EFFECT OF SEMINAL PLASMA ON EQUINE SPERM QUALITY: PREPARATION AND STORAGE TECHNIQUES

A Thesis

by

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ABSTRACT

This study had four experimental aims. We investigated whether fresh (unfrozen) or snap-frozen homologous seminal plasma yielded similar sperm quality in cool-stored semen. We compared sperm quality following exposure to homologous versus heterologous seminal plasma. Various freezing methods for long-term storage of seminal plasma were also tested to identify any impacts on longevity of sperm quality. Finally, we adapted a freeze-drying protocol originally developed for human blood plasma for use with stallion seminal plasma with the goal of comparing sperm quality in cooled-stored semen prepared with fresh, frozen/thawed or lyophilized seminal plasma. Prior to the lyophilization study, we evaluated different vials and rubber stoppers to identify the most appropriate storage container for this purpose. Experimental endpoints for sperm quality included percent total motility (TMOT), percent progressive motility (PMOT), curvilinear velocity (VCL; μm/s), straightness of track trajectory (([straight-line velocity/average-path velocity] x 100); %), percent viable (VIAB), percent acrosome intact (AI), and percentage of sperm with abnormal sperm DNA (COMP; %) Motility values were obtained using a computerized sperm motility analyzer, whereas values for VIAB, AI, and COMP were obtained using a flow cytometer.

No significant difference was detected between fresh and frozen/thawed seminal plasma for any experimental endpoint ($P>0.05$). Sperm from two of three stallions yielded similar values for sperm quality with homologous versus heterologous seminal plasma ($P>0.05$), whereas PMOT and STR in the remaining stallion were greater in

heterologous seminal plasma, as compared to homologous seminal plasma ($P<0.05$). Various methods for processing and freezing seminal plasma prior to use resulted in only minor differences in sperm quality following cooled storage in extender. Prolonged exposure of sperm to chlorobutyl-isoprene blend rubber stoppers resulted in lower values for TMOT, PMOT, and VCL, as compared to chlorobutyl rubber stoppers or plastic vials ($P<0.05$); therefore, chlorobutyl stoppers were used in the lyophilization experiment. Lyophilization of seminal plasma resulted in similar values for TMOT, PMOT, VCL, VIAB, AI, and COMP, as compared to fresh or frozen/thawed seminal plasma ($P>0.05$). Variable STR was slightly lower with lyophilized seminal plasma, as compared to fresh or frozen/thawed seminal plasma ($P<0.05$).

I dedicate this work to:

Mom and Dad, for their continuous love,

and

My husband, for his unwavering support.

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NOMENCLATURE

196	Seminal plasma stored at -196°C
20	Seminal plasma stored at -20°C
80	Seminal plasma stored at -80°C
4-196	Seminal plasma held at 4°C for 24 h, then stored at -196°C
4-20	Seminal plasma held at 4°C for 24 h, then stored at -20°C
4-80	Seminal plasma held at 4°C for 24 h, then stored at -80°C
AI	Percentage of acrosome intact sperm
COMP	Percentage of sperm cells with fragmented DNA
CASA	Computer Assisted Sperm Analysis
h	Hour(s)
Heterol	Heterologous seminal plasma
Homol	Homologous seminal plasma
min	Minute(s)
mTorr	Millitorr, a measure of pressure
PBS++	Phosphate buffered saline with $MgCl_2$ and $CaCl_2$
PI	Propidium Iodide
PMOT	Percentage of progressively motile sperm
PSA	Lectin from *Pisum sativum* FITC conjugate, lyophilized powder
PSA/PI	*Pisum sativum* agglutinin / Propidium iodide

RW-196	Raw semen stored for 24 h at 4°C, then processed for seminal plasma and stored at -196°C
RW-20	Raw semen stored for 24 h at 4°C, then processed for seminal plasma and stored at -20°C
RW-80	Raw semen stored for 24 h at 4°C, then processed for seminal plasma and stored at -80°C
SAS	Statistical Analysis Software®
SCSA	Sperm Chromatin Structure Assay
SP	Seminal plasma
STR	Straightness (%) of sperm trajectory
TMOT	Percentage of motile sperm
VCL	Curvilinear velocity (μm/s)
VIAB	Viability, also referred to as sperm membrane integrity

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1. INTRODUCTION

Seminal plasma is the fluid component of ejaculated semen, and consists of fractions from the epididymis, testis, and accessory sex glands. Seminal plasma increases the volume of the sperm-rich portion of the ejaculate that aids in depositing semen in the uterus and subsequent sperm transport through the female tract [1]. In addition, seminal plasma provides proteins, electrolytes and minerals that facilitate sperm capacitation and eventual fertilizing capacity of the male gamete. Seminal plasma ensures that sperm survive to reach the site of fertilization by modifying the female's innate immune response, as well as protecting the sperm from the harsh and foreign environment in the female tract [2]. Conflicting views concerning seminal plasma and its interaction with sperm have sparked numerous studies that have evaluated effects of concentration, removal and/or replacement of seminal plasma on sperm longevity and resultant fertility of individual stallions [3].

As sperm progress from their storage site in the epididymis through the remaining excurrent duct system of the male reproductive tract until expulsion into the female reproductive tract, various fractions of seminal plasma are secreted from the accessory sex glands. Kareskoski et al. [4] provides a concise summary of this process. Prior to ejaculation, the bulbourethral gland secretes fluid for the pre-sperm component that is followed by prostatic and ampullar secretions just before ejaculation occurs. The ampulla and prostate continue to secrete fluid along with the epididymal cells to make up the first fraction of the ejaculate. The final ejaculatory fraction is primarily

comprised of fluid expelled from the seminal vesicles. These vesicular gland secretions commence after the prostate gland ceases activity. Any disruption in the function of these glands can alter the chemical composition of the ejaculate. For example, measurement of alkaline phosphatase activity in seminal plasma is a method to verifying that an ejaculate contains fluid contributions from the epididymis and testis. [5]. If alkaline phosphatase activity is high in the absence of sperm in the ejaculate, a testicular dysfunction is suspected. However, low alkaline phosphatase activity paired with no spermatozoa present can be linked to either a failure to ejaculate or blockage of the duct system resulting in the absence of testicular and epididymal secretions in the ejaculate [6]. Based on results from these diagnostic tests, a plan can be created and implemented to aid in the improvement of the stallion's reproductive performance.

Although not all proteins in stallion seminal plasma have been identified thus far, work has been conducted to identify and evaluate the correlation of known proteins to sperm longevity. These proteins are separated into three main families, which include the fibronectin-type II modules (Fn-2), the cysteine-rich secretory proteins (CRISPs), and the spermadhesins [7]. Horse Seminal Proteins (HSP) comprise the majority of proteins in stallion seminal plasma and belong to the Fn-2 family. These proteins help orchestrate the capacitation of sperm based on their heparin-binding abilities and tight association with the sperm membrane [8]. The protein, HSP-1, may be the equine homologue of osteopontin, which has been previously identified as beneficial to fertility [9]. Contradictory findings from Novak et al. [10] hypothesize that HSP-1 and HSP-2 actually bind to sperm and serve as protection from the female tract thereby reducing

their fertilizing capacity. These investigators propose that this negative relationship may be a reflection of the relative abundance of the proteins in their samples rather than a function of a physiological relationship. In 2011, Kareskoski et al. [11] produced results that strengthened the findings previously published by Brandon et al. [9] concerning the positive correlation of HSP-1 and HSP-2 with fertility in the stallion. Surface proteins on the acrosome associate with seminal plasma molecules after ejaculation to protect the sperm from the environment prior to entry into the female tract. Once inside the uterus, these seminal plasma and surface proteins are removed to facilitate binding with the oocyte's zona pellucida for the initiation of the acrosome reaction [11].

Pores on the sperm head are created over the duration of the acrosome reaction to enable enzymes to pass from the sperm to the egg to facilitate further penetration into the female gamete [1]. Damage to the acrosome or the absence of components necessary to initiate this reaction can decrease a stallion's fertility and result in decreased pregnancy rates. The inhibition of capacitation or the acrosome reaction of sperm have been linked to bovine seminalplasmin, human antifertility factor, decapacitation factors and rabbit acrosome stabilizing factor [9]. Heparin binding proteins aid in ensuring sperm viability and fertilization, while boar spermadhesins serve to induce capacitation factors and male and female gamete interaction [9]. A member of the CRISP family, HSP-3 may be linked to a gene polymorphism that improves fertility in the stallion [11]. Related to sperm motility, HSP-4 is a presumed homologue of a calcitonin gene product found in infertile males. Homologous to a human prostate-specific antigen, HSP-6 and HSP-8 are suspected to be two isoforms of a kallikrein-like protein [7]. As the only spermadhesin

protein in the horse found thus far, HSP-7 binds to the oocyte's zona pellucida to aid in the fertilization capacity of the sperm. Although labeled and identified based on molecular weight, HSP-5 has yet to have its physiological function determined in the stallion [11].

Not only does seminal plasma serve as an aqueous medium for sperm as they progress through the female tract, it is also essential for protection and maintenance of sperm viability as well as modulation of the female reproductive tract's immune response [10,11]. The female reproductive tract is protected against foreign particles (i.e. bacteria and semen) by lymphocytes and natural killer cells. After insemination, via natural service or artificial means, the mare reproductive tract undergoes a sperm-induced transitory inflammatory response. The innate immune response is initiated and a cascade of events occurs to introduce inflammatory intermediates and increased numbers of inflammatory cells into the uterine lumen for phagocytosis of sperm cells. Accompanied by an increase in blood flow, the uterus also experiences a wave of uterine contractions in a mechanical attempt to flush out foreign particles. On average, less than one percent of sperm successfully reach the oviducts for fertilization after natural insemination [12]. A decrease in viable sperm significantly reduces the chances of fertilization. The presence of seminal plasma in an ejaculate reduces the degree of uterine inflammation while protecting spermatozoa [13]. An insemination dose of raw semen, or seminal plasma devoid of sperm resulted in a significant increase in uterine blood flow following insemination for both groups, indicating that some of the components of seminal plasma interact with the uterine environment to modulate post-

breeding endometritis. In a pregnancy trial, Heise et al. [14] discovered that exposing epididymal sperm to seminal plasma prior to artificial insemination yielded better pregnancy rates than when epididymal sperm was not exposed to seminal plasma. This finding supports the hypothesis that seminal plasma is one of many factors that contributes to maintaining the integrity of sperm in an ejaculate for fertilization. The addition of seminal plasma before insemination with frozen/thawed spermatozoa may provide the semen with the protective qualities of the seminal plasma as well as aid in the transport of processed sperm through the mare's tract [2, 13, 14].

1.1. Background

Seminal plasma concentrations in extended semen typically vary from 5-25%. [15]. Several studies have compared sperm motility among ejaculates exposed to varying percentages of seminal plasma to determine an optimal concentration [3, 16]. The composition of the extender determines the optimal amount of seminal plasma that is needed to maintain high motion characteristics following cooled-storage. It has been shown that extenders containing Tyrode's medium are the most effective for sperm preservation if seminal plasma is largely removed using centrifugation techniques, while skim milk-glucose extenders lacking this supplement need seminal plasma to maintain sperm quality [16].

1.1.1. Homologous and heterologous seminal plasma

Stallions are generally selected for breeding based on their individual athletic performance and pedigree, and not on reproductive potential; therefore, there are numerous stallions used for breeding purposes that do not produce high quality semen. In some cases, semen handling and/or processing can be altered to improve semen quality and fertility. Seminal plasma components differ depending on which fraction of the ejaculate they accompany. Proteins and electrolyte concentrations vary between the sperm-rich fraction of an ejaculate as well as the sperm-poor fraction. One study [17] evaluated sperm motion characteristics and plasma membrane intactness in sperm exposed to various treatments, which included exposure to seminal plasma from the sperm-rich fraction of an ejaculate, seminal plasma from the sperm-poor fraction of an ejaculate, heterologous seminal plasma or an absence of seminal plasma. When exposed to seminal plasma from the sperm-poor fraction, motility values for both homologous and heterologous seminal plasma were higher than those for sperm exposed to seminal plasma filtered from the sperm-rich fraction. Certain stallion's sperm preferred their own seminal plasma, while others showed better motion characteristics when placed in another stallion's seminal plasma [17]. The removal of seminal plasma prior to cooled storage in extender resulted in the highest values for motility after 24 h of storage. A small amount of seminal plasma appears important for optimizing sperm motility, with some stallions responding well to treatment with heterologous seminal plasma [17]. Results from another study [18] also showed that sperm motility benefits from

centrifugation and extension of sperm in a milk-based extender with very little seminal plasma prior to cooled storage.

1.1.2. Processing techniques

Sperm quality appears to suffer in the presence of high concentrations of seminal plasma during cooling and/or freezing of extended semen [19]. One study examined the relationship between almost zero percent and twenty percent seminal plasma in cooled-stored equine semen [20], and found cooled-stored semen containing seminal plasma resulted in more damage to sperm DNA integrity and lower total and progressive sperm motility than cooled-stored semen having seminal plasma removed. Brinsko et al., [21] reported similar findings when seminal plasma was partially removed from dilute ejaculates by centrifugation before cooling, with reduction in the amount of seminal plasma resulting in improved progressive sperm motility both before and after cooled storage. Fresh epididymal sperm that had been exposed to pooled seminal plasma prior to artificial insemination yielded the higher pregnancy rates (75%) than fresh epididymal sperm that never contacted seminal plasma (22% pregnancy rate) [14]. These findings suggest that at least short-term exposure to seminal plasma may be important to sperm function. Long-term exposure of sperm to high concentrations of seminal plasma is deleterious to sperm longevity. Raw semen has the highest ratio of seminal plasma to sperm. Inadequate dilution with extender results in a high percentage of seminal plasma in the extended semen. Regarding frozen semen, some stallions produce semen that survives the freezing-thawing process well, and others do not. Aurich et al. [22] tested

the effects of centrifugation of semen from good- and poor-cooling stallions, removal of homologous seminal plasma, followed by replacement with either homologous or heterologous seminal plasma. These workers found that adding seminal plasma harvested from stallions that produced good quality frozen semen to sperm of stallions that produced poor quality frozen semen increased the post-thaw motility of the sperm from the poor-freezing stallions. They also noted that adding seminal plasma from stallions that produced poor quality frozen semen lowered post-thaw sperm motility of stallions that otherwise produced good post-thaw sperm motility. Whether this type of processing would actually improve fertility remains unstudied. However, direct comparisons among studies may not be warranted, but rather serve as a possible resource from which to base conclusions [3].

The centrifugation process alone may reduce (i.e. without the removal of seminal plasma) sperm motility, while removing most of the seminal plasma by centrifugation followed by suspension in extender resulted in higher sperm motility rates after 24 h of cooled storage for certain stallions [23]. Single layer centrifugation using a density gradient colloid, with resuspension in extender, resulted in improved sperm motion characteristics and chromatin integrity following 24 h of cooled-storage. The addition of five percent seminal plasma prior to artificial insemination yielded the highest motility values when compared to ten and twenty percent seminal plasma added [24]. The significant improvement in sperm quality following brief exposure to seminal plasma after cooled-storage strengthens the need for an effective and efficient method of seminal plasma removal and storage for addition to an ejaculate later. Homologous

seminal plasma may be effective for maintaining sperm motion characteristics [24]; however, ejaculates of individual stallions can be evaluated to determine the most beneficial concentration and source (i.e. homologous or heterologous) of seminal plasma. Centrifugation techniques separate sperm from the seminal plasma fraction of an ejaculate after a brief period of exposure. Consistently high/improved sperm recovery rates, motility, morphology and DNA integrity implies that it might be beneficial to sperm to separate them from seminal plasma. It has been shown that centrifugal fractionation can improve total and progressive sperm motility, morphology and DNA integrity. Initial centrifugation followed by processing with a gradient solution can improve semen quality of some stallions with decreased fertility [25]. One study demonstrated that the fertility of some sub fertile stallions could be improved with this technique [26]. Pregnancy rates in mares following deep horn insemination with sperm that was obtained via simple centrifugation or centrifugation with a density-gradient silane-coated silica colloid solution were evaluated in another study [27]. This team also added five percent seminal plasma from a stallion with high fertility to the oligospermic ejaculate to examine heterologous seminal plasma effects. Overall, the separation of sperm from an ejaculate following density-gradient centrifugation yielded improved sperm motion characteristics and yielded higher pregnancy rates than simple centrifugation. The addition of five percent seminal plasma from a stallion with high fertility prior to insemination did not significantly improve pregnancy rates [27]. The addition of heterologous seminal plasma from a fertile stallion to the sperm from a stallion with low fertility does not always improve the sub fertile stallion's measures of

fertility [27]. For some stallions, it appears that the relationship between sperm concentration and percentage of seminal plasma is critically important for maintenance of sperm motility in cooled-stored extended semen.

1.1.3. Interaction with glass and plastic vials

The type of container in which semen is stored may have an effect on resulting sperm motion characteristics. Laboratories generally use tubes made from various plastics or glassware to process an ejaculate. Glass centrifugation tubes have yielded greater motility values than polypropylene or polystyrene plastic centrifugation tubes, with a negative chemical interaction between the sperm and the plastic contributing to this decrease in sperm motility [28]. In another study using human semen [29], storage in glass tubes resulted in higher motility values; however, among the plastic tubes evaluated, storage in polypropylene yielded higher motility values than storage in either polyethylene or polyurethane tubes. Once again, a toxic interaction between the sperm and plastic were suspected for the significant decreases in motility values. Borosilicate glass tubes were compared to polystyrene Petri dishes in a study conducted by Bedford and colleagues [30]. Although storage in the plastic dishes containing semen yielded higher motion characteristics, the shape of the container may have contributed to these results. In the glass tubes, the sperm sank to the bottom and were exposed to unequal concentrations of extender whereas the sperm on the Petri dishes were exposed more evenly to the extender [30]. Polypropylene syringes can have either a rubber or a plastic plunger, which also may affect sperm motion characteristics. When extended semen

was incubated with latex-free synthetic rubber syringe plungers with silicon lubricant, sperm motility decreased more than when semen was incubated with polypropylene syringe plungers [31]. A toxic environment may be caused by the rubber syringe plunger because , , sperm motion characteristics improved after fresh extender was added to the exposed semen [31]. Current recommendations are for laboratories to use plastic syringe plungers to avoid exposing semen to potentially toxic conditions.

