Abstract
The need for improvement in pregnancy rates and litter sizes has resulted in ever evolving theriogenology techniques. Over the past two decades, artificial insemination in the canine has progressed immensely. This reproductive technique has allowed increased genetic diversity and international breeding in multiple species. Another important advantage of artificial insemination is reduced disease transmission among breeding animals. Chilled transported semen is typically cooled to four to five degrees Celsius and can be conserved for up to 120 hours. A number of studies have evaluated various canine semen extenders and cooling techniques, with no single extender or technique found to be clearly superior. Methods for assessment of spermatozoa motility and fertility parameters include manual evaluation with a standard light microscope, various staining techniques, and computer assisted evaluation. These techniques vary in the degree of spermatozoa manipulation, technician experience required, and expense incurred.

Keywords: Canine, semen, semen extenders, shipping containers, CASA

Introduction
The initial description of the spermatozoa was provided by Leeuwenhoek in 1679. Approximately 100 years later, the first artificial insemination in the canine species was performed by abbé Lazarro Spallanzani in Italy. It was not until the early 1950’s that artificial insemination in the dog using cooled extended semen resulted in a successful pregnancy. The technique of artificial insemination with cryopreserved semen was not allowed by the American Kennel club until 1982. Interestingly, the registration of offspring by extended cooled semen was not recognized until 1986.

Pre-collection evaluation
Initial reproductive assessment of the canine patient should always begin with a thorough historical examination. The animal’s age is important due to associated increased incidence of reproductive problems in the aged dog and transient infertility in the prepubertal dog. Rijsselaere, et al. reported that age was significantly correlated with the percentage of normal spermatozoa but did not influence motility characteristics. The reproductive history of the male, including the number of bitches bred, conception rate, litter size, and date of last collection or breeding, can assist with establishing the presence and duration of a reproductive problem. Serial breeding soundness examinations can also be useful for determination of changes in fertility and semen characteristics. If available, the breeding history of the dog’s sire, dam, and littermates should also be evaluated.

It is also important to discuss the animal’s previous medical history, attempting to identify any problems which could negatively influence fertility. Environmental, toxic, or physical insults can cause abnormal spermatozoal production for up to three months, potentially resulting in infertility during this time. Hereditable conditions such as hip dysplasia and degenerative myelopathy should also be investigated through assessment of the animal’s pedigree and screening tests. Brucella canis testing and all routine vaccinations should be current in any breeding canine.

Physical examination of the male should be performed prior to collection. Thorough evaluation of the external genitalia and the prostate gland should be conducted. A rectal examination should always be performed to ensure the prostate gland is symmetrical in size and no pain is elicited upon digital palpation. Any abnormalities noted should be further investigated as they could suggest prostatic pathology such as benign prostatic hypertrophy, prostatic neoplasia, or prostatitis. Ultrasonography of the scrotum is beneficial for evaluation of the internal architecture of the testicles and epididymis. The testicular and epididymal texture and total scrotal width should be noted. Abnormalities such as adhesions, non-uniformity in size, fibrosis, and softness should also be recorded and investigated further.

Semen collection
It is important to note the date of the last ejaculation. If ejaculation has not occurred within the past ten days, secondary abnormalities associated with sperm cell aging and delayed spermatozoal transport, such as distal cytoplasmic droplets, could be observed in the first ejaculate. Frequently, a second collection following 30 minutes of sexual rest can result in a reduction in the number of these observed defects.
Prior to semen collection all equipment should be properly prepared. Microscope supplies, pipettes, and collection vessels should be warmed to 37 degrees Celsius to prevent excessive temperature fluctuations during semen handling. Rapid temperature changes can result in a loss of sperm motility and fertilizing ability. If lubricants are needed, small amounts of non-spermicidal lubricants or petroleum jelly are best due to the toxic effects of water soluble lubricants on spermatozoa. The extender should be prepared for use according to manufacturer recommendations and the collection vessel properly assembled prior to beginning the collection.

