

For each of the assays the components of variation were determined using stallion, ejaculate within a stallion, and straw within an ejaculate as the areas examined. The means and standard errors are listed in Table 2 to represent the magnitude of these values for comparison with further analysis. For each of the assays stallion accounted for the highest amount of the variation (Table 3). Ejaculate accounted for the least amount of variation and R123 and PI were the two assays that contained the least variation in ejaculate or straw (Table 3). The data was analyzed using nested random effects analysis of variance and significance was determined as $p < 0.05$.

Correlation of the flow cytometric assays and conventional methods

The interassay correlations were performed to determine the relationship in seminal traits measured differently for all of the assays excluding EMA. Significance was established as $p < 0.05$. Table 4 displays the correlations between all of the assays. Graphic representation of the correlations between the flow cytometric assays and the conventional methods of semen evaluation (motility and morphology) are seen Figures 2-7. The only flow cytometric assay that correlated with a conventional method was Rhodamine 123 with post-thaw motility ($r = 0.76$, $p < 0.0001$). Morphology was not correlated with any of the assays in this experiment (Figure 2-7).

Within the flow cytometric assays R123 and PI had a negative correlation ($r = -0.63$, $p < 0.0001$). The COMPαt had a positive correlation with PI and negative with mean green fluorescence ($r = 0.82$, $p < 0.0001$; $r = -0.86$, $p < 0.0001$ respectively). Mean green fluorescence also had correlations with R123 and PI ($r = 0.54$, $p < 0.0006$; $r = -0.95$, $p < 0.0001$ respectively). The only other correlation was between the Xαt and SDαt ($r = 0.72$, $p < 0.0001$).

Table 1: The stallion distribution from Experiment 1

STALLION	MEAN MOTILITY (%)	MOTILITY RANGE (%)	MEAN MORPHOLOGY (%)	MORPHOLOGY RANGE (%)
1	37.5 ± 5.57	30-47	77.33 ± 9.52	62-94
2	7.33 ± 6.10	1-16	77.82 ± 1.89	75-81
3	21 ± 10.97	11-44	79 ± 6.4	70-90
OVERALL	21.94 ± 14.69	1-47	78.03 ± 6.65	62-94

The mean values above are displayed as mean ± standard deviation. Morphologic percentages indicate the percent normal spermatozoa as scored by light microscopy; n = 36.

Table 2: Mean values of the variables used in Experiment 1

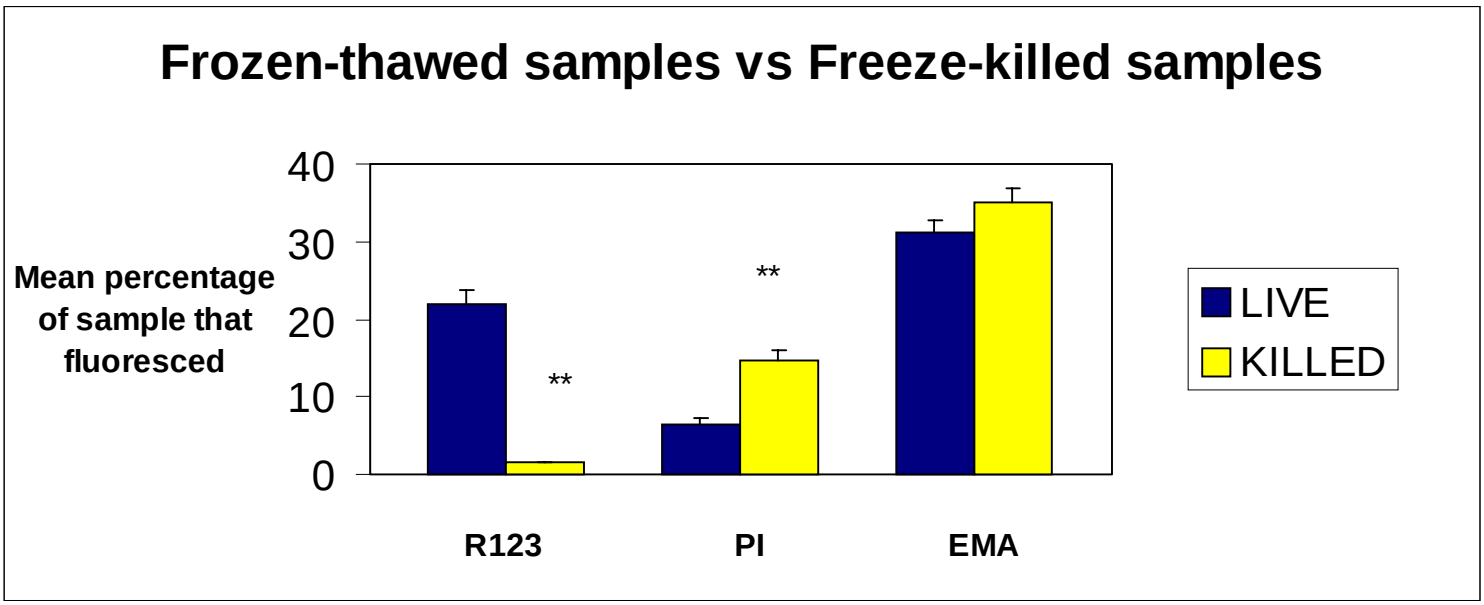
Variable n = 36	Mean	Standard Deviation	Minimum	Maximum
Motility	21.94±8.72	14.68	1	47
Morphology	78.03±0.49	6.65	62	94
R123 only	21.88±11.21	16.70	2.95	50.2
PI only	6.41±4.48	6.59	0.55	19.8
COMPαt	19.81±5.45	9.22	2.64	43.42
Xαt	228.95±1.16	4.97	218.87	237.67
SDαt	39.28±0.77	8.40	22.37	59.37
Mean green	539.60±15.58	23.47	500.1	573.4

Values are mean ± SEM.

Alpha-t variables from the sperm chromatin structure assay. Xαt , SDαt , COMPαt , and mean green refer to mean, standard deviation, cells outside the main population, and average fluorescent wavelength of the cells respectively of the alpha-t distribution.

Mean and morphology are based on 100 %. Morphology is the percentage of normal spermatozoa.

Figure 1: Frozen-thawed compared to Freeze-killed samples in Experiment 1



Samples from each straw were taken and divided in half, centrifuged to remove the freezing extender. Samples from the same straw were frozen-thawed three times in liquid nitrogen to give the killed sample. The means of live and killed samples represent duplicate observations on each of 6 ejaculates from 3 different stallions (n = 36). The mean percentage of the sample that fluoresced for each assay is out of a possible 100%. There was no autofluorescence of extender or semen in this experiment.
R123 = Percentage of Rhodamine 123 staining only
PI = Percentage of Propidium iodide staining only
EMA = Percentage of Ethidium monoazide staining
Bars = Standard error
**Significantly different ($p < 0.0001$) from zero

Table 3: The estimate and percentage of variation for each assay

	STALLION		EJACULATE		ERROR*	
	Variation Estimate	Percent of the total	Variation Estimate	Percent of the total	Variation Estimate	Percent of the total
MOTILITY	218	76% **	51	18% **	17	6%
MORPHOLOGY	-2.4	0%	-11	0%	57	100%
R123	374	94% **	7	2%	13	4%
PI	60	96% **	2	4% **	-0.1	0%
COMPαt	87	77% **	-0.2	0%	26	23%
SDαt	- 4.9	0%	6.17	8%	67.95	92%
Xαt	2.11	8%	-0.40	0%	23.6	92%
MEAN GREEN	723	93% **	7	1%	48	6%

Three stallions were collected for six ejaculates and two straws were randomly chosen from each ejaculate. Duplicate samples were run per straw and a total of 72 observations were recorded for each assay.

αt = ratio of red to total (red + green) fluorescence. COMPαt, SDαt, Xαt, and Mean green are all components of the sperm chromatin structure assay. COMPαt, SDαt, and Xαt are defined as cells outside the main population, standard deviation, and mean, respectively, of the αt distribution. Mean green is the mean green fluorescent wavelength emitted from each sample.

*Error encompasses residual variation including variation due to each straw per ejaculate.

** Significantly different ($p < 0.01$) from zero.

Table 4: Correlation coefficients between the assays used in Experiment 1

	MOTILITY	MORPHOLOGY	R123	PI	COMPαt	SDαt	Xαt	MEAN GREEN
MOTILITY		0.016	0.760 **	-0.173	0.122	-0.132	-0.029	0.104
MORPHOLOGY			-0.055	0.174	0.131	-0.055	-0.037	-0.108
R123				-0.631 **	-0.325	-0.083	-0.113	0.544 *
PI					0.824 **	0.047	0.311	0.950 **
COMPαt						0.026	0.171	-0.863 **
SDαt							0.727 **	-0.280
Xαt								-0.022
MEAN GREEN								

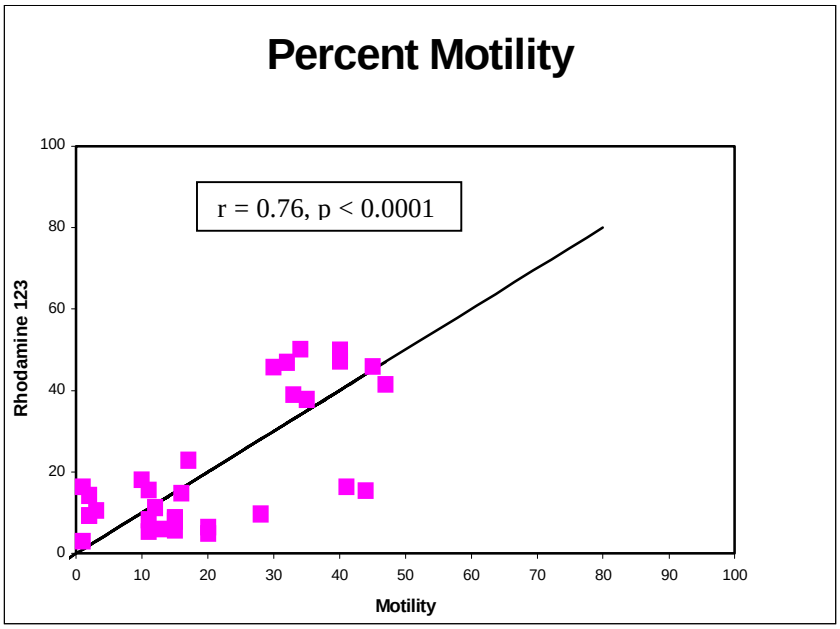
A total of 36 observations were taken for each assay. Motility and morphology were recorded as the percent total motility and normal spermatozoa respectively. R123 and PI were a combination assay and the percent that fluoresced green (live) and the percent that fluoresced red (dead) were recorded respectively. COMPαt, SDαt, Xαt, and Mean green are all components of the sperm chromatin structure assay. COMPαt, SDαt, and Xαt are defined as cells outside the main population, standard deviation, and mean, respectively, of the αt distribution. Mean green is the mean green fluorescent wavelength emitted from each sample.