1.2. Lyophilization

Freeze-drying, also known as lyophilization, is an actively evolving method used to preserve biological cells and fluids, and has become a preferred method of storage for delicate, solvent-impregnated materials and pharmaceuticals. The freeze-drying process has been described as time-consuming [32], with even the shorter drying periods ranging from 30-44 or more h [33, 34]. Prolonged processing times can result in product backlog and subsequent shipping delay. Added production costs, such as freeze-dryer maintenance and energy fees, are other drawbacks to this technology [35]. The increased cost and time associated with formulation of a proper lyophilization protocol also limits the availability of including this technique in the equine industry since a trial and error approach is employed to establish an effective lyophilization process for each stallion's lot of product [33, 35]. This approach depletes necessary resources, especially when working with a set amount of fluid like seminal plasma, and can incur even more costs for the production of an elegant cake (i.e. the dried portion of the lyophilizate), the desired product following lyophilization. Since the protein composition and

concentration vary among stallions and ejaculates of an individual stallion [36], a definitive protocol to freeze-dry all products remains to be established. This lack of homogeneity in processing creates difficulties when it comes to repeating the prescribed measures for a freeze-drying protocol [32]. Any deviation from the drying time or an increase in temperature could compromise the integrity of the final cake and result in discarding the entire batch, thereby resulting in lost time and money in the process [35].

The freeze-drying process itself can actually alter the final composition, such as an increase in pH or reduction in pCO_2, and yield a product unsuitable to achieve its end goal [37]. Careful evaluation of the reconstituted lyophilized product is necessary to ensure the retention of physiological and chemical properties. Various stabilizing agents may have to be added to the product to stabilize proteins that may be degraded. Mannose, sucrose, glycine and various other surfactant proteins have been proposed to serve as effective stabilization additives to prevent denaturation of proteins during the lyophilization process; however, each biological product must be analyzed to determine which stabilizer will provide the required protection for the compound specific proteins [37, 38]. More economically sound and simpler lyophilization techniques have been proposed as an option for sperm preservation of bovine semen [39].

Although lyophilization presents numerous obstacles, the values and advantages gained from investing in this technology could provide many benefits to the equine industry. Freeze-drying extracts water from the product using negative pressure and temperature in a method that reduces damage of the crystalline structure of the product, helping to maintain the physiological and biochemical properties inherent in the product.

Prior to reconstitution, sterile water being the most easily accessible and effective diluent, the lyophilized product is purported to be in a preserved state that prevents degradation over time [40]. Studies note that the improved solubility of the lyophilized product improves the outcome of freeze-drying aqueous solutions and biological fluids, like blood [34, 41]. Possibly, this technology could be applied to seminal plasma. Ideally, the stabilized product will have a markedly increased shelf life in as simple a storage unit as a common refrigerator. A powdered seminal-plasma cake that is lightweight and more stable at lower or ambient temperatures would not require expensive liquid nitrogen for storage or transport [35, 42]. Since the restrictions that come with shipping liquid nitrogen would no longer be in effect, the cost of shipping would decrease and the availability of shipping facilities might increase, thereby allowing smaller breeding operations greater access to high quality marketed seminal plasma. The prospect of being able to store high quality seminal plasma that maintains its biochemical integrity in a powder cake form and the ability to store these products in a laboratory refrigeration unit makes the exploration of lyophilization with equine seminal plasma a venture worthy of the time and effort expended.

Live offspring have been obtained in the horse, mouse, rat, and rabbit using lyophilized sperm through intracytoplasmic sperm injection techniques, while viable blastocyst formation has been achieved in cattle and pigs [42]. This technique for the long-term storage of seminal plasma has been contemplated by our laboratory; however, no results have been published to date. The goal of this novel conservation method is to allow for storage and banking of superior quality stallion seminal plasma at simple

refrigeration (4°C) for long periods without the denaturation of proteins that might occur in cryopreserved seminal plasma. Lyophilization of seminal plasma would also enable transport and exchange between breeding facilities without the needed addition of liquid nitrogen for shipment purposes. One of many hurdles to overcome before lyophilized seminal plasma is introduced to the equine industry involves determining a freeze-drying protocol for this protein-rich fluid. Extensive research concerning the freeze-drying of blood plasma has been conducted in the military for transport to the battlefield in the Middle East without compromising the integrity of the hemostatic properties [43]. Our laboratory has considered following the protocol set forth by Bakaltcheva et al. [37] to evaluate the possibility of creating a viable and undamaged freeze-dried seminal plasma product in the stallion. By utilizing a protocol tested with protein complex human blood plasma, we hope to protect these molecules present in stallion seminal plasma. Upon completion of this research, stallion seminal plasma may be an excellent candidate for lyophilization to provide an invaluable storage method of superior donor stallion's seminal plasma in order to improve fertility in a less fertile, yet highly desirable, recipient sire.

The protocol utilized by Bakaltcheva et al. [37] for the freeze-drying of human blood plasma will be followed for the lyophilization of stallion seminal plasma as a starting point for any future studies concerning the freeze-drying procedure. Determining which type of seminal plasma (fresh, frozen at different temperatures, or freeze-dried) yields the highest laboratory parameters for semen quality, along with the use of appropriate controls, will allow us to assess whether biological function has been

maintained in the processed seminal plasma. Any variation between homologous and heterologous seminal plasma and sperm function will also be evaluated. These results will further elucidate the practicality of using seminal plasma to improve cooled-stored or frozen semen quality/longevity.

Our hypothesis is that frozen seminal plasma, regardless of method of storage, will yield similar sperm quality endpoints after being added to sperm as compared to fresh seminal plasma.

This study had four experimental aims. We investigated whether fresh (unfrozen) or snap-frozen homologous seminal plasma yielded similar sperm quality in cooled-stored semen. We compared sperm quality following exposure to homologous versus heterologous seminal plasma. Various freezing methods for long-term storage of seminal plasma were also tested to identify any impacts on longevity of sperm quality. Finally, we adapted a freeze-drying protocol originally developed for human blood plasma for use with stallion seminal plasma. We compared sperm quality in cooled-stored semen prepared with fresh, frozen/thawed or lyophilized seminal plasma.

2. MATERIALS AND METHODS

2.1. Animals

Sexually active and mature stallions were used to conduct the experiments reported herein. All animals were fed a pelleted diet with access to fresh water and roughage and were in good body condition. They were kept in stalls with occasional turn out in paddocks. Two to three daily ejaculates from each stallion were collected in an artificial vagina to reduce extragonadal reserves prior to collection of semen and/or seminal plasma for the experimental procedures that are detailed in the following sections. Specifically, three Quarter Horse and two Arabian stallions (11 to 24 years of age) were used as donors of both seminal plasma and sperm for all of the experiments reported herein. Seven Quarter Horse stallions (9 to 20 years of age) were used as donors only of seminal plasma in Experiment 1.

2.2. Semen collection

Semen was collected using a non-spermicidal lubricated Missouri-model artificial vagina (Nasco, Ft. Atkinson, WI, USA) fitted with a semen receptacle (Animal Reproduction Systems, Chino, CA, USA) and a nylon mesh in-line filter (Animal Reproduction Systems, Chino, CA, USA) to separate gel-free and gel-containing fractions of the ejaculate. Stallions were exposed to an ovariectomized mare to stimulate penile erection. The erect penis was rinsed with warm water and then patted dry with

clean, disposable towels. After penile cleaning, stallions were teased further and, when fully stimulated, were allowed to mount a breeding dummy for semen collection [6].

2.3. Semen processing

Immediately after collection of semen, the in-line filter was removed from the semen receptacle and the amount of gel present in the filter was estimated. The liner was then discarded. The gel-free semen volume was measured by weight using a pre-tared scale and recorded in mL (1 g ≈ 1 mL). Sperm concentration was determined using a cell counter (NucleoCounter® SP-100™, Chemometec, Allerød, Denmark). An aliquot of gel-free semen was diluted to 30 x 10^6 sperm/mL in pre-warmed INRA 96 extender (IMV Technologies, Maple Grove, MN, USA), then incubated for 15 min at 37^{o}C, and initial sperm motion characteristics were assessed using a computerized motility analyzer.

Remaining gel-free semen was extended in INRA 96 and then subjected to cushioned centrifugation, using 40-mL capacity glass nipple-bottom centrifuge tubes [43]. To prepare glass nipple tubes for centrifugation, 30 µL of cushion fluid (Minitube of America, Inc., Verona, WI, USA) was pipetted into the bottom of the nipple underneath 1 mL of INRA 96 extender. Following extension of semen to a concentration of 30 x 10^6sperm/mL in INRA 96, 1 x 10^9 sperm were carefully layered on top of the INRA-96 in the nipple tubes. Loaded nipple tubes were centrifuged at 400 x g for 20 min at room temperature. The supernatant was aspirated and the resulting sperm pellet was resuspended in INRA 96 extender, then transferred to a 50-mL conical-bottom

tube and further extended to reach a final concentration of approximately 37.5 x 10^6 sperm/mL as evaluated by the cell counter.

2.4. Seminal plasma processing

Semen used for harvesting sperm-free seminal plasma was collected as described above. The gel-free raw semen was centrifuged at 2000 x g for 10 min at room temperature using 15-mL plastic conical-bottom tubes (VWR International, LLC, Radnor, PA, USA). The seminal plasma was decanted and filtered through tandem syringe filters (5.0 and 1.2 µm pores, Spectrum Chemical Manufacturing Corp., New Brunswick, NJ, USA) to remove any residual sperm. Freshly filtered seminal plasma was kept at room temperature in a 15-mL plastic conical tube until divided into 1.0- to 1.8-mL aliquots in sterile 2- mL plastic cryovials and stored according to the experimental specifications.

2.5. Sperm motion characteristics analysis

Sperm motion characteristics were analyzed using a computerized motility analyzer (IVOS CASA system, version 14, Build 008, Hamilton Thorne Biosciences, Beverly, MA, USA). An aliquot of gel-free semen was diluted to 30 x 10^6 sperm/mL with pre-warmed INRA 96 extender and incubated for 15 min at 37 ^{o}C. A 6.0-µL sample was loaded on a two-chamber microscope slide (Leja Products, B.V., Nieuw-Vennep, The Netherlands) and placed onto the viewing platform for insertion into the instrument. Ten different fields were assessed and a minimum of 500 cells were

analyzed. The instrument settings for the IVOS system are as followed: frames acquired = 45; frame rate = 60 Hz; minimum contrast = 60; minimum cell size = 4 pixels; minimum static contrast = 30; straightness threshold for progressive motility = 50.0%; path velocity threshold for progressive motility = 30 μm/s; path velocity threshold for static cells = 15.0 μ/s; cell intensity = 106; static head size = 0.59 to 2.99; static head intensity = 0.68 to 1.74; static elongation = 12 to 97; magnification = 1.89; LED illumination intensity = 2393; temperature = 37.0°C. Values were recorded for subsequent analysis. Percentage of total sperm motility (TMOT), percentage of progressively motile sperm (PMOT), mean curvilinear velocity (VCL; μm/s) and straightness (STR; %; [mean straight-line velocity/average-path velocity] x 100) were measured in this study.

2.6. Flow cytometric analysis

All flow cytometric analyses in this study were performed using a FACScan analytic flow cytometer (Bectin-Dickinson Immunocytometry Systems, San Jose, CA, USA), equipped with three-color detection abilities and a fixed 488-nm laser. Sample data were saved in List-mode until later analysis using WinList™ software (Verity Software House, Topsham, ME, USA). Each sample was equilibrated for 30 sec prior to the acquisition of 5000 events. Sperm membrane integrity and sperm DNA integrity were evaluated with this flow cytometer.

2.6.1. Pisum sativum agglutinin (PSA)/Propidium iodide (PI) protocol

Sperm membrane integrity (SMI; %) and acrosomal intactness (AI; %) were evaluated using a staining protocol that incorporated fluorescein-labeled *Pisum sativum* agglutinin (PSA) and propidium iodide (PI) [44]. Stock solutions of 0.25 mM PSA-FITC conjugate (Sigma-Aldrich, St. Louis, MO, USA) and 2.4 mM PI stain (Invitrogen Molecular Probes, Eugene, OR, USA) were frozen in 100 µL aliquots until they were thawed at room temperature prior to use. Four-hundred (400) µL of phosphate buffered saline containing calcium and magnesium (PBS++) was added to 100 µL of thawed PSA stock solution. A 133-µL aliquot of PBS++) was pipetted into a 5-mL Falcon Tube (BD Biosciences, San Jose, CA, USA) and 50 µL of each semen sample was added along with 3 µL of 2.4 mM PI solution and 10 µL of 0.48 mM PSA conjugate. Tubes were incubated in a dark cabinet for 10 min at room temperature (approximately 37°C). After incubation, 40-µL aliquots of semen-buffer mixtures were mixed with 500 µL of PBS++ in a new 5-mL Falcon tube and then analyzed using the flow cytometer. Final working concentrations of PSA and PI were 6.4 µM and 0.0387 mM, respectively.

A scatter gram displaying four distinct sperm populations was generated from collected data and used to sperm membrane integrity and acrosome intactness: non-viable/acrosome intact sperm (Region 1), non-viable/acrosome damaged sperm (Region 2), viable/acrosome intact sperm (Region 3), and viable/acrosome damaged sperm (Region 4) (Fig. 1). Regions 2 and 4 were combined for determination of percent viable sperm (VIAB).

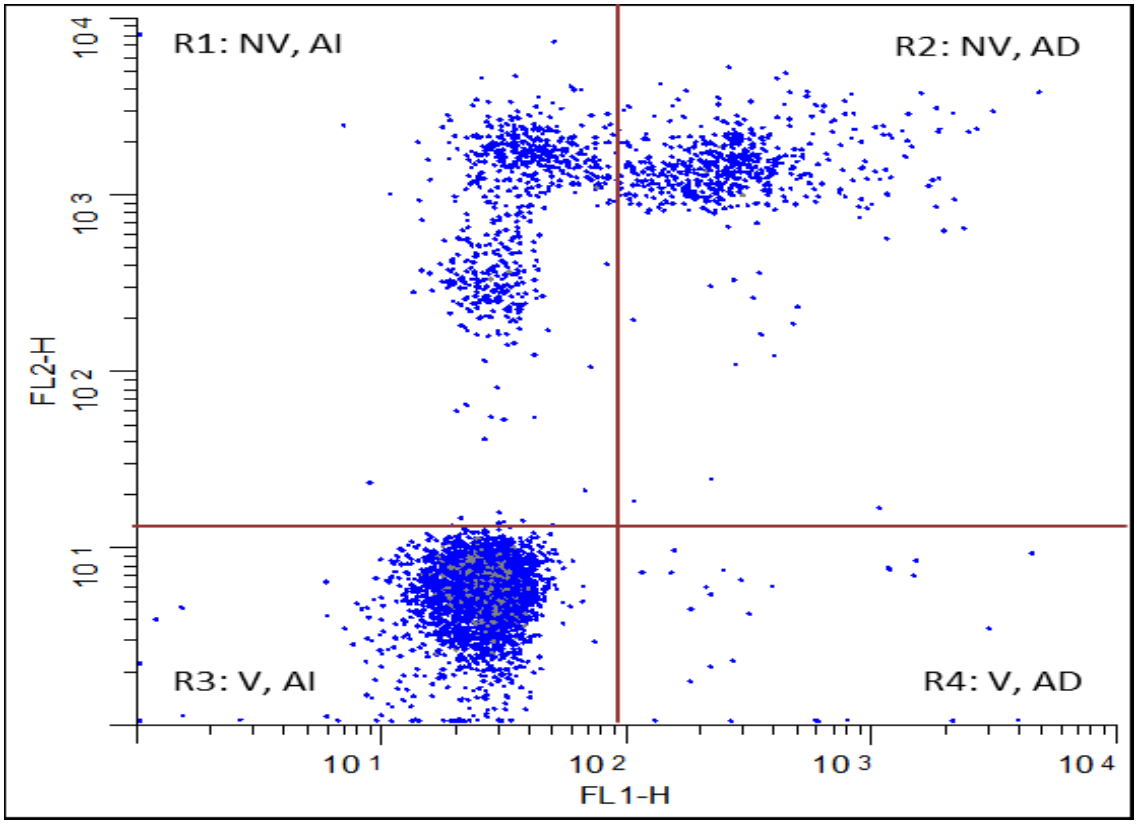

Fig. 1. Example scatter gram showing the four distinct regions of sperm membrane integrity and acrosome intactness following flow cytometric analysis with the PSA/PI staining technique. Non-viable/acrosome intact sperm in region 1(R1:NV,AI), non-viable/acrosome damaged sperm in region 2 (R2:NV,AD), viable/acrosome intact sperm in region 3 (R3:V,AI), and viable/acrosome damaged sperm in region 4 (R4:V,AD).

2.6.2. Sperm Chromatin Structure Assay (SCSA) protocol

The SCSA protocol was conducted as previously described [45, 46]. Fifty (50)-µL aliquots of semen were stored at -80°C until analysis was performed. All stock solutions ((buffer solution (TNE; pH 7.4; 0.19 g disodium EDTA, 0.79 g Tris-HCl, 4.380 g NaCl in 500 mL deionized water), Triton-X (2.19 g NaCl, 1.0 mL of 2N HCl solution, 0.25-mL Triton-X, qs. 250 mL with deionized water), and acridine orange (pH 6.0; 3.8869 g citric acid monohydrate, 8.9429 g Na_2HPO_4, 4.3850 g NaCl, 0.1700 g

disodium EDTA, 4.0 µg/mL acridine orange stock solution (1.0 mg/mL), qs. 500 mL water)) were kept on ice throughout the duration of the procedure.

