Clinical perspectives on the stallion
Paul R. Loomis
Select Breeders Service, Inc., Chesapeake City, MD

Introduction

Stallions are presented to Select Breeders Service laboratories for a variety of reasons including; training for semen collection, breeding soundness examination, semen evaluation and cooled and frozen semen processing. This review will outline the process that we use when presented with a stallion for semen collection and processing of frozen semen and our approach to troubleshooting stallions that exhibit abnormal semen characteristics or poor results from standard preservation protocols.

Standard workup for new stallions

As a facility approved for semen collection and storage for the European Union and a variety of other countries that is inspected twice yearly by the USDA, we have implemented some basic health screening requirements for all stallions entering our facility. These include a negative EIA within 12 months and negative EVA and CEM within 30 days of entry. Stallions seropositive for arteritis virus must show proof of vaccination (and appropriate boosters) or have a negative virus isolation on the semen to confirm that they are not shedding virus in the semen.

Our approach for the first collection attempt is different for a stallion that has previous experience mounting a phantom than it is for a novice. Briefly, if it is a novice stallion, we will introduce him to the breeding shed environment without a stimulus mare, allow him to see and approach the tease rail and the phantom and get comfortable with the environment before bringing in an estrus stimulus mare. We then introduce him to the stimulus mare to elicit an erection and wash the penis with warm water and cotton and at this time examine the external genitalia for any abnormalities. Testicular palpation and measurements are performed after successful collection. Novice stallions are then brought to the phantom with the stimulus mare positioned alongside and attempts are made to encourage the stallion to mount the phantom. This process may take ten minutes or several days depending on the stallion but the key is patience on the part of the stallion handler and collector and consistent positive reinforcement during the training process.

For experienced stallions, we will generally start with a stimulus mare in the shed and take them straight to the phantom after washing, with or without a stimulus mare positioned near the phantom based on the stallion’s behavior and libido.

Prior to commencing on a freezing program with a new stallion we will collect and evaluate the semen. If the stallion is sexually rested or if we have no real history, we plan on performing a minimum of three daily collections to deplete extragonadal sperm reserves and evaluate semen quality. If the semen quality is satisfactory and stabilized after three collections, we then give the stallion one day of sexual rest and perform a split-ejaculate “test-freeze” procedure. If sperm numbers are not stabilized or semen quality is poor (low progressive motility and/or poor morphology) we may continue to deplete the stallion’s sperm reserves until they are stable. For standard evaluation, sperm concentration is measured using a Nucleocounter, sperm motility is evaluated using a computer assisted sperm analyzer (CASA) and morphology is assessed on stained samples of 200 total sperm using 1000X oil immersion phase contrast microscopy.

Basic test freeze procedure

The number of protocols evaluated in a typical split-ejaculate test freeze depends on the total number of sperm available. If sufficient sperm are available, we will evaluate four different protocols in the first test freeze. The protocols differ in the extender used and the cooling/freezing rate employed. If there are not sufficient sperm in the ejaculate to evaluate all four treatments we will use two or three, select the treatment that provided the best post-thaw motility and then compare that treatment to additional treatments in subsequent split-ejaculate test freezes. Following determination of concentration and motility, semen is diluted with a centrifugation extender and centrifuged on a cushion in 50 ml
conical bottom tubes. The supernatant is then removed as well as the cushion fluid and the sperm are re-
suspended in the appropriate freezing extender. The concentration of sperm in the extended semen is
again counted using a Nucleocounter and final adjustments are made to achieve the desired concentration
before loading the semen into 0.5 ml straws. Straws are sealed, racked and frozen using a controlled rate
cell freezer. For each treatment and all ejaculates, two straws are thawed and evaluated. The contents of
the two straws are combined in a sterile tube and a volumetric loop is used to obtain a sample for
bacteriology on a blood agar plate. An aliquot of the thawed semen is diluted to approximately 25-30
x10^6 per ml with pre-warmed extender (of the same type used for centrifugation of the raw semen) and
the extended semen is incubated at 37°C for 30 minutes before being analyzed for motility using CASA.
The technician performing the motility analysis is blinded as to the stallion and treatment and post-thaw
evaluations are done in batches which include different stallions and treatments to minimize the potential
for bias. Based on the results of the test freeze procedure(s) one of the protocols is selected and used for
subsequent freezes on ejaculates typically collected three times per week until the desired number of
acceptable doses is achieved.