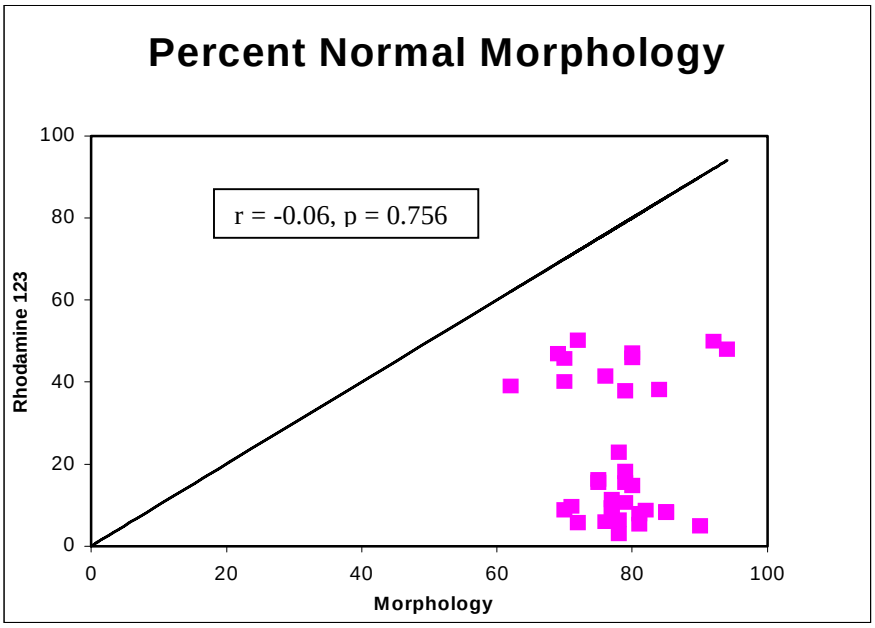
αt = ratio of red to total (red + green) fluorescence

*Significantly different ($p < 0.05$) from zero.

**Significantly different ($p < 0.0001$) from zero.

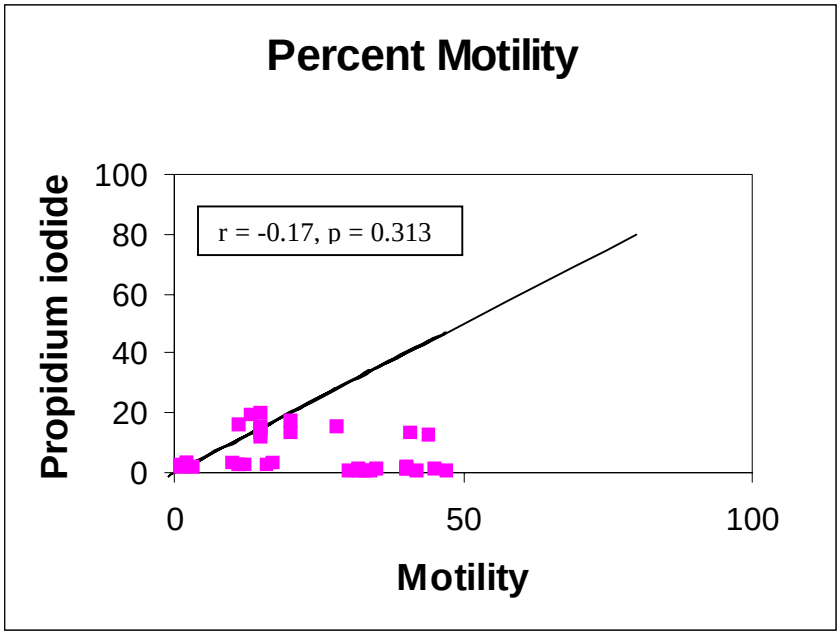
Figure 2: Correlation analysis of Rhodamine 123 (R123) to motility and morphology

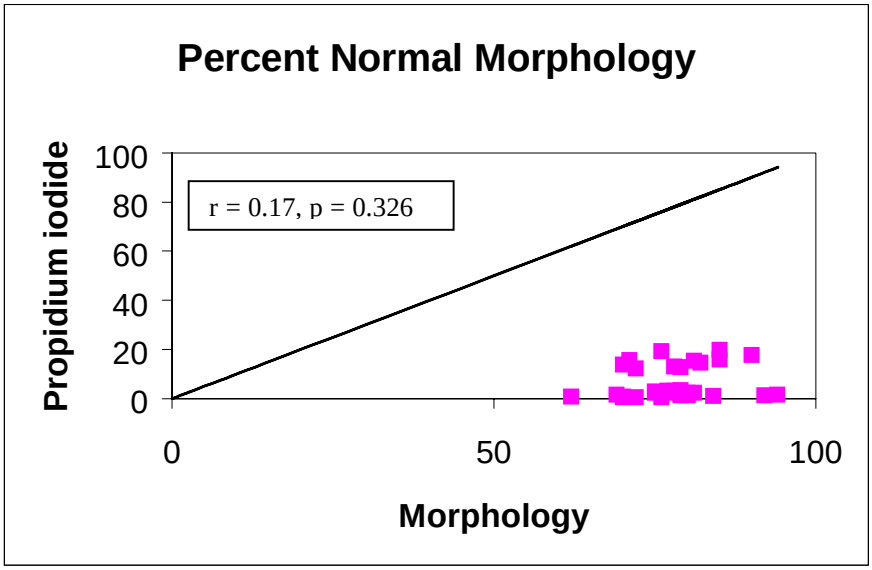




R123 measures the percentage of sperm with green fluorescence. Motility and morphology are also recorded as a percentage. n =36

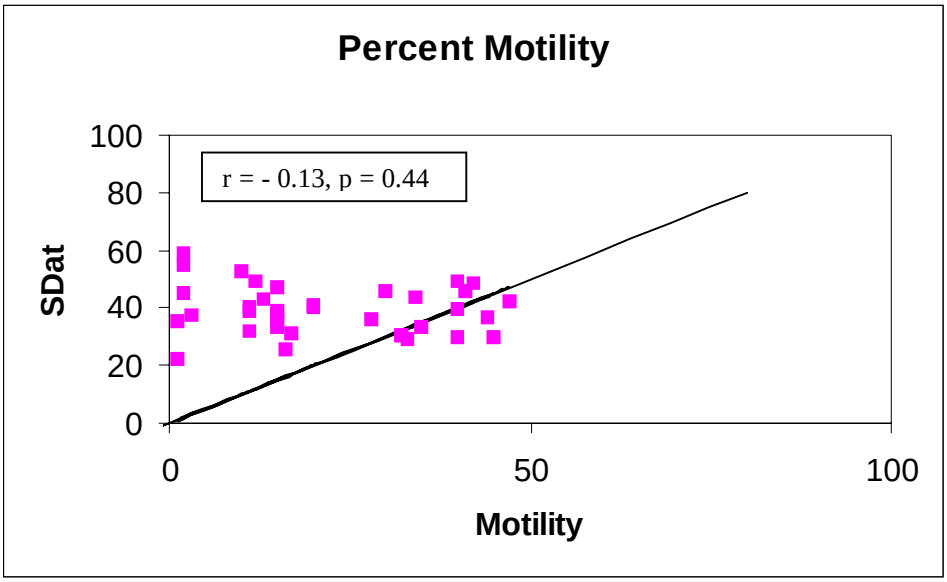
Figure 3: Correlation analysis of Propidium iodide (PI) to motility and morphology

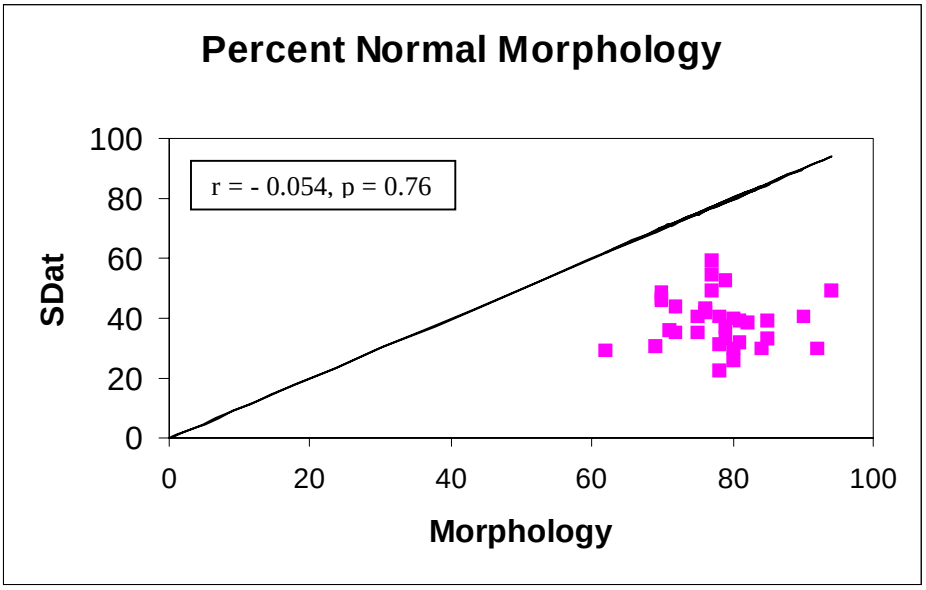




PI measures the percentage of sperm with red fluorescence. Motility and morphology are also recorded as a percentage. n = 36

