Canine Semen Evaluation

The following interview was originally released as a podcast on September 24, 2015.

In this podcast interview with reproductive specialist Dr. Cheryl Lopate of Reproductive Revolutions and Wilsonville Veterinary Clinic in Wilsonville, Oregon we will be discussing canine semen evaluation. Dr. Lopate received her Master's degree in reproductive physiology and her DVM from The Ohio State University. She completed a residency in comparative theriogenology (reproduction) at Purdue University and has been board certified in Theriogenology since 1997. She has worked in a variety of practice settings including general mixed practice, referral practice and academia. She believes strongly in providing client education and speaks at breed group meetings regularly. She also speaks at many veterinary conferences and has written many journal articles and book chapters on a variety of reproductive topics. Welcome Dr. Lopate.

AKC Canine Health Foundation (CHF): What are the reasons that a semen evaluation might be requested?

Dr. Cheryl Lopate (Lopate): There are many reasons that we receive requests for semen evaluation – the most common reasons are for young males to assess fertility prior to breeding; any dog with a breeding imminent that hasn't been used in the last month or two with a confirmed pregnancy; after a dog misses with one or more bitches or goes from having normal sized litters to small litters; prior to an upcoming chilled semen breeding; or prior to freezing semen.

CHF: What is involved in a routine semen evaluation?

Lopate: It is very important to do a complete semen evaluation anytime one is requested. Gross assessment or eyeballing of sperm numbers, motility or morphology from a wet mount smear can be tremendously misleading. For example, sperm can be very motile, yet be abnormal in shape or function, making the dog infertile. Further, proper motility evaluation is crucial because if the ejaculate is very concentrated, normal sperm will push the dead ones around making them appear motile when they are not. We can talk about each of the components later in our talk today.

So each semen evaluation should include assessment of the male's libido and the ejaculatory process; a semen volume, motility assessment (both total and progressive), sperm count, and a stained morphology (assessment of how normal the individual sperm cells are).

CHF: Can you discuss each of these in more detail? What does each test tell us about the male's fertility and the quality of the semen?

Lopate: First - Libido and ejaculatory process - We always recommend having a bitch in season in front of a male we are collecting because this will provide us with the most representative ejaculate we can get. Many dogs that have high libido or are used to being collected may be able to ejaculate without a teaser, but there is no doubt that sperm numbers will be highest if he is stimulated with an estrus bitch prior to ejaculation. If an estrus bitch is not available, swabs or pads, stored in the freezer, can be used

to provide scent for a non-estrus bitch and she can just stand in front of the male to provide a visual cue. There are some estrus pheromones available as well, but they are not as good as the real thing.
We want to assess how easily he was to stimulate to erection, whether the erection was normal (remained engorged the entire time with no waxing or waning of the erection while actively being collected), and whether all 3 fractions of the ejaculate were produced and were normal in gross appearance – that is, no blood or urine in the ejaculate. This gives us some assessment of whether he will be able to successfully cover a bitch naturally.

Collection may be performed using a collection sleeve (disposable or disinfected latex rubber), funnel system, or even a clean zip lock baggie if nothing else is available. Care should be taken if one is not using a soft collection sleeve to not damage the penis on the edges of the collection device during thrusting. If the dog has long hair, it may be necessary to clip the hairs along the edge of the prepuce to prevent trauma to the penis or contamination of the sample with urine or smegma. If the dog has a large amount of smegma at the end of the prepuce, this should be wiped away with a paper towel prior to collection. Further, if one finds many WBC in the ejaculate, it is important to determine if these came from the interior genitourinary tract versus contamination from smegma. It may take another collection several days later, with inspection of the prepuce carefully prior to collection to make this determination. Since some dogs do not clean themselves very well, there can be significant accumulation of WBC that are not pathologic and these should not be confused with infection resulting in the dog being treated unnecessarily.