Digital manipulation is the most widely used method for semen collection in the dog and involves a collection vessel with or without a collection cone. The collection cone is usually composed of a sterilized plastic funnel-shaped sleeve that is attached to the collection vessel. The cone is occasionally not used because it has been associated with increased ejaculate contamination and increased difficulty of semen fractionation.

The canine artificial vagina is comprised of a rubber cone with a collection vessel attached to the funneled end. In one study, the rubber liner used in artificial vaginas resulted in deleterious effects on canine spermatozoa. However, this study did provide data suggesting decreased toxic effects with repeated washing of the liner. Electroejaculation is another collection method but is not commonly used in the canine patient due to the necessity of anesthesia for this procedure.

Urination should be prevented immediately prior to beginning ejaculation to avoid urine residue in the urethra. The animal should be restrained by a leash in a slip-free area that is void of distractions such as other animals and people. Although not necessary for males accustomed to manual stimulation, the presence of an estral bitch can assist with the collection process. Responsiveness to manipulation and a higher total number of spermatozoa in the ejaculate are commonly found when a teaser bitch is used. If an estral bitch is unavailable, cotton tipped swabs which have been previously collected and frozen from a Brucella canis negative estral bitch can be used for olfactory stimulation.

The libido of the canine should be assessed as the collector begins to digitally manipulate the caudal aspect of the bulbus glandis. Pelvic thrusting normally begins quickly following manipulation. The collector should ensure that the preputial sheath is retracted fully caudal to the bulbus glandis prior to complete engorgement of the penis. If this is not possible, the collection process should be halted, the dog removed from the area until full detumescence is observed, and semen collection attempted again.

With retraction of the prepuce caudal to the bulbus glandis, the collector should slide the collection device over the penis, ensuring that there is no contact of the glans penis to the collection receptacle. A steady, firm pressure should be held caudal to the engorged bulbus glandis for the remainder of the collection. Allowing the male to step over the collector’s arm or beginning collection with the penis directed caudally between the dog’s rear legs enables a more normal ejaculation by stimulating a tie. If reluctance to ejaculate is encountered, the thumb of the hand holding the collection vial can be introduced into the fossa of the glans and pulsatile pressure applied to the urethral process against the wall of the vial. To prevent injury and drying of the penile tissues, it is important to ensure full detumescence and full retraction of the penis into the preputial sheath following collection.

The ejaculate should be observed as it is deposited into the receptacle to determine the color and amount of each fraction. The first fraction, originating from the prostate gland, varies in volume, but is normally one to five milliliters. This fraction is clear to slightly opaque. The second fraction, also known as the sperm rich fraction, is between one to three milliliters and is cloudy-white in color. The third fraction, which is also prostatic in origin, is from two to 40 milliliters in volume and can be easily distinguished from the second fraction by its clearness. During discharge of the third fraction of the ejaculate, pulsations of the urethral sphincter can be observed by palpation of the penile shaft. It is important to note that in the canine the volume of the ejaculate does not correlate with the quality of the semen.

Depending on which extender is used or the purpose of the ejaculate, the three fractions may need to be separated during the collection process. It has been shown that the first and third ejaculate fractions are detrimental to spermatozoal motility and morphology during long term storage. As a result, some practitioners prefer to only extend and ship the sperm rich, second fraction of the ejaculate. Fractionation of the ejaculate can be accomplished by forgoing usage of a collection cone and swapping pre-warmed collection vessels between the ejaculate fractions as they are collected. Separating the first and second fractions without partial mixing can be difficult, so these two fractions are often combined.

If there is uncertainty of ejaculation of the sperm rich fraction, alkaline phosphatase levels can be evaluated on the sample collected. This enzyme is produced in the epididymis and elevated levels in the sample validate presence of the second fraction. Seminal plasma alkaline phosphatase values suggestive of presence of the sperm rich fraction of the ejaculate are greater than 10,000 units per liter or a total measurement of greater than 30,000 units.11
Semen evaluation

A thorough semen evaluation includes assessment of the ejaculate volume, color, motility, concentration, and morphology. There are various procedures available to evaluate membrane integrity which can be associated with fertility. Ensuring collection equipment is clean, preventing heat and cold shock, and minimizing exposure to toxic insults will help ensure the findings of all examinations are reliable.