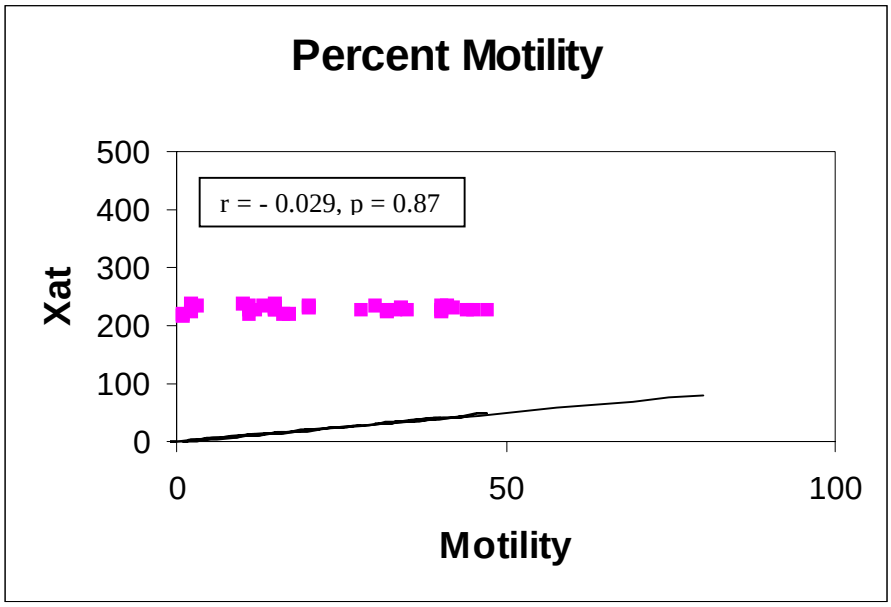
Figure 4: Correlation analysis of the Standard deviation (SDαt) to motility and morphology

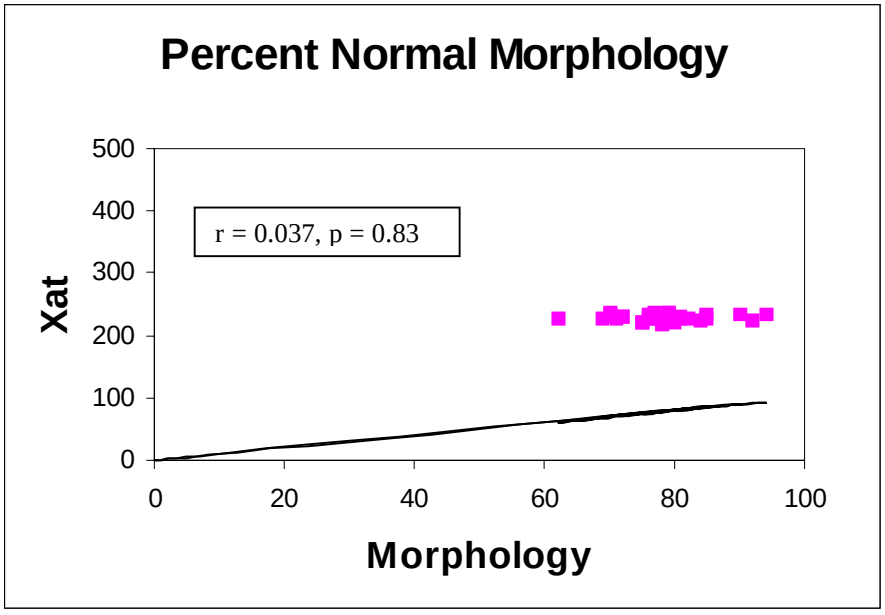




SDαt measures the standard deviation of alpha-t. Alpha-t is the ratio of red to total (red+green) fluorescence. n = 36

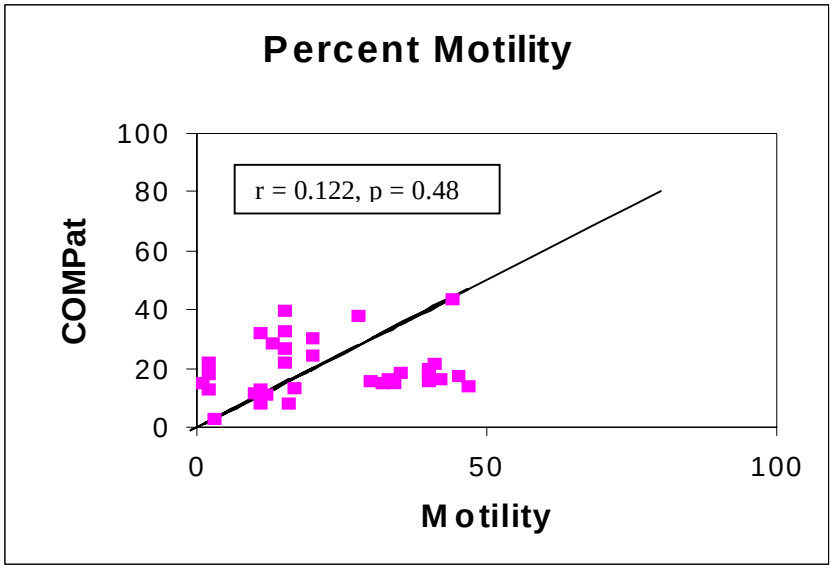
Figure 5: Correlation analysis of the Mean (Xαt) to motility and morphology

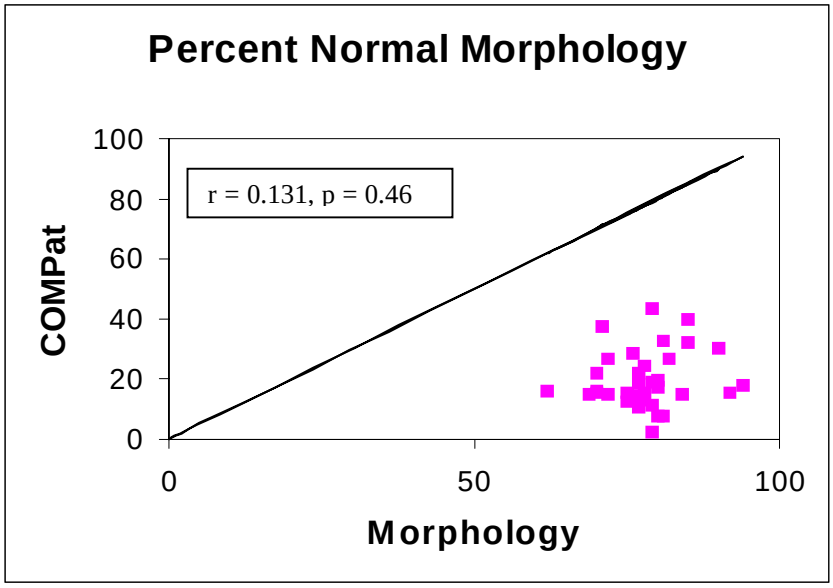




Xαt measures the mean of alpha-t. Alpha-t is the ratio of red to total (red+green) fluorescence. n = 36

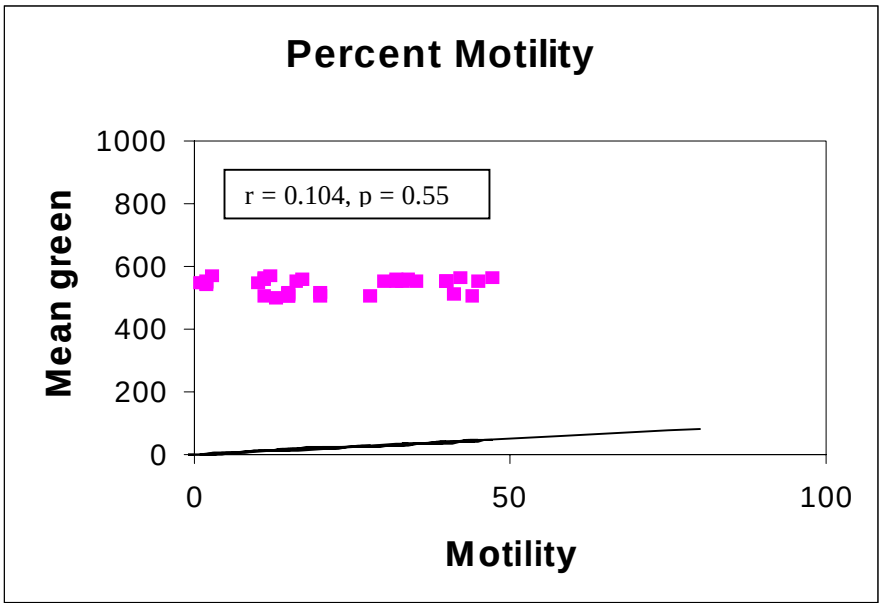
Figure 6: Correlation analysis of the COMPαt to motility and morphology