Immediately prior to analysis, semen samples were thawed in a 37°C water bath. Based on sperm concentration of each sample, 1 to 9 µL of semen (Table 1) were aliquoted into a 5-mL Falcon tube and diluted to 200 µL with the TNE solution. Then 400 µL of Triton-X was added and the mixture was placed on ice for 30 sec. A 1.2 mL aliquot of acridine orange stain was then pipetted into the 5-mL Falcon tube. The mixture was analyzed via flow cytometry using the following settings: mean green fluorescence at 500 channels (FL-1: 500) and mean red fluorescence at 150 channels (FL-3: 150). All samples underwent an equilibrium period of 30 seconds prior to analysis. Five-thousand (5000) events were recorded at a rate of at least 200 events/sec. Cells Outside the Main Population (COMP) was the endpoint and represents the percentage of sperm cells outside the main population when compared to the total number of sperm cells evaluated.

Table 1
Sperm concentration and corresponding aliquot of semen used for SCSA evaluation.

Sperm Concentration ($x10^6$/mL)	**Aliquot of semen (µL)**
300	1
200	2
100	3
50	5
30	9

2.7. Statistical analysis

Percentage data were arc sine-root transformed for normalization prior to statistical analysis using SAS® (SAS Institute Inc., Cary, NC, USA). Statistical tests were conducted on transformed data. Analysis-of-variance (ANOVA) procedures were used for data analysis, with the Tukey test used for mean separation when treatment F ratios were significant ($P<0.05$). Level of significance was set at $P < 0.05$.

2.8. Experiment 1

Experiment 1 evaluated sperm motion characteristics and viability following exposure to various seminal-plasma treatments. Treatment comparisons included fresh versus frozen-thawed seminal plasma, homologous versus heterologous seminal plasma, and various methods for processing seminal plasma prior to frozen storage. Seminal plasma from each of seven stallions was processed as described above. Aliquots (1.8 mL) of freshly filtered seminal plasma were pipetted into 2-mL plastic cryovials (Sigma-Aldrich, Co., St. Louis, MO, USA) and frozen according to Table 2, with the initial temperature listed marking the first 24 h of storage and the second value noting the long-term storage temperature.

Table 2
Treatment groups for long-term storage of seminal plasma

Initial 24 h at 4°C	Storage °C for 9 months	Treatment group
None	-20, -80, -196	20, 80, 196
Seminal plasma	-20, -80, -196	4-20, 4-80, 4-196
Raw semen	-20, -80, -196	RW-20, RW-80, RW-196

Six mL aliquots of raw semen from each of seven stallions were stored in plastic cryovials for 24 h at 4°C. These samples were then processed for seminal plasma as follows: each sample was centrifuged at 2000 x g for 10 min then filtered for seminal plasma using tandem filters (5.0 and 1.2 µm, respectively). The sperm-free seminal plasma was divided into 6 1.8 mL aliquots and one sample was stored at each of the following temperatures (-20°C, -80°C, and -196°C) for nine months before completion of this experiment. Resulting treatment groups for seminal plasma included the following: Group 20 (seminal plasma processed immediately following collection and frozen at -20°C); Group 80 (seminal plasma processed immediately following collection and frozen at -80°C); Group 196 (seminal plasma processed immediately following collection and frozen at -196°C); Group 4-20 (seminal plasma processed immediately following collection, stored at 4°C for 24 h and then frozen at -20°C); Group 4-80 (seminal plasma

processed immediately following collection, stored at 4°C for 24 h and then frozen at -80°C); Group 4-196 (seminal plasma processed immediately following collection, stored at 4°C for 24 h and then frozen at -196°C); Group RW-20 (raw semen stored at 4°C for 24 h, then processed for seminal plasma and frozen at -20°C); Group RW-80 (raw semen stored at 4°C for 24 h, then processed for seminal plasma and frozen at -80°C); Group RW-196 (raw semen stored at 4°C for 24 h, then processed for seminal plasma and frozen at -196°C).

To test the effects of the source of the seminal plasma and the method of storage on semen quality, seven ejaculates from each of three sperm-donor stallions were collected. The volume and concentration of gel-free semen in each ejaculate was recorded prior to further processing of the samples. Ejaculates were maintained in a 37°C incubator during initial processing. Fifty (50)-µL aliquots of raw gel-free semen were placed in 0.6-mL Eppendorf tubes and immediately frozen (-80°C) until analyzed for sperm DNA integrity, using the Sperm Chromatin Structure Assay (SCSA). Two 0.5-mL aliquots of gel-free semen were diluted with INRA 96 extender to obtain a sperm concentration of 30 x 10^6 sperm/mL and placed in labeled tubes for initial (T0) evaluation and evaluation following 24 h of cooled storage (T24). Remaining raw semen was centrifuged to obtain fresh seminal plasma or subjected to centrifugation following initial dilution in INRA 96 extender, using the method described above, and the resulting sperm pellet was re-suspended with INRA96 extender containing 20% seminal plasma from various treatment groups to obtain a final sperm concentration of 30 x 10^6sperm/mL. Treatment groups included fresh homologous seminal plasma

(Group Fresh Hom), flash-frozen/thawed homologous seminal plasma (Group Frozen Hom), and Groups 20, 80, 196, 4-20, 4-80, 4-196, RW-20, RW-80, RW-196 for heterologous seminal plasma.

All samples were capped and mixed gently then wrapped in two 60-mL ballast bags and stored for 24 h at approximately 4°C in an Equitainer (Hamilton Research, Inc., Ipswitch, MA, USA).

Following cooled-storage, extended semen samples were evaluated by CASA and flow cytometric analysis for experimental endpoints (TMOT, PMOT, VCL, STR, VIAB, AI and COMP).

2.9. Preliminary experiment 2.1

This preliminary experiment was conducted to evaluate sperm motion characteristics of extended semen following exposure to glass borosilicate vials with chlorobutyl-isoprene blend rubber stoppers and plastic polypropylene cryovials after 24 h of cooled-storage in various vial orientations. Three ejaculates from each of five stallions were collected as previously described. The gel and gel-free portion of each ejaculate was recorded and concentration was evaluated using a cell counter (NucleoCounter® SP-100™, Chemometec, Allerød, Denmark). In four borosilicate glass vials (Wheaton Scientific, Millville, NJ, USA), 2.5 mL of raw semen was diluted to a concentration of 30 x 10^6 sperm/mL with INRA 96 extender and in four plastic cryovials, 2.0 mL of raw semen was diluted to a final concentration of 30 x 10^6 sperm/mL using the same extender. Vials were then capped and stored in an Equitainer.

Vial orientation was either upright to minimize contact with the vial cap or upside down to maximize exposure to the vial cap. After 24 h of storage, all samples were mixed well and analyzed for sperm motion characteristics.

2.10. Preliminary experiment 2.2

This preliminary study was conducted to evaluate two different rubber stoppers, a chlorobutyl-isoprene blend rubber and a chlorobutyl only rubber, for use in the glass vials for the lyophilization experiment below. One ejaculate from each of four stallions was processed and extended to a final concentration of 30 x 10^6 sperm/mL as described in preliminary Experiment 2.1 using six borosilicate glass vials and one plastic cryovial for a control group. Three glass vials were capped with a chlorobutyl-isoprene blend rubber stopper (Wheaton Scientific, Millville, NJ, USA) and the remaining three glass vials were capped with a chlorobutyl rubber stopper (Wheaton Scientific, Millville, NJ, USA). For each rubber stopper type, one vial was stored upright to prevent exposure of the extended semen to the rubber stopper; one vial was stored upside down to maximize exposure time to the rubber stoppers; and the one vial was rotated ten times prior to upright storage to expose the extended semen to the rubber stopper for a brief period of time. Following 24 h of cooled-storage in an Equitainer, all samples were evaluated for sperm motion characteristics. The vials stored upright were mixed using a pipette to avoid sample contact with the cap while the upside-down vials and vials inverted prior to storage were mixed by inversion just prior to evaluation.

2.11. Experiment 2.3

This experiment was conducted to evaluate the effects of homologous seminal plasma processing technique (fresh, frozen/thawed, or lyophilized) on sperm motion characteristics and viability, and DNA quality immediately following sperm exposure (T0) and following 24 h of cooled storage (T24). One ejaculate from each of five stallions was processed for seminal plasma as described above following three semen collections to deplete extragonadal reserves. For preparation of lyophilized and frozen-thawed seminal plasma, 1-mL aliquots of fresh seminal plasma were transferred to 2-mL capacity borosilicate glass vials and covered with Parafilm (American National Can™, Neenah, WI, USA) prior to freezing (-80°C). A subset of these frozen samples were transferred to a freeze dryer (Advantage; VirTis Industries, Gardiner, NY, USA). The glass vials were placed directly on a pre-cooled to -80°C shelf inside the freeze-drying chamber and chlorobutyl rubber stoppers were inserted partially into the vial necks. The vials underwent a 46 h lyophilization cycle with a vacuum of 200 mTorr, condenser temperature at -50°C, and shelf temperatures of -40, -30, 0, +20, and +25°C for 1200, 360, 180, 360, and 660 min respectively. A more detailed description of freeze-dryer settings are in Appendix A and B.

Following completion of the lyophilization process, the glass vials were vacuum sealed in the chamber and then an overlying metal cap was applied and hand-crimped (Fig. 2). All vials were stored at 4°C for three months until used in this experiment.

To determine the amount of laboratory-grade deionized water needed for reconstitution, 30 glass vials containing 1.0 mL of seminal plasma were weighed using a

pre-tared scale prior to the lyophilization process and then weighed after lyophilization. The weights were averaged and the mean difference between the two indicated the amount of water needed for reconstitution to 1.0 mL of seminal plasma. The lyophilized seminal plasma was reconstituted by injecting 786 µL of deionized water into each vial and then swirled gently until the sample was a fully reconstituted fluid (Fig. 3).

The sperm donor samples were prepared as described above using three ejaculates from each of five stallions. Seminal plasma was processed (as outlined above) to provide a fresh source of seminal plasma as one of the treatments. A 400-µL aliquot of extended semen was added to 100-µL aliquots of seminal plasma from each treatment group (fresh, frozen/thawed, and reconstituted lyophilized seminal plasma) to yield 20% seminal plasma in the extended semen samples. All samples were inverted to mix thoroughly then evaluated at T0 and T24 for TMOT, PMOT, VCL, STR, VIAB and, COMP.

Fig. 2. Photograph of lyophilized seminal plasma in a borosilicate glass vial.

Fig. 3. Photograph of reconstituted lyophilized seminal plasma in a borosilicate glass vial.

3. RESULTS

3.1. Experiment 1: Comparison of fresh versus frozen homologous seminal plasma

No significant differences ($P>0.05$) were noted between the fresh and frozen/thawed seminal plasma treatment groups (Table 3); nor were any stallion-by-treatment effects detected. There were significant differences ($P<0.05$) for individual stallions for all laboratory parameters (Table 4). One stallion (Apollo) had a lower TMOT than the other two stallions ($P<0.05$). Variable PMOT was lower for Rock than the other two stallions. ($P<0.05$). Variables VIAB, STR and AI differed significantly among all three stallions ($P<0.05$). Variable VIAB was higher for Rock than the other stallions ($P<0.05$). Variable COMP was significantly lower (better) for one stallion (Spencer) than the remaining stallions ($P<0.05$).

Table 3
Main effect of fresh versus frozen/thawed homologous seminal plasma on measures of sperm quality (mean ± SEM) for three stallions following 24 h of cooled storage (n = 7 ejaculates).

	Treatment	
Laboratory parameter*	Fresh seminal plasma	Frozen/thawed seminal plasma
TMOT (%)	80 ± 1.8	79 ± 2.0
PMOT (%)	49 ± 1.7	48 ± 1.5
VCL (μm/s)	183 ± 6.8	187 ± 6.9
STR (%)	64 ± 2.0	62 ± 2.1
VIAB (%)	85 ± 0.6	84 ± 0.7
AI (%)	88 ± 1.0	88 ± 1.1
COMP (%)	10 ± 0.8	9 ± 0.9

*TMOT = total sperm motility (%); PMOT = progressive sperm motility (%); VCL = curvilinear velocity (μm/s) STR = straightness (%); VIAB = sperm with intact plasma membrane (%); AI = sperm with intact acrosomal membrane (%); COMP = percentage of sperm with αt value outside the main population (%). Percentage data (TMOT, PMOT, STR, VIAB, AI, and COMP) were arc sine-root transformed for normalization prior to statistical analysis. Untransformed values for mean and SEM are presented in table to ease interpretation but statistical tests were conducted on transformed data. For each dependent variable, treatment differences were not detected (P>0.05).

Table 4
Main effect of stallion on measures of sperm quality (mean ± SEM) following 24 h of cooled storage (n = 7 ejaculates).

	Stallion		
Laboratory parameter*	Spencer	Rock	Apollo
TMOT (%)	85 ± 0.7[a]	82 ± 1.6[a]	71 ± 2.4[b]
PMOT (%)	51 ± 1.6[a]	44 ± 1.4[b]	52 ± 2.2[a]
VCL (μm/s)	187 ± 7.1[b]	215 ± 2.8[a]	152 ± 2.3[c]
STR (%)	61 ± 1.6[b]	54 ± 0.8[c]	74 ± 0.6[a]
VIAB (%)	84 ± 0.8[b]	87 ± 0.4[a]	83 ± 0.8[b]
AI (%)	88 ± 0.9[b]	92 ± 0.6[a]	83 ± 0.8[c]
COMP (%)	7 ± 0.5[b]	10 ± 1.1[a]	11 ± 1.1[a]

*TMOT = total sperm motility (%); PMOT = progressive sperm motility (%); VCL = curvilinear velocity (μm/s) STR = straightness (%); VIAB = sperm with intact plasma membrane (%); AI = sperm with intact acrosomal membrane (%); COMP = percentage of sperm with αt value outside the main population (%). Percentage data (TMOT, PMOT, STR, VIAB, AI, and COMP) were arc sine-root transformed for normalization prior to statistical analysis. Untransformed values for mean and SEM are presented in table to ease interpretation but statistical tests were conducted on transformed data
[a-c] Within row, means with different superscripts differ (P<0.05).

3.2. Experiment 1: Comparison of homologous versus heterologous frozen-thawed seminal plasma

Table 5 illustrates the main effects of homologous and heterologous seminal plasma on experimental endpoints. No significant differences (P>0.05) were detected between homologous and heterologous treatment groups for variables TMOT, VIAB, AI and COMP. Heterologous seminal plasma yielded a lower VCL when compared to homologous seminal plasma (P<0.05). For variables PMOT and STR, heterologous seminal plasma yielded significantly higher values than did homologous seminal plasma (P<0.05). Stallion-by treatment interactions were detected for PMOT and STR. Both variables were lower for homologous seminal plasma than heterologous seminal plasma

for one stallion (P<0.005 or 0.05?). Treatment differences were not detected for the other two stallions (P>0.05; Table 6).

Significant differences (P<0.05) were noted among individual stallions (Table 7) for some variables. One stallion (Apollo) yielded lower TMOT than the other stallions (Spencer and Rock). There were no differences among the variables PMOT and COMP for three stallions. Two stallions (Spencer and Rock) were not different from each other; however, these two stallions yielded a lower STR value than the remaining stallion (Apollo). Measures of VIAB were similar between Spencer and Apollo, but were lower than the VIAB for the remaining stallion (Rock). Variables AI and VCL were higher for Rock than the other stallions (P<0.05).

Table 5
Main effect of frozen/thawed homologous versus heterologous seminal plasma on measures of sperm quality (mean ± SEM) for three stallions following 24 h of cooled storage (n = 7 ejaculates).

	Treatment	
Laboratory parameter*	Homol	Heterol
TMOT (%)	79 ± 2.0[a]	82 ± 1.7[a]
PMOT (%)	48 ± 1.5[b]	54 ± 1.9[a]
VCL (μm/s)	187 ± 6.9[a]	176 ± 7.8[b]
STR (%)	62 ± 2.1[b]	67 ± 2.0[a]
VIAB (%)	84 ± 0.7[a]	85 ± 1.2[a]
AI (%)	88 ± 1.1 [a]	88 ± 1.1[a]
COMP (%)	9 ± 0.9[a]	8 ± 0.5[a]

*TMOT = total sperm motility (%); PMOT = progressive sperm motility (%); VCL = curvilinear velocity (μm/s) STR = straightness (%); VIAB = sperm with intact plasma membrane (%); AI = sperm with intact acrosomal membrane (%); COMP = percentage of sperm with αt value outside the main population (%). Percentage data (TMOT, PMOT, STR, VIAB, AI, and COMP) were arc sine-root transformed for normalization prior to statistical analysis. Untransformed values for mean and SEM are presented in table to ease interpretation but statistical tests were conducted on transformed data
[a,b] Within row, means with different superscripts differ ($P<0.05$).

Table 6
Effect of frozen/thawed homologous versus heterologous seminal plasma on measures of sperm quality (mean ± SEM), as sorted by stallion, following 24 h of cooled storage (n = 7 ejaculates).

Laboratory Parameter*	Stallion					
	Spencer		Rock		Apollo	
	Homol	Heterol.	Homol.	Heterol.	Homol.	Heterol.
TMOT (%)	85 ± 1.0[a]	85 ± 1.0[a]	81 ± 3.2[a]	86 ± 1.3[a]	71 ± 3.7[a]	73 ± 2.9[a]
PMOT (%)	49 ± 2.3[a]	53 ± 3.5[a]	44 ± 2.1[b]	55 ± 2.8[a]	52 ± 2.7[a]	53 ± 3.7[a]
VCL (μm/s)	191 ± 9.7[a]	187 ± 8.0[a]	218 ± 4.0[a]	208 ± 7.6[a]	152 ± 2.7[a]	134 ± 3.4[a]
STR (%)	59 ± 2.1[a]	62 ± 2.6[a]	54 ± 1.3[b]	62 ± 2.2[a]	74 ± 0.5[a]	77 ± 1.6[a]
VIAB (%)	84 ± 1.2[a]	83 ± 1.7[a]	87 ± 0.5[a]	88 ± 1.6[a]	82 ± 1.4[a]	84 ± 2.3[a]
AI (%)	89 ± 1.2[a]	88 ± 1.2[a]	92 ± 0.9[a]	92 ± 0.7[a]	82 ± 1.4[a]	84 ± 2.3[a]
COMP (%)	6 ± 0.5[a]	8 ± 1.0[a]	10 ± 1.0[a]	9 ± 0.7[a]	12 ± 1.9[a]	8 ± 0.7[a]

*TMOT = total sperm motility (%); PMOT = progressive sperm motility (%); VCL = curvilinear velocity (μm/s) STR = straightness (%); VIAB = sperm with intact plasma membrane (%); AI = sperm with intact acrosomal membrane (%); COMP = percentage of sperm with αt value outside the main population (%). Percentage data (TMOT, PMOT, STR, VIAB, AI, and COMP) were arc sine-root transformed for normalization prior to statistical analysis. Untransformed values for mean and SEM are presented in table to ease interpretation but statistical tests were conducted on transformed data
[a,b] Within stallion and laboratory parameter, means with different superscripts differ ($P<0.05$).