Troubleshooting the “problem stallion”

The following sections describe our systematic approach to addressing abnormal findings in our
basic semen evaluation or initial freezing attempts to design a customized program that achieves
acceptable results for the stallion.

Observation: low sperm numbers

When low total sperm numbers are observed in the collected semen one should first determine if
the sperm output is or isn’t consistent with the expected sperm production based on examination of the
testicles and calculation of testicular volume. If the low numbers are due to small or degenerated
testicles, then there is very little to be done to increase sperm production. If sperm output is less than
expected based on testicular volume and there are no abnormalities observed in the testicles upon
ultrasound examination, then possibilities include incomplete ejaculation, retrograde ejaculation into the
bladder or spermiosgenesis (blockage of sperm in the reproductive tract), typically in the ampulla. If the
sample contains no sperm but the stallion appears to have ejaculated, then it can be assayed for the level
of alkaline phosphatase, a testicular component of the ejaculate. If alkaline phosphatase is low, then a
complete blockage is suspected. If high, then the semen does contain the testicular contribution and
severe testicular dysfunction is suspected. Palpation or ultrasonic examination of the ampullae per rectum
can often detect enlarged ampullae with distended lumen. Treatment for blocked ampullae is repeated
collections sometimes aided by aggressive massage of the ampullae per rectum and administration of
oxytocin (10-20 IU) or cloprostenol (25-125ug) given 5-10 minutes before semen collection.

Observation: excessive gel fraction in ejaculate

The gel fraction is typically the last component of the ejaculate and is contributed by the seminal
vesicles. Some stallions produced little or no gel fraction while others produce a consistent 5 to 20 ml of
viscous gel that is easily filtered out using an in-line nylon gel filter. Occasionally, a stallion is presented
that produces copious quantities of gel and sometimes this gel is not easily filtered and can mix with the
gel-free portion of the ejaculate. Semen which contains thin gel that is not filtered out is very difficult to
accurately pipette and analyze sperm quality and in some cases may adversely affect sperm quality. Like
other accessory sex gland fluids, excessive stimulation tends to increase the amount of gel produced. In
these cases, management should minimize stimulation of the stallion by housing him where there is little
other horse activity and schedule collecting the stallion early in the day before any other stallions are
collected. When this fails to alleviate the issue the ejaculate can be fractionated and only the gel-free jets
collected which prevents the gel fraction from mixing with the semen and usually results in improved
semen quality.
In some rare cases the contaminating gel is very toxic to the sperm causing a rapid loss of sperm motility. Below is a description of one such case of extremely toxic and difficult to filter gel which prevented this stallion from being used in a cooled or frozen semen AI program.

Our approach was to use an open-ended artificial vagina for collection which allowed for the stallion’s glans penis to protrude through the back of the AV so that individual jets could be isolated and collected. We used a 19 inch Colorado model AV. The inner latex bladder was thoroughly cleaned and disinfected using our standard protocol for reusable latex AV liners. The AV is filled with warm (50°C water and the bladder lubricated using a non-spermicidal lubricant. A sterile fingerless palpation sleeve is affixed to a wire loop with a handle to catch the sperm-rich fractions. The stallion mounts the phantom as usual and the collector places the AV on the penis. A second technician positions the collection device at the back of the open-ended AV to visualize the glans penis and the jets of semen as they are ejaculated. The collector places two fingers on the base of the penis to feel the urethra and alert the second technician as to when the jets were happening. The collector calls out the jets and after jets 1, 2, and 3 are collected the second technician closes off the collection bag and removes the device allowing for the remaining gel-contaminated jets to fall to the ground. For diagnostic purposes the remaining individual fractions can be collected by quickly exchanging a second collection bag/device after the sperm-rich fractions were collected. Using this technique, we could properly evaluate the semen from this stallion, prevent any gel contamination and successfully manage him in a cooled transported and frozen semen AI program.