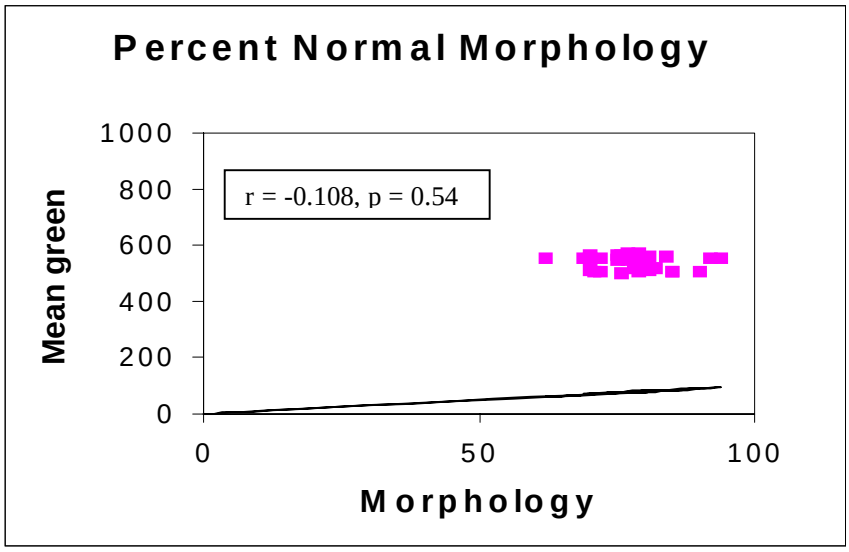




COMPαt is the percentage of cells outside the main population (alpha-t). Alpha-t is the ratio of red to total (red+green) fluorescence. n = 36

Figure 7: Correlation analysis of the mean green fluorescence to motility and morphology





Mean green is the mean green fluorescence of the cells in the sperm chromatin structure assay. The green fluorescence represents the double stranded DNA population of a spermatozoon. n = 36

Experiment 2

Fertility determination

Thirty-three stallions were used in this experiment. Of the thirty-three stallions fertility data was obtained from only twenty-nine of the stallions. Overall pregnancy rate and first cycle conception rates were accumulated but only overall pregnancy rate was used in the calculation of the regression model since it was the most consistent parameter collected. Overall pregnancy rate was defined as number of mares that became pregnant divided by the total number of mares bred during the breeding season. This does not take into account number of times bred per cycle, age of the mares, time of year the mare was bred, and post-breeding problems. Overall pregnancy rate ranged from 0-100 % with a mean of 50.7% and a standard deviation of 14.5. Two graphs of the fertility data and number of mares bred by each stallion is seen in Figure 2 A & B.

Analysis of the data

Each assay component was analyzed for mean, standard deviation, and range of data points and results summarized in Table 3. Also pair wise correlations and variation inflation factors were evaluated for collinearity and high influence observations indicating high variability. Standard deviation of αt (SDαt) and mean of αt (Xαt) had a high correlation with each other ($r=0.93$, $p< 0.0001$) and visual examination of the data found that three stallions were skewed with high SDαt values and abnormal αt populations. The original data was reevaluated under the WinList program with no changes made to the data or gates. The αt population and corresponding SDαt values were back within a normal range. It is unknown why the computer originally gave abnormal αt values but no manipulations were made to the data to make it fit. Correlations were calculated again and no significant correlations were found and the variation

inflation factors were also not significant. There was no significant difference between the three freezing centers used in this experiment.

Regression model using assays

Our ultimate goal was to determine which assays explain the majority of the variation in fertility. All of the components of each assay were considered as one (e.g. SCSA assay = Xαt, SDαt, COMPαt, mean green fluorescence). Each assay was first analyzed alone using regression analysis to determine which components of each assay accounted for the largest variation in fertility and which combination of components create the best model for fertility. For the SCSA assay the Xαt parameter alone accounted for 11% of the variation in fertility (Table 5). However the best combination was of the Xαt, COMPαt, and SDαt and accounted for 27% of the variation in fertility ($p = 0.04$)(Table 5). The Rhodamine 123-Propidium iodide combination assay (R123-PI) accounted for 48% of the variation in fertility ($p = 0.0002$); however the PI only staining alone accounted for 47% of the variation ($p = 0.0003$) (Table 4). Neither post-thaw motility nor morphology was able to determine variation in fertility accounting for only 5% ($p = 0.22$) and 2% ($p = 0.46$) respectively.

All possible subsets of assays were analyzed to determine which combination of assays produced the largest amount of variation in fertility. The results are summarized in Table 6 but briefly the SCSA assay and R123-PI combination accounted for the largest variation in fertility with the lowest error ($R^2 = 0.65$, $p = 0.0003$). The SCSA, R123-PI, and Morphology assay as well as the SCSA, R123-PI, and Motility assay combination also accounted for high variation in fertility and low error. However, by adding morphology or motility there was less than a 6 % increase in variation that could be explained ($R^2 = 0.68$, $p = 0.0004$; $R^2 = 0.70$, $p = 0.0003$ respectively).

Regression model with the best fit for fertility prediction

The assays that provided the largest variation were determined. However, a model was needed to determine whether motility and morphology did not account for a significant amount of the variation due to interactions with some of the components in the other assays. A regression model was analyzed forcing in all of the components of the two assays of significant variation (SCSA and R123-PI). These components included R123 staining only, PI staining only, Xαt, SDαt, COMPαt, and mean green. These components were analyzed with all possible interactions between components to determine if more variation could be explained by any interactions in components of the assays that may change our model. Our model determined that only the interactions between mean green and SDαt (mean green_SDαt) and mean green and R123 only staining (mean green_R123 only) accounted for an additional 12% rise in the variation of fertility estimate and the best fit model was obtained. The equation is in the form of:

$$Y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \ldots \beta_7 x_7 + \varepsilon,$$

where Y = overall pregnancy rate

β_0 = y intercept which in this case is centered on the mean pregnancy rate

β_1, β_2, … β_7 = parameter estimates for each component of the assays, respectively

x_1, x_2, … x_7 = is each of the components

ε = unexplained variation

The final best fit model is the following:

Overall predicted pregnancy rate = 50.71 + 27.03 Xαt – 21.32 SDαt – 9.35 COMPαt – 2.97 mean green – 8.95 R123 only – 23.30 PI only – 10.54 mean green_SDαt + 10.87 mean green_R123 only (n = 29, R^2 = 0.77, adj R^2 = 0.68, mean square error = 62.46).

Figure 8: Fertility and mare data from Experiment 2

Figure A

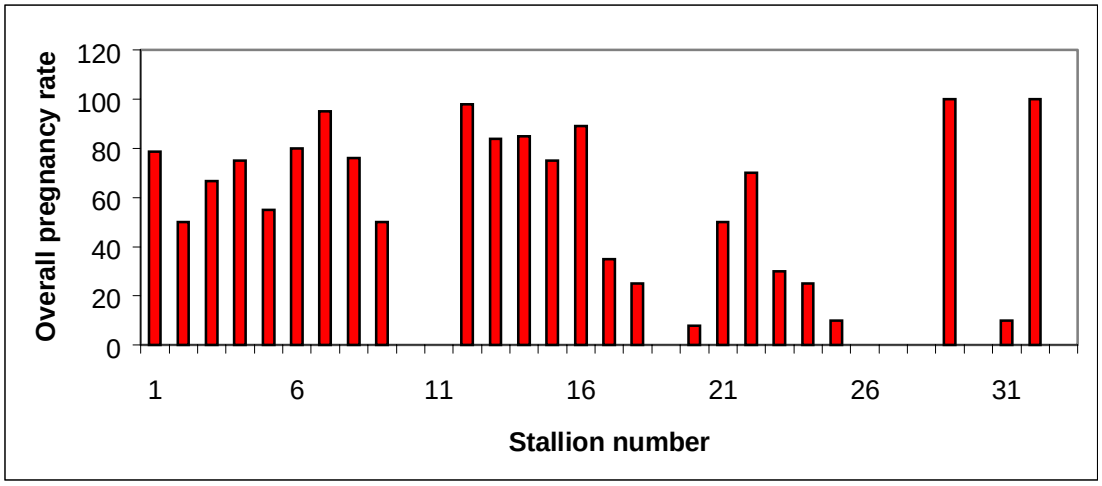
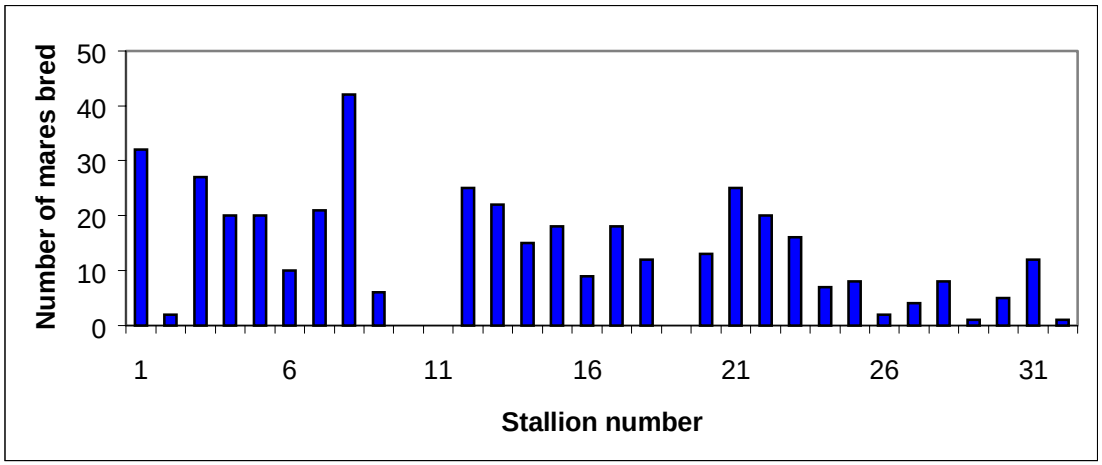


Figure B



Thirty-three stallions were used in Experiment 2. The fertility data was based on overall pregnancy rate and was weighted for determination of the regression model. Overall pregnancy rate = number of mares pregnant/ number bred.

