

“IT’S ALL IN THE TIMING” - FRESH-CHILLED VERSUS FROZEN ARTIFICIAL INSEMINATION IN THE BITCH

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Introduction

Great advances in canine assisted reproduction techniques have been achieved during the past 20 years. In addition to natural breeding, artificial inseminations with fresh “neat” (unextended) semen, fresh-chilled extended semen and frozen semen are now viable breeding options. With each technological advance, so too come more manipulations by human hands and an increased risk for human error in semen processing and insemination.

When good quality, fresh semen is used for breeding with either natural mating or “side by side” artificial inseminations, there is much forgiveness with respect to breeding timing. This is because fresh semen remains highly viable and “fertilizable” for 5 to 6 days once deposited *in utero*. Live spermatozoa have been recovered from the uterus up to 11 days post-breeding. In contrast, survival of fresh, “neat” semen in *extrauterine* environments is poor.

Advances in preserving extrauterine longevity of spermatozoa include both reducing energy consumption and increasing energy sources by and for the sperm cells. Lowering the temperature of sperm cells slows metabolism and reduces their energy consumption. Replacing seminal plasma with extender bathes sperm cells in an energy-rich environment. However, even optimally extended and cooled spermatozoa fail to survive nearly as long as their fresh, intrauterine counterparts.

Fresh-chilled sperm cells have a relatively short life span. Fresh-chilled semen, once warmed to body temperature and *in utero*, lives about 24 to 72 hours. Properly frozen sperm cells can be stored **indefinitely** in liquid nitrogen at -322°F (-180°C). Once thawed, however, sperm cells have an ultra-short life span. Frozen-thawed spermatozoa live an average of 12 hours, 24 hours maximum! Curtailed sperm cell longevity necessitates precise ovulation timing and breeding.

Ovulation Timing

Ovulation timing (OVT) is an art as well as a science. Unlike other species in which ovulation occurs in an estrogen environment, ovulation in the canine occurs only after progesterone levels have risen to >4ng/ml. Another unique feature is that the bitch ovulates ova in the primary oocyte stage. These primary oocytes must undergo another meiotic division and further maturation before they can be fertilized. Unlike most species in which breeding/insemination is timed to coincide with ovulation, insemination in the bitch is performed 2 to 4 days after ovulation. It is important to remember that, in the bitch, the critical timing events occur 4 to 6 days *before* the optimal breeding dates.

A number of tools are available for ovulation timing. These include LH (luteinizing hormone) assays, qualitative and quantitative progesterone assays, vaginal epithelial cytology, vaginoscopy, examination of external genitalia and behavioral assessment. Success in breeding is optimized when as many tools as possible are utilized to time ovulation. While ovulation timing constitutes an entire discussion of its own, a brief description of each tool follows.

Estrogen

Estrogen levels rise, peak and begin to decline during proestrus. Under the influence of estrogen, the vulva and vagina become edematous, a serosanguinous vulvar discharge (of uterine origin) is present, the vaginal epithelium becomes thickened and cornified and the bitch exhibits behavioral signs of estrus. However, blood estrogen levels are highly variable, do not accurately predict ovulation and are not useful as a tool for timing ovulation.

LH (Luteinizing Hormone)

Hypophyseal LH is the biological trigger for the cascade of events that culminate in ovulation. Following a variable period (1 to 21 days) of estrogen level elevation, the LH surge signals the transition from proestrus to estrus. LH stimulates follicular granulosa cells to secrete progesterone. The LH surge is the key timing event dictating days of peak fertility. Ovulation begins 2 days post-LH surge and continues for another day or so. Ova mature and are capable of fertilization 2 days later. Mature ova live another 1-3 days. Optimal breeding times are day 4, 5, and 6 post-LH surge.

Pinpointing the day of the LH surge is cumbersome. The LH surge is short-lived, typically lasting only 12 to 24 hours. Therefore, daily blood testing is required. LH is a species specific hormone and assays are not currently available from commercial laboratories in the US. A qualitative (test kit) assay is available for in-hospital use, but it has a short shelf life (3 months maximum). LH assays are most often used in the timing for breedings with frozen semen.

Progesterone

Progesterone's *initial rise* occurs concomitantly with the LH surge. At that time, baseline progesterone levels (<1.5ng/ml) rise to 1.5-2.0 ng/ml. After the initial rise, progesterone continues to rise and may reach levels of 10-15 ng/ml by the end of the fertile period. Ovulation occurs when progesterone levels are between 4 and 10 ng/ml. Plan breedings 4 to 6 days after the initial rise, and 2 to 4 days after the onset of ovulation. Since baseline and initial rise levels can vary from individual to individual, it is important to start testing early enough to define the baseline progesterone level.