Color

Discoloration of the ejaculate is commonly associated with pathology. However, it is important to remember that color evaluation is a very subjective test. Semen with a yellowish hue may indicate urine contamination. An increased amount of white blood cells in the ejaculate can also result in semen with a slightly yellow appearance. A red to brown discoloration represents blood in the ejaculate which can be associated with prostatic disease or trauma prior to or during collection.

Motility

Motility, as an expression of viability and structural integrity, is one of the most important characteristics associated with the fertilizing ability of sperm. The motility of the ejaculate should be observed as soon after collection as possible. The most commonly used method of motility analysis involves manual evaluation using a conventional light microscope at 100 to 200 times magnification. This is performed by placing a drop of semen on a microscopic slide and applying a warmed coverslip. Another commonly utilized method of motility assessment is the computer assisted semen analyzer (CASA). This equipment will be discussed in detail later in this manuscript.

To subjectively assess sperm motility, total and progressive motility should be evaluated by observing at least five fields within five minutes of placing the ejaculate on a warmed slide. Normal motility for canine patients is greater than 70 percent for both total and progressive motility. Progressive motility should be interpreted as spermatozoa that are moving forward in the field with speed. The percentage of progressively motile sperm has been positively correlated with the percentage of morphologically normal spermatozoa in dogs. Mickelsen, et al. reported that canine pregnancy rates are significantly influenced by the total number of progressively motile or morphologically normal spermatozoa per ejaculate.

Concentration

There is little value in attempting to use concentration alone as a correlation to semen quality. The concentration of spermatozoa produced in each ejaculate varies greatly among dogs due to age, genetics, and environmental factors. Also, the total number of spermatozoa in an ejaculate is decreased with frequent semen collection. Normal concentration of canine semen also depends on the weight of the dog and the breed, with a range of four to 400 million sperm per milliliter of ejaculate. Mixed breeds tend to have higher sperm concentration.

The recommended insemination dose for fresh or chilled semen is 150 to 200 million progressively motile spermatozoa. With advances in artificial insemination procedures, the amount of spermatozoa inseminated can be decreased to as low as 10 million progressively motile fresh spermatozoa with acceptable pregnancy rates. Due to the amplification of knowledge in artificial insemination in the canine, a single ejaculate can be used to inseminate multiple bitches with good pregnancy results.

Determination of spermatozoal concentration can be performed using optical density measurement, manual calculation using a hemacytometer, or CASA equipment. The hemacytometer technique is considered the gold standard. Each side of the hemacytometer should be evaluated to ensure that a concentration with less than ten percent variation is observed. Accurate calculation of concentration using the CASA system is a problem in every species. In the canine, the CASA system has been shown to overestimate concentration by 1.7 times. Thus, the author does not recommend the use of the CASA system for determination of semen concentration in the dog.

Morphology

Morphology is a vital part of a complete semen evaluation. Oettlé reported that less than 60 percent normal morphology can adversely affect fertility. Age, environment, and physical injury can result in a decrease in morphologically normal spermatozoa. The standard canine morphology guidelines allow for no less than 70 percent normal spermatozoa.