Table 5: Corresponding statistics for the assay components in Experiment 2

Variable	Mean	Standard error	Standard deviation	Range
Motility	38.85	2.20	12.63	59.37
Morphology	76.48	1.87	10.71	40.75
Mean green	534.94	5.28	30.35	166.77
COMPαt	13.80	1.58	9.10	44.67
Xαt	221.48	4.73	23.34	117.36
SDαt	62.56	3.64	18.67	93.57
R123 only	29.15	2.60	14.94	47.68
PI only	2.80	0.47	2.67	8.68

Motility, morphology, R123 only, and PI only are measured as a percentage. Mean green is the mean fluorescent wavelength of each sample. Xαt and SDαt are statistics on the population αt. αt is measured as red fluorescence/total red + green fluorescence. COMPαt, SDαt, and Xαt are defined as cells outside the main population, standard deviation, and mean, respectively, of the αt distribution. n = 33

Table 6: Assessment of each assay using multiple linear regressions

Variable	Parameter Estimate	R-square	Adjusted R-square	p-value
SCSA		**0.28**	**0.16**	**0.08**
Mean green	**-3.39**	0.03	-0.002	0.59
COMPαt	**-13.52**	0.006	-0.03	0.15
SDαt	**-21.37**	0.0002	-0.04	0.03
Xαt	**23.92**	0.11	0.08	0.008
R123/PI		**0.48**	**0.44**	**0.0002**
R123 only	**-2.56**	0.13	0.10	0.59
PI only	**-21.30**	0.47	0.45	0.0003
Motility		**0.05**	**0.02**	**0.23**
Morphology		**0.02**	**-0.02**	**0.46**

The bold face values are those of the assay with all the components of the assay included as one. The parameter estimates determine the magnitude and direction each component has on fertility. Each component of the assays was analyzed as to their contribution to the variation in fertility and is seen in the regular face type.

Table 7: Assay combinations and their variation in fertility

Assays	R-square	Adjusted R-square	p-value	Mean Square Error
S	0.28	0.16	0.08	10,127
R	0.48	0.44	0.0002	6788
Mot	0.05	0.02	0.23	11,869
Mor	0.02	-0.02	0.46	12,286
S x R	**0.65**	**0.56**	**0.0003**	**5316**
S x Mot	0.32	0.18	0.08	7922
S x Mor	0.34	0.20	0.07	9719
R x Mot	0.54	0.49	0.0002	6184
R x Mor	0.48	0.42	0.0008	7036
Mot x Mor	0.07	-0.01	0.41	12,158
S x R x Mot	**0.70**	**0.60**	**0.0003**	**4891**
S x R x Mor	**0.68**	**0.57**	**0.0004**	**5147**
S x Mot x Mor	0.37	0.20	0.09	9728
R x Mot x Mor	0.54	0.47	0.0006	6441
S x R x Mot x Mor	**0.71**	**0.60**	**0.0004**	**4842**

The abbreviation for the assays are as follows: S = SCSA, R = R123/PI, Mot = motility, and Mor = morphology. This table evaluates the assays individually and all possible subsets to determine which combination accounts for the highest variation in fertility. All regressors have been standardized in order to equally compare all of the different assays that may be in different units. n = 29

Discussion

The introduction of the stallion breeding soundness examination in 1983 by Kenney established the first parameters to evaluate stallion semen to assist in the identification of stallions with satisfactory breeding potential.[60] Since the use of frozen-thawed semen has gained popularity there has been a further reduction in fertility of some stallions. It has been speculated that motility, morphology, and the other parameters identified in the routine breeding soundness exam may not adequately identify damage that may occur during cryopreservation . This has stimulated interest in determining what other compartments may be damaged that are not evaluated with post-motility and morphology but may be better predictors of fertility. It is important to remember that the stallion populations used in experiment two were chosen based on having passed a breeding soundness exam. This correlates with what will be seen in the field with only stallions passing a breeding soundness exam having semen frozen.

Cryopreservation has been shown to damage ultrastructural compartments of the spermatozoon. Tests have been developed over the years using specialized instruments such as fluorescence microscopy, electron microscopy, and flow cytometry to evaluate these other compartments. Through experiments some flow cytometric tests have been found to be good predictors of fertility.[117, 119] It was the main objective of this project to determine a combination of assays that may best predict fertility in stallion frozen-thawed semen. We used a combination of conventional tests (motility and morphology) and flow cytometric tests (SCSA, R123-PI, and EMA) to analyze frozen-thawed semen in 33 breeding stallions.

In experiment one we evaluated three mature breeding stallions to determine if the flow cytometric tests could differentiate between live and freeze-killed samples. Of the flow cytometric stains used in experiment one only the sperm chromatin structure assay (SCSA) has previously been evaluated using stallion frozen-thawed semen and found to be able to distinguish differences between stallions.[121] Therefore, this experiment did not reevaluate the ability of the sperm chromatin structure assay to distinguish between the live and freeze-killed samples. The other flow cytometric assays in this experiment rhodamine 123 (R123), propidium iodide (PI), and ethidium monoazide (EMA) were subjected to validation for stallion frozen-thawed semen.

Ethidium monoazide was originally used in human semen to enable samples to be fixed without stain leaching.[105] Henley and colleagues determined in their experiment that EMA was more stable in fixed samples for evaluating membrane damage than PI. It was suspected that cryopreservation may also allow PI to leach out with time so a comparison between EMA and PI's ability to distinguish between live frozen-thawed samples and freeze-killed samples was evaluated. The technique used in experiment one was similar to Henley and colleagues excluding the fixing of samples.[105] The technique was more time consuming than the assay using PI and it was determined that EMA could not consistently differentiate between the two samples (Figure 1). The reason for this is unclear but may be due to technique differences needed to evaluate stallion spermatozoa. The cryopreserved sample may interact differently than a fresh sample. The EMA concentration used for human spermatozoa may not be high enough to adequately stain stallion spermatozoa or photoactivation time may need to be longer. When compared to a sample that is not photoactivated, a peak of fluorescence in the range characteristic for EMA was noted in the photoactivated sample and not the control. Also a

control sample that contained extender only did not autofluoresce. EMA needs to be evaluated further, testing concentration and photoactivation time ranges, to determine if EMA is an adequate stain for evaluating stallion frozen-thawed semen. Since PI was an easy protocol to follow it was felt that EMA did not need to be examined further at this time.

Rhodamine 123 and propidium iodide have been evaluated as dual stains in multiple species to evaluate mitochondrial activity and plasma membrane damage.[4, 111] In this project a protocol adapted from Karabinus and colleagues was used to assess stallion frozen-thawed semen.[4] This specific technique had not been used in the stallion, so it was again important to determine the dual stain's ability to distinguish between live thawed samples and freeze-killed samples. Unlike EMA both PI and R123 were able to adequately differentiate between the two samples (Figure 1). Fluorescent stains were added to the freezing extender to determine if the extender alone contains some fluorescent properties that may confound the data. It was determined that extenders used in this project did not contain fluorescent properties. Frozen-thawed samples also did not have any autofluorescence either. The technique for PI and R123 used in this project was quick, repeatable, and able to distinguish between different samples. This combination assay is known throughout this paper as R123-PI assay.

In order to be able to use these tests to evaluate frozen-thawed stallion semen it is important to determine how many samples are needed to adequately evaluate the semen. Since semen samples may vary from ejaculate to ejaculate and straw to straw, it was necessary to determine the possibility of taking a straw at random and evaluate the semen using conventional and flow cytometric tests. Three stallions were used and after having depleted their extragonadal reserve the semen in six consecutive ejaculates was frozen in 0.5 ml straws. Each

stallion had two random straws from each ejaculate evaluated to determine if ejaculate or straw attributed to a considerable portion of the variance between samples. Each straw was run in duplicate to give 72 total observations to analyze. It was determined as seen in Table 1 that stallion accounted for most of the variation noted. This was expected since each stallion can have vastly different semen profiles. This was not the case for morphology where all stallions had a high percentage of normal spermatozoa even with other tests showing vast differences between the stallions. This was similar for Xαt and SDαt that had the majority of the variation due to error mainly from a small range in values. Differences between ejaculate provided the least amount of explained variation between the samples. Based on these results one or two straws taken at random from a stallion can be used to evaluate the semen for SCSA, R123-PI, motility, and morphology.

Some of the assays used in experiment one measured similar parameters as the conventional tests of motility and morphology. This is especially the case for R123 measuring mitochondrial activity, the powerhouse of the flagellum, and PI analyzing damaged plasma membranes. It was important to determine if any of the flow cytometric assays correlated with motility and morphology. If motility and morphology can account for alterations seen in the flow cytometric assays then the flow cytometric tests would be unnecessary. Correlation coefficients were calculated comparing all components of each assay to each other and motility and morphology. Rhodamine 123 and motility had a high positive correlation, which was expected since the mitochondria provide the ATP necessary for spermatozoal propulsion. Morphology did not correlate with any assay components. Therefore, the flow cytometric assays provide unique information that cannot be described through morphologic evaluation. Some of the of the assay components such as COMPαt and mean green fluorescence had

correlations with each other, but it was more important to determine if the assays provided unique information that the conventional tests could not explain. There was concern about some of the correlations since only three stallions were used. It was expected that experiment two would provide important information on correlations through usage of a larger population size. Therefore it was decided that even with the high correlation between R123 and motility all of the components of the assays were to be used in experiment two.

Fertility prediction is the ideal endpoint to many semen cryopreservation research projects. The downside is the difficulty in obtaining consistent and accurate pregnancy data. Many stallions are not bred to a large enough population of mares to establish statistical significance. Many facilities do not have the time or money to house enough mares and stallions to obtain this degree of significance so the researchers must rely on other facilities and researchers for collaboration. When evaluating frozen-thawed semen even the timing of insemination and fertility of the mare can result in confounding data. This was the concern in experiment two of this project. Some of these factors were taken into consideration through choosing equine freezing centers that might be able to provide better fertility data. Also surveys were designed to ask such questions as mare age, insemination timing, post-breeding complications, etc. Surveys were sent with each batch of semen. However, despite the effort less than 10% of the cards were returned. The most consistent data retrieved was number of mares bred, overall pregnancy rate, and first cycle conception rate. Overall pregnancy rate was chosen as the endpoint to the model in this experiment. Overall pregnancy rate is defined as the total number of mares pregnant at the end of the breeding season divided by the number of mares bred by the end of the breeding season. Number of times bred per cycle or per pregnancy was not evaluated in this experiment due to lack of information. This may play a role in lower

results in fertility, but it can be assumed that most mares are bred once per cycle due to costs and timing of ovulation. Of the thirty-three stallions evaluated only twenty-nine had pregnancy data. Some of the stallion frozen-thawed semen was used to breed only a few mares so it was decided to weight the data based on number of mares bred to help add significance to the data. However, data was not obtained from the mares, which could have contributed to some of the low pregnancy rates seen in some stallions. The mean overall pregnancy rate for the twenty-nine stallions was 51% with fourteen stallions having pregnancy rates lower and fifteen stallions having pregnancy rates higher. The overall pregnancy rate ranged from 0-100% giving a range of fertility in which to evaluate the assays.

