

The value of canine semen evaluation for practitioners

M.V. Root Kustritz *

University of Minnesota, College of Veterinary Medicine, 1352 Boyd Avenue, St. Paul, MN 55108, United States

Abstract

Compared to human medicine, little is known in canine medicine regarding specific findings on semen evaluation and their correlation with fertility. Suggestions to optimize quality of semen evaluation in veterinary practice include creating standardized protocols for evaluation of all semen parameters and updating those protocols as needed; creating some form of quality control for the clinic laboratory; educating owners about our inability to predict with 100% accuracy whether dogs with poor semen quality never could impregnate a bitch or whether dogs with excellent semen quality always could impregnate a bitch; and generating protocols for further diagnostic work-up for those dogs with abnormal semen quality.

© 2007 Elsevier Inc. All rights reserved.

Keywords: Semen evaluation; Spermatozoa; Breeding soundness; Dog; Canine

1. What do the numbers mean?

Veterinary practitioners may perform semen analysis as part of a complete breeding soundness examination, to evaluate suitability of semen for AI, preservation by chilling or freezing, or to investigate subfertility or infertility. Unfortunately, there is a paucity of data associating the parameters measured during semen evaluation with the issues veterinarians really need to evaluate, i.e. testicular function, fertilizing capability of spermatozoa, and likelihood that pups will develop normally [1,2]. One author suggested that semen analysis only can be reliably predictive of fertility if the semen quality is either very good or very bad [3].

Ejaculated spermatozoa examined *in vitro* do not exhibit the characteristics they will take on as they traverse the reproductive tract of the female after insemination. For a spermatozoon to fulfill its role, it must develop properly in the testis, undergo maturation in the epididymis, pass through the cervix and uterus,

aided by uterine contractions, undergo capacitation and the acrosome reaction as it passes from the seminal fluid into secretions from the female reproductive tract, and bind to the epithelium of the uterus or distal uterine tube before detaching at the correct time, moving into the uterine tube, penetrating the cumulus cells surrounding the ovum, and binding to the zona pellucida [4–6]. Results of tests that may be performed in a semen evaluation are influenced by sample collection technique and timing, concentration of spermatozoa in the sample, amount of time from sample collection to evaluation, temperature at which the sample was held, equipment used, and many other factors [7–9].

This paper is a review of tests commonly performed as part of a semen evaluation in dogs, with comparison to what is done in human medicine. Furthermore, there will be recommendations regarding interpretation of results (relating semen quality to ability to impregnate females).

2. Collection and evaluation of semen

Collection of canine semen by manual ejaculation will not be described. The reader is asked to review

* Tel.: +1 612 624 7290; fax: +1 612 624 7290.

E-mail address: rootk001@umn.edu.

pertinent literature regarding appropriate equipment, technique, and environment to enhance collection of semen from dogs. Although it is generally accepted that a dog should not be condemned on the results of one semen evaluation, there are no published guidelines for timing of semen collection relative to sexual activity, as there are in humans. The World Health Organization, to promote standardization and quality control in human andrology laboratories, produces a regularly updated handbook detailing semen evaluation techniques [9]. In humans, it is recommended that semen be collected when the man has had sexual rest for at least 2 but no more than 7 days, and that two separate samples, collected 7–21 days apart, be evaluated before any recommendations are made.

3. Volume

Dog semen is ejaculated in three fractions. The first (pre-sperm) fraction is small in volume and contains few to no spermatozoa. The second (sperm-rich) fraction comes from the epididymes and testes. The third (prostatic) fraction consists solely of prostatic fluid and also contains few to no spermatozoa. The volumes of the first and third fractions, especially the latter, are variable, and the volume of the third fraction is controlled by the person collecting the sample, as they choose to collect more or less of the cell-free prostatic fluid. Volume is not an indicator of semen quality in dogs. However, the volume measurement is part of the calculation of total number of spermatozoa in the sample, which is one indicator of semen quality. Note the volume collected before removing samples from the collection vessel.

4. Color

Color evaluation is subjective but may guide the clinician toward other tests to be performed. A clear sample contains no spermatozoa. Cloudy or milky samples probably contain spermatozoa but always should be checked microscopically; occasionally, a dog with azoospermia will shed excessive numbers of fat droplets into the sample, giving the appearance of normal semen. Yellow semen is indicative of urine contamination and is also seen in humans with icterus or after ingestion of certain vitamins. Brown discoloration is indicative of old digested blood and red discoloration is indicative of fresh blood. The most common causes of hemospermia in dogs are prostatic disease and penile trauma. In humans, hemospermia also may be associated with urinary calculi, sexually transmitted

diseases such as syphilis and gonorrhea, spermatocele and hydrocele, and treatment with anticoagulant medications [10].