As the dog is collected, the first few jets of ejaculate should be allowed to drip onto the floor – this first fraction is produced by the prostate and the urethral glands as a method to clear the urethra and distal prepuce of urine, cellular debris, dead sperm, and WBC. It also serves as lubrication during a natural breeding to facilitate intromission. We do not want to contaminate the ejaculate we evaluate with this fluid. This first fraction is produced until the dog obtains a full erection. There may be 2-8 ml or more of fraction 1.

The second fraction, the sperm-rich fraction, comes next and originates from the epididymis (the storage site for sperm lying on the top of the testicle). The second fraction is usually emitted once there is full swelling of the bulbus and thrusting motion ceases. Often the dog will step his leg over the collector's arm just before fraction 2 is emitted. This fraction is generally quite small, usually ½ - 2 ml. Some collector's will try to fractionate the ejaculate and so will capture fraction 1 in a separate tube from fraction 2 and another for fraction 3. This usually requires having an assistant available or a rack to quickly swap the collection vessels. The benefit of fractionation is that if there is contamination in the ejaculate with urine or inflammatory cells, the source of the fraction that they derived is more easily detectable, allowing for localization of diagnostics and treatments. If semen is to frozen or shipped chilled, the prostatic fluid components are removed prior to cooling and fractionation decreases the amount of centrifugation that the sperm will have to undergo and this may reduce damage to some sperm cells.

The third fraction is the largest in volume and derives from the prostate only. Its purpose is to flush the urethra of all sperm that have been ejaculated, to provide a medium for sperm to swim in and to fill the vagina with fluid during a natural breeding to facilitate the sperm reaching their destination at the cervix. The vagina of the bitch is VERY long and the end of the penis is normally a few to several inches away from the cervix, so the volume of the third fraction is important to make sure there is enough fluid

for sperm to be able to reach the front, or proximal , end of the vaginal canal, called the fornix. If a dog has an outside tie, there may not be enough fraction 3 emitted to facilitate the sperm's journey. During ejaculation of fraction 3, active pulsations can be seen around the dog's anus and along the perineum (the area between the anus and the scrotum).

Loss of erection, called detumescence, occurs after the collector releases the penis behind the bulbus and can occur rapidly or may take many minutes to occur. Typically, fraction 3 is emitted as long as there is an erection present. The presence of prostate disease may be discovered during production of the 3rd fraction, and may be seen either as a pinkish; blood-red; or coffee – ground brown color fluid. Presence of any abnormal color of fraction 3 should instigate further evaluation of the prostate gland. The source of any fresh blood collected with the ejaculate must be differentiated from damage to a vessel on the outside of the penis from hair, hand, or the collection vessel vs. from the prostate or less commonly the urethra.

Once the ejaculate is collected, a volume should be obtained using a syringe with no rubber stopper as the latex in the stopper can be spermicidal. The volume collected has no impact on the quality of the ejaculate. Many breeders are disappointed when only a milliliter of ejaculate is collected – but they shouldn't be, because all the volume is used for during semen evaluation is as a multiplier to determine the total sperm in the ejaculate. Volumes from <0.5 ml to 80+ ml may be collected depending on how long the collector holds onto the dog's penis maintaining the erection and continues to collect the 3rd fraction.

An accurate semen evaluation can be performed with minimal equipment. A collection device, a slide warmer, microscope slide, cover slips, pipettes or syringes without latex, a slide warmer, a counting device (discussed later) and some type of morphology stain. Once the semen is collected it may be allowed to sit at room temperature, but everything that contacts the sperm should be warmed to body temperature (37C). All glassware (slides, coverslips, pipettes) should be warmed before use. Having a warm microscope stage is ideal but if it is not available having a slide warmer or using a warm bag of IV fluids for your slide warmer, should be used to frequently rewarm the sample during evaluation, particularly of motility.