Although phase contrast microscopy is considered the gold standard, staining procedures with evaluation under light microscopy is the most commonly used method for morphology assessment. Stains that are customarily used for morphological examination are eosin-nigrosin and modified Wright’s Giemsa. Eosin-nigrosin slides are prepared by mixing one drop of semen with one drop of stain and utilizing a “pusher” slide to spread the mixture.
into a thin film. Spermatozoa that are stained with eosin-nigrosin appear white against a dark purple to black background. This stain is considered a vital stain due to the uptake of eosin by spermatozoa with non-intact or damaged membranes and these cells appear pink in color. Modified Wright’s Giemsa stained slides are prepared by placing one drop of the semen sample on a slide which is then spread into a thin film. The sample slide is allowed to air dry and is then immersed into each of the three sequential stains for five minutes before rinsing with water. Wright’s Giemsa stained spermatozoa are purple to pink in color on a white background. Acrosomal defects cannot be visualized using the modified Wright’s Giemsa stain. A major benefit of using the modified Wright’s Giemsa stain is that this staining procedure allows the differentiation of round cells, unlike when using eosin-nigrosin staining.

Examination for morphological characteristics is performed by observing the prepared slide under 1000 times magnification. Evaluation of at least 100 cells should be performed and the sperm should be divided into normal, primary abnormality, and secondary abnormality categories. Primary abnormalities are associated with spermatogenesis and include abnormalities such as proximal cytoplasmic droplets, bent midpieces, and defects involving the head of the spermatozoa. Secondary abnormalities occur as a result of faulty maturation or problems during epididymal transport. Such defects include, but are not limited to, distal cytoplasmic droplets, retroflexed tails, and detached heads.

Hypo-osmotic swelling test

Integrity of the plasmalemma has been associated with spermatozoal viability; thus, the hypo-osmotic swelling test is one method available to evaluate membrane integrity. An intact plasma membrane is important for sperm metabolism and changes in membrane properties that must occur for capacitation and the acrosome reaction to progress normally.20

The hypo-osmotic swelling test is a simple and inexpensive procedure. Various solutions including sucrose, fructose, and sodium citrate are utilized for this assay. Fructose is the most commonly used hypo-osmotic solution at a concentration ranging from 60 to 200 milliosmoles. One tenth of a milliliter of the semen sample is combined with one milliliter of the preferred hypo-osmotic solution. This mixture is incubated from one to 60 minutes at 37 degrees Celsius and is then evaluated under light microscopy or phase contrast microscopy. At least 100 sperm cells should be assessed for reaction to the hypo-osmotic media.

The spermatozoa respond to the hypo-osmotic solution by transporting water across the plasmalemma, resulting in swelling and curling if the membrane is intact during incubation. The sperm tail is particularly susceptible to changes in osmotic pressure; thus, most spermatozoa observed with a positive reaction to the hypo-osmotic swelling test reveal variations of tail curling. This affirmative reaction can range from curling of only the tip of the tail to a tightly coiled tail and suggests that the spermatozoal plasma membrane is intact or undamaged. Various studies have reported positive correlation between the hypo-osmotic swelling test response and sperm motility and viability in canines.21,22 The hypo-osmotic swelling test has also been used effectively by Kumi-Diaka to observe subfertility in the canine patient.20,23

Computer assisted semen analysis

Computer assisted semen analysis was first described in the canine by Günzel-Apel, et al. in 1993.24 With conventional microscopic analysis, variation in motility parameters of the same ejaculate can differ due to the subjective nature of this method. The usage of CASA systems provides an objective assessment of semen parameters by rapidly and accurately determining sperm motility and morphology. Although this equipment has great advantages, few practitioners have access to this method of analysis due to the cost and maintenance of the CASA system.

The CASA system produces sperm analysis results in the form of tables, graphs, and digital images of sperm cell tracks. This system can detect more subtle motion changes than conventional semen analysis. Computer assisted semen analysis also allows assessment of velocity parameters, linearity, straightness,beat cross frequency, and amplitude of lateral head displacement. As discussed previously, determination of semen concentration using CASA is not reliable.

The CASA system can evaluate semen parameters in any species, including humans, but the standardized system settings for each species should always be used. Several authors have reported high correlation between CASA results and conventional microscopic evaluation.19,24 The accuracy of the resulting data depends greatly on the training and familiarity of the individual processing the samples. Another important factor in assessment of sperm motility parameters is the temperature of the sample at the time of evaluation. Iguer-Ouada and Verstegen revealed that motility parameters were decreased at 30 degrees Celsius, which corresponds with semen temperature
after ejaculation. At 38 degrees Celsius, correlating with physiologic uterine temperature, motility parameters were more optimal.