5. pH

Controversy exists regarding the value of measurement of pH in canine semen. Reported values for normal pH in non-fractionated canine semen vary from 6.4 to 6.8 [11–13]. One author recommended that evaluation of pH be performed immediately after collection using accurate equipment (presumably a pH meter) and strongly discouraged use of a “dipstick” method [14]. In humans, it is recommended that pH be evaluated within an hour of semen collection and that pH paper be used [9]. The author is unaware of published data comparing pH analysis techniques for canine semen or specifically describing the clinical value of pH measurement.

6. Motility

For canine semen, motility is better maintained if samples are kept at room temperature than at body temperature [11,14]. Quick temperature fluctuations should be avoided. Room temperature may play a factor; one report of canine semen evaluation in a tropical region suggested that lack of motility in some samples was due to lack of air conditioning in the room where the sample was collected [12]. Percentage progressively motile spermatozoa from a given dog is not affected by frequency of semen collection [15,16]. Age may be a factor in motility; in humans, motility has been documented to decline at rate of 0.27%/year in men > 45 years old [17].

To evaluate motility, place a drop of semen on a clean glass slide. In human andrology laboratories, 10 μ L of semen is pipetted onto a clean glass slide with a positive displacement pipette and the drop covered with a 22 mm $\times$ 22 mm coverslip. This consistently spreads the sample to a depth of about 20 μ m [9]. Under 100–200 $\times$ magnification, view at least five fields to classify at least 200 spermatozoa. Subjectively assess percentage of spermatozoa moving forward and total motility. In veterinary medicine, normal percentage progressively motile spermatozoa is 70% or greater. Speed or quality of motility also may be assessed; a canine spermatozoon with normal motility should traverse the microscopic field of view in 2–3 s [14].

Percentage progressively motile spermatozoa is positively correlated with percentage morphologically normal spermatozoa in dogs [18,19]. Morphologically

normal spermatozoa that are immotile may have been collected or evaluated using contaminated equipment, may have undergone too rapid or extreme temperature fluctuations, or, rarely, may have abnormal flagella.

In human andrology laboratories, motility of individual spermatozoa is graded as A (=rapid progressive motility), B (slow or sluggish motility), C (non-progressive motility), and D (complete lack of motility). A second sample of 200 spermatozoa is evaluated and percentage from each motility grade compared between the two specimens. If the difference is greater than expected by statistical random variation, two additional slides are evaluated from that semen sample [9].

Computer-based automated semen analysis (CASA) systems also have been described for assessment of motility in dogs [19–26]. Few veterinary practitioners have such systems available to them. Use of such systems allows andrologists to evaluate many more motility parameters than those described above, including curvilinear velocity, straight-line velocity, and amplitude of lateral head displacement. Such parameters can be incorporated into detailed mathematical formulas to produce factors such as a Semen Quality Score [19] or Sperm Motility Index [23]. The author is unaware of any published reports associating such values with fertility in dogs. Another potential value of these sperm motility factors is recognition of sub-populations of spermatozoa within the sample that may react differently to cryopreservation or other manipulations of spermatozoa [21,22]. Clinical relevance of these sub-populations in canine theriogenology has not yet been described.

Subjective analysis of percentage progressively motile spermatozoa has been demonstrated to be well correlated with computer-based analysis [20,26]. In one study, correlation between subjective analysis and analysis with a CASA system was very high ($r = 0.924$), with least consistency in the mid-range of 34–57% [26].

7. Morphology

Percentage morphologically normal spermatozoa (MNS) is not altered in dogs with frequent semen collection [16]. Age may have a role; in humans, percentage MNS declines after 45 years of age [17].