Next is motility. Motility is evaluated as total and progressive. Total motility is the number of sperm moving; while progressive motility is the number of sperm moving in a straight line across the field of view in the microscope. Initially, motility may be assessed at low power, but before a final determination is made, higher power magnification should be used. If one cannot see individual sperm movement clearly, the sample should be diluted (often just a drop of semen and a small volume of extender is used), allowed to equilibrate and then be re-evaluated. This is one of the most common mistakes made by evaluators. It is VERY easy to overestimate sperm motility due to high concentration. Use of more sophisticated microscopes (phase contrast or differential interference microscopy) will improve accuracy as well but may not be available everywhere. There are very few dogs that will have a progressive motility > 85%, yet it is very common to see interpretations of motility being >90%. This most likely indicates a lack of evaluation of individual sperm movement. The reason there are so many sperm produced in an ejaculate is that some will not be normal and that some will not arrive at their destination (either due to abnormality, lack of energy, or poor directional movement). So it is not realistic to believe that most dogs would have progressive motility > 90%. The more accurate we are with semen evaluation, the better we can determine the potential fertility of the ejaculate.

In addition to the number of sperm moving, the speed at which they move is also assessed – this is called velocity of movement. A grading scale of 0 – 5 is typically used, with 0 being dead sperm and 5 being those that are moving as fast as is possible across the field. Most dogs are in the 3-4 range. Velocity needs to be assessed after the sperm are re-warmed to body temperature.

Recently, a new device has been introduced into commercial practice. It is called a CASA machine (computer assisted sperm analysis) and some reproductive practices will have them. The CASA machine was first produced to provide objective data on sperm motility for research purposes, but recently the price of the equipment has been decreasing making it more reasonable for some practices to incorporate into their evaluations. There is a special cassette that is loaded with a specific volume of fluid and the sperm's movement is recorded and analyzed. Because the computer can analyze all dimensions of sperm head movement (forward, reverse or sideways movement), much more accurately than the naked eye, it can provide a more accurate assessment of both total and progressive motility. It should be noted that experienced clinicians, with a properly diluted sample, may be able to be fairly close to the results of the CASA machine, but this does require significant practice and taking the time to properly dilute, equilibrate, warm and then evaluate the sample. We'll talk more about CASA machines as we continue.

The next step is to determine a sperm concentration per milliliter. There are numerous methods used to make this determination. The gold standard of concentration determination is use of a device called a hemocytometer. This is a cassette that contains 2 small identical counting grids, a channel for sperm to be applied to the chamber and special cover slip that provides specific weight to be applied to the fluid. This allows a very specific amount of fluid to be contained under the coverslip. The grids have scored lines and the number of sperm within a specific number of squares is counted. Since there is an exact volume of fluid each time the hemocytometer is loaded, the concentration can be determined quite accurately. The hemocytometer must be loaded correctly to obtain accurate. A specific amount of ejaculate (typically 200 microliters) is diluted into a specific volume of solution (typically 2 ml) that kills the sperm to immobilize them so they can be counted. There are commercial diluents available or they can be made in-house depending on the practice. The hemocytometer can be used with raw or extended semen, and both very concentrated and very dilute samples can be counted with accuracy. The presence of cellular debris (epithelial cells or WBC) can be easily differentiated from sperm, and thus does not interfere with the count.

Other counting devices include densimeters, which measure the density of a fluid. These must be used with raw semen only because extenders will have components which add to their optical density, thus affecting the sperm count (falsely elevating it). Blood or pus will also falsely elevate sperm counts with densimeters. These devices can provide very inaccurate results, so require the operator to evaluate the sample and make sure that the count provided appears accurate. If there is significant cellular debris or the semen has been extended, a different counting method is needed.

Two other devices have been introduced into commercial practice to provide concentration. The first is the CASA machine and the second is a machine called the nucleocounter. The CASA machine must be used with raw semen – it is not accurate with extended semen, and the ejaculate must not be too concentrated or the count will be inaccurate. One of the most critical factors associated with accuracy on the sperm count is how the machine is set up in terms of what it is measuring. The computer is looking for cells with a specific dimension that have been programmed into the computer. The head size

and shape of sperm from every species is different, so the machine must be calibrated for canine sperm before use. We often allow for some variation in head size or shape in the computer's analysis, and this means that other cells that may fit into this size range and may also be counted as sperm cells depending on how the practice has the machine set up. The machine will not necessarily differentiate a sperm from an epithelial cell, WBC or RBC or from debris, if it fits the size parameters programmed into the machine. For this reason, we find that counts on CASA machines are not as accurate as those taken by the hemocytometer method.