Observation: poor initial sperm motility

1. Start by obtaining a thorough history on the stallion:
   Has he experienced a testicular insult or swelling of the scrotum? Is the stallion suffering from extreme heat stress or has he been in heavy training in a hot climate? Is there a possible history of steroid or other drug use? Has the stallion been ill or had a fever in the last two to three months?
2. Next, rule out the possibility of damage introduced during the collection process:
   Was the semen exposed to thermal stress? Was it trapped in a hot AV too long? Was the semen cold shocked from contact with cold collection vessel or exposure to cold ambient temperature? Was the semen contaminated with a non-isosmotic lubricant or excessive bacteria from dirty collection equipment? Did the stallion require several mounts before ejaculating? If so, was the collection vessel changed between mounts and/or was the pre-ejaculatory fluid removed. If not the semen is likely contaminated with debris and pre-ejaculatory fluid. Was the semen exposed to toxic residues on improperly rinsed collection equipment?
3. Rule out damage introduced during the initial semen handling and processing:
   Was the temperature of the water bath, incubator, extender, microscope slides and stage correct? Are the slides free of residues? (Even “pre-cleaned” slides often have a film on them than can affect sperm motility). Are you using a good quality extender to dilute the semen? Is it mixed properly? Did you check the motility of the raw semen? If it appears to be very active and the extended semen is very poor, you may have an issue with the extender.
   If you suspect an issue with the extender, then it could be a sensitivity to the antibiotics in the extender or the type of extender. We have experienced stallions that have sensitivity to most milk based extenders and some with sensitivity to certain antibiotics. Try extending the semen on the next collection in a variety of different extenders with and without antibiotics and evaluate motility over time. We have also used complex freezing extenders which contain egg yolk and very little milk protein but without the cryoprotectant added to extend and chill semen from certain stallions that would not survive cooling using conventional extenders.

   Once convinced that the poor motility was not induced by the collection process or analysis conditions there are a few diagnostic observations that can be made to try and better understand the cause of the poor motility.
   1. Perform a morphology evaluation to determine if there are a high percentage of morphologically abnormal sperm in the ejaculate that could explain the poor motility.
2. Increase the collection frequency, perhaps even two collections an hour apart. Some stallions seem to accumulate sperm rapidly that deteriorate quickly with residence in the distal reproductive tract and exhibit much better motility when collected more frequently.

3. Some stallion’s sperm are highly sensitive to exposure to their own seminal plasma. Collecting these stallions directly into extender placed inside the collection vessel may improve semen quality. In this situation, the sperm rich jets are diluted in the extender and somewhat protected from the toxic effects of the seminal plasma before the seminal plasma mixes with the sperm. You can also remove most of the seminal plasma by centrifuging the diluted semen, discarding the supernatant and replacing with extender for chilling semen. In cases of extreme seminal plasma toxicity, it may also be beneficial to collect fractionated ejaculates as described above.

4. Perform bacterial cultures on the semen to rule out a heavy bacterial load which can negatively impact sperm quality.

When these diagnostics do not provide an explanation of the poor initial sperm motility we advise the client to re-evaluate in two to three months to rule out a possible effect of season or transient poor semen quality due to some undetected illness or testicular insult that may have temporarily impacted spermatogenesis. If this is not an option, then we will suggest using a sperm selection technique such as single layer colloid centrifugation using EquiPure or Androcoll to separate the normal from abnormal sperm in the ejaculate and process only the enriched population. While this technique will result in an enriched population of good quality sperm, the recovery rate is limited by the number of functionally normal sperm in the ejaculate to begin with.