Preparation of the morphology slide and staining technique artifactually alter fertility [27–31]. One study compared preparation of slides by placing a drop of semen on a glass slide, laying another slide directly on top of it, and pulling the two slides apart, as in a

squash prep; putting a drop of semen on a slide and using a “pusher” slide at a 45° angle as for preparation of a blood smear; distributing a drop of semen over a slide using a camel’s hair brush; and pouring diluted semen over a slide and allowing it to dry. In that study, the first two techniques were determined to be superior, with the third associated with a greater percentage of spermatozoa with detached heads and the fourth associated with increased percentage of abnormal spermatozoa of various types [31]. Compared to evaluation using phase contrast microscopy, staining techniques may increase acrosome defects [28], or defects of the head, midpiece, and tail [30]. This may be due to alterations in osmolarity or pH of the stain used [29]. Although phase contrast microscopy is the gold standard, the lack of availability of phase contrast microscopes in practice requires staining and examination of morphology of canine spermatozoa using light microscopy. Serial evaluations should be performed using a consistent technique. The author prepares slides as for a blood smear and after drying, immerses the slides in fixative, safranin, and crystal violet, in that order, for 5 min each (modified Giemsa stain, DiftQuik™; Baxter Healthcare, Miami FL, USA).

In human medicine, recommended preparation of slides for morphology assessment includes cleaning of glass slides with 70% ethanol, application of either 5 µL of semen (for samples with concentration greater than 20 million/mL) or 10–20 µL (for samples with concentration less than 20 million/mL), and preparation as for a squash preparation. Spermatozoa should be evaluated under oil immersion. Precision is increased as a higher number of spermatozoa are assessed [32]; the recommended minimum number in human medicine is 200 spermatozoa (on two separate slides). The sum and difference for the two values of percentage MNS are compared to determine if re-evaluation is required to enhance accuracy [9]. Spermatozoa in the middle third of the slide are least likely to be altered by preparation technique [28].

Morphologic abnormalities can be classified as primary (occurring during spermatogenesis) or secondary (occurring during maturation or sample preparation), or as major or minor [33]. The author is uncomfortable with the latter nomenclature because it implies scientific knowledge of effect of morphologic defects on fertility in dogs. Some andrologists may wish to identify specific abnormalities rather than broad classes. This may be advantageous when attempting to identify morphologic abnormalities that may be hereditary, such as knobbed acrosomes, round heads,

the “Dag” defect of the midpiece, and the “tail stump” defect [34,35].

Morphology scores described in human medicine include the teratozoospermic index (TZI) and sperm deformity index (SDI). The TZI is the total number of morphologic defects divided by the total number of defective spermatozoa. This varies from 1 to 3; values above 1.6 are associated with lower pregnancy rates [36]. The SDI is the total number of morphologic defects divided by the total number of spermatozoa; values above 1.6 are associated with failure of IVF [37].

The most important parameter identified is percentage MNS. Percentage MNS has been demonstrated not be influenced by investigator regardless of staining preparation technique used [30]. In dogs, percentage MNS below which fertility is affected is 60% [38]. CASA systems have been described for assessment of percentage MNS in dogs. Positive correlation between subjective evaluation and CASA systems is high, reported as $r = 0.82$ in one study [39].

8. Concentration and total number

Concentration is the parameter measured when performing semen evaluation in dogs but has little value as an indicator of semen quality. Concentration is inversely related to volume collected [40]. Concentration multiplied by volume is the total number of spermatozoa in the ejaculate; total number of spermatozoa is dependent on testicular size [41]. In dogs, normal total number of spermatozoa is greater than 300 million.

Total number of spermatozoa decreases with frequent semen collection, presumably as epididymal reserves are depleted [15,16]. In humans, concentration and total number decrease with age, declining 2.1%/year after 45 years of age [17]. Normal dogs may ejaculate oligozoospermic or azoospermic samples due to apprehension or pain [3]. In human medicine, apparently azoospermic samples are centrifuged at greater than $3000 \times g$ for 15 min and all the fluid systemically examined before that sample is declared completely deficient of spermatozoa [9].

Both CASA systems and optical density measurement have been described for assessment of concentration of spermatozoa in dogs [20,42–44]. The traditional technique for spermatozoal concentration assessment is use of a hemacytometer. The hemacytometer technique has been reported to be equally accurate or more accurate than CASA systems and is considered the gold standard.

To determine concentration of canine spermatozoa using a hemacytometer, semen is drawn up into the 20 μL pipette of the Unopette WBC system (Becton-Dickinson, Rutherford, NJ, USA) and dispensed into the diluent chamber. The coverslip specific to that hemacytometer should be used. The edges of the coverslip are lightly moistened and the coverslip pressed to the hemacytometer firmly, until iridescence is seen. Diluted semen is dispensed under the coverslip by capillary action. Allow spermatozoa to settle for about 5 min before counting under light microscopy [9]. Two techniques are described for assessment of concentration. In the first, all spermatozoa visible in one of the large nine squares on the hemacytometer grid are counted; that number is the concentration. A second technique requires counting of all spermatozoa visible in three of the nine squares. The total in all three squares is designated as S. Concentration equals the quantity 3S multiplied by 0.1 added to 3S with that quantity divided by 10 [24].