The nucleocounter is a machine that evaluates the nucleus of the cells it evaluates. There is a specific amount of DNA within a sperm head (the sperm head is all DNA, while most other cells have a smaller amount of DNA and other cellular components surrounding the nucleus). The sperm are stained with a fluorescent dye to make the nuclear material stand out, thereby allowing a more accurate count. Another benefit of the nucleocounter is that it can be used on raw or extended samples with accuracy and on samples that are contaminated by RBC (no DNA in a RBC), WBC (different shape and size nuclei) or epithelial cells (different shape and size nuclei) because of the fluorescent stain used to highlight the DNA of the sperm head. It is likely that the nucleocounter will soon replace the hemacytometer as the gold standard for sperm counting.

Once the concentration per milliliter is established, the total sperm/ejaculate is calculated by multiplying the concentration/ml by the volume. This number is recorded.

The last step of the standard semen evaluation is evaluation of sperm morphology or cell shape. In order to evaluate morphology, the sperm need to be stained and evaluated at very high magnification or special microscopes can be used with unstained but dead sperm (phase contrast or differential interference contrast). There are numerous stains that can be used, but the 2 most common are eosin-nigrosin and Wright-Giemsa. The eosin-nigrosin stain would be the preferred one of the 2. This stain not only used for morphology but is also considered a vital stain. If it is applied correctly to the sperm, it can be used not only to evaluate sperm morphology but also viability. Sperm that are alive when the stain is mixed will allow no stain to diffuse across their membranes, so they appear white under the microscope; while sperm that are dead when the stain is mixed will allow a pink dye to cross the cell membrane, so they appear pink under the microscope.

Eosin-nigrosin stain will allow for evaluation of sperm head size and shape and duplication; some, but not all, abnormalities of the DNA may be visualized; the midpiece, or motor apparatus of the sperm, can be evaluated for normal thickness, length and shape, attachment, and the presence of cytoplasmic droplets; and the sperm tail, or propeller, can be assessed for shape and duplication. This stain provides a dark background and lighter staining sperm, allowing for ease of visualization. Round cells in the ejaculate (WBC or germ cells) cannot be differentiated with this stain.

While eosin-nigrosin is probably preferred, practices that don't do a lot of reproduction may not have it and may use Wright-Giemsa stain instead. This stain is used for almost all general cytology evaluation in most veterinary practices so is almost always on hand. This stain doesn't allow for good assessment of nuclear defects, the acrosomes, and some midpieces defects (like presence of cytoplasmic droplets) are not detected at all – so will not provide as accurate an evaluation as eosin-nigrosin. It also has a very light background making details of sperm shape sometimes harder to detect. It can be used to evaluate the origin of round cells in the ejaculate. There are other stains available that some practices may have

and use that provide some details that eosin-nigrosin won't, and so some practices may do additional staining if there is a fertility issue that is not detectable by normal staining – we will not go into detail on these stains during this podcast.

Accurate morphologic assessment takes considerable practice and requires the operator to take the time to evaluate each sperm individually. It is a critical part of the semen evaluation, because motile sperm may not be normal, so without this piece of the puzzle, a fertility issue may go undetected.

CHF: So once the standard semen evaluation is performed, how do we know if the dog is considered a satisfactory potential breeder?

Lopate: Accepted normal parameters in dogs are as follows:

Motility: Greater than 70% progressive motility, with a velocity of at least 3/5
Total sperm/ejaculate: A bare minimum of 10 million/sperm/pound bodyweight (i.e. a 30# dog will have at least 300 million sperm). Most normal dogs exceed these numbers by 2-3x or more.
Morphology: Greater than 70% morphologically normal sperm

CHF: How long is a semen evaluation good for? Or, how quickly can semen quality change?