The gold standard for determining progesterone levels is quantitative measurement by radioimmunoassay. Results are reported in ng/ml. Progesterone, like all steroid

hormones, is not species-specific and can be measured commercially by human and veterinary laboratories alike.

Several semiquantitative ELISA progesterone kits are available, Status-Pro [Synbiotics], Target [Biometallics], and PreMate [Camelot Farms], for example. Results are interpreted from a color change in the test well or membrane. Hemolyzed blood samples will falsely lower the result. If test kit components are not warmed to room temperature, then a falsely high result will be interpreted. The general consensus is that test kits are usually adequate for most natural breedings, but are not accurate enough when planning a fresh chilled or frozen breeding.

Recommendations for progesterone testing include starting to test around day 5 or 6 from onset of sign of proestrus, followed by testing every other day until ovulation has been confirmed. However, some females have very short seasons and require testing at the first sign of proestrus. Others may not ovulate until after 21 or more days. When in doubt, start testing early.

Evaluation of External Genitalia

During proestrus, the vulva is swollen and a sanguinous vulvar discharge (of uterine origin) is present. As proestrus transitions to estrus, vulvar edema diminishes and the vulvar discharge becomes straw colored. Note however, that some normal bitches may have a hemorrhagic discharge that persists throughout estrus. With the onset of diestrus, vulvar edema subsides completely.

Behavior

The proestrous bitch attracts males but will not stand to be mounted. During estrus, the bitch will stand, “flag” and allow the dog to mount and intromit. With the onset of diestrus, the bitch will refuse to stand and be mounted.

Exfoliative Vaginal Cytology

During early proestrus, estrogen concentrations are low, and cytology specimens show parabasal and intermediate cells, red blood cells, neutrophils and bacteria. As proestrus progresses, estrogen levels rise and the vaginal epithelium thickens. The number of superficial cells increases and the number of red blood cells gradually decreases. By the end of proestrus, more than 80% of the epithelial cells are cornified red blood cells. During estrus, vaginal smears contain >90% cornified superficial epithelial cells, red blood cells are few in number and neutrophils are absent. With the onset of diestrus, vaginal cytology abruptly changes from that seen during estrus to one showing few superficial cells, a predominance of parabasal and intermediate cells and many neutrophils.

Practically, vaginal cytology samples are useful for identifying inflammation in the reproductive tract. If a large number of neutrophils are seen on an estrus smear, then a

vaginal culture and sensitivity may be indicated. Cytology is also useful for differentiating proestrus, estrus and diestrus. It is not useful for determining ovulation date within the estrous period.

Vaginoscopy

Vaginoscopy is a useful tool for estimating the LH surge and optimally fertile period. During proestrus, the vaginal mucosa becomes edematous and has a billowing pillow appearance on vaginoscopy. Around the time of the LH surge, vaginal edema decreases and the vaginal folds become wrinkled or crenulated. Maximal crenulation occurs during the optimal fertile period several days later. As diestrus approaches, the vaginal mucosa takes on a blotchy white and pink appearance.

Tips for Success With Fresh Chilled Semen

Planning the Breeding

1. Don't commit to organizing and performing a breeding if you cannot be available on weekends and holidays.
2. Plan the breeding with young, healthy fertile individuals. Since this is almost always out of the clinician's control, special measures may need to be taken if either the bitch or dog is aged or known to be subfertile. For example, fresh chilled semen of marginal quality may necessitate surgical rather than vaginal insemination to achieve pregnancy.
3. Recommend that the bitch owner always have a Plan B ready to be implemented in the event that the scheduled breeding encounters a "hitch".
4. Recommend that the dog have had a negative test result for Brucella canis within 30 days or since his last natural breeding, whichever is the most recent. Be aware that Brucellosis, often thought to be only a sexually transmitted disease, can also be transmitted by casual contact.
5. Start ovulation timing early, particularly in females who have not had their cycles "tracked" before.
6. If the dog has never been collected and shipped before, a trial collection and "chill check" is advisable at least a week before to the anticipated shipping date. This ensures that he can be collected and that his semen quality is maintained with addition of buffer and chilling.