Other techniques reported for concentration evaluation include use of standardized photographs and spermatocrit. In the former technique, bull and ram semen samples with known concentrations of 100, 200, 400, 800, and 1500 million spermatozoa/mL were diffused into a 10 μm chamber and photographed to create standardized concentration distributions. Semen of unknown concentration was placed in the same 10 μm chamber and concentration evaluated by comparison with the standardized photographs, with estimation if the observed sample fell between two of the standardized photographs. This was compared to measurement of concentration by optical density. Difference between the two techniques averaged less than 15.2% [45]. Spermatocrit is determination of concentration by evaluation of percentage solids when semen is centrifuged in a hematocrit tube; this technique is not accurate in dogs [46].

9. Miscellaneous tests

None of the techniques described below are useful for semen evaluation by the average practitioner, with the possible exception of assessment for other cell types and the hypo-osmotic swelling test. Some, such as use of centrifugation gradients, can be employed to enhance quality of semen before chilling or freezing.

10. Assessment for other cell types

Other cell types that may be present in semen include prostatic or urethral epithelial cells, immature germ

cells, red blood cells, and inflammatory cells. Quantification of epithelial cells may be accomplished by determining number of the cells of interest (N) per 100 spermatozoa. Concentration of the cell of interest in millions/mL equals the number of the cells of interest times concentration of spermatozoa in that sample divided by 100 [9].

As many as 2000 white blood cells/ μ L may be present in the first and second fractions of the ejaculate in normal male dogs [47]. Presence of inflammatory cells is not well correlated with lack of significant bacterial growth from semen samples in dogs; 44% of dogs with no inflammatory cells in their semen had significant aerobic bacterial growth from that semen in one study [48].

11. Live-dead staining

Live-dead staining relies on variable appearance of spermatozoa that take up stain. It is assumed that spermatozoa that take up stain have damaged plasma membranes and are therefore designated as non-functional, or dead. Eosin-nigrosin stains are those most commonly described. Problems with this test include inability to consistently classify spermatozoa with partial staining and interference with staining if glycerol or fat globules are present in the seminal fluid [49]. The author is unaware of published reports documenting correlations between live-dead staining and fertility in dogs.

12. Hypo-osmotic swelling (HOS) test

The hypo-osmotic swelling test involves submersion of spermatozoa into a hypo-osmotic medium. Those spermatozoa that have intact plasma membranes will swell as fluid moves into the sperm cell; this will cause swelling and coiling of the tail [3]. Hypo-osmolar solutions described include sodium citrate (7.35 g) and fructose (13.51 g) in 1000 mL of distilled water, and 100 mM sucrose solution [50,51]. Incubation time before examination varies. In one study, maximum percentage swollen spermatozoa was observed after 45–60 min of incubation of spermatozoa at 37 °C in the hypo-osmotic solution; in another study, there was no difference in percentage of swollen spermatozoa between those samples incubated for <1 min and those incubated for 60 min [51,52].

In human medicine, the HOS test only is performed on semen samples containing fewer than 50% motile spermatozoa [9]. Remember to evaluate the percentage of spermatozoa with coiled tails before performing the

HOS test; the initial value must be subtracted from the percentage of spermatozoa with coiled tails after incubation to get the true percentage of spermatozoa with presumed intact plasma membranes as determined by this test [9]. Studies differ regarding correlation of percentage spermatozoa with coiled tails in the HOS test with motility and morphology in dogs [50,53,54]. The author is unaware of published reports correlating results of the HOS test with fertility in dogs.

13. Measurement of components of seminal fluid

The components of seminal fluid reported as measured as a component of semen evaluation in dogs include glandular products, proteins, and electrolytes [1,55–59]. Components may break down at variable rates after semen collection due to metabolism by spermatozoa and enzyme degradation, and may vary with interval from previous ejaculation, degree of sexual excitement, and health status of accessory sex glands [1].

Heparin-binding proteins have been identified in semen of several species including bulls and dogs [57–59]. These proteins may be correlated with fertility, perhaps at the level of the acrosome [57,59]. Identification of these proteins in canine seminal fluid has been published recently; work regarding correlation with fertility is ongoing.