Dilution of the sample to approximately 50 million cells per milliliter is preferred to ensure adequate evaluation of individual spermatozoa and their motility and velocity components. Assessment of at least 200 cells reduces variability and misinterpretation of results. Semen should not be extended in whole milk or non-clarified egg yolk extenders when using CASA. These extenders contain particles approximately the same size as spermatozoal heads and can result in inaccurate motility assessment. If these extenders must be used, DNA staining procedures can assist with differentiation between live sperm cells and inorganic particles.

**Semen extenders**

Canine artificial insemination with adequate numbers of freshly collected sperm can result in fertility rates equal to those obtainable with natural service. Pinto, et al. reported that pregnancy rates and litter sizes were not different when dogs were bred with fresh or chilled extended semen that was stored for up to 48 hours. The major objective of semen extension is conservation of spermatozoal motility and fertility during temperature changes and stress during shipment. Verstegen, et al. observed the ability to conserve chilled canine semen for up to 27 days, with addition of new semen extender at days 11, 21, and 27. Spermatozoal motility was noted for up to 16 days with no exchange of semen extender. In that study, motility was not significantly different than initial values up to day ten of evaluation and fertility was preserved for up to 11 days.

Semen extenders provide energy, stabilize the pH and osmolarity, preserve cellular integrity during cooling of the sample, and can include antibiotics to assist with prevention of bacterial growth during transport or storage. There is a variety of commercially available semen extenders, including egg yolk and milk based solutions. The addition of egg yolk to extenders has a protective effect by preserving motility parameters and is commonly added to extenders for semen cryopreservation. The major effect of glucose and fructose in semen extenders is to support spermatozoal motility and movement patterns during storage. Antibiotics are especially important when using an egg yolk based extender due to the increased risk of bacterial growth. The two most frequently used antibiotics in semen extenders are amikacin and gentamicin which provide protection against gram negative bacterial growth. In 1992, Bjurström and Linde-Forsberg reported that the most common organisms cultured from preputial samples were *Pasteurella multocida*, β-hemolytic streptococci, and *Escherichia coli*. They also stated that the most commonly cultured bacteria from semen were *Pasteurella multocida* and β-hemolytic streptococci.

Extender preparation varies due to manufacturer and extender type. Some commercially available extenders should be warmed to room temperature prior to combining with the ejaculate, versus 37 degrees Celsius as recommended by other semen extender manufacturers. Some manufacturers recommend extending only the second ejaculate fraction to achieve maximal fertility and motility throughout storage or shipment. The need for equilibration is another component of the extension process that varies depending on the semen extender type and manufacturer. Homemade extenders can also be used but require increased preparation time and there is variable consistency between preparations. Thus, commercially available extenders with less strict instructions on preparation, collection, and equilibration could be considered more efficient and user friendly.

Most commercially available extenders suggest a dilution ratio of at least one part semen to four parts extender for maximal motility and fertility during cooled shipment or storage. Depending on the concentration of the ejaculate, centrifugation and re-extension of the sample might be necessary prior to packaging. This should be decided on an individual case basis as the need arises. It is important to ensure the insemination volume is between five and ten milliliters for vaginal artificial insemination and approximately two milliliters for intrauterine insemination. This volume can be adjusted in relation to breed and size of the bitch.

It is beneficial to evaluate the semen of a new patient in different extenders and shipment containers to determine which technique or product is best for that particular patient or the situation of insemination or shipment. This is especially important in animals with known subfertility or infertility.

**Semen shipment**

Semen transport for insemination purposes has become very common over the past two decades due to convenient overnight delivery services. Artificial insemination allows national and international breeding with chilled or cryopreserved shipped semen. Studies have previously found pregnancy rates of approximately 80 percent with natural matings, good quality fresh, chilled extended semen, or combinations of the above.