Veterinarians want to know what tests are cost effective and reliably predict fertility. Researchers want to know what components of each assay give us the most solid information for prediction of fertility. Using multiple linear regression in experiment two an attempt was made to satisfy both needs. In stallion frozen-thawed semen the determination of a multiple assay model for prediction of fertility has not been established. Before a model could be chosen it was important to make sure no one component of an assay could be explained by another, thus negating its usefulness. Pair-wise correlations were run on the samples to assess collinearity. At first two variables had a very high correlation: mean of alpha-t ($X\alpha t$) and standard deviation of alpha-t ($SD\alpha t$). During graphical assessment of the data it was determined that only three stallions accounted for this correlation. Further investigation led to the discovery that all three were analyzed on the same day and their alpha-t peaks appeared abnormal. To ensure this was not a technical error, the stored data was re-analyzed using the WinList program (Verity Software House, Inc.). A technical error was detected and the problem was corrected. The correlations were reevaluated and no statistical significance was

noted. This contradicts what was observed in experiment one. One reason for this may simply be the number of stallions evaluated (3 versus 33). The correlations from experiment one assessed correlations between multiple ejaculates and straws from the same stallion and in experiment two only two straws from each stallion were evaluated. Therefore within a stallion, a correlation between motility and R123 across multiple ejaculates may be seen, but across stallions of varying semen profiles we detected no correlation.

Each time an assay is run usually more than one value is generated. For example for the SCSA a sample readout will give mean green fluorescence, Xαt, SDαt, and COMPαt. It was therefore more important to determine which assays need to be run and their interactions determined rather than evaluating each component of an assay individually. Each assay was first evaluated using multiple linear regression to establish which components within each assay explain most of the variation in overall pregnancy rate. Motility and morphology were analyzed and neither contributed a significant amount of variation in fertility ($p > 0.05$). This contradicts previous reports on correlation with fertility in fresh or cooled semen.[74] However this is what was speculated when using frozen-thawed semen based on a past experiment by Voss and colleagues.[66] It was thought that other compartments were damaged during the cryopreservation process that motility and morphology do not describe. It may also be due to the fact that stallions were pre-selected based on having passed a breeding soundness exam.

In experiment one it was determined that the flow cytometric assays provide unique information and may adequately explain fertility. Most of the stallions in experiment two had a morphology count that was greater than 75% normal spermatozoa but pregnancy rates that varied greatly. This agrees with the suspicion that morphology may be adequate but fertility poor. Post-thaw motility can vary greatly depending on circumstances. In experiment two post-

thaw motility appeared to be extremely low and the R123 staining was high. This may represent the difference in evaluation of each assay. The semen was split to remove extender from the portion that was analyzed by the flow cytometer. Even though efforts were made to keep lab settings the same it is still possible that some of the variation in motility may be due to technical error. Handling of the semen to evaluate motility could have led to shock to the spermatozoa and reduced motility; however, the mitochondria may still be functional. This is why we chose to evaluate motility using the most conventional method, a slide and microscope. This is the way motility is evaluated by most veterinarians in the field and we felt this would be more realistic than computerized methods of motility evaluation. It is thought that R123 gives a more sensitive assessment of actual flagellar activity and may best represent the activity in vivo. This was also speculated by Papaioannou in 1997.[111] Even though there was a large range in motility values, motility still accounted for a small amount of the variation in fertility (Table 4).

The sperm chromatin structure assay (SCSA) has been used in the past to evaluate denaturability of DNA in spermatozoa of multiple species.[119, 121, 128] This assay measures the shift in chromatin from double stranded to single stranded through the addition of acid. The acid added in this assay tests the denaturability of the DNA. In more susceptible spermatozoa the chromatin denatures into single stranded DNA. This is seen as a shift in fluorescence from green fluorescence to red, double stranded and single stranded DNA respectively. A population expressed as the alpha-t population (red fluorescence/total red + green fluorescence), is constructed.[113] Several important components of this assay have been noted in early testicular damage and infertility.[33, 34] The standard deviation (SDαt) of the alpha-t population and the population of cells outside the main population of alpha-t (COMPαt) have been considered important parameters in predicting fertility.[119] Also COMPαt and mean

(Xαt) of the alpha-t population have been considered an early indicator of DNA shift that precede changes in SDαt.[34] Mean green fluorescence was also evaluated to determine if changes in this may also correlate well with fertility. Mean green fluorescent wavelength in the SCSA is indicative of the percentage of double stranded DNA.

In experiment two, SCSA was first run alone to determine how much variation in fertility this assay may explain. It was concluded that this assay alone accounts for 28% of the variation in fertility (Table 5). Within the SCSA the component that accounted for most of the variation was the Xαt (11%, Table 4). The remaining components had minimal contribution and mean green fluorescence was not significant. Mean of alpha-t's contribution to fertility determination in this study contradicts past experiments. Ballachey and colleagues determined that SDαt was the best predictor of fertility in mature bulls based on non-return rates.[119] In another study by Karabinus and colleagues scrotal insulation was used in the bull to mimic heat stress. In that study COMPαt was the first parameter to change quickly followed by Xαt.[34] Love and Kenney in 1999 did not see this early rise in any of the SCSA values associated with epididymal transit time in stallions. They also found Xαt and COMPαt to rise before SDαt.[33] Our project found results similar to Love and Kenney in that Xαt appeared to be more important than SDαt. Some of these stallions may be showing early signs of testicular degeneration or damage thus explaining why Xαt accounts for most of the variation in fertility. It appears through our experiment and the previous experiment by Love and Kenney that Xαt is more indicative of testicular alterations in the stallion than SDαt as seen in the bull.

Unfortunately even though the flow cytometer is normally considered objective when analyzing the SCSA it has some subjectivity. In the list mode programs a gate must be chosen

to separate the alpha-t population and the cells outside (COMPαt) which leads to a subjective choosing of boundaries for each population. This does not change the overall trend in the data with multiple samples but each machine is setup different so no one value can be set as a value indicative of infertility. A trend can be described but each flow cytometer must establish its own limits for fertile stallions.

A combination assay of R123 and PI, also known as R123-PI, was also used in this project. The technique was similar to the Karabinus article using frozen-thawed bull semen.[4] This assay evaluates the mitochondrial activity and plasma membrane damage of spermatozoa. Numerous experiments have evaluated the use of R123-PI in combination or PI with other stains. These two stains work well together since R123 measures in the green fluorescent range and PI in the red. In this experiment it was determined that the combination assay explained 48% of the variation in fertility, which is better than the SCSA. Within this assay the percentage of spermatozoa that stained with PI only, damaged spermatozoa with no functional mitochondria, attributed to the highest amount of variation (47%, Table 4). Plasma membrane damage is the one compartment of the spermatozoa most susceptible to cryopreservation damage. This project confirmed that damage to this compartment plays a large role in determining alterations in fertility.

Once the assays were run separately and it was determined that only SCSA and the R123-PI assay accounted for significant variation in stallion frozen-thawed fertility. The components of these two assays were forced to be considered as one unit and all possible assay combinations were analyzed to determine which combination accounted for the highest variation in fertility. The results are summarized in Table 5. It was determined that three different combinations accounted for about the same level of fertility variation. However by

adding either post-thaw motility or morphology only up to 7% additional variation in fertility was explained. It was felt that this is not a large enough increase in variation to warrant including these two tests unless they have already been run. Therefore the SCSA and R123-PI combination accounted for 65% of the variation in fertility.

We were able to conclude using the assays that SCSA and R123-PI explain the largest amount of variation in fertility. In order to predict fertility it was necessary to look at interactions between the components of each assay. It was thought that post-thaw motility and morphology may not be considered significant due to interactions between these tests and components in other assays. All interactions were considered to determine the best fit model to predict fertility. From that analysis the only interactions that were important to include were the ones between mean green fluorescence and R123 staining as well as mean green fluorescence and SDαt. These three components are already included in the model with the assays SCSA and R123-PI. It was appropriate to add these interactions since no new test was added to the model in order to incorporate the SDαt_R123 interaction. It was found that with low levels of mean green pregnancy rate decreases with increasing R123. However, with high levels of mean green there is almost no effect on pregnancy rate with increasing R123. High levels of mean green also interact with SDαt where pregnancy rate increases with increasing SDαt. Also with low levels of mean green pregnancy rate increases with SDαt. So the final best fit model to predict fertility was to use the SCSA and R123-PI assays as well as to examine the interactions between mean green and SDαt as well as mean green and R123 thus accounting for 77% of the variation in fertility of stallion frozen-thawed semen.

It has been determined that post-thaw motility and morphology are poor predictors of fertility in frozen-thawed stallion semen. It is therefore necessary to perform other tests to determine the amount of damage incurred on the spermatozoon from cryopreservation. Through this project it has been determined that the SCSA and R123-PI assays can be used to successfully predict fertility in stallion frozen-thawed semen. Overall these tests are relatively inexpensive and easy to perform. However, these tests must be performed at centers containing flow cytometry. These tests should be added to the routine evaluation of stallion frozen-thawed semen.

Conclusions

Cryopreservation damages ultrastructural compartments that may or may not be adequately evaluated by post-motility or morphology. Some of these include plasma membrane damage and a reduction in mitochondrial activity. Also changes in the chromatin structure that may be early indicators of testicular damage or degeneration have been evaluated. The flow cytometer is one instrument that has been incorporated to evaluate these compartments. In this project several flow cytometric tests were validated for use with stallion-frozen thawed semen and correlated with conventional tests. Once this was completed a model for prediction of fertility was determined using multiple linear regression.