Semen Collection, Processing and Packaging

1. Schedule collections to minimize the collection to insemination time interval. Shipment usually involves an overnight FedEx service.
2. Use an estrous teaser bitch whenever possible. Spermatozoa number and quality are maximized when a teaser bitch is available. As much as a four-fold increase in spermatozoa number can be realized when using a teaser bitch. General guidelines for adequate semen quality include >70% motility, >70% morphologically normal spermatozoa, few inflammatory cells, and a normal

sperm count. Sperm count should approximate 10 million spermatozoa per pound of body weight.

7. If collected sperm numbers are marginal (<250 million sperm), wait 45 minutes and collect the dog a second time. If the total number of sperm cells remains low, recommend that the dog owner allow you to call the bitch owner to confirm that he or she still wants to have the suboptimal collection shipped.
8. When collecting the dog, collect only the sperm-rich second fraction of the ejaculate. Excess prostatic fluid is deleterious to semen quality. If excess prostatic fluid is present, centrifuge the ejaculate, decant off the supernatant and resuspend the sperm pellet in an appropriate extender. Follow semen processing directions carefully. Many extenders are available, each with its own handling instructions.
9. When extending semen, it is important to know how the insemination will be performed. Surgical insemination requires much less total volume than does vaginal insemination. For routine vaginal insemination, the total volume should be appropriate for the size of the bitch. Generally, 2 to 3 mls is adequate for small breeds, 4 mls for medium breeds, 6 mls for large breeds and up to 10 mls for giant breeds.
10. Send the extended semen sample in a conical shaped centrifuge tube with a screw cap. Make sure the tube does not leak. It is also a good idea to wrap the cap with a layer of parafilm. Placing the tube inside a whirl pack or sealable plastic bag allows for recovery of semen should a leak occur.
11. A number of shipping containers are available commercially, including Equitainers and Camelot Farms shippers. Styrofoam boxes also work adequately in most instances. In Europe, insulated Thermos-type containers are popular. The bagged tube is placed in the box along with 1 or 2 **frozen** gel pack or Kool-It brick. The tube should not be placed in direct contact with the frozen coolant. Make sure that the tube is separated from the brick by at least 4 to 6 layers of paper towel or newspaper. The tube can be wrapped with an insulating material or the tube can be placed in a smaller Styrofoam box. More newspaper (sufficient to fill the dead space) is packed between the brick and the tube. Alternatively, one **refrigerated** brick can be placed between the frozen brick and the tube, still using newspaper to fill dead space.
12. Most fresh chilled semen is shipped via FedEx. UPS can also be used. It is important to make sure that the package can be shipped overnight with an AM delivery the next day. When dealing with weekend inseminations, make sure that package can be delivered on Saturday mornings. There are often special boxes on the shipping form that need to be checked for AM and Saturday delivery.
13. Prior to the 9/11 terrorist attack, **counter to counter** shipment of semen offered a convenient option when weekend inseminations were placed. With a counter to counter shipment, the dog owner typically picked up the package from the collecting veterinarian's office and took it to the nearest airport where it was put on a flight to the airport designated by the bitch owner. Delta Dash and US Air both offer reliable services. The bitch owner would pick up the package from the airport and take it to the inseminating veterinarian's hospital. Since 9/11, counter

to counter shipments are problematic because the sender must be a “known shipper” to be granted shipping privileges by airlines.

14. The collecting veterinarian should call and give the inseminating veterinarian the FedEx or UPS tracking number of the shipment.
15. The cost of semen collection and shipping is typically the bitch owner’s responsibility. It is helpful to get the bitch owner’s credit card information so that services and FedEx fees can be billed directly to them.