Table 7
Main effect of frozen-thawed homologous versus heterologous seminal plasma on measures of sperm quality (mean ± SEM) for individual stallions following 24 h of cooled storage (n = 7 ejaculates).

Laboratory Parameter*	Stallion		
	Spencer	Rock	Apollo
TMOT (%)	85 ± 0.7[a]	83 ± 1.8[a]	72 ± 2.3[b]
PMOT (%)	51 ± 2.1[a]	50 ± 2.3[a]	52 ± 2.2[a]
VCL (μm/s)	189 ± 6.1[b]	213 ± 4.3[a]	143 ± 3.3[c]
STR (%)	61 ± 1.7[b]	58 ± 1.7[b]	76 ± 0.9[a]
VIAB (%)	83 ± 1.0[b]	88 ± 0.8[a]	83 ± 1.3[b]
AI (%)	88 ± 0.8[b]	92 ± 0.6[a]	83 ± 1.3[c]
COMP (%)	7 ± 0.6[a]	9 ± 0.6[a]	10 ± 1.1[a]

*TMOT = total sperm motility (%); PMOT = progressive sperm motility (%); VCL = curvilinear velocity (μm/s) STR = straightness (%); VIAB = sperm with intact plasma membrane (%); AI = sperm with intact acrosomal membrane (%); COMP = percentage of sperm with αt value outside the main population (%). Percentage data (TMOT, PMOT, STR, VIAB, AI, and COMP) were arc sine-root transformed for normalization prior to statistical analysis. Untransformed values for mean and SEM are presented in table to ease interpretation but statistical tests were conducted on transformed data
[a-c] Within row, means with different superscripts differ (P<0.05).

3.3. Experiment 1: Storage methods for frozen preservation of heterologous seminal plasma

Data regarding the effect frozen storage methods for heterologous seminal plasma on semen quality are provided in Tables 8-10. A main effect of treatment was detected (P<0.05) for all sperm motion variables. Variable TMOT was higher for Group 4-80 as compared to Group RW-20 (P<0.05), but TMOT for both treatment groups was similar to that of the remaining treatments groups (P>0.05; Table 8). Group 20 exhibited higher PMOT than RW-80 and RW-196 (P<0.05), but did not differ from the other treatment groups (P>0.05). Variable VCL was higher in Groups 4-80, and 80 than in

Groups 20, 4-20, RW80 and RW-196 ($P<0.05$). Mean VCL for Group RW-20 was lower than that of all other treatment groups ($P<0.05$). Variable STR was higher for Groups 20, 4-20,and RW-20 than for Groups 4-196, 4-80, 80, RW-80, and 196 ($P<0.05$). Main effects of treatment were not detected for variables VIAB, AI, and COMP ($P>0.05$).

A main effect of stallion was detected for all treatment variables (Table 9). Variables TMOT, VCL, VIAB, and AI differed among all three stallions ($P<0.05$). Variable PMOT was higher for Rock than the other two stallions. Variable STR was higher for Apollo than the remaining two stallions ($P<0.05$). Variable COMP was higher for Apollo than for Spencer.

A treatment-by-stallion interaction was detected for variables PMOT and STR ($P<0.05$). Variable PMOT was similar among treatment groups for two of three stallions ($P>0.05$). For the remaining stallion (Rock), PMOT was higher in Group 20 than in Groups 4-20, 80, 4-80, 196, RW-196 and RW-196. Variable STR was similar among treatment groups for one stallion (Apollo). For stallion Rock, STR was higher in Group 20 than in Groups 80, 4-80, 196, 4-196, and RW-196. For stallion Spencer, STR was higher in Group 4-20 than in Groups 80, 4-80, RW-80, 196, 4-196, and RW-196 ($P<0.05$).

Table 8
Main effect of frozen-thawed seminal plasma that had previously been processed in various manners and stored for 9 months at various freezing temperatures on measures of sperm quality (mean ± SEM) following 24 h of cooled storage with heterologous sperm (n = 7 stallions).

Laboratory	Treatment‡								
Parameter*	20	80	196	4-20	4-80	4-196	RW-20	RW-80	RW-196
TMOT (%)	81 ± 2^{ab}	82 ± 2^{ab}	82 ± 2^{ab}	82 ± 2^{ab}	83 ± 2^{a}	83 ± 2^{ab}	80 ± 2^{b}	82 ± 2^{ab}	80 ± 2^{ab}
PMOT (%)	58 ± 2.3^{a}	55 ± 2.1^{ab}	54 ± 1.9^{ab}	57 ± 1.8^{ab}	55 ± 1.9^{ab}	57 ± 2.3^{ab}	54 ± 2.1^{ab}	53 ± 2.4^{b}	53 ± 2.1^{b}
VCL (μm/s)	166 ± 7.4^{c}	177 ± 7.5^{a}	176 ± 7.8^{ab}	166 ± 7.6^{c}	178 ± 8.3^{a}	177 ± 7.7^{ab}	155 ± 7.4^{d}	168 ± 8.5^{bc}	164 ± 7.6^{c}
STR (%)	70 ± 1.7^{a}	67 ± 2.1^{c}	67 ± 2.0^{c}	70 ± 1.8^{ab}	67 ± 2.2^{c}	68 ± 2.2^{c}	70 ± 1.5^{a}	67 ± 1.9^{c}	68 ± 1.9^{bc}
VIAB (%)	85 ± 0.7^{a}	85 ± 0.8^{a}	85 ± 1.2^{a}	84 ± 1.4^{a}	86 ± 0.7^{a}	86 ± 0.8^{a}	86 ± 0.9^{a}	87 ± 0.7^{a}	86 ± 0.6^{a}
AI (%)	89 ± 0.9^{a}	89 ± 0.8^{a}	88 ± 1.1^{a}	89 ± 0.9^{a}	89 ± 0.8^{a}	89 ± 0.8^{a}	89 ± 0.9^{a}	89 ± 0.8^{a}	89 ± 0.8^{a}
COMP (%)	10 ± 0.8^{a}	9 ± 0.7^{a}	8 ± 0.5^{a}	9 ± 0.5^{a}	11 ± 0.7^{a}	10 ± 0.6^{a}	10 ± 0.6^{a}	10 ± 0.6^{a}	9 ± 0.5^{a}

*TMOT = total sperm motility (%); PMOT = progressive sperm motility (%); VCL = curvilinear velocity (μm/s) STR = straightness (%); VIAB = sperm with intact plasma membrane (%); AI = sperm with intact acrosomal membrane (%); COMP = percentage of sperm with αt value outside the main population (%). Percentage data (TMOT, PMOT, STR, VIAB, AI, and COMP) were arc sine-root transformed for normalization prior to statistical analysis. Untransformed values for mean and SEM are presented in table to ease interpretation but statistical tests were conducted on transformed data

†20 = seminal plasma stored only at -20°C; 80 = seminal plasma stored only at -80°C; 196 = seminal plasma stored only at -196°C; 4-20 = seminal plasma held at 4°C for 24 h and then stored at -20°C; 4-80 = seminal plasma held at 4°C for 24 h and then stored at -80°C; 4-196 = seminal plasma held at 4°C for 24h and then stored at -196°C; RW-20 = raw semen sample stored for 24 h at 4°C and then processed for seminal plasma which was then stored at -20°C; RW-80 = raw semen sample stored for 24 h at 4°C and then processed for seminal plasma which was then stored at -80°C; RW-196 = raw semen sample stored for 24 h at 4°C and then processed for seminal plasma which was then stored at -196°C.

[a-c] Within row, means with different superscripts differ (P<0.05).

Table 9
Main effect of stallion on frozen/thawed seminal plasma that had been processed by various methods and stored for 9 months in various freezing temperatures on measures of sperm quality (mean ± SEM) following 24 h of cooled storage with heterologous sperm (n=7 ejaculates).

Laboratory Parameter*	Stallion		
	Spencer‡	Rock	Apollo
TMOT (%)	85 ± 0.4[b]	86 ± 0.4[a]	74 ± 1.0[c]
PMOT (%)	54 ± 1.3[b]	58 ± 0.9[a]	53 ± 1.4[b]
VCL (μm/s)	174 ± 2.8[b]	204 ± 2.3[a]	131 ± 2.1[c]
STR (%)	64 ± 1.0[b]	64 ± 0.7[b]	77 ± 0.5[a]
VIAB (%)	83 ± 0.6[c]	87 ± 0.4[a]	86 ± 0.4[b]
AI (%)	88 ± 0.4[b]	92 ± 0.3[a]	86 ± 0.4[c]
COMP (%)	9 ± 0.4[b]	9 ± 0.3[ab]	10 ± 0.4[a]

*TMOT = total sperm motility (%); PMOT = progressive sperm motility (%); VCL = curvilinear velocity (μm/s) STR = straightness (%); VIAB = sperm with intact plasma membrane (%); AI = sperm with intact acrosomal membrane (%); COMP = percentage of sperm with αt value outside the main population (%). Percentage data (TMOT, PMOT, STR, VIAB, AI, and COMP) were arc sine-root transformed for normalization prior to statistical analysis. Untransformed values for mean and SEM are presented in table to ease interpretation but statistical tests were conducted on transformed data
[a-c] Within row, means with different superscripts differ ($P<0.05$).

Table 10
Effect of treatment of frozen-thawed heterologous seminal plasma on measures of sperm quality (mean ± SEM), as sorted by stallion, following 24 h of cooled storage at different temperatures (n = 7 ejaculates).

Treatment Group†	Laboratory Parameter					
	PMOT (%)*			STR (%)*		
	Spencer	Rock	Apollo	Spencer	Rock	Apollo
20	57 ± 3.4[a]	64 ± 3.2[a]	52 ± 4.4[a]	66 ± 2.9[ab]	68 ± 2.3[a]	77 ± 1.8[a]
80	53 ± 4.7[a]	55 ± 2.6[b]	56 ± 3.8[a]	62 ± 3.2[c]	62 ± 2.3[c]	77 ± 1.8[a]
196	53 ± 3.5[a]	55 ± 2.8[b]	53 ± 3.7[a]	62 ± 2.6[c]	62 ± 2.2[c]	77 ± 1.6[a]
4-20	57 ± 3.4[a]	58 ± 2.0[b]	56 ± 4.2[a]	67 ± 2.6[ab]	64 ± 2.2[abc]	78 ± 1.8[a]
4-80	54 ± 4.0[a]	56 ± 2.2[b]	55 ± 4.0[a]	63 ± 3.4[c]	61 ± 2.3[c]	78 ± 1.8[a]
4-196	55 ± 4.9[a]	57 ± 3.5[b]	57 ± 4.0[a]	62 ± 3.3[c]	62 ± 2.2[c]	78 ± 1.4[a]
RW-20	54 ± 3.2[a]	60 ± 2.8[ab]	48 ± 4.0[a]	68 ± 2.2[a]	66 ± 1.9[ab]	77 ± 1.2[a]
RW-80	52 ± 5.3[a]	58 ± 2.0[ab]	50 ± 4.4[a]	63 ± 3.2[bc]	63 ± 2.0[bc]	76 ± 1.7[a]
RW-196	53 ± 3.9[a]	57 ± 2.0[b]	50 ± 4.6[a]	63 ± 3.1[bc]	65 ± 2.3[abc]	76 ± 1.8[a]

* PMOT = progressive sperm motility (%);STR = straightness (%). Percentage values were arc sine-root transformed for normalization prior to statistical analysis. Untransformed values for mean and SEM are presented in table to ease interpretation but statistical tests were conducted on transformed data

†20 = seminal plasma stored only at -20°C; 80 = seminal plasma stored only at -80°C; 196 = seminal plasma stored only at -196°C; 4-20 = seminal plasma held at 4°C for 24 h and then stored at -20°C; 4-80 = seminal plasma held at 4°C for 24 h and then stored at -80°C; 4-196 = seminal plasma held at 4°C for 24h and then stored at -196°C; RW-20 = raw semen sample stored for 24 h at 4°C and then processed for seminal plasma which was then stored at -20°C; RW-80 = raw semen sample stored for 24 h at 4°C and then processed for seminal plasma which was then stored at -80°C; RW-196 = raw semen sample stored for 24 h at 4°C and then processed for seminal plasma which was then stored at -196°C.

[a-c] Within column , means with different superscripts differ ($P<0.05$).

3.4. Preliminary experiment 2.1: Preliminary evaluation of using glass or plastic vials for use in lyophilization of seminal plasma

A main effect of vial composition was detected, with greater values for TMOT, PMOT, VCL (73 ± 1.9, 46 ± 2.1, and 185 ± 8.5, respectively) obtained by use of plastic vials rather than borosilicate glass tubes (57 ± 4.1, 35 ± 3.2, and 155 ± 9.3, respectively; $P<0.05$). A main effect of vial orientation was also detected, with upright orientation yielding higher values for TMOT, PMOT, and VCL (75 ± , 46 ±, and 190 ± , respectively) than inverted (55 ± 3.9, 35 ± 3.3, and 151 ± 8.9, respectively; $P<0.05$). Variable STR was higher in inverted vials (68 ± 2.0) than in upright vials (65 ± 2.2; $P<0.05$). A main effect of stallion was not detected for any experimental endpoint ($P>0.05$). A tube-by-orientation interaction was detected for variables TMOT, PMOT, and VCL ($P<0.05$). For glass vials, upright orientation yielded higher values for TMOT, PMOT, and VCL than inverted ($P<0.05$; Table 11). For plastic vials, TMOT was higher in upright tubes than inverted tubes ($P<0.05$); however the difference was not as dramatic as for glass vials (Table 11). Variables PMOT and VCL were not different between the two treatment groups ($P>0.05$; Table 11).

A stallion-by-vial orientation interaction was detected for PMOT, VCL, and STR ($P<0.05$; Table 12). Three of the five stallions (Apollo, Christy, and Rock) did not display differences between treatment groups for these laboratory parameters ($P>0.05$). Spencer had lower VCL values for the inverted vials when compared to the upright vials ($P<0.05$). Mean PMOT, and VCL were lower for the inverted vials for Smooth

(P<0.05), while STR was higher for the inverted vials when compared to the upright vials (P<0.05).

3.5. Preliminary experiment 2.2: Preliminary comparison between two types of rubber stoppers for use in lyophilization of seminal plasma.

Mean TMOT and PMOT were lower for semen stored in borosilicate glass vials with chlorobutyl-isoprene blend stopper and inverted for storage, as compared to all other treatment groups (P<0.05; Table 13). Mean VCL and STR were not different among treatments (P>0.05).

Table 11
Effects of type of vial composition and orientation on sperm motion characteristics (mean ± SEM) following 24 h of cooled-storage (n = 15).

Laboratory Parameter *	Tube Type			
	Borosilicate glass†		Plastic†	
	UP	INVERT	UP	INVERT
TMOT (%)	75 ± 2.6[a]	40 ± 4.3[b]	75 ± 2.5[a]	71 ± 2.9[b]
PMOT (%)	46 ± 2.8[a]	24 ± 4.2[b]	47 ± 3.0[a]	45 ± 3.4[a]
VCL (μm/s)	192 ± 11.4[a]	119 ± 5.7[b]	188 ± 12.1[a]	183 ± 12.2[a]
STR (%)	64 ± 3.1[a]	70 ± 2.4[a]	66 ± 3.3 [a]	66 ± 3.3[a]

*TMOT = total sperm motility (%); PMOT = progressive sperm motility (%); VCL = curvilinear velocity (μm/s) STR = straightness (%). Percentage data (TMOT, PMOT, and STR) were arc sine-root transformed for normalization prior to statistical analysis. Untransformed values for mean and SEM are presented in table to ease interpretation but statistical tests were conducted on transformed data
†Borosilicate glass vial contained chlorobutyl-isoprene blend stopper; Plastic vials were composed of polypropylene plastic.
UP = vial stored in upright position; INVERT = vial stored upside down.
[a,b] Within tube type and dependent variable, means with different superscripts differ (P<0.05).

Table 12
Effects of vial orientation on sperm motion characteristics (mean ± SEM) following 24 h of cooled-storage (n=3).

Laboratory Parameter *	Stallion			
	Smooth		Spencer	
	UP	INVERT	UP	INVERT
PMOT (%)	55 ± 2.3[a]	50 ± 2.2[b]	43 ± 3.7[a]	25 ± 8.3[a]
VCL (μm/s)	145 ± 3.4[a]	128 ± 2.1[b]	215 ± 15.0[a]	156 ± 24.3[b]
STR (%)	77 ± 0.8[b]	81 ± 0.9[a]	58 ± 1.2[a]	58 ± 2.0[a]

*TMOT = total sperm motility (%); PMOT = progressive sperm motility (%); VCL = curvilinear velocity (μm/s) STR = straightness (%). Percentage data (TMOT, PMOT, and STR) were arc sine-root transformed for normalization prior to statistical analysis. Untransformed values for mean and SEM are presented in table to ease interpretation but statistical tests were conducted on transformed data

†UP = vial stored in upright position; INVERT = vial stored upside down.

[a,b] Within stallion and dependent variable, means with different superscripts differ ($P<0.05$).

Table 12 Continued

Laboratory Parameter *	Stallion					
	Apollo		Christy		Rock	
	UP†•	INVERT†	UP	INVERT	UP	INVERT
PMOT (%)	52 ± 2.0[a]	33 ± 9.0[a]	35 ± 3.3[a]	33 ± 3.3[a]	50 ± 5.2[a]	34 ± 9.0[a]
VCL (μm/s)	157 ± 6.4[a]	132 ± 10.7[a]	249 ± 2.4[a]	189 ± 25.4[a]	185 ± 14.6[a]	149 ± 22.2[a]
STR (%)	77 ± 1.1[a]	73 ± 2.7[a]	47 ± 1.5[a]	61 ± 14.4[a]	66 ± 3.0[a]	67 ± 2.5[a]

*TMOT = total sperm motility (%); PMOT = progressive sperm motility (%); VCL = curvilinear velocity (μm/s) STR = straightness (%). Percentage data (TMOT, PMOT, and STR) were arc sine-root transformed for normalization prior to statistical analysis. Untransformed values for mean and SEM are presented in table to ease interpretation but statistical tests were conducted on transformed data

†UP = vial stored in upright position; INVERT = vial stored upside down.