As more breed associations accept the usage of frozen-thawed semen more stallions may be labeled as "poor freezers." It is important to identify these stallions prior to stallion and mare owners spending a lot of time and money with no return. There is also not a good way to determine if new methods of cryopreservation in stallions produce too much damage to the spermatozoon. This project demonstrated that post-thaw motility and morphology, although important in assessment of semen in the routine breeding soundness exam; did not adequately explain the variation in fertility seen with frozen-thawed semen. However, the flow cytometric assays examined may not only help to predict fertility prior to usage of stallion frozen-thawed semen, but may also be valuable tools to use when evaluating new cryopreservation techniques. Through the addition of SCSA and R123-PI to the routine post-thaw evaluation of stallion frozen-thawed semen time and money will be saved for those clients whose ultimate goal is a live foal.

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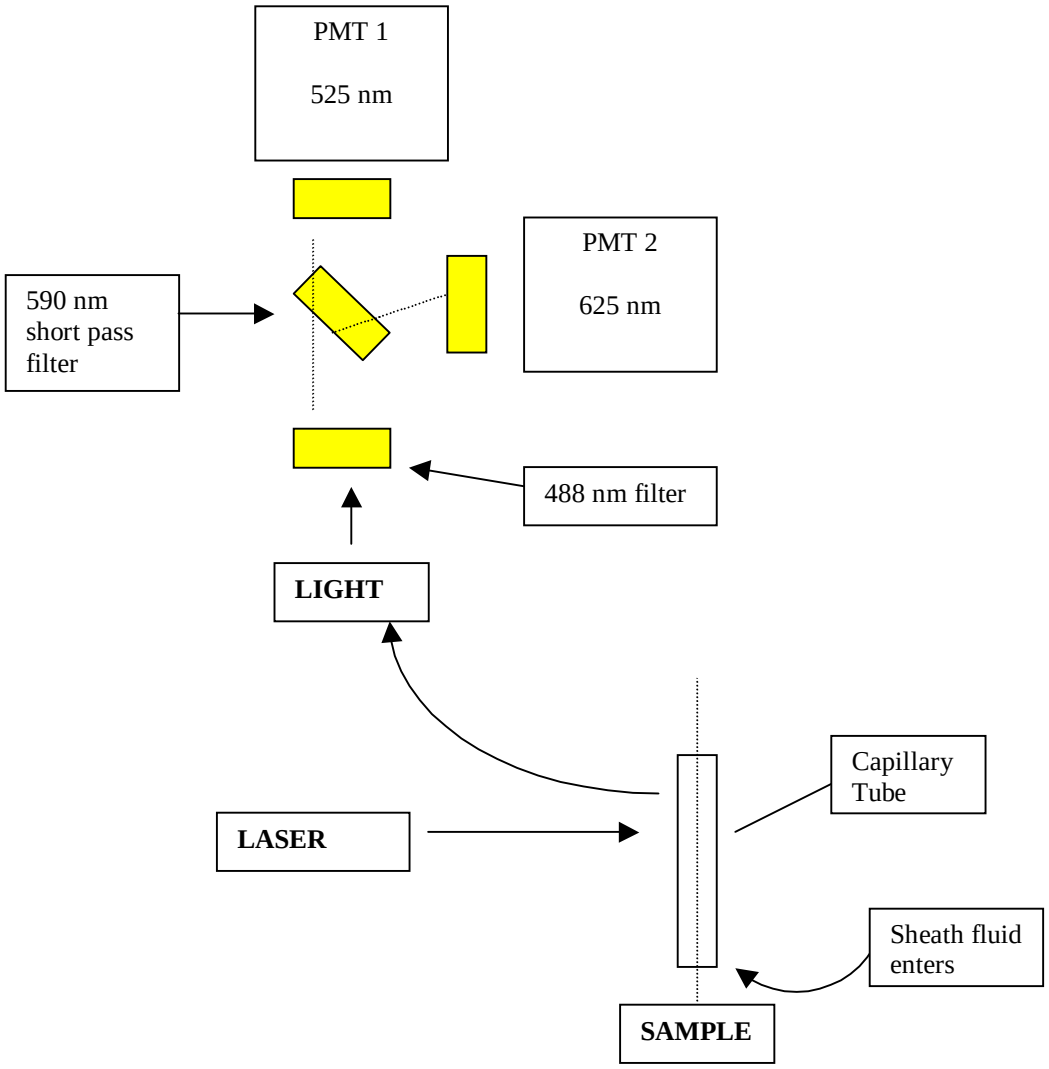
Appendices

Appendix A

Basic Flow Cytometer Design

(See text for more thorough description)



Appendix B

Freezing Extender Composition

Ingredient	Amount
Glucose	4.9 g
Non fat dried skim milk	2.4 g
Distilled water	100 ml
Egg yolk	4 ml
Glycerol	4 ml

Combine the glucose, skim milk, and distilled water. Remove 8 ml of the solution and add the egg yolk. Centrifuge the solution at 500 x g for 20 minutes. Add the glycerol and adjust the pH to 6.8-7.0.

Appendix C

Modified Tyrode's Medium (MTM) Composition

Ingredient	Concentration (mM)
$NaHCO_3$	25
NaCl	120
KCl	2.68
Na_2PO_4	0.15
$CaCl_2$	1.8
$MgCl_2$	0.49
$NaC_3H_3O_3$	0.5
$C_6H_{12}O_6$	5.56

Tyrode's salt solution (T2397) was purchased from Sigma as was all chemicals that had to be added to achieve these concentrations.

pH 7.3 Osmolality 300 mOsm

Appendix D

Acridine Orange Medium (AO) Composition

Ingredient	Concentration
Acridine orange	20 μg/ml
Na_2HPO_4	0.2 M
Citric acid	0.1 M
EDTA	0.001 M
NaCl	0.15 M
pH	6.0

Appendix E

Examples of printouts from the R123/PI assay

Figure A

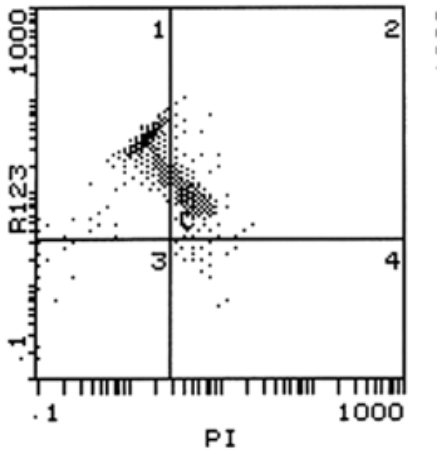


Figure B

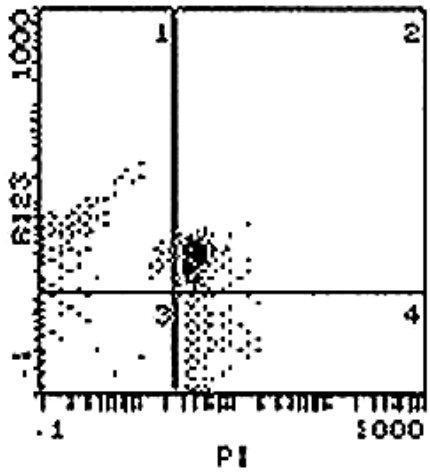


Figure A is a sample with high R123 staining. Figure B is a sample with high PI staining. Quadrant 1 is the live population with R123 staining only. Quadrant 2 is the moribund population, R123 and PI staining. Quadrant 3 is debris and quadrant 4 is PI only staining.

Appendix F

Examples of printouts from the sperm chromatin structure assay

Figure A

Figure B

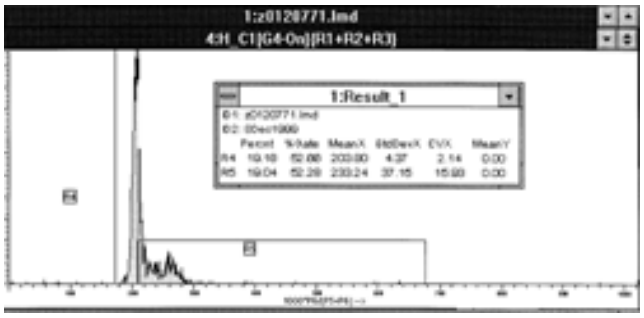


Figure A is a sample with low COMPαt. Figure B is a sample with high COMPαt. The tall peak is the main population (alpha-t) and R4 is gated on this population. R5 gates the cells outside of the main population (COMPαt). Alpha-t is the measure of red fluorescence/total fluorescence (red + green). As more cells move across the x-axis this indicates a higher percentage of cells with more single stranded DNA after being subjected to acidic conditions.