Semen Delivery, Handling and Insemination

1. On the day of scheduled semen collection, call the collecting veterinarian’s office and obtain the tracking number for the shipment.
2. When fresh chilled semen package arrives, it should be opened immediately. Attention should be paid to the "impression of coldness." The ice packs should be at least cold, if not still frozen.
3. The tube containing the semen should be removed from the packaging material (usually newsprint). The tube should contain the extended semen in a liquid state. Unfortunately, occasional mishandling by the shipping company or by the shipper placing the semen package in a non-pressurized compartment of the airplane will cause the sample to arrive frozen. The freezing kills the sperm cells and renders the sample useless.
4. One drop of the sample should be placed on a warmed microscope slide. The rest of the sample should be refrigerated. Allowing the chilled sample to warm to room temperature only allows the sperm cells to speed up, using precious energy and shortening their life span.
5. Semen arriving looking “DOA” may still be viable. Some extenders render the sample immotile and additional of an activator buffer is required to restore sperm cell motility. Warming or transferring apparently lifeless semen into fresh extender may bring it back to life. Perform test manipulations on only a drop of semen. As the semen drop warms, side to side motility becomes noted. The continued warming eventually shows the cells to have achieved a normal forward progression. As the drop warms on the slide, the accompanying paperwork with the semen collector's evaluation of the semen quality. Time of collection and post-collection motility should be studied. The semen drop is then analyzed and compared to the collector's evaluation.
6. If no motility is noted after 15 minutes, the sample is most likely non-viable. If this occurs, the collector of the semen should be contacted to determine, if possible, the cause of the semen's demise. In other cases where only partial semen recovery is noted, the inseminator must use judgment based on the concentration of the semen, estimated total spermatozoa numbers and the percent recovered. It may also be necessary to alter the insemination method to that of an intra-uterine deposition of the fresh chilled sperm to further reduce sperm cell stress and to aid its arrival at the fallopian tubes.
7. The refrigerated fresh chilled sample need not be warmed to room temperature or body temperature before insemination. Having the sample in the uterus as it warms makes maximum use of the conserved energy. All fresh chilled semen

- samples are handled in a similar manner, however, many different commercial companies sell packaging kits and extenders. One should always read their instructions for any specific handling recommendations before using the semen.
8. Most often, fresh chilled semen is inseminated vaginally. The pregnancy rate for vaginal inseminations using good quality semen should be at least 80%. Surgical insemination or TCI (transcervical insemination) can be performed to increase pregnancy rate. Deposition of the semen directly into the uterus improves pregnancy rate when chilled semen quality is marginal. Uterine deposition of semen also improves pregnancy rate in very small and giant breeds of dogs, which are reported to have less success with vaginal inseminations.
 9. To perform a vaginal insemination, the pipette is introduced through the dorsal vulvar commissure, directed dorsocranially through the vagina until it enters the pelvic vagina. Then the pipette is directed cranially as far as possible, ideally to the external cervical os. The bitch's hind end is elevated to a 45 degree angle while the semen is injected through the pipette. The dorsal vaginal wall is gently "feathered" for a moment or two while the hind end remains elevated for 10 minutes. Care is taken not to place pressure on the caudal abdomen during this time.
 10. Recent reports indicate that elevating the hind end for longer than one minute offers no advantage over a one minute elevation.
 11. Save the semen tube for DNA verification of paternity should that become necessary.

Tips for Success With Frozen Semen

Planning the Breeding

1. Again, if you agree to plan and perform an insemination with frozen semen, you must be available to perform the insemination should it need to be performed on a weekend or holiday.
2. Insemination with frozen semen necessitates precise timing of ovulation. All available tools should be utilized to pinpoint the period of optimal fertility.
3. In planning a breeding involving international shipment of semen, care must be taken to ensure that all the receiving country and kennel club requirements are met. This often involves several forms, health certificates and blood tests. The addresses and information for a large number of Kennel Clubs world-wide can be found on the website (www.fci.be) of the Fédération Cynologique Internationale.
4. Dog semen is allowed entry into the United States provided it is accompanied by a certificate endorsed by the animal health official in the country of origin. The certificate must certify either that the semen extender does not contain milk products **OR** that if milk products were used, they originated from a country recognized as free of foot-and-mouth disease by the USDA. Requirements for semen import into the United States can be obtained from the United States Drug Administration (USDA) P.O. Box 3220, Minneapolis, MN 55403-1503, USA, website (www.aphis.usda.gov).

5. The AKC requests a prior application to permit AI by imported semen. They also request a DNA sample, which can be ordered via E-mail: dna@akc.org. It is taken with the aid of a special cheek-swab kit supplied by the AKC. The AKC can also be reached at 5580 Centerview Drive, Raleigh, NC 27606-3390, USA. Phone: 919-233-9767 or 919-854-0124; Fax: 919-233-3627 or 919-854-0102; website: www.akc.org.