[a,b] Within stallion and dependent variable, means with different superscripts differ ($P<0.05$).

Table 13
Effect of vial orientation and rubber stopper composition on measures of sperm motion characteristics (mean ± SEM) following 24 h of cooled storage (n=5).

Laboratory Parameter *	Stopper Composition						
	Polypropylene	Chlorobutyl-isoprene blend			Chlorobutyl		
	CONTROL	INVERT	ROTATE	UP	INVERT	ROTATE	UP
TMOT (%)	75 ± 4.5[a]	32 ± 7.1[b]	76 ± 4.0[a]	76 ± 2.5[a]	76 ± 6.0[a]	77 ± 4.1[a]	78 ± 2.9[a]
PMOT (%)	40 ± 3.9[a]	15 ± 6.9[b]	41 ± 5.0[a]	43 ± 1.1[a]	43 ± 8.0[a]	40 ± 4.7[a]	42 ± 1.3[a]
VCL (μm/s)	192 ± 31.3	96 ± 13.1	189 ± 34.7	190 ± 21.2	188 ± 33.9	191 ± 32.1	199 ± 27.8
STR (%)	60 ± 3.4	63 ± 4.4	59 ± 3.7	61 ± 4.6	61 ± 2.7	58 ± 3.1	59 ± 4.3

*TMOT = total sperm motility (%); PMOT = progressive sperm motility (%); VCL = curvilinear velocity (μm/s) STR = straightness (%). Percentage data (TMOT, PMOT, and STR) were arc sine-root transformed for normalization prior to statistical analysis. Untransformed values for mean and SEM are presented in table to ease interpretation but statistical tests were conducted on transformed data

††CONTROL = polypropylene vial stored upright; INVERT = borosilicate vial stored upside down; ROTATE = contents of borosilicate glass vial inverted ten times then stored upright; UP = borosilicate vial stored in upright position..

[a,b] Within row, means with different superscripts differ ($P<0.05$).

3.6. Experiment 2: Evaluation of lyophilization as a method for preservation of seminal plasma

At T0, mean values for TMOT, PMOT, VCL, STR, and COMP were not different among treatment groups ($P>0.05$; Table 14). Mean VIAB was lower in Group UC, as compared to Groups Fresh, Frozen-Thaw and Lyo ($P<0.05$). Mean AI was lower in Group UC than in Group Lyo ($P<0.05$). Mean AI was similar among Groups Fresh, Frozen-Thaw, and Lyo ($P>0.05$; Table 14). A stallion-by-treatment interaction was detected only for variable VCL ($P<0.05$). Mean VCL was similar among treatments for four of five stallions. For one stallion (Rock), mean VCL was lower in Group UC (206 ± 7.6), than in Groups Fresh, Frozen-Thaw, and Lyo (236 ± 1.9, 227 ± 6.5;and 232 ± 3.7, respectively; $P<0.05$). A main effect of stallion was detected for all experimental endpoints ($P<0.05$).

At T24, mean values for TMOT, PMOT, and VCL were similar among treatment groups ($P>0.05$; Table 15). Mean STR was lower in Group Lyo than in other treatment groups ($P<0.05$). Mean VIAB was lower in Group UC, than Group Fresh ($P<0.05$). Mean AI was lower in Group UC than in other treatment groups ($P<0.05$). Mean COMP was lower (better) in Group Lyo than all other treatment groups, and also lower in groups Fresh and Frozen-Thaw than in Group UC ($P<0.05$). A main effect of stallion was detected for all except COMP ($P<0.05$); however, stallion-by-treatment interactions were not detected for any experimental endpoint ($P>0.05$).

Table 14
Effect of different seminal plasma processing techniques on semen quality (mean ± SEM) immediately following sperm exposure to seminal plasma (T0; n= 15).

Laboratory parameter*	Uncentrifuged seminal plasma	Fresh seminal plasma	Frozen/thawed seminal plasma	Lyophilized seminal plasma
TMOT (%)	82 ± 3.2	82 ± 3.3	82 ± 3.5	82 ± 3.5
PMOT (%)	49 ± 4.5	51 ± 4.9	51 ± 4.8	51 ± 5.1
VCL (μm/s)	232 ± 7.3	239 ± 4.6	229 ± 4.9	231 ± 5.0
STR (%)	59 ± 2.3	58 ± 2.8	60 ± 2.6	59 ± 2.8
VIAB (%)	69 ± 3.7[b]	74 ± 3.6[a]	73 ± 3.5[a]	73 ± 3.6[a]
AI (%)	87 ± 3.5[b]	88 ± 3.4[a]	88 ± 3.4[a]	89 ± 3.5[a]
COMP (%)	21 ± 2.4	21 ± 2.8	20 ± 3.0	21 ± 3.2

*TMOT = total sperm motility (%); PMOT = progressive sperm motility (%); VCL = curvilinear velocity (μm/s) STR = straightness (%); VIAB = sperm with intact plasma membrane (%); AI = sperm with intact acrosomal membrane (%); COMP = percentage of sperm with αt value outside the main population (%). Percentage data (TMOT, PMOT, STR, VIAB, AI, and COMP) were arc sine-root transformed for normalization prior to statistical analysis. Untransformed values for mean and SEM are presented in table to ease interpretation but statistical tests were conducted on transformed data
[a,b] Within row, means with different superscripts differ ($P<0.05$).

Table 15
Effect of different seminal plasma processing techniques on semen quality (mean ± SEM) following 24 h of cooled storage (T24; n= 15).

Laboratory parameter*	Uncentrifuged seminal plasma	Fresh seminal plasma	Frozen/thawed seminal plasma	Lyophilized seminal plasma
TMOT (%)	75 ± 2.5	78 ± 3.3	75 ± 3.8	74 ± 3.8
PMOT (%)	44 ± 2.8	45 ± 4.5	44 ± 4.4	38 ± 3.4
VCL (μm/s)	198 ± 7.7	210 ± 8.3	204 ± 9.2	211 ± 9.6
STR (%)	61 ± 2.3[a]	59 ± 3.2[a]	59 ± 3.0[a]	55 ± 2.7[b]
VIAB (%)	69 ± 3.6[b]	73 ± 3.4[a]	71 ± 3.8[a]	72 ± 3.9[a]
AI (%)	86 ± 3.3[b]	88 ± 3.2[a]	88 ± 3.4[a]	89 ± 3.5[a]
COMP (%)	30 ± 3.1[a]	25 ± 3.2[a]	25 ± 3.3[b]	23 ± 3.1[d]

*TMOT = total sperm motility (%); PMOT = progressive sperm motility (%); VCL = curvilinear velocity (μm/s) STR = straightness (%); VIAB = sperm with intact plasma membrane (%); AI = sperm with intact acrosomal membrane (%); COMP = percentage of sperm with αt value outside the main population (%). Percentage data (TMOT, PMOT, STR, VIAB, AI, and COMP) were arc sine-root transformed for normalization prior to statistical analysis. Untransformed values for mean and SEM are presented in table to ease interpretation but statistical tests were conducted on transformed data
[a,b] Within row, means with different superscripts differ ($P<0.05$).

4. DISCUSSION

This study evaluated the effect of seminal plasma processing and freezing temperature on sperm quality. Seminal plasma is often removed from a stallion's ejaculate via centrifugation and then added back in varying concentrations to yield better sperm motion characteristics for an ejaculate. An optimal seminal plasma concentration, or method for preparation, has yet to be established, but the literature generally notes that between 5 to 20% [15] is commonly used. Our results indicate that seminal plasma can be processed and stored in different conditions while still yielding high sperm quality.

There was no difference between fresh and snap-frozen/thawed seminal plasma, which suggests that seminal plasma can be processed for immediate use, or it can be stored for later use. The finding allows the clinician more flexibility when processing and storing seminal plasma for either fresh, cooled, or cryopreserved semen.

We evaluated the effects of homologous versus heterologous seminal plasma on sperm semen quality to determine if the stallion from which the seminal plasma originated would affect sperm quality. Clinically, there are anecdotal reports of stallions whose seminal plasma exerts a depressing effect on sperm quality; yet when seminal plasma from another stallion is substituted, sperm quality improves. In this study,, heterologous seminal plasma was generally similar to homologous seminal plasma for maintaining sperm motion characteristics and membrane integrity after 24 h of cooled-storage. However, we did note a stallion effect, whereby semen from one stallion

exhibited improved quality following cooled storage when mixed with heterologous seminal plasma, as compared to homologous seminal plasma. This finding supports the work of Aurich et al. [22] where the seminal plasma from stallions with good post-thaw sperm quality improved sperm quality of stallions with poor post-thaw sperm quality. All sperm donors in this study have high sperm motion characteristics and VIAB, so we were unable to evaluate a stallion with low fertility and the interaction with a good quality stallion's seminal plasma. In addition, we did not test cryosurvival of sperm. Individual stallions' ejaculates differed; however, a stallion-based variation is to be expected since there are differences for laboratory parameters not only among different stallions, but also among ejaculates from a given stallion. The fact that semen from one stallion in our study did show a preference for heterologous seminal plasma for two motion characteristics (PMOT and STR) suggests that it may be appropriate to test semen from stallions in the clinical setting to determine whether heterologous seminal plasma may be more appropriate than homologous seminal plasma when processing semen for cooled storage.

We used various processing methods and freezing temperatures for seminal plasma in an effort to determine the most appropriate technique for accomplishing this task while optimizing semen quality following cooled storage. Although we hypothesized that sperm quality would not be affected by the stored freezing temperature of seminal plasma, we did note differences among treatment groups (Table 8). Sperm motion characteristics displayed the most variability among freezing methods. Based on experimental results, we do not recommend that semen be stored in the raw form and

then processed for seminal plasma, as sperm motility values were slightly suppressed when compared to the other treatment groups. The seminal plasma samples in this study were stored for nine months prior to use. From this study we found it is feasible for a clinic or lab to store seminal plasma using a conventional freezer (-20C), especially if liquid nitrogen or dry ice is not readily accessible. Our data support the notion that laboratories and clinics can use freezers they already have and do not need to purchase new freezing units to store seminal plasma for an extended time. However, we did not test freezers with automatic defrost (frost-free) systems. It is possible that defrost cycles of such freezers would be deleterious to some components of seminal plasma.

This study also evaluated the effect of freeze-drying seminal plasma as a method for long-term preservation. Commonly, biologic products are freeze-dried in glass vials with a rubber stopper that vacuum seals the container to prevent contamination [37]. Previous studies, indicated that rubber plungers in syringes reduce stallion sperm quality, therefore, we wanted to investigate whether the type of vial or rubber stopper would affect sperm quality. We wanted to select the most appropriate vial to use for seminal plasma storage following lyophilization without harming sperm. To evaluate any deleterious effects on sperm motion characteristics, we compared borosilicate glass vials with polypropylene plastic vials commonly used in our lab. In addition, we also evaluated whether continued exposure to the rubber stopper would reduce sperm quality. To mimic disturbances to products during shipping, we had an inverted treatment group to expose the semen to the cap of the vial for 24 h. No differences were detected among stallions; however, significant differences were detected between vial type and

orientation of the vial (Table 11). The inverted glass vials (semen in contact with rubber stopper) yielded the lowest motility values. We suspected a toxic environment for sperm was caused by exposure to the rubber stopper, resulting in significantly decreased motility. Previous studies have noted a decrease in sperm motion characteristics following exposure to different rubber plungers and syringes [29] and indicate rubber toxicity as the cause. Others have reported that the negative effects of the toxic environment can be reversed following removal of old extender and addition of fresh extender [31]. The chlorobutyl-isoprene blend in the rubber stoppers used in this experiment may be harmful to sperm and should not be used in procedures where sperm contacts the rubber. The upright glass and plastic vials yielded sperm motion characteristics not significantly different, and the sperm were not exposed to the caps in these treatments.

We compared two commercially available rubber stoppers for use with the lyophilized borosilicate glass. One stopper was the chlorobutyl-isoprene blend from the previous study and the second stopper was a chlorobutyl rubber. Sperm motion characteristics were significantly decreased when the semen was in contact with the chlorobutyl-isoprene blend stopper for 24 h when compared to the chlorobutyl or polypropylene stopper (Table 13). The toxic effect was caused by exposure of sperm to the isoprene-blended component of the rubber since the chlorobutyl rubber treatment did not result in a decrease in sperm motion characteristics. To avoid a toxic environment in the lyophilized seminal plasma vials, we elected to use the chlorobutyl rubber stoppers. Contact between sperm and rubber stopper were minimized by injecting the

reconstituted seminal plasma to the extended sperm in a separate vial that has not been exposed to a rubber stopper. We did not evaluate sperm membrane intactness or chromatin quality following sperm exposure to rubber stoppers, so are unable to comment on these potential effects.

Determining the final freeze-drying protocol for the lyophilized seminal plasma treatment group was somewhat problematic. To our knowledge, based on literature research, no one has reported on lyophilization of seminal plasma. We modified a protocol used for freeze-drying human blood plasma, using a trial and error approach [35] to establish a protocol for use with seminal plasma. We selected a blood plasma-based protocol since this biological fluid has been successfully lyophilized and shipped without compromising the hemostatic properties and biological integrity of its final product [43].

Preliminary test freezes were conducted on stallion seminal plasma to evaluate visually the resultant product and make modifications to the protocol (i.e. the programmed freeze-drying cycle). At shorter drying times, the product remained in a crystalline state and did not form the desired powder cake. To avoid this incomplete freezing and ensure extraction of water to form a powdered substance, the primary and secondary drying times were increased [47]. Following this change, a powdered cake was obtained. Location on the shelf in the drying chamber also affected the final product. Vial placement near the chamber door or near the edges of the shelf resulted in products that remained liquid after undergoing the lyophilization procedure. To correct

for this edge effect, vials were lined up in a uniform 3 x 3 configuration around the thermocouple in the center of the shelf to achieve successful lyophilization runs.

Test freezing seminal plasma was time consuming, with cycle times ranging from 35 to 46 h. This extensive drying period is a major drawback to this technology [32] and the lengthy lyophilization process for each test run took about three weeks before a final protocol was selected. These cycles were run multiple times and the unit had to cool down between runs to avoid overheating the vacuum pump. Equipment malfunction, breakdown of the vacuum pump, repair time, and costs were other issues that arose during this project and these setbacks are definite issues with the lyophilization process that are not specific to this lab [35]. Despite these obstacles, a successful and repeatable lyophilization process was achieved. Although the freeze-drying process can alter the composition of the final product [37], we did not evaluate the pH or protein composition for this experiment. Our immediate goal was to obtain a white powdered cake. We also did not incorporate a stabilizing agent, like glycine, to reduce protein denaturation [38] because we evaluated the effects of unaltered seminal plasma on sperm motion characteristics and VIAB.

Prior to use in Experiment 2, lyophilized seminal plasma was stored in a refrigerator at 4°C for two weeks. No differences among processing methods of seminal plasma (i.e., fresh, frozen/thawed, or lyophilized) on subsequent semen quality parameters were detected immediately following exposure to seminal plasma (Table 14). Mean VIAB and AI were lower in Group UC compared to the other three treatment groups, and we suspect that the variability in seminal plasma concentrations of simple

dilutions for Group UC may have contributed to this finding. Following 24 h of cooled storage, the three treatment groups (fresh, frozen/thawed, and lyophilized seminal plasma) yielded similar mean values for TMOT, PMOT, and VCL (Table 15). Mean STR was lower in Group Lyo than the other treatments; however, the relevance of the decreased STR to fertility is unknown. Deviation from a straight path of motion for a sperm cell may be needed to navigate the female tract to reach the site of fertilization. An aquaculture study conducted in 2004 [48] evaluated the circular trajectory of sperm and noted that a sperm must make contact with the surface of the egg. A nonlinear path is necessary to ensure sperm swim in a circular fashion around the egg, rather than in a straight line [48]. Another study compared variations in sperm motion characteristics in antelope species [49]. These researchers found discrepancies in STR values among species and concluded that each species requires different degrees of STR and other measures of sperm motion (i.e. VCL) for optimal fertilizing capacity [49]. Therefore, faster, or a higher percent of STR, may not necessarily be an indication of greater fertility. Further research needs to be conducted in the stallion to evaluate the interaction between fertility and track trajectory.

Centrifugation and filtration methods were used in this set of experiments to remove seminal plasma from the sperm portion of an ejaculate. Twenty-percent seminal plasma was added to Groups Fresh, Frozen-Thaw, and Lyo. A wide range of seminal plasma concentration (6 to 44%) existed in simple dilutions, due to variability in stallion and ejaculate, of the extended semen from the uncentrifuged control group (Group UC). At Time 24, Group UC was lower for VIAB, AI, and COMP (Table 15). Any DNA

damage that was present at the initial evaluation did not change following cooled-storage (i.e., mean COMP remained consistent between the two evaluation periods). While there was a decrease in VIAB, AI, and COMP over time for these five stallions, this finding is to be expected. Our findings suggest that it is beneficial for maintaining intact sperm membranes to centrifuge an ejaculate, remove excess seminal plasma, and then add back an appropriate amount of seminal plasma to achieve the desired concentration. In these experiments, 20% seminal plasma was used to maintain semen quality [23]. Possibly, a further reduction in seminal plasma concentration in the extended semen may have enhanced sperm quality following cooled storage.

Sperm quality, when subjected to cooled storage in lyophilized seminal plasma, did not differ substantively from the other two treatment groups; thus, we suspect that biological function of the seminal plasma was not compromised by the lyophilization process. Presently, we consider it acceptable to freeze-dry seminal plasma as another method of processing and preservation; however, cost of equipment, staff, and time requirements may limit its feasibility in a commercial setting. From a clinical standpoint, seminal plasma can be processed fresh and subsequently used or stored according to the equipment and skills available at a laboratory without significantly decreasing the sperm quality when added back to an ejaculate.