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Education:

Biochemistry Major	Matriculated to DVM program Virginia Polytechnic Institute and State University	8/90 - 5/92
DVM	Virginia-Maryland Regional College of Veterinary Medicine	8/92 - 5/96
Masters (in progress)	Virginia-Maryland Regional College of Veterinary Medicine	7/96 – 6/00

Professional activities:

Externships	New Bolton Center, NICU Kennet Square, PA	June 1995
	Ashby Herd Health Services, Inc. Harrisonburg, VA	July 1995
	Peterson, Smith, Matthews, Hahn & Slone, P. A. Equine Hospital Ocala, FL	January 1996
	Great Plains Veterinary Educational Center Clay Center, NE	March 1996
	Texas A&M University, Theriogenology College Station, TX	April 1996
	Colorado State University, Theriogenology Fort Collins, CO	August 1997

Resident, Theriogenology	Virginia-Maryland Regional College of Veterinary Medicine Blacksburg, VA	July 1996- 1999

Scientific Organizations:

Alpha Zeta Fraternity, Member (1991- 1993)
Golden Key Honor Society, Member (1991- 1993)
Phi Sigma Pi National Honors Fraternity, Member (1991-1993)
Alpha Psi Fraternity, Member (1993- 1996)
Society for Theriogenology, Member (1994-)
American Veterinary Medical Association, Member (1996-)
American Association of Equine Practitioners, Member (1997-)
Society for the Study of Reproduction, Member (1998-)
International Embryo Transfer Society, Member (1999-)

Elections, Appointments, Honors:

Recipient, Cyrus McCormick Merit Scholarship, 1990 academic year
Honor's Program, Virginia Polytechnic Institute and State University, 1991 academic year
Recipient, Florence A. Nocka Scholarship, 1993 academic year
Recipient, Roanoke Valley Horseman's Association, Inc. Scholarship, 1993 academic year
Recipient, Virginia Veterinary Medical Association Auxiliary Scholarship, 1994 academic year
Recipient, Vaughan Memorial Scholarship, 1995 & 1997 academic years
Recipient, Pauline Willson-Gun Scholarship, 1996, 1997, & 1998 academic years
Appointment, Graduate Honor Board of Virginia Polytechnic Institute and State University, 1997
Graduate/Resident abstract competition – Society for Theriogenology 3rd place December 1998
Appointment, Graduate and Research Board, VA-MD Regional College of Vet. Med., 1999

Continuing Education Programs attended:

Annual meeting of the Society for Theriogenology , Kansas City, Kansas - August 15-17, 1996.
Reproductive Pathology Symposium, Montreal, Quebec - September 14-16, 1997.
Annual meeting of the Society for Theriogenology, Montreal, Quebec - September 17-20, 1997.
Stallion Reproduction Symposium, Baltimore, Maryland – December 2-4, 1998.
Annual Meeting of the Society for Theriogenology, Baltimore, Maryland – December 5-6, 1998.
Annual Meeting of the American Association of Equine Practitioners, Baltimore, Maryland – December 6-9, 1998.
Annual Meeting of the Society for Theriogenology, Nashville, Tennessee – September 23-25, 1999.

Funded Research:

Ley WB, Bowen JM, DiGrassie WA. Evaluation of SCSA and heparin binding in stallion sperm with respect to fertility. Clinical Research Grant, VMRCVM, $3748, 1996.

Parker NA, Ley WB, Purswell BJ, Buechner-Maxwell V, DiGrassie WA. Peripartum risk assessment of the equine fetus associated with oxytocin induced parturition. Clinical Research Grant, VMRCVM, $1218, 1996.

Freeman LE, Bailey TL, DiGrassie WA. Vascular studies of the bovine penis. Clinical Research Grant, VMRCVM, $1575, 1998.

Ley WB, DiGrassie WA. Relationship between conventional tests, flow cytometric tests, and fertility in stallion frozen-thawed semen. VA Horse Industry Board, Richmond, VA $1000, 1999.

Scholarly Writings:

Publications:

DiGrassie WA, Wallace MA, Sponenberg DP. Multicentric lymphosarcoma with ovarian involvement in a Nubian goat. Can Vet J 1997; 38:383-384.

DiGrassie WA and Ley WB. Induction of parturition. J Equine Vet Science 1997; 17(10):528-529.

Dascanio JJ, Parker NA, Purswell BJ, DiGrassie WA, Bailey TL, Ley WB, Bowen JM. Diagnostic procedures in mare reproduction: basic evaluation. Compend Cont Ed Pract Vet 1997; 19(8):980-985.

Dascanio JJ, Parker NA, Ley WB, Bailey TL, Purswell BJ, Bowen JM, DiGrassie WA. Diagnostic procedures in mare reproduction: uterine evaluation, hysteroscopy, oviductal patency, and scintigraphy. Compend Cont Ed Pract Vet 1997; 19(9):1069-1075.

Bailey TL, Dascanio JJ, Parker NA, Purswell BJ, Ley WB, Bowen JM, DiGrassie WA. Diagnostic procedures in mare reproduction: hormonal evaluation and genetic testing. Compend Cont Ed Pract Vet 1997; 19(10):1183-1189.

Bowen JM, Walker WL, DiGrassie WA, Ley WB. The use of a Newton® PDA in an integrated veterinary record keeping system. J Equine Vet Science 1997; 17(9):476.

Newman SJ, Bailey TL, Jones JC, DiGrassie WA, Whittier WD. Multiple congenital anomalies in a calf. J Vet Diag Invest 1999; 11:368-371.

DiGrassie WA, Dascanio JJ, Ley WB, Newman SJ, Prater DA. Theriogenology question of the month: Abortion in a mare due to placental insufficiency and villous atrophy. J American Veterinary Medical Association 2000; 216(6):833-835.

Ley WB, Bowen JM, Purswell BJ, Dascanio JJ, Parker NA, Bailey TL, DiGrassie WA. Modified technique to evaluate uterine tubal patency in the mare. 44th Annual American Association of Equine Practitioners Convention Proceedings, Baltimore, Maryland, December 1998: 56-59.

Ley WB, Parker NA, Bowen JM, DiGrassie WA, Jack NE. How we induce a normal mare to foal. 44th Annual American Association of Equine Practitioners Convention Proceedings, Baltimore, Maryland, December 1998: 194-197.

Abstracts:

DiGrassie WA, Bailey TL, Carlin S, Gwazdauskas F, Robertson J, Walker WL, Parker NA, Dascanio JJ, Irby M, Bethard R. Classification of ovarian histology following ultrasound-guided transvaginal follicular aspiration in 8 adult Holstein cows. Proceedings for the Annual Meeting of the Society for Theriogenology, Montreal, Quebec, September 1997.

Bowen JM, Walker WL, DiGrassie WA, Ley WB. The use of a Newton® PDA in an integrated veterinary record keeping system. The World Equine Veterinary Association Congress, Padova, Italy, September 1997.

DiGrassie W, Ley W, Loomis P, Bowen J, Bailey T, Kalnitsky J. The validation of several flow cytometric tests to be used in the assessment of stallion frozen-thawed semen. Proceedings for the Annual Meeting of the Society for Theriogenology, Baltimore, Maryland, December 1998.

Ley W, DiGrassie W, Boon D, Dunnington E, Jack N. Pre-foaling mammary secretion sodium and potassium in relation to fetal readiness for birth. Proceedings for the Annual Meeting of the Society for Theriogenology, Nashville, Tennessee, September 1999.

Professional/Non-refereed Publications:

DiGrassie WA and Ley WB. Induction of Parturition in the Mare. Society for Theriogenology Newsletter 1997; 20(3):9-10.

Ley WB, Bowen JM, Walker WL, DiGrassie WA. The use of an Apple MessagePad 2000® personal digital assistant in an integrated veterinary invoicing and medical records keeping system. Proc Ann Mtg American Veterinary Medical Association, Baltimore, Maryland July 25-29 1998:138-139.

Ley WB, Bowen JM, Walker WL, DiGrassie WA, Fahl J. The use of FileMaker Pro® to maintain and deliver preventive medicine and herd health information for both restricted and public use over intra- and internet networks. Proc Ann Mtg American Veterinary Medical Association, Baltimore, Maryland July 25-29 1998:340-342.

Professional Presentations:

DiGrassie WA, Bailey TL, Carlin S, Gwazdauskas F, Robertson J, Walker WL, Parker NA, Dascanio JJ, Irby M, Bethard R. Classification of ovarian histology following ultrasound-guided transvaginal follicular aspiration in 8 adult Holstein cows. Annual Meeting of the Society for Theriogenology, Montreal, Quebec, September 1997.

DiGrassie W, Ley W, Loomis P, Bowen J, Bailey T, Kalnitsky J. The validation of several flow cytometric tests to be used in the assessment of stallion frozen-thawed semen. Proceedings for the Annual Meeting of the Society for Theriogenology, Baltimore, Maryland, December 1998.

Instruction:

Graduate Lectures:

Uterine conditions and surgery (3 hours), Advanced Urogenital Surgery, Fall 1996.

The relationship of catecholamines, lactate, glucose and cortisol in the exercising horse (3 hours), Endocrinology, Spring 1997.

Evaluation of Molecular and Structural Abnormalities and Heparin Binding in Stallion Sperm with Respect to Fertility (2 hours), Membrane Physiology, Spring 1997.

Professional student lectures:

Bison and Bear reproduction (1 hour), Theriogenology, Fall 1997.

Gestation and twin management (1 hour), Advanced Equine Reproduction, Spring 1999.

Stallion Reproduction (1 hour), Horse Production, Spring 1999.

Male and Female Reproductive Histology (2 hours), Histology, Fall 1999.

Laboratories:

Equine clinical techniques (12 hours), second year veterinary students, Clinical Techniques, Fall 1996.

Theriogenology (32 hours), third year veterinary students, Theriogenology, Fall 1996 & 1997, Spring and Fall 1998.

Equine dental lab (4 hours), third year veterinary students, Digestive System II, Fall 1996 and Fall 1997.

Ovine lab (1 week), second year veterinary students, Clinical Nutrition, Spring 1997.

Equine palpation (8 hours), first year veterinary students, Physiology II, Spring 1997.

Equine palpation lab, VMRCVM student chapter AAEP, Fall 1996, Fall 1997, Spring and Fall 1998.

Parturition (6 hours), animal science students, Horse Production, Spring 1999.

Stallion breeding soundness exam (8 hours), third year veterinary students and animal science students, Advanced Equine Reproduction and Horse Production, Spring 1999.

Histology (4 hours/week), first year veterinary students, Histology, Fall 1999.

General Pathology (4 hours/week), first year veterinary students, General Pathology, Spring 2000.

Extension activities:

Bull breeding soundness exam lab, Beef Producers Conference, Spring 1997.

Hormonal testing lab, Small Animal Theriogenology continuing education course, Spring 1997.

Postpartum problems in beef cows lab, Inservice Training for Extension Agents. Spring 1997.

Current Topics in Equine Medicine – Reproduction, Equine continuing education course, Spring 1999.

Stallion Breeding Soundness Exam, SAVMA 2000 conference, Spring 2000.