14. Filters, centrifugation gradients, fluorescent staining, and DNA analysis

Various filters have been described for use in evaluation and improvement of canine semen quality [60–62]. Filters may be used to assess for normal function of spermatozoa by evaluating motility via determination of extent to which spermatozoa can penetrate the filter, or by binding abnormal spermatozoa. While percentage MNS may be much better in semen after filtration, total number of spermatozoa present in the filtrate may be much lower than that in the original sample. In one study evaluating semen from four dogs before and after passage through a glass-wool filter, average concentration of spermatozoa in the unfiltered samples as 445.7 million/mL and average concentration of spermatozoa in the filtered samples was 53.3 million/mL [62].

Centrifugation gradients are media layered in differing concentrations in a test tube over which a layer of semen is laid and the sample centrifuged [61,63–65]. Abnormal spermatozoa and other cells are bound while normal spermatozoa move through and

accumulate as a pellet in the bottom of the tube. Canine spermatozoa have been demonstrated to be not damaged by centrifugation [63]. These techniques greatly improve percentage MNS in the centrifuged samples, with type of morphologic defect most improved variable by product. Digested and fresh blood also may be removed using some systems [65].

Fluorescent staining is used to evaluate integrity of the plasma membrane and capacitation status in canine spermatozoa [49]. A variety of stains can be used simultaneously and the cells sorted by flow cytometry. This technique is not commonly available to practitioners.

Analysis of DNA structure can be used to try to predict future fertility; in humans, reduced evidence of DNA denaturation in spermatozoa was associated with higher probability of pregnancy maintenance [66]. A sperm chromatin structural assay has been described for use in dogs; one study suggested that some morphometric parameters assessed using a computer-based system could be associated with DNA denaturation in canine semen [66,67].

15. Quality control

Standardization and quality control within laboratories enhances accuracy of analysis. Standardization among laboratories better allows researchers and practitioners to compare laboratory practices and to determine if evidence in the veterinary literature from different laboratories is comparable. Commercial veterinary laboratories work hard to ensure continuing quality control. Individual practices rarely address this issue.

Types of error that may occur include random error, which are chance differences in the sample or its assessment that may be identified by repeating the test, and systematic error (i.e. a bias such that change occurs in one direction only and cannot be detected by repeating the test). Semen analysis is subject to a large amount of error because relatively few cells are being evaluated in any given analysis. As an example, if 100 spermatozoa are counted in an assessment of concentration of spermatozoa, the standard error of the count is 10%, with a 95% confidence interval such that the number of spermatozoa that may be reported varies from 80 to 120 spermatozoa. If 200 spermatozoa are counted, standard error of the count at the 95% confidence interval declines to 7%, such that the reported number varies from 172 to 228. To get to 2.5% error with a 95% confidence interval, one would need to count 1600 spermatozoa; at some point, enhanced

accuracy of the count is offset by technician fatigue [9]. Additional random errors include loss of a portion of the sample in collection or assessment equipment, poorly mixed samples, technician stress or lack of adequate training, poor technique, and worn or poorly calibrated equipment [1,9]. Although few practitioners have the desire or ability to perform the statistical tests required to document quality control in the laboratory, requiring proper training of all individuals involved in semen analysis, including proper techniques and use of equipment, can easily be incorporated into the policies and procedures for that facility. The more rigid guidelines described as used in human andrology laboratories may be appropriate for use in small animal theriogenology but no research exists at this writing to support or refute that notion.

16. Interpretation of findings

Values determined in semen analysis by any means, computer-aided or more subjective, are meaningless unless taken in context. Factors that must be considered include the signalment of the animal; inbred status of the animal; time since semen was last collected; and perhaps season of the year. With this background knowledge, conclusions may be drawn regarding the likelihood of a dog being able to impregnate females.

Age was associated with fertility in humans; semen quality declined after 45 years of age [17]. There certainly are reports of men siring children when much older than this, but they are the exception rather than the rule and their success may not have been predicted based on semen analysis alone. Two studies by the same investigators identified 6 years of age as the cut-off after which there was a decline in sperm output, percentage motile spermatozoa, and percentage MNS in Dalmatians and Rottweilers [68,69]. Breed also is a factor in canine semen analysis, since total number of spermatozoa in the ejaculate is based on testicular size. The author is unaware of published breed-specific guidelines for total number of spermatozoa in the ejaculate in dogs.