5. SUMMARY

In this project we evaluated the effects of using fresh, frozen/thawed and lyophilized seminal plasma on sperm quality following cooled-storage. We also compared various seminal plasma processing and frozen storage methods to examine sperm motion characteristics as well as membrane and DNA integrity after 24 hours of cooled-storage. We hypothesized that there would be no differences among treatment groups on sperm quality regardless of the type of seminal plasma used.

In summary, fresh and frozen seminal plasma yielded similar results for sperm quality, regardless of freezing temperature for storage. Seminal plasma can be banked for future use rather than processing for a fresh sample when needed. However, we do not recommend storing raw semen for 24 hours prior to processing for seminal plasma. Certain stallions may benefit from using heterologous seminal plasma. Differences detected among processing/freezing methods were slight, suggesting considerable flexibility in freezing temperature and processing method might be acceptable. Each method can be used successfully based on available equipment and demand. Borosilicate glass vials with chlorobutyl rubber stoppers should be used for the lyophilization of seminal plasma to avoid exposure of sperm to a toxic environment, like the one found following 24 hour contact with a chlorobutyl-isoprene blend rubber stopper. Lyophilization of seminal plasma appears to be a viable option for long-term preservation of seminal plasma; however, cost and time consumption presently limit its practicality.

6. FUTURE AIMS

In this project, we evaluated different processing methods and storage temperatures of seminal plasma to maintain the highest possible sperm longevity characteristics. It will be important to the industry to determine an optimal method for preserving seminal plasma from stallions that produce seminal plasma that improves the ability of other stallions' sperm to survive cooled-storage or freezing. A fertility trial using the same seminal plasma processing techniques should also be conducted. This was the first study to attempt lyophilization of equine seminal plasma. We successfully lyophilized seminal plasma for reconstitution and addition back to an ejaculate while maintaining sperm motion characteristics, plasma membrane and acrosomal membrane integrity, and DNA integrity. The lyophilization process used was time consuming, so optimization of a shorter freeze-drying protocol would make this processing technique more cost-effective and efficient for use in the equine industry. Long term storage of lyophilized seminal plasma trials need to be conducted to evaluate whether or not this technology is a feasible option for prolonged preservation with retention of biological properties of seminal plasma. Storage of the lyophilized seminal plasma samples at different temperatures needs to be conducted to determine if samples can be maintained at ambient temperature as well as at 4°C.

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APPENDIX A

LYOPHILIZATION PROTOCOL

1. Turn on lyophilizer using switch on the back of the machine.
2. Pre-cool the chamber to approximately -80°C by activating the condenser.
3. Remove vials from the -80°C freezer, place on dry ice, then transport to the pre-cooled chamber.
4. Arrange vials around thermocouple in the center of the shelf.
5. Select "recipe 05" from the Recipe tab.
    a. Parameters for recipe 5 are outlined in Appendix B
6. Hit "run" to activate lyophilization process
7. Once cycle is complete, vacuum seal the vials as follows:
    a. Turning on the gas regulator to 10 PSI
    b. Open the valve on the back side of the machine
    c. Change stopper valve on the front of the machine to the "down" position
    d. Monitor the descending shelf to properly seal the vials
    e. Change the stopper valve on the front of the machine to "up" to raise the shelf
8. De-pressurize the chamber and then remove the lyophilized vials
9. Allow chamber to air dry and then wipe clean with a paper towel.

APPENDIX B

RECIPE 5 INSTRUMENT SETTINGS

Thermal Treatment Steps

Step #	*Temperature*	*Time*	*R/H*
01	00	0000	H

Freeze, Condenser, Vacuum
Freeze = -40°C
Additional time = 0600 min
Condenser = -50°C
Vacuum = 0200 mTorr

Drying Cycle Steps

Step #	*Temperature (°C)*	*Time (min)*	*R/H*	*Vacuum (mTorr)*
01	-40	0600	H	0100
02	-30	0360	H	0100
03	00	0180	H	0100
04	20	0360	H	0100
05	25	0240	H	0100

Secondary Drying
Secondary set point = +25°C
Post Heat Settings
Temperature = +25°C
Time = 0420 min
Vacuum = 0100 mTorr

APPENDIX C

NOMENCLATURE FOR RAW DATA SETS

CLB	Chlorobutyl rubber stopper
CLB_DN	Chlorobutyl rubber stopper stored upside down
CLB_INV	Chlorobutyl rubber stopper inverted 10 times prior to upright storage
CLB_UP	Chlorobutyl rubber stopper stored upright
EJAC	Ejaculate number
FRSH	Fresh seminal plasma
F_T	Frozen/thawed seminal plasma
HET	Heterologous seminal plasma
HOM	Homologous seminal plasma
ISO	Chlorobutyl-isoprene blend rubber stopper
ISO_DN	Chlorobutyl-isoprene blend rubber stopper stored upside down
ISO_INV	Chlorobutyl-isoprene blend rubber stopper inverted 10 times prior to upright storage
ISO_UP	Chlorobutyl-isoprene blend rubber stopper stored upright
Obs	Observation
PLAS	Polypropylene plastic cryovial
PLAS _UP	Polypropylene plastic cryovial stored upright as a control group
SP1	Seminal plasma donor stallion
SP2	Seminal plasma donor stallion

SPM	Sperm donor stallion (TAMU)
STOPPER	Rubber stopper type evaluated
TREAT	Treatment group
TREAT2	Second treatment group
V_AI	Viable and acrosome intact sperm

APPENDIX D

RAW DATA FOR EXPERIMENT 1: FRESH VERSUS SNAP/FROZEN SEMINAL PLASMA

Obs	SPM	SP1	SP2	EJAC	TREAT	TIME	TMOT	PMOT	VCL	STR	COMP	V_AI	VIAB	AI
1	Apollo	Hancock	Apollo	1	FRSH	T24	56	38	141.2	71	8.56	77	79	79
2	Apollo	Hancock	Apollo	1	F_T	T24	52	40	146.9	76	14.16	77	78	78
3	Apollo	King	Apollo	2	FRSH	T24	65	44	139.1	71	13	84	85	85
4	Apollo	King	Apollo	2	F_T	T24	68	49	155.9	73	6.04	86	87	87
5	Apollo	Blue	Apollo	3	FRSH	T24	69	48	153	73	12.08	83	84	84
6	Apollo	Blue	Apollo	3	F_T	T24	71	47	147.8	73	11.14	84	85	85
7	Apollo	Strait	Apollo	4	FRSH	T24	83	67	166.9	79	13.56	87	87	87
8	Apollo	Strait	Apollo	4	F_T	T24	79	56	153.9	73	10.8	86	87	87
9	Apollo	Yellow	Apollo	5	FRSH	T24	76	58	163.9	75	11.16	82	83	83
10	Apollo	Yellow	Apollo	5	F_T	T24	80	61	160.5	75	11.26	79	80	80
11	Apollo	Jack	Apollo	6	FRSH	T24	75	50	146.7	72	9.18	81	82	82
12	Apollo	Jack	Apollo	6	F_T	T24	71	51	140.5	75	21.06	78	79	79
13	Apollo	Slick	Apollo	7	FRSH	T24	73	55	151.7	75	4.94	81	82	82
14	Apollo	Slick	Apollo	7	F_T	T24	78	58	158.6	75	6.84	81	81	81
15	Rock	Hancock	Rock	1	FRSH	T24	85	49	205.1	58	9.3	88	89	94
16	Rock	Hancock	Rock	1	F_T	T24	78	42	208.6	56	8.72	88	89	94
17	Rock	King	Rock	2	FRSH	T24	80	41	205.4	53	16.02	85	86	92
18	Rock	King	Rock	2	F_T	T24	79	35	218.3	49	10.28	86	87	92
19	Rock	Blue	Rock	3	FRSH	T24	81	41	214.9	54	15.34	87	88	95
20	Rock	Blue	Rock	3	F_T	T24	85	44	233.1	50	15.06	84	85	95

21	Rock	Strait	Rock	4	FRSH	T24	85	50	224.5	57	17.98	86	86	92
22	Rock	Strait	Rock	4	F_T	T24	88	53	209.7	58	7.06	88	88	93
23	Rock	Yellow	Rock	5	FRSH	T24	84	51	203.5	58	5.48	86	87	89
24	Rock	Yellow	Rock	5	F_T	T24	64	47	205.7	57	9.62	87	87	90
25	Rock	Jack	Rock	6	FRSH	T24	81	44	227.1	54	6.72	87	88	93
26	Rock	Jack	Rock	6	F_T	T24	86	43	229.4	53	7.66	86	86	93
27	Rock	Slick	Rock	7	FRSH	T24	80	37	203.7	52	6.54	84	84	88
28	Rock	Slick	Rock	7	F_T	T24	86	43	218.5	52	8.66	85	86	88
29	Spencer	Hancock	Spencer	1	FRSH	T24	89	55	210.8	61	6.3	82	83	85
30	Spencer	Hancock	Spencer	1	F_T	T24	85	49	219.8	57	5.86	82	83	88
31	Spencer	King	Spencer	2	FRSH	T24	83	53	149.4	68	8.68	84	85	88
32	Spencer	King	Spencer	2	F_T	T24	83	47	172.7	61	6.26	85	86	90
33	Spencer	Blue	Spencer	3	FRSH	T24	83	61	153.7	71	10.48	85	86	91
34	Spencer	Blue	Spencer	3	F_T	T24	87	60	166.1	67	6.84	85	85	90
35	Spencer	Strait	Spencer	4	FRSH	T24	87	52	179.3	61	9.38	89	90	94
36	Spencer	Strait	Spencer	4	F_T	T24	88	53	188	60	5.8	87	87	93
37	Spencer	Yellow	Spencer	5	FRSH	T24	87	47	218.6	54	8.12	81	83	85
38	Spencer	Yellow	Spencer	5	F_T	T24	84	45	202.2	55	9.18	85	86	90
39	Spencer	Jack	Spencer	6	FRSH	T24	87	42	209.8	52	8.36	79	80	84
40	Spencer	Jack	Spencer	6	F_T	T24	86	41	225.9	51	4.72	78	78	84
41	Spencer	Slick	Spencer	7	FRSH	T24	85	52	163.7	64	6.6	82	83	87
42	Spencer	Slick	Spencer	7	F_T	T24	80	50	161.9	65	5.24	82	82	85

APPENDIX E

RAW DATA FOR EXPERIMENT 1: HOMOLOGOUS VERSUS HETEROLOGOUS SEMINAL PLASMA

Obs	SPM	SP1	SP2	EJAC	TREAT	TIME	TMOT	PMOT	VCL	STR	COMP	V_AI	VIAB	AI
1	Apollo	Hancock	Hancock	1	HET	T24	62	46	128.4	79	6.62	75	76	76
2	Apollo	Hancock	Apollo	1	HOM	T24	52	40	146.9	76	14.16	77	78	78
3	Apollo	King	King	2	HET	T24	71	48	147.8	71	8.7	85	86	86
4	Apollo	King	Apollo	2	HOM	T24	68	49	155.9	73	6.04	86	87	87
5	Apollo	Blue	Blue	3	HET	T24	74	58	133.7	79	6.02	88	88	88
6	Apollo	Blue	Apollo	3	HOM	T24	71	47	147.8	73	11.14	84	85	85
7	Apollo	Strait	Strait	4	HET	T24	84	68	143.4	81	7.76	90	90	90
8	Apollo	Strait	Apollo	4	HOM	T24	79	56	153.9	73	10.8	86	87	87
9	Apollo	Yellow	Yellow	5	HET	T24	75	51	120.8	78	6.36	85	86	86
10	Apollo	Yellow	Apollo	5	HOM	T24	80	61	160.5	75	11.26	79	80	80
11	Apollo	Jack	Jack	6	HET	T24	66	39	131.1	70	11.1	74	75	75
12	Apollo	Jack	Apollo	6	HOM	T24	71	51	140.5	75	21.06	78	79	79
13	Apollo	Slick	Slick	7	HET	T24	80	61	133.3	79	6.62	87	88	88
14	Apollo	Slick	Apollo	7	HOM	T24	78	58	158.6	75	6.84	81	81	81
15	Rock	Hancock	Hancock	1	HET	T24	92	62	212.9	64	7.12	89	90	94
16	Rock	Hancock	Rock	1	HOM	T24	78	42	208.6	56	8.72	88	89	94
17	Rock	King	King	2	HET	T24	86	43	238.3	52	9.22	87	97	93
18	Rock	King	Rock	2	HOM	T24	79	35	218.3	49	10.28	86	87	92
19	Rock	Blue	Blue	3	HET	T24	86	55	225.7	60	6.96	87	87	94
20	Rock	Blue	Rock	3	HOM	T24	85	44	233.1	50	15.06	84	85	95

21	Rock	Strait	Strait	4	HET	T24	87	62	180.7	69	10.48	88	88	93
22	Rock	Strait	Rock	4	HOM	T24	88	53	209.7	58	7.06	88	88	93
23	Rock	Yellow	Yellow	5	HET	T24	82	59	188.7	67	12.2	85	86	91
24	Rock	Yellow	Rock	5	HOM	T24	64	47	205.7	57	9.62	87	87	90
25	Rock	Jack	Jack	6	HET	T24	87	58	202.1	63	9.96	86	86	91
26	Rock	Jack	Rock	6	HOM	T24	86	43	229.4	53	7.66	86	86	93
27	Rock	Slick	Slick	7	HET	T24	82	47	204.6	58	9.46	85	85	89
28	Rock	Slick	Rock	7	HOM	T24	86	43	218.5	52	8.66	85	86	88
29	Spencer	Hancock	Hancock	1	HET	T24	82	58	170.8	67	7	83	84	89
30	Spencer	Hancock	Spencer	1	HOM	T24	85	49	219.8	57	5.86	82	83	88
31	Spencer	King	King	2	HET	T24	86	41	216.9	52	5.7	83	84	88
32	Spencer	King	Spencer	2	HOM	T24	83	47	172.7	61	6.26	85	86	90
33	Spencer	Blue	Blue	3	HET	T24	87	63	180.2	67	10.94	81	82	88
34	Spencer	Blue	Spencer	3	HOM	T24	87	60	166.1	67	6.84	85	85	90
35	Spencer	Strait	Strait	4	HET	T24	89	65	187.4	67	5.52	90	90	94
36	Spencer	Strait	Spencer	4	HOM	T24	88	53	188	60	5.8	87	87	93
37	Spencer	Yellow	Yellow	5	HET	T24	84	54	159.7	66	6.78	84	84	89
38	Spencer	Yellow	Spencer	5	HOM	T24	84	45	202.2	55	9.18	85	86	90
39	Spencer	Jack	Jack	6	HET	T24	87	43	213	52	11.94	78	78	85
40	Spencer	Jack	Spencer	6	HOM	T24	86	41	225.9	51	4.72	78	78	84
41	Spencer	Slick	Slick	7	HET	T24	82	50	178.5	61	10.06	75	76	84
42	Spencer	Slick	Spencer	7	HOM	T24	80	50	161.9	65	5.24	82	82	85

APPENDIX F

RAW DATA SET FOR EXPERIMENT 1: VARIOUS STORAGE FREEZING TEMPERATURES

Obs	SPM	SP1	SP2	EJAC	TREAT	TIME	TMOT	PMOT	VCL	STR	COMP	V_AI	VIAB	AI
1	Apollo	Hancock	Hancock	1	4_20	T24	79	62	133.5	81	7.12	82	83	83
2	Apollo	Hancock	Hancock	1	4_80	T24	60	43	120.9	77	12.18	83	84	84
3	Apollo	Hancock	Hancock	1	4_196	T24	61	43	119.1	80	10.76	83	84	84
4	Apollo	Hancock	Hancock	1	20	T24	58	39	119.9	76	8.2	81	82	82
5	Apollo	Hancock	Hancock	1	80	T24	59	42	131.1	75	11.26	82	83	83
6	Apollo	Hancock	Hancock	1	196	T24	62	46	128.4	79	6.62	75	76	76
7	Apollo	Hancock	Hancock	1	RW_20	T24	68	47	121.7	79	17.76	83	85	85
8	Apollo	Hancock	Hancock	1	RW_80	T24	59	42	128	75	9.16	85	86	86
9	Apollo	Hancock	Hancock	1	RW_196	T24	58	41	120	77	10.42	83	84	84
10	Apollo	King	King	2	4_20	T24	62	42	131.3	72	9.22	87	88	88
11	Apollo	King	King	2	4_80	T24	76	49	151.9	69	10.1	86	88	88
12	Apollo	King	King	2	4_196	T24	76	58	166.5	75	11.7	85	86	86
13	Apollo	King	King	2	20	T24	62	37	117.3	70	10.06	82	84	84
14	Apollo	King	King	2	80	T24	72	47	151.8	70	7.16	85	86	86
15	Apollo	King	King	2	196	T24	71	48	147.8	71	8.7	85	86	86
16	Apollo	King	King	2	RW_20	T24	65	42	119.7	73	11.14	84	85	85
17	Apollo	King	King	2	RW_80	T24	69	46	146.9	72	10.48	87	88	88
18	Apollo	King	King	2	RW_196	T24	62	36	120.2	69	9.08	85	86	86
19	Apollo	Blue	Blue	3	4_20	T24	83	72	157.7	83	12.02	89	89	89
20	Apollo	Blue	Blue	3	4_80	T24	79	68	171.2	82	9.62	87	88	88