Lopate: Semen evaluation is a like a snapshot in time. Because sperm production is a never ending process and can be interrupted at any stage of development by scores of factors, like temperature, physical stress or illness, travel, age, cancer, infection, just to name a few, semen quality has the potential to change significantly within just a day or two of an insult to the testicles. For this reason it is always a good idea to have a semen evaluation performed close to or at the time of breeding, to ensure semen quality is adequate for normal pregnancy rates. If semen quality is found to be low, a different sire may be substituted in time for the breeding to occur.

CHF: Is there any bloodwork that is recommended at the time of a semen evaluation?

Lopate: Brucellosis testing is recommended either every 2 months for males being bred frequently or within 2 months of breeding for less frequently used males. Many reproductive clinics run brucellosis tests in-house so they only take a few minutes to run and can be performed immediately prior to semen collection.

CHF: How should semen be evaluated if the intention is to ship chilled semen someplace?

Lopate: If we are doing a semen evaluation prior to a chilled semen breeding, a small aliquot of semen should be diluted with extender in a very dilute manner (25-100 million/ml) and another sample may be tested at a more concentrated dilution (200 – 300 million/ml). Using 2 dilutions allows the collector to determine if the particular dog's sperm metabolism is too high to ship in a concentrated manner. If the semen stores better more dilutely a small aliquot of fresh extender can be shipped along with the semen for the recipient to use after re-centrifugation. When prepping semen for longevity exam, the semen should be centrifuged and all the prostatic fluid removed prior to extension, to mimic what will be done when the semen is actually shipped. The semen should be stored in the refrigerator for a minimum of 96 hours, but some will continue to evaluate until the motility drops below 30%

progressively motile, which may be 7 or more days for some dogs. This longevity exam not only provides the bitch owner and their veterinarian with a sense of how well the dog chills, which helps them formulate a breeding management plan, but also ensures that the dog will chill well in the extender used. Occasionally, a dog will chill poorly in a particular extender, and finding this out before the semen is shipped for the breeding is preferable to the finding out after the semen is shipped.

CHF: What is the difference between a semen evaluation done as a pre-breeding or fertility exam vs. that done after semen is frozen?

Lopate: When semen is frozen, a full semen evaluation should be performed prior to freezing. If semen quality is poor it should not be frozen. Once the semen is frozen, a small aliquot should be thawed for post thaw analysis. This ensures that an appropriate breeding dose is being shipped to the bitch owner. Some dogs freeze better than others and so sometimes the drop in motility after a freeze is much higher than expected resulting in the need to adjust what makes up a breeding dose (either adding more pellets or more straws). Minimally, a post-thaw motility examination (total and progressive, with velocity) should be evaluated on each ejaculate frozen. Even if a dog is collected several days in a row, the freeze quality may vary over time, so every single freeze should have a post-thaw evaluation. Some additional testing may also be performed on post thaw samples including HOST testing (hypo-osmotic swelling test) which evaluates membrane damage during the freeze; acrosome staining to assess hyperactivation or damage to the acrosomal cap during freezing, or viability testing (using the nucleocounter or other fluorescent staining techniques) to determine how many sperm survived the freeze process.

CHF: What if everything is normal on a semen evaluation yet the male is still having fertility issues? Is there anything else that can be done?

Lopate: There are advanced diagnostic tests that can be used in cases where semen evaluation appears normal but a dog still has poor fertility: 1) sperm chromatin structure analysis to look at how the DNA is packaged in the sperm head; 2) acrosomal staining for integrity of the enzyme cap needed to digest the egg membrane so the sperm can enter it to fertilize it; 3) viability testing to determine if cells are surviving cooling or freezing; 4) HOST testing to assess sperm membrane integrity; and 5) electron microscopy to look for defects in the sperms structure that are not visible with a standard light microscope.

CHF: How often should a semen evaluation be performed on an intact male?

Lopate: Generally speaking, semen evaluation should be performed at least once annually for any breeding male, along with a brucellosis test. In some cases evaluation will be performed just prior to an anticipated breeding to make sure the dog is acceptable for breeding. They should be performed at the time of every semen shipment or freezing. They also may be performed because the dog has a fertility issue or is showing signs of possible prostate or reproductive tract disease.