Observation: good motility but high volume, low concentration ejaculates

This type of ejaculate present a unique set of problems when freezing semen. We always dilute raw semen a minimum of 1:1 for centrifugation and when the sperm concentration is low (say <100 x10⁶/ml), there may only be 2 x10⁹ total sperm in each centrifuge tube. After aspirating the supernatant the volume of the sperm pellet and residual supernatant contributes significantly to the final volume of the extended semen even when the final desired concentration is only 200 x10⁶/ml. The problem is even more pronounced when freezing at higher concentrations such as 400 x 10⁶/ml as is standard in many laboratories. In these cases, adding extender that contains a concentration of cryoprotectant at 3-4% may result in the final extended sperm suspension having a cryoprotectant concentration as low as 1.5-2% which is likely too low to provide optimum protection for the sperm.

This is another reason why a high concentration, low volume ejaculate is desired for semen freezing and managing the stimulation of the stallion and the collection process to minimize seminal plasma is critical. In addition to managing the stallion to minimize gel production as discussed above, voiding the pre-ejaculatory fluid by deflecting the stallion’s penis away from the AV after mounting and before inserting into the AV can significantly reduce the volume of the ejaculate resulting in a higher sperm concentration. Despite these measures some stallions will still produce ejaculates that have very low sperm concentration. In these instances one can adjust the final cryoprotectant concentration in the extended semen by adding supplemental cryoprotectant, either to the extender or to the extended semen after final dilution. This can be very difficult to do because the cryoprotectants are very viscous and difficult to accurately pipette in such small volumes.

Observation: decreased post-centrifugation motility

After centrifugation and resuspension in freezing extender, we remove an aliquot of the extended semen, dilute in the centrifugation extender, incubate 5 min and re-evaluate the motility on CASA. For most stallions, motility after centrifugation is similar to the fresh semen and sometimes better. However, occasionally the post-centrifugation motility is significantly reduced. In this case, first rule out any damage that could be related to improper analysis conditions or semen handling as discussed above. Next, try diluting another aliquot in freezing extender and re-evaluate. If the decrease is not seen when
diluted in freezing extender, there may be a problem related to cryoprotectant efflux from the cells following dilution in a cryoprotectant free extender. Further discussion on this point will follow.

On the next freeze try splitting the semen and diluting in different centrifugation extenders. Keep the original as one of the treatments as a control. If a cushioned centrifugation technique was used and the sperm pellets were difficult to re-suspend, try reducing the g-force or time. You can also try centrifugation without a cushion at lower g-force (300-350 x g, for 10-12 min). This technique will result in lower sperm recovery but may be less damaging to the sperm.

Observation: poor post-thaw motility

If the results of the initial freezing attempt are poor, efforts should be made to adjust the protocol to find a technique that may provide acceptable results for the client. We believe that there is a definite extender/protocol preference between stallions and this is a reason why we always perform split-ejaculate test freezes to evaluate different protocols on the initial attempts for new stallions. Dismissing a stallion as a poor candidate for a frozen semen program based on the negative results of a single test freeze using one standard protocol or extender type will eliminate several stallions that could be used successfully if efforts to customize the protocol had been employed.

Use of extenders containing lower molecular weight cryoprotectants such as ethylene glycol, propylene glycol and amides (methyl formamide and dimethyl formamide) alone or in combination can be beneficial for many stallions. Other variables in extender composition such as the types of sugars used, the source and amount of lipids and protein (milk and egg yolk) and the addition of buffers, chelating agents, and antioxidants can influence results. Also, the optimum rate at which sperm are cooled prior to freezing may be stallion or extender dependent.