21	Apollo	Blue	Blue	3	4_196	T24	86	75	160.2	84	16.26	86	86	86
22	Apollo	Blue	Blue	3	20	T24	80	69	168.7	83	7.42	85	85	85
23	Apollo	Blue	Blue	3	80	T24	79	68	156.8	82	7.56	88	88	88
24	Apollo	Blue	Blue	3	196	T24	74	58	133.7	79	6.02	88	88	88
25	Apollo	Blue	Blue	3	RW_20	T24	81	68	143.1	82	11.2	88	88	88
26	Apollo	Blue	Blue	3	RW_80	T24	81	69	146	82	9	85	86	86
27	Apollo	Blue	Blue	3	RW_196	T24	84	74	159.5	83	9.96	88	88	88
28	Apollo	Strait	Strait	4	4_20	T24	80	58	117.7	82	7.3	90	91	91
29	Apollo	Strait	Strait	4	4_80	T24	83	67	128.5	83	15.04	88	88	88
30	Apollo	Strait	Strait	4	4_196	T24	81	62	129.3	81	13.72	90	90	90
31	Apollo	Strait	Strait	4	20	T24	81	63	132.5	81	14.14	89	90	90
32	Apollo	Strait	Strait	4	80	T24	82	70	140.1	84	18.36	90	90	90
33	Apollo	Strait	Strait	4	196	T24	84	68	143.4	81	7.76	90	90	90
34	Apollo	Strait	Strait	4	RW_20	T24	73	48	115.6	79	12.38	89	89	89
35	Apollo	Strait	Strait	4	RW_80	T24	80	60	124.9	81	13.7	90	90	90
36	Apollo	Strait	Strait	4	RW_196	T24	77	52	112.2	79	4.62	89	89	89
37	Apollo	Yellow	Yellow	5	4_20	T24	78	59	121.5	81	7.28	85	85	85
38	Apollo	Yellow	Yellow	5	4_80	T24	80	60	128	81	10.26	86	86	86
39	Apollo	Yellow	Yellow	5	4_196	T24	80	61	128.5	80	12.22	86	87	87
40	Apollo	Yellow	Yellow	5	20	T24	76	55	117.6	79	12.76	85	85	85
41	Apollo	Yellow	Yellow	5	80	T24	79	58	117.2	80	8.3	83	84	84
42	Apollo	Yellow	Yellow	5	196	T24	75	51	120.8	78	6.36	85	86	86
43	Apollo	Yellow	Yellow	5	RW_20	T24	69	36	94.9	77	12.7	85	86	86
44	Apollo	Yellow	Yellow	5	RW_80	T24	74	45	105.7	76	9.36	87	87	87
45	Apollo	Yellow	Yellow	5	RW_196	T24	78	53	116.6	80	12.16	86	86	86
46	Apollo	Jack	Jack	6	4_20	T24	66	40	110.6	72	10.7	80	81	81

47	Apollo	Jack	Jack	6	4_80	T24	66	43	138	75	12.06	80	80	80
48	Apollo	Jack	Jack	6	4_196	T24	70	47	131.3	74	7.78	79	80	80
49	Apollo	Jack	Jack	6	20	T24	71	48	110.4	76	11.34	83	84	84
50	Apollo	Jack	Jack	6	80	T24	74	55	152.8	74	8.96	83	84	84
51	Apollo	Jack	Jack	6	196	T24	66	39	131.1	70	11.1	74	75	75
52	Apollo	Jack	Jack	6	RW_20	T24	66	41	110.5	75	8.24	81	82	82
53	Apollo	Jack	Jack	6	RW_80	T24	67	34	106.5	69	15.52	83	84	84
54	Apollo	Jack	Jack	6	RW_196	T24	71	45	119.2	74	6.68	82	83	83
55	Apollo	Slick	Slick	7	4_20	T24	81	57	122.6	78	10.64	89	89	89
56	Apollo	Slick	Slick	7	4_80	T24	82	58	122.6	78	10.42	88	89	89
57	Apollo	Slick	Slick	7	4_196	T24	79	54	127.3	75	7.16	88	88	88
58	Apollo	Slick	Slick	7	20	T24	76	53	154.6	72	9.64	86	86	86
59	Apollo	Slick	Slick	7	80	T24	78	55	125.6	77	9.48	90	90	90
60	Apollo	Slick	Slick	7	196	T24	80	61	133.3	79	6.62	87	88	88
61	Apollo	Slick	Slick	7	RW_20	T24	76	55	141.7	74	7.7	86	86	86
62	Apollo	Slick	Slick	7	RW_80	T24	79	51	121.1	75	10	87	88	88
63	Apollo	Slick	Slick	7	RW_196	T24	75	50	131.4	73	13.78	88	89	89
64	Rock	Hancock	Hancock	1	4_20	T24	87	60	205.2	65	9.68	90	90	94
65	Rock	Hancock	Hancock	1	4_80	T24	87	53	215	60	11.42	90	91	94
66	Rock	Hancock	Hancock	1	4_196	T24	89	57	217.1	61	11.06	90	91	94
67	Rock	Hancock	Hancock	1	20	T24	93	63	214	64	8.84	90	90	94
68	Rock	Hancock	Hancock	1	80	T24	83	52	203.7	61	7.96	90	90	95
69	Rock	Hancock	Hancock	1	196	T24	92	62	212.9	64	7.12	89	90	94
70	Rock	Hancock	Hancock	1	RW_20	T24	90	64	206.2	65	7.16	100	100	100
71	Rock	Hancock	Hancock	1	RW_80	T24	91	61	212.9	63	11.08	91	91	95
72	Rock	Hancock	Hancock	1	RW_196	T24	88	58	206.4	61	7.58	88	89	93

73	Rock	King	King	2	4_20	T24	92	51	230.9	55	7.84	86	87	93
74	Rock	King	King	2	4_80	T24	90	46	239.5	52	13.16	86	87	93
75	Rock	King	King	2	4_196	T24	80	40	227.2	52	13.8	84	85	93
76	Rock	King	King	2	20	T24	82	50	215.8	59	6.84	86	86	93
77	Rock	King	King	2	80	T24	88	42	239.5	51	7.02	86	87	92
78	Rock	King	King	2	196	T24	86	43	238.3	52	9.22	87	97	93
79	Rock	King	King	2	RW_20	T24	83	49	212.7	58	8.92	86	87	93
80	Rock	King	King	2	RW_80	T24	90	49	223.1	54	8.66	87	87	93
81	Rock	King	King	2	RW_196	T24	85	48	225	55	6.24	86	87	92
82	Rock	Blue	Blue	3	4_20	T24	87	55	226.2	60	7.2	87	88	94
83	Rock	Blue	Blue	3	4_80	T24	88	58	227.5	59	14.5	88	89	95
84	Rock	Blue	Blue	3	4_196	T24	90	58	226.1	59	7.2	89	90	95
85	Rock	Blue	Blue	3	20	T24	91	71	202.2	71	10.42	89	90	95
86	Rock	Blue	Blue	3	80	T24	88	57	227	61	7.92	87	87	94
87	Rock	Blue	Blue	3	196	T24	86	55	225.7	60	6.96	87	87	94
88	Rock	Blue	Blue	3	RW_20	T24	85	57	204.6	63	6.4	86	87	94
89	Rock	Blue	Blue	3	RW_80	T24	90	62	219.8	63	6.56	88	89	94
90	Rock	Blue	Blue	3	RW_196	T24	83	63	198.8	70	7.16	86	87	94
91	Rock	Strait	Strait	4	4_20	T24	86	65	182.8	71	10.66	87	88	92
92	Rock	Strait	Strait	4	4_80	T24	85	64	198.1	69	12.3	88	88	92
93	Rock	Strait	Strait	4	4_196	T24	89	67	205.2	68	5.64	87	88	92
94	Rock	Strait	Strait	4	20	T24	91	75	187.3	74	9.3	88	88	93
95	Rock	Strait	Strait	4	80	T24	85	61	194.8	69	11.04	88	88	92
96	Rock	Strait	Strait	4	196	T24	87	62	180.7	69	10.48	88	88	93
97	Rock	Strait	Strait	4	RW_20	T24	84	62	175	71	10.44	86	86	92
98	Rock	Strait	Strait	4	RW_80	T24	85	65	181.9	71	6.14	89	90	92

99	Rock	Strait	Strait	4	RW_196	T24	86	63	184.7	70	7.36	86	87	92
100	Rock	Yellow	Yellow	5	4_20	T24	83	58	182.4	67	9.56	82	84	89
101	Rock	Yellow	Yellow	5	4_80	T24	83	61	185.1	69	11.92	86	86	91
102	Rock	Yellow	Yellow	5	4_196	T24	87	64	181.8	69	7.9	87	87	92
103	Rock	Yellow	Yellow	5	20	T24	83	66	172.1	74	14.16	85	86	90
104	Rock	Yellow	Yellow	5	80	T24	85	63	188.3	69	8.12	86	87	92
105	Rock	Yellow	Yellow	5	196	T24	82	59	188.7	67	12.2	85	86	91
106	Rock	Yellow	Yellow	5	RW_20	T24	92	72	165	72	9	88	89	91
107	Rock	Yellow	Yellow	5	RW_80	T24	84	58	184.6	67	7.88	86	87	91
108	Rock	Yellow	Yellow	5	RW_196	T24	80	60	174.4	71	8.8	84	85	90
109	Rock	Jack	Jack	6	4_20	T24	84	63	181.4	70	12.54	83	84	90
110	Rock	Jack	Jack	6	4_80	T24	87	54	203	61	9.92	86	86	92
111	Rock	Jack	Jack	6	4_196	T24	92	64	208.2	64	7.15	85	86	90
112	Rock	Jack	Jack	6	20	T24	83	65	199.3	69	14.08	83	84	89
113	Rock	Jack	Jack	6	80	T24	85	55	209.9	62	10.5	85	86	90
114	Rock	Jack	Jack	6	196	T24	87	58	202.1	63	9.96	86	86	91
115	Rock	Jack	Jack	6	RW_20	T24	84	61	178.4	69	6.78	82	83	89
116	Rock	Jack	Jack	6	RW_80	T24	85	55	212.5	61	6.7	86	86	91
117	Rock	Jack	Jack	6	RW_196	T24	86	54	190.2	62	8.16	88	88	91
118	Rock	Slick	Slick	7	4_20	T24	80	52	205.6	62	10.38	83	84	89
119	Rock	Slick	Slick	7	4_80	T24	85	54	216.4	60	15.08	84	84	88
120	Rock	Slick	Slick	7	4_196	T24	82	52	202.6	61	8.46	82	83	88
121	Rock	Slick	Slick	7	20	T24	86	57	210.4	62	6.18	83	84	87
122	Rock	Slick	Slick	7	80	T24	84	53	206.5	60	7.84	85	85	90
123	Rock	Slick	Slick	7	196	T24	82	47	204.6	58	9.46	85	85	89
124	Rock	Slick	Slick	7	RW_20	T24	82	55	197.7	63	9.82	84	84	89

125	Rock	Slick	Slick	7	RW_80	T24	89	59	212.5	61	10.46	84	85	89
126	Rock	Slick	Slick	7	RW_196	T24	83	55	197.1	63	8.6	82	82	88
127	Spencer	Hancock	Hancock	1	4_20	T24	84	61	165.9	68	9.16	60	61	88
128	Spencer	Hancock	Hancock	1	4_80	T24	92	63	182.4	66	8.04	85	86	89
129	Spencer	Hancock	Hancock	1	4_196	T24	87	63	179.9	66	11.04	79	80	89
130	Spencer	Hancock	Hancock	1	20	T24	84	62	168.8	69	6.52	85	86	90
131	Spencer	Hancock	Hancock	1	80	T24	83	59	183.7	67	14.86	87	87	91
132	Spencer	Hancock	Hancock	1	196	T24	82	58	170.8	67	7	83	84	89
133	Spencer	Hancock	Hancock	1	RW_20	T24	86	64	150.7	72	10.16	84	85	90
134	Spencer	Hancock	Hancock	1	RW_80	T24	83	58	167.3	67	8.1	86	87	90
135	Spencer	Hancock	Hancock	1	RW_196	T24	87	60	164.4	67	8	87	88	91
136	Spencer	King	King	2	4_20	T24	86	48	183.1	59	3.96	82	83	89
137	Spencer	King	King	2	4_80	T24	89	46	210.3	54	5.88	85	85	89
138	Spencer	King	King	2	4_196	T24	85	40	207.6	51	10.94	85	86	90
139	Spencer	King	King	2	20	T24	87	45	199	54	21.42	84	85	89
140	Spencer	King	King	2	80	T24	85	38	211	50	7.08	84	85	89
141	Spencer	King	King	2	196	T24	86	41	216.9	52	5.7	83	84	88
142	Spencer	King	King	2	RW_20	T24	81	46	178	59	11.86	85	86	89
143	Spencer	King	King	2	RW_80	T24	83	40	202.1	53	14.4	86	87	91
144	Spencer	King	King	2	RW_196	T24	85	43	186.3	55	7.72	83	84	88
145	Spencer	Blue	Blue	3	4_20	T24	80	65	163.3	75	8.78	81	83	88
146	Spencer	Blue	Blue	3	4_80	T24	84	64	148.9	74	4.04	84	85	90
147	Spencer	Blue	Blue	3	4_196	T24	87	68	182.6	70	6.7	83	84	89
148	Spencer	Blue	Blue	3	20	T24	84	67	146.1	76	10.76	83	83	90
149	Spencer	Blue	Blue	3	80	T24	89	69	162.8	71	8.4	83	84	89
150	Spencer	Blue	Blue	3	196	T24	87	63	180.2	67	10.94	81	82	88

151	Spencer	Blue	Blue	3	RW_20	T24	84	63	145.3	75	11.8	81	81	89
152	Spencer	Blue	Blue	3	RW_80	T24	92	74	159	73	9.7	81	82	88
153	Spencer	Blue	Blue	3	RW_196	T24	84	63	144.1	73	7.46	81	82	88
154	Spencer	Strait	Strait	4	4_20	T24	87	66	163.8	71	8.48	86	87	95
155	Spencer	Strait	Strait	4	4_80	T24	90	64	181.2	69	7.2	89	90	94
156	Spencer	Strait	Strait	4	4_196	T24	91	72	178.7	70	10.12	91	91	95
157	Spencer	Strait	Strait	4	20	T24	87	65	167.7	70	9	90	91	95
158	Spencer	Strait	Strait	4	80	T24	87	68	164.4	71	11.8	85	85	94
159	Spencer	Strait	Strait	4	196	T24	89	65	187.4	67	5.52	90	90	94
160	Spencer	Strait	Strait	4	RW_20	T24	87	59	141.4	71	11.46	90	91	95
161	Spencer	Strait	Strait	4	RW_80	T24	89	64	166.5	69	6.92	90	91	95
162	Spencer	Strait	Strait	4	RW_196	T24	88	63	156.5	70	11.86	86	86	94
163	Spencer	Yellow	Yellow	5	4_20	T24	85	62	143.9	72	5.46	81	82	87
164	Spencer	Yellow	Yellow	5	4_80	T24	86	58	161.1	67	5.28	83	83	88
165	Spencer	Yellow	Yellow	5	4_196	T24	85	54	168	64	10.08	84	85	89
166	Spencer	Yellow	Yellow	5	20	T24	87	63	146.8	71	6.68	78	79	86
167	Spencer	Yellow	Yellow	5	80	T24	81	50	163.2	64	6.96	80	81	88
168	Spencer	Yellow	Yellow	5	196	T24	84	54	159.7	66	6.78	84	84	89
169	Spencer	Yellow	Yellow	5	RW_20	T24	82	55	132.2	70	9.8	85	85	89
170	Spencer	Yellow	Yellow	5	RW_80	T24	82	50	142.3	67	7.44	85	85	89
171	Spencer	Yellow	Yellow	5	RW_196	T24	82	51	153.6	67	8.66	84	85	90
172	Spencer	Jack	Jack	6	4_20	T24	81	42	184.5	56	7.18	72	73	80
173	Spencer	Jack	Jack	6	4_80	T24	87	37	230.8	48	9.2	79	80	84
174	Spencer	Jack	Jack	6	4_196	T24	85	39	214.6	49	7.86	78	79	85
175	Spencer	Jack	Jack	6	20	T24	82	47	177	59	10.16	79	80	85
176	Spencer	Jack	Jack	6	80	T24	83	40	211.3	52	5.38	73	74	81

177	Spencer	Jack	Jack	6	196	T24	87	43	213	52	11.94	78	78	85
178	Spencer	Jack	Jack	6	RW_20	T24	78	44	171.4	61	7.46	78	79	83
179	Spencer	Jack	Jack	6	RW_80	T24	81	34	210.6	50	11.06	79	80	83
180	Spencer	Jack	Jack	6	RW_196	T24	83	37	208.6	50	6.08	77	78	82
181	Spencer	Slick	Slick	7	4_20	T24	88	58	174.1	65	10.34	81	82	85
182	Spencer	Slick	Slick	7	4_80	T24	85	49	177.4	60	13	82	82	86
183	Spencer	Slick	Slick	7	4_196	T24	81	50	160.6	65	7.4	81	82	85
184	Spencer	Slick	Slick	7	20	T24	84	52	167.9	64	8.24	82	83	86
185	Spencer	Slick	Slick	7	80	T24	84	49	184.7	61	5.48	81	82	85
186	Spencer	Slick	Slick	7	196	T24	82	50	178.5	61	10.06	75	76	84
187	Spencer	Slick	Slick	7	RW_20	T24	76	46	144.3	68	6.78	84	85	87
188	Spencer	Slick	Slick	7	RW_80	T24	79	45	162.6	64	8.34	80	81	85
189	Spencer	Slick	Slick	7	RW_196	T24	82	48	169.9	62	7.94	81	82	85