Semen Collection and Freezing

1. Semen is collected in routine fashion.
2. Semen is frozen. There are many methods of freezing, each with its own set of buffers, cryoprotectants, and freezing protocol. Some methods are proprietary and require franchise purchase. Proprietary agencies include Canine Cryobank, CLONE, International Canine Semen Bank (ICSB), and Synbiotics. Non-proprietary systems, with published protocols, include the Norwegian Tris extender, the Uppsala-Equex extender and its modification, the Uppsala-Equex 2 extender.
3. Semen is frozen in either straw or pellet form. The semen is usually frozen so that the final number of spermatozoa per straw or pellet is between 100 and 200 million. Depending on the semen quality, usually 2-4 straws are used for each AI. In smaller breeds producing fewer spermatozoa per ejaculate it may be desirable to freeze a less concentrated semen to obtain more straws.
4. The semen sender must provide the inseminating practitioner with adequate information about the quality of the semen they send and, in the case of frozen semen, information about how the semen should be thawed, because the method of thawing is dependent on how the freezing was done, and the methods vary among freezing facilities.
5. If, however, the semen is of unsatisfactory quality it should not be shipped unless the bitch owner or importer is informed about the situation and has given consent.

Semen Shipping and Thawing

1. To ship frozen semen a liquid nitrogen container is required. The container must maintain the temperature at around -197°C. Today most semen freezing facilities use dry-shippers, called dewars, which absorb the liquid nitrogen into a porous material in their walls. These will not spill and therefore need not to be shipped as dangerous goods, which is more expensive. They should, however, always be sent as fragile goods, because they are easily broken by rough handling. The tank is usually shipped in a plastic box for protection.
2. If the inseminating veterinarian has a liquid nitrogen storage tank, then the frozen semen can be shipped in a dry shipper or dewar well in advance of its anticipated date of use. This relieves the angst associated with overnight shipments at the last minute. If a storage tank is not available, most dewars can safely store semen for up to 4 to 5 days.

3. When removing semen from the storage tank, check the labeling to ensure that the information on the straw or vial matches that on the accompanying paperwork, and that the paperwork is as expected (correct name, breed, etc. of dog).
4. Because of the many freezing methods employed today, it is critically important to follow the accompanying handling and thawing instructions implicitly.
5. Straws are thawed in a water bath. Common thaw methods include immersion at 70°C for 8 seconds, 50°C for 10 seconds or 37°C for 30 - 60 seconds. Thawing is complete when the bubble rises to the top of the straw. After thawing, any water remaining on the outside of the straw is carefully wiped off before opening the straw. Some methods may direct you to empty straws into pre-warmed thaw medium.
6. Semen frozen in pellets is stored in vials. When removing a vial from the storage tank, care must be taken to carefully loosen the vial cap and let all liquid nitrogen vaporize prior to removing the cap. Pellets are then quickly transferred and sealed in a whirl pack bag which is plunged into a water bath. Pellets are massaged through the bag to aid in the rapid thaw process.
7. Save the straws or whirl pack bag so that cells are available for DNA verification should that become necessary.

Insemination of Frozen Semen

1. Normally, frozen semen is inseminated directly into the uterus. At present, there are three methods for depositing semen directly into the uterus: surgical insemination, blind transcervical passage of a rigid catheter into the uterus, and endoscopic transcervical passage of a catheter into the uterus.
2. Surgical insemination is performed with the bitch under general anesthesia. The uterus is exteriorized through a ventral midline laparotomy approach. The thawed semen, contained in a sterile syringe, is injected into the uterine horn at a 45° angle, with a 22 or 23 gauge needle, bevel up. Half of the volume is injected into each uterine horn. After the needle is withdrawn, a saline-moistened gauze sponge is held over the injection site until hemostasis is achieved.
3. Both Scandinavian and Norwegian rigid catheters are used for blind transcervical insemination. To perform the insemination, the outer sheath is passed as far as possible cranially into the vagina, the internal stainless steel catheter is advanced into the fornix adjacent to the external cervical os, the cervix is palpated through the abdomen, grasped and manipulated onto the end of the catheter, then the catheter is advanced through the cervix. This method requires considerable training, practice and skill. Anesthesia of the bitch is not required.
4. Endoscopic transcervical insemination is referred to as the New Zealand method. The equipment needed includes a 5 mm diameter, 36 cm long rigid endoscope with a 30° viewing angle, a sheath for the catheter, a light source and an optional viewing monitor. An 8 French polypropylene urinary catheter is inserted into the channel on the sheath and the endoscope/sheath/catheter combination is passed as a unit into the cranial vagina. The external cervical os is visualized and passage of the catheter into the cervix can be confirmed. Anesthesia is not required.

5. With both transcervical techniques, semen should not be thawed until correct catheter placement is achieved. These can be lengthy procedures and sometimes catheter passage is not achieved, thereby necessitating a change in plans to surgical insemination.

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