One of the most significant stresses to sperm during freezing and thawing occurs after the sperm suspension is thawed. As mentioned above, when thawed sperm are placed in an extender that does not contain any cryoprotectant for post-thaw motility evaluation, the penetrating cryoprotectant diffuses out of the cell at a much slower rate than water moves into the cell to balance the osmotic gradient. This leads to an increase in cell volume and swelling of the plasma membrane that can be very damaging to sperm from some stallions. Differences in membrane composition (particularly the cholesterol to phospholipid ratio) between species and males within species may contribute to the ability of sperm from certain species and individuals to withstand the stresses of cryopreservation better than others. When a significant decrease is seen between pre-freeze and post-centrifugation or post-thaw motility for a stallion with good fresh semen quality, it may be a result of damage from this rapid increase in cell volume.

A way to test this theory is to evaluate motility of sperm diluted after thawing in extender containing cryoprotectant and compare with that of sperm diluted in extender without cryoprotectant. We do this by diluting sperm post-thaw to a concentration of 25 to 30 x10⁶/ml in standard milk based centrifugation extender with or without cryoprotectant at the same concentration as the freezing extender. Because we have also seen instances of stallions whose semen does not survive well during post-thaw incubation in milk based extenders, we will also dilute aliquots of the thawed semen in freezing extender and freezing extender that does not contain cryoprotectant. If the motility is better in the extenders containing cryoprotectant, we suspect a sensitivity to cryoprotectant efflux. If the motility is better in the freezing extenders (with or without cryoprotectant) than in the milk based extenders (with or without cryoprotectant) then we suspect an issue with incubation in milk based extenders and will perform centrifugation and post-thaw motility evaluation using cryoprotectant-free freezing extender.

If the issue is cryoprotectant efflux one can perform a serial dilution of the cryoprotectant by exposing the thawed sperm to extender with increasingly lower concentrations of cryoprotectant resulting in a series of several smaller volume changes over time as opposed to a single large volume change. If this protocol results in a better retention of sperm motility, one can implement a similar post-thaw treatment to sperm prior to insemination since presumably the same issue occurs when thawed sperm are exposed to cryoprotectant-free uterine fluids following insemination.

When discussing the relative “freezability” of individual stallions it is important to consider the decrease in any measure of semen quality relative to the value for the unfrozen fresh sample. All
cryopreservation protocols will damage some sperm from all stallions and the change in quality between pre-freeze and post-thaw samples is a measure of how good sperm from that stallion/ejaculate survived the process. A stallion (stallion A) that has a pre-freeze progressive motility of 70% and a post-thaw progressive motility of 35% is very different than one (stallion B) that had a pre-freeze progressive motility of 45% and a post-thaw value of 35%. Even though the post-thaw motility and the number of progressively motile sperm are the same for each stallion and meet the suggested minimum standards for commercial use, sperm from stallion B appears to be more resistant to damage from cryopreservation than stallion A. Furthermore, one may suspect that because of the significant drop in motility for stallion A, the sperm that remain progressively motile may have also suffered sub-lethal damage that could impact longevity in the female reproductive tract and fertility. This is another reason to strive to obtain the best possible quality for each individual stallion by customizing protocols to minimize the damage to sperm during cryopreservation.

I have referenced motility as the primary assay for measuring sperm quality throughout this manuscript but it is important to remember that motility is only one functional attribute of sperm required for fertilization. However, objective, accurate and repeatable measures of motility still seem to be the best and most practical single assay of relative cell health that can be readily performed on all batches of frozen semen. Additional assays of sperm function such as flow cytometric measures of sperm membrane integrity, mitochondrial function and DNA integrity in combination with motility may provide additional value to determine the potential fertility of a given sample of frozen semen. These assays should be considered for stallions whose fertility with frozen semen is poor even though post-thaw motility is good.

One should also consider whether the defective sperm in the dose are suffering from a defect that is compensable by increasing the total number of sperm in the insemination dose. If the damaged sperm do not compete with the fully functional sperm for fertilization, then acceptable fertility may be achieved by increasing the number of total sperm in the insemination dose. Rectally guided, low dose insemination in situations where the number of functional sperm are reduced may also increase fertility and lastly, the technique of intracytoplasmic injection ICSI provides an option for achieving pregnancies from even the poorest quality frozen semen.