Extended semen should be placed in plastic syringes or other plastic or glass vessels with secure lids for storage. A study performed by England and Allen revealed toxic effects of disposable plastic syringe components, particularly rubber plungers. Due to this finding, washing syringes or using syringes without rubber components are common practices when storing or transporting semen.
A study by Michael, et al. revealed that during shipment, semen quality always deteriorated in a gradual, constant, and expected manner. Chilling the sample during storage lowers the metabolic rate, resulting in increased spermatozoal longevity. Sperm quality and longevity following storage can be affected by the temperature to which the semen is cooled. Semen being transported for insemination purposes should be chilled to four to five degrees Celsius, as determined by various studies throughout the last decade. Canine semen stored at four degrees Celsius has a significantly longer life span than semen stored at 22 degrees Celsius. Semen parameters are also maintained for longer periods when stored at 22 degrees Celsius than at 37 degrees Celsius.

The amount of time the sample is stored prior to insemination is also an important factor in semen quality following storage. Although a variety of investigators have found viability of spermatozoa for approximately four to five days, semen can retain good quality for at least 24 hours under ideal packaging and shipment conditions. Due to this finding, most shipped semen is used within the first 48 hours of storage.

The maintenance of semen quality throughout shipment or storage is also affected by the shipment container employed. There are various commercially available semen transport containers, including the vacuum flask (Thermos®, Thermos LLC, Rolling Meadows, IL), extruded polystyrene foam (Styrofoam®, Dow Chemical, Midland, MI) box, and plastic box, such as the Equitainer® (Hamilton Research, Inc, South Hamilton, MA). Although reuse is possible with proper care, both the vacuum flask and extruded polystyrene foam box are considered single use shipment containers. Shipment using the vacuum flask is inexpensive due to the low purchase cost and the small size of the container. Vacuum flasks have no defined cooling rates or temperature holding ability because of variability in models and sample preparation. The extruded polystyrene foam box is fairly inexpensive and can be purchased from various companies. The Equitainer® is the only semen shipment container available that is marketed for reuse. This container is associated with increased initial purchase expense and shipping costs due to the increased weight.

A single shipment container has not been proven as the gold standard for maintenance of semen quality following storage. A study performed by Lopes, et al. demonstrated advantages of the Equitainer® over the extruded polystyrene foam box and the vacuum flask when transporting chilled canine semen for more than 48 hours. A higher percentage of progressive motility was observed when using the Equitainer® in this study. Another report evaluating the Equitainer® and a low cost extruded polystyrene foam box using equine semen revealed that the low cost system was satisfactory for cooling and preserving equine semen for up to 48 hours of storage.

Semen transport is a very common procedure in canine theriogenology. There are various commercially available and homemade semen extenders that allow preservation of semen during transport or storage. Semen shipment containers available for chilled transport include the standard vacuum flask, various extruded polystyrene foam boxes, and the Equitainer®. Although previous studies have found one extender or shipment container to be superior, without uniformity in the resulting data, an ultimate semen extender and shipment container have not been defined.

Conclusion

Semen transport is a very common procedure in canine theriogenology. There are various commercially available and homemade semen extenders that allow preservation of semen during transport or storage. Semen shipment containers available for chilled transport include the standard vacuum flask, various extruded polystyrene foam boxes, and the Equitainer®. Although previous studies have found one extender or shipment container to be superior, without uniformity in the resulting data, an ultimate semen extender and shipment container have not been defined.
Although the best evaluation of semen extenders and shipping techniques is conception rates after well-timed matings, the prolonged interretrial interval of the canine patient prevents substantial fertility research in this species. The various components of standard semen evaluation can assist in determining the fertility potential of breeding canines. Although computer assisted semen analysis is an expensive procedure and requires a skilled operator, utilization of this objective assessment of motility and morphology is becoming a mainstay for semen evaluation in theriogenology. This system coupled with inexpensive and simple microscopic evaluation can result in a thorough evaluation of semen quality in the canine patient.

References