APPENDIX G

RAW DATA FOR PRELIMINARY EXPERIMENT 2.1: VIAL AND ORIENTATION COMPARISON

Obs	STUD	TIME	EJAC	TUBE	ORIENT	TEMP	TMOT	PMOT	VCL	STR
1	Apollo	24	1	Glass	Up	4	66	49	150.9	74
2	Apollo	24	1	Glass	Invert	4	28	15	122	69
3	Apollo	24	1	Plastic	Up	4	61	46	140	77
4	Apollo	24	1	Plastic	Invert	4	48	35	136.4	76
5	Christy	24	1	Glass	Up	4	72	25	239.9	45
6	Christy	24	1	Glass	Invert	4	43	29	153.6	73
7	Christy	24	1	Plastic	Up	4	77	25	255.6	43
8	Christy	24	1	Plastic	Invert	4	68	21	249.8	43
9	Rock	24	1	Glass	Up	4	88	56	229.1	61
10	Rock	24	1	Glass	Invert	4	49	28	123.5	72
11	Rock	24	1	Plastic	Up	4	90	60	225.2	64
12	Rock	24	1	Plastic	Invert	4	85	56	236.5	63
13	Smooth	24	1	Glass	Up	4	67	55	145.9	75
14	Smooth	24	1	Glass	Invert	4	53	44	122.8	82
15	Smooth	24	1	Plastic	Up	4	66	55	129.6	78
16	Smooth	24	1	Plastic	Invert	4	62	50	129.5	78
17	Spencer	24	1	Glass	Up	4	93	51	256	56
18	Spencer	24	1	Glass	Invert	4	17	4	93.7	57
19	Spencer	24	1	Plastic	Up	4	83	40	236.1	52
20	Spencer	24	1	Plastic	Invert	4	77	35	225.5	53
21	Apollo	24	2	Glass	Up	4	65	50	149.8	80
22	Apollo	24	2	Glass	Invert	4	38	20	106.6	71
23	Apollo	24	2	Plastic	Up	4	67	52	153.7	80
24	Apollo	24	2	Plastic	Invert	4	67	55	157.6	81
25	Christy	24	2	Glass	Up	4	83	33	249.6	45
26	Christy	24	2	Glass	Invert	4	63	40	141.4	70
27	Christy	24	2	Plastic	Up	4	90	41	242.9	48
28	Christy	24	2	Plastic	Invert	4	87	43	232.4	51
29	Rock	24	2	Glass	Up	4	77	62	178.3	75
30	Rock	24	2	Glass	Invert	4	38	21	112.7	71

31	Rock	24	2	Plastic	Up	4	68	54	176.3	76
32	Rock	24	2	Plastic	Invert	4	76	61	182.6	75
33	Smooth	24	2	Glass	Up	4	57	45	145.4	77
34	Smooth	24	2	Glass	Invert	4	52	43	128.3	82
35	Smooth	24	2	Plastic	Up	4	69	57	147.7	79
36	Smooth	24	2	Plastic	Invert	4	60	49	130.1	80
37	Spencer	24	2	Glass	Up	4	86	49	228.9	59
38	Spencer	24	2	Glass	Invert	4	51	19	163.9	53
39	Spencer	24	2	Plastic	Up	4	86	52	227.2	60
40	Spencer	24	2	Plastic	Invert	4	89	58	224.4	64
41	Apollo	24	3	Glass	Up	4	77	55	162.08	74
42	Apollo	24	3	Glass	Invert	4	26	10	101.2	62
43	Apollo	24	3	Plastic	Up	4	76	60	185.7	75
44	Apollo	24	3	Plastic	Invert	4	78	63	165.1	76
45	Christy	24	3	Glass	Up	4	84	39	251.1	51
46	Christy	24	3	Glass	Invert	4	41	30	106.8	78
47	Christy	24	3	Plastic	Up	4	86	44	252	52
48	Christy	24	3	Plastic	Invert	4	78	37	248.7	51
49	Rock	24	3	Glass	Up	4	66	34	158.4	59
50	Rock	24	3	Glass	Invert	4	6	2	86	60
51	Rock	24	3	Plastic	Up	4	69	34	140.4	62
52	Rock	24	3	Plastic	Invert	4	68	35	153.7	62
53	Smooth	24	3	Glass	Up	4	71	57	155.2	74
54	Smooth	24	3	Glass	Invert	4	65	56	122.7	84
55	Smooth	24	3	Plastic	Up	4	72	62	142.9	78
56	Smooth	24	3	Plastic	Invert	4	66	55	136.2	80
57	Spencer	24	3	Glass	Up	4	76	36	178.7	59
58	Spencer	24	3	Glass	Invert	4	24	5	92.9	63
59	Spencer	24	3	Plastic	Up	4	66	30	160.7	59
60	Spencer	24	3	Plastic	Invert	4	61	27	138.2	60

APPENDIX H

RAW DATA FOR PRELIMINARY EXPERIMENT 2.2: RUBBER STOPPER COMPARISON

Obs	STUD	TIME	EJAC	STOPPER	TREAT	TREAT2	TMOT	PMOT	VCL	STR
1	Clifford	24	1	PLAS	CTRL	PLAS_UP	83	45	200	57
2	Clifford	24	1	ISO	UP	ISO_UP	75	40	211	53
3	Clifford	24	1	ISO	INVERT	ISO_DN	50	34	133	72
4	Clifford	24	1	ISO	ROTATE	ISO_INV	84	47	218	54
5	Clifford	24	1	CLB	UP	CLB_UP	84	41	216	52
6	Clifford	24	1	CLB	INVERT	CLB_DN	82	48	211	58
7	Clifford	24	1	CLB	ROTATE	CLB_INV	82	42	218	54
8	Rock	24	1	PLAS	CTRL	PLAS_UP	78	40	260	53
9	Rock	24	1	ISO	UP	ISO_UP	83	44	225	57
10	Rock	24	1	ISO	INVERT	ISO_DN	22	6	83	64
11	Rock	24	1	ISO	ROTATE	ISO_INV	78	40	265	51
12	Rock	24	1	CLB	UP	CLB_UP	80	42	257	54
13	Rock	24	1	CLB	INVERT	CLB_DN	84	48	256	55
14	Rock	24	1	CLB	ROTATE	CLB_INV	82	43	257	52
15	Apollo	24	1	PLAS	CTRL	PLAS_UP	62	29	108	69
16	Apollo	24	1	ISO	UP	ISO_UP	72	45	129	74
17	Apollo	24	1	ISO	INVERT	ISO_DN	19	4	73	51
18	Apollo	24	1	ISO	ROTATE	ISO_INV	65	27	103	66
19	Apollo	24	1	CLB	UP	CLB_UP	70	39	124	71
20	Apollo	24	1	CLB	INVERT	CLB_DN	58	20	95	64
21	Apollo	24	1	CLB	ROTATE	CLB_INV	65	27	106	65
22	Spencer	24	1	PLAS	CTRL	PLAS_UP	76	46	198	61
23	Spencer	24	1	ISO	UP	ISO_UP	73	42	195	58
24	Spencer	24	1	ISO	INVERT	ISO_DN	36	16	96	66
25	Spencer	24	1	ISO	ROTATE	ISO_INV	75	49	168	64
26	Spencer	24	1	CLB	UP	CLB_UP	78	45	197	58
27	Spencer	24	1	CLB	INVERT	CLB_DN	79	57	190	67
28	Spencer	24	1	CLB	ROTATE	CLB_INV	79	49	184	62

APPENDIX I

RAW DATA FOR EXPERIMENT 2: FRESH VS. FROZEN/THAWED VS. LYOPHILIZED SEMINAL PLASMA

Obs	STUD	EJAC	TIME	TREAT	TMOT	PMOT	VCL	STR	VIAB	AI	VIAB_AI	COMP
1	Spencer	1	0	UC	90	62	246.7	64	80.36	94.78	79.12	14.94
2	Spencer	1	24	UC	84	53	215.2	62	82.72	93.14	81.22	19.68
3	Spencer	1	0	FRSH	92	65	235.5	67	83.84	95.14	83.28	14.46
4	Spencer	1	24	FRSH	90	64	195.3	67	87.06	95	86.34	14.1
5	Spencer	1	0	F_T	90	61	229.8	64	82.96	94.2	82.24	13.66
6	Spencer	1	24	F_T	86	58	213.9	64	85.14	93.96	84.52	16.1
7	Spencer	1	0	LYO	88	58	230.3	63	84.32	95.68	83.74	13.9
8	Spencer	1	24	LYO	89	55	230.7	60	85.84	94.74	85.06	13.84
9	Spencer	2	0	UC	83	48	215	59	70.04	91.84	68.36	16.52
10	Spencer	2	24	UC	82	53	172.6	66	76.16	88.68	73.28	22.88
11	Spencer	2	0	FRSH	93	67	212.3	66	80.72	93.44	79.26	8.38
12	Spencer	2	24	FRSH	87	58	172.9	65	82.32	92.6	80.14	18.2
13	Spencer	2	0	F_T	93	67	219.1	64	81.7	91.18	80.18	12.32
14	Spencer	2	24	F_T	94	65	211.1	63	81.12	92.36	80.12	20.88
15	Spencer	2	0	LYO	92	64	241.3	62	80.96	92.42	79.92	8.9
16	Spencer	2	24	LYO	93	55	223	58	81.18	93.02	80.2	14.24
17	Spencer	3	0	UC	90	58	246.4	62	69.58	92.78	68.04	13
18	Spencer	3	24	UC	83	56	219	64	75.42	91	72.64	26.12
19	Spencer	3	0	FRSH	84	59	215.7	65	74.98	92.9	74.06	18.56

20	Spencer	3	24	FRSH	84	51	216.6	59	77.14	92.14	75.8	24.92
21	Spencer	3	0	F_T	85	63	223.4	65	69.44	92.3	68.08	9.48
22	Spencer	3	24	F_T	84	52	203.6	60	73.68	86.5	72.32	21.58
23	Spencer	3	0	LYO	84	59	218.2	63	71.46	92.58	70.56	12.72
24	Spencer	3	24	LYO	84	47	219	57	76.4	91.68	75.32	22.86
25	Apollo	1	0	UC	89	59	275.9	65	72.92	95.6	71.94	18.6
26	Apollo	1	24	UC	74	60	194.4	77	68.38	94.54	67.58	25.66
27	Apollo	1	0	FRSH	87	63	276.3	65	80.66	94.08	79.98	16.88
28	Apollo	1	24	FRSH	77	65	178.9	79	80.08	93.14	79.06	18.96
29	Apollo	1	0	F_T	89	65	251.1	66	78.36	96.18	77.72	16.92
30	Apollo	1	24	F_T	71	58	161.1	79	76.48	93.82	75.54	20.34
31	Apollo	1	0	LYO	90	68	270.6	66	79.62	98.38	79.2	19.38
32	Apollo	1	24	LYO	74	56	156	77	75.28	96.66	74.14	21.18
33	Apollo	2	0	UC	94	71	266.5	70	84.86	96.22	84	11.8
34	Apollo	2	24	UC	68	45	107.7	78	85.28	93.94	83.76	16.6
35	Apollo	2	0	FRSH	86	69	243.9	72	85.06	96.78	84.56	10.5
36	Apollo	2	24	FRSH	77	61	167.3	78	77.38	96.72	76.88	12.62
37	Apollo	2	0	F_T	94	82	235.4	76	85.32	96.86	84.7	10.38
38	Apollo	2	24	F_T	64	48	147.6	76	75.5	96.5	74.94	9.46
39	Apollo	2	0	LYO	90	75	227	76	85.36	97.36	84.92	10.78
40	Apollo	2	24	LYO	54	35	147.1	71	79.44	97.46	79.06	7.7
41	Apollo	3	0	UC	95	75	278.9	72	79.34	96.94	78.06	12.26
42	Apollo	3	24	UC	79	56	193.3	70	74.62	95.3	72.64	25.98
43	Apollo	3	0	FRSH	90	72	252.6	71	86.8	97.04	86.1	13.06
44	Apollo	3	24	FRSH	88	72	175.3	78	84.62	96.74	84	14.48
45	Apollo	3	0	F_T	83	70	217.5	74	87.02	97.16	86.3	8.96

46	Apollo	3	24	F_T	88	69	163.8	75	85.2	96.8	84.36	18.4
47	Apollo	3	0	LYO	93	78	225.8	75	86.42	97.92	85.9	9.42
48	Apollo	3	24	LYO	69	42	165.1	67	86.48	97.28	85.46	17.22
49	Rock	1	0	UC	92	64	214.2	66	81.6	95.08	80.14	13.92
50	Rock	1	24	UC	84	50	202.6	61	83.64	94.82	82.36	22.58
51	Rock	1	0	FRSH	91	61	233.2	62	86.26	95.66	85.64	17.76
52	Rock	1	24	FRSH	87	58	218.2	63	87.58	95.5	86.78	16.2
53	Rock	1	0	F_T	93	64	239.6	63	83.94	97.22	83.6	15.3
54	Rock	1	24	F_T	85	48	223.3	58	88.04	96.86	87.4	23.56
55	Rock	1	0	LYO	92	65	238.2	65	86.68	97.8	86.38	16.32
56	Rock	1	24	LYO	87	51	222.5	58	87.58	96.14	86.76	16.04
57	Rock	2	0	UC	86	53	191.1	63	77.1	91.44	72.84	25.1
58	Rock	2	24	UC	81	42	208.8	57	75.3	90.52	69.96	28.08
59	Rock	2	0	FRSH	90	63	235.6	65	80.64	94.86	77.08	16.26
60	Rock	2	24	FRSH	86	39	237.6	49	80.88	93.04	76.88	21.88
61	Rock	2	0	F_T	89	55	220.7	64	78.66	94.26	76.24	19.24
62	Rock	2	24	F_T	84	47	235.9	56	80.98	93.46	78.12	19.5
63	Rock	2	0	LYO	89	58	225.5	64	74.54	93.24	71.46	20.48
64	Rock	2	24	LYO	84	41	231.1	52	79.72	92.56	76.44	22.1
65	Rock	3	0	UC	88	56	213.3	63	78.44	90.96	76.82	18.12
66	Rock	3	24	UC	79	49	219.9	58	77.06	89.4	74.74	23.46
67	Rock	3	0	FRSH	91	66	239.8	67	81.06	98.82	80.24	16.02
68	Rock	3	24	FRSH	83	42	232.6	53	79.26	97.48	78.28	25.94
69	Rock	3	0	F_T	86	58	219.7	63	80.74	92.14	80.06	16.18
70	Rock	3	24	F_T	85	63	200	66	81.2	96.7	80.56	19.66
71	Rock	3	0	LYO	91	65	232	66	78.38	99.36	78	18.12

72	Rock	3	24	LYO	85	46	218.6	54	81.7	92.66	79.62	16.2
73	Christy	1	0	UC	62	23	209.8	48	34.12	44.82	29.28	48.72
74	Christy	1	24	UC	56	37	195.2	60	39.62	47.2	33.64	58.66
75	Christy	1	0	FRSH	57	20	248.3	43	42.36	45.78	36.82	49.58
76	Christy	1	24	FRSH	54	29	222.7	54	46.4	48.72	39.86	52.02
77	Christy	1	0	F_T	59	26	240.1	48	42.32	47.54	38.06	50.64
78	Christy	1	24	F_T	52	26	199.7	52	46.56	45.06	35.22	56.88
79	Christy	1	0	LYO	56	22	227.5	48	43.38	45.68	37.44	51.7
80	Christy	1	24	LYO	58	22	227.8	46	46.26	43.6	35.22	52.2
81	Christy	2	0	UC	84	33	231.6	48	60.44	71.58	55.28	21.76
82	Christy	2	24	UC	79	36	236	51	63.48	76.2	58.74	28.16
83	Christy	2	0	FRSH	87	33	252.9	42	77.14	80.48	72.36	19.02
84	Christy	2	24	FRSH	88	32	287	43	77.18	82.4	72.62	18.62
85	Christy	2	0	F_T	86	35	256.1	45	72.42	78.2	68.56	15.9
86	Christy	2	24	F_T	83	30	278.1	43	74.5	80.42	69.28	19
87	Christy	2	0	LYO	87	32	254.4	42	79.14	81.96	74.84	16.96
88	Christy	2	24	LYO	86	29	281.1	42	76.54	83.22	72.2	19.16
89	Christy	3	0	UC	85	38	262.7	47	61.38	75.34	57.2	23.9
90	Christy	3	24	UC	79	31	213.8	51	60.34	72.12	58.24	32.78
91	Christy	3	0	FRSH	85	36	253	48	73.1	77.04	68.5	21.72
92	Christy	3	24	FRSH	83	33	228.8	46	71.78	79	69.52	25.76
93	Christy	3	0	F_T	90	33	263.8	44	70.26	74.52	65.3	21.08
94	Christy	3	24	F_T	84	31	255.5	45	68.52	77.96	65.66	28.44
95	Christy	3	0	LYO	87	33	258.3	44	68.38	75.18	65.24	19.76
96	Christy	3	24	LYO	84	25	259.3	41	70.02	77.84	67.34	26
97	Clifford	1	0	UC	78	44	205.8	54	75.48	89.34	73.72	17.68

98	Clifford	1	24	UC	75	40	182.2	55	72.32	89.44	70.32	27.04
99	Clifford	1	0	FRSH	76	34	206.9	49	73.82	86.46	71.44	19.2
100	Clifford	1	24	FRSH	75	32	190.7	48	66.3	87.62	63.48	24.68
101	Clifford	1	0	F_T	83	36	218.1	49	73.28	88.02	71.06	18.4
102	Clifford	1	24	F_T	70	30	206.9	48	60.84	86.64	57.26	20.46
103	Clifford	1	0	LYO	77	33	213.9	47	73.5	89.94	71.96	22.3
104	Clifford	1	24	LYO	66	28	211	48	65.64	87.94	62.22	16.4
105	Clifford	2	0	UC	67	24	209.6	47	57.56	88.18	55.94	22.12
106	Clifford	2	24	UC	77	37	212.7	52	61.64	85.04	57.88	35.06
107	Clifford	2	0	FRSH	55	21	237.9	45	51.8	88.2	49.28	37.3
108	Clifford	2	24	FRSH	58	22	230.1	48	52.8	87.6	49.8	46.18
109	Clifford	2	0	F_T	56	25	213.5	52	55.88	88.66	53.92	37.18
110	Clifford	2	24	F_T	51	18	197.1	46	46.56	83.96	42.82	39.58
111	Clifford	2	0	LYO	60	26	214.7	51	51.3	90.44	48.56	37.82
112	Clifford	2	24	LYO	51	21	199.5	49	41.34	89.28	37.58	30.12
113	Clifford	3	0	UC	52	20	213.5	50	46.3	84.14	42.92	30.48
114	Clifford	3	24	UC	52	21	195.2	51	42.2	86.56	39.9	54.74
115	Clifford	3	0	FRSH	63	30	237.1	49	53.26	87.12	50.98	33.16
116	Clifford	3	24	FRSH	53	17	197.7	48	51.3	87.5	49.02	44.36
117	Clifford	3	0	F_T	54	29	190.1	57	50.86	91.88	48.8	30.88
118	Clifford	3	24	F_T	49	21	168.3	53	46	93.94	43.38	47.98
119	Clifford	3	0	LYO	55	27	192.7	55	50.66	92.06	48.32	38.4
120	Clifford	3	24	LYO	53	20	178	47	49.56	94.08	46.88	45.8