Inbred status of the dog may be responsible for variations in semen quality. In a study evaluating 14 outbred and four inbred male foxhounds bred to 544 outbred and 51 inbred bitches, sperm count, motility, conception rate, and number of pups born alive was decreased in males with inbreeding coefficients of 0.125–0.558 [70].

The owner should be asked how long prior to the evaluation the dog was used for natural service or had semen collected by manual ejaculation. Ejaculation

more frequently than every 48 h is associated with a decline in sperm output as epididymal reserves are depleted [71,72]. However, more frequent semen collection also may be associated with increased percentage motility and percentage MNS [73]. Another aspect of time is recognition that semen quality varies over time in dogs; in one study in which semen was collected from six young adult dogs monthly for 6 months, there was considerable monthly variation in total number of spermatozoa, percentage motile spermatozoa and percentage MNS [74]. Finally, after prolonged sexual rest, there may be a concern that the ejaculate will contain a greater percentage of morphologically abnormal spermatozoa due to presence of aged spermatozoa from epididymal storage. This has not been rigorously evaluated in dogs and is not supported by research evaluating 6 weeks sexual rest [71].

Season of the year has been documented as a possible factor in several studies. Work done in Japan and England demonstrated best semen quality with highest sperm count in the spring, and an increase in percentage abnormal morphology in the summer [75–77]. Region of the world has not been demonstrated to play a role with semen collection in a tropical country, similar to reports of semen quality from temperate regions [78].

Comparison of values assessed during semen evaluation to recognized normal values in dogs must be tempered with the above factors to determine whether or not a male dog may be considered likely to be able to achieve pregnancy. Insemination dose reported in dogs for fresh semen is 220–250 morphologically normal spermatozoa per ejaculate [79,80]. Number of morphologically normal spermatozoa is calculated as total number of spermatozoa in the ejaculate multiplied by percentage MNS. Recognition of this valuable number, rather than strict attention to individual parameters, permits use of dogs with low percentage MNS if they have a very high total number of spermatozoa in the ejaculate. However, in one study of 28 dogs with average total number of spermatozoa in the ejaculate of 332.8 million (range 36–630 million), average percentage motile spermatozoa of 89.5% (range 65–95%), and average percentage MNS of 78.2% (range 62–90%), all of the dogs used for breeding ($n = 25$) successfully sired litters, despite many of these dogs having semen quality well below what is considered normal in our testing scheme [2].

The author suggests the following:

- Create standardized protocols for evaluation of semen volume, semen color, percentage progressively motile

spermatozoa, percentage MNS, and concentration/total number of spermatozoa in your practice's laboratory, based on the human and veterinary literature and updated accordingly. Consider some form of quality control for your laboratory outside of repeating tests on individual dogs. For example, keeping a laboratory notebook in which all values are recorded may identify variation in technique of individuals in the laboratory or trends in values suggesting abnormalities of reagents or aging of equipment.

- Acquaint owners with the recommended insemination dose of 220–250 million normal spermatozoa per ejaculate and do the mathematical calculations necessary to determine if the dog under investigation can achieve that number. Educate owners about our inability to predict with 100% accuracy whether dogs with poor semen quality never could impregnate a bitch or whether dogs with excellent semen quality always could impregnate a bitch.
- If abnormalities are identified, consider factors that may have altered semen quality and collect another sample after an appropriate interval of time or further diagnostic testing.

References

- [1] Amann RP. A critical review of methods for evaluation of spermatogenesis from seminal characteristics. *J Androl* 1981;2:37–58.
- [2] England GCW, Allen WE. Semen characteristics and fertility in dogs. *Vet Rec* 1989;125:399.
- [3] Martinez ALP. Canine fresh and cryopreserved semen evaluation. *Anim Reprod Sci* 2004;82–83:209–24.
- [4] Verstegen JP, Iguer-Ouada M. Oestrus cycle stage dependent effects of male and female blood plasma and vaginal fluid on dog semen motility parameters. *Theriogenology* 2005;64:810 [Abstract].
- [5] England GCW, Burgess CM, Freeman SL, et al. Relationship between the fertile period and sperm transport in the bitch. *Theriogenology* 2006;66:1410–8.
- [6] Irvine DS. Tests of sperm function and male infertility. *Res Reprod* 1986;18:2–3.
- [7] Rui H, Morkas L, Purvis K. Time- and temperature-related alterations in seminal plasma constituents after ejaculation. *Int J Androl* 1986;9:195–200.
- [8] Verstegen J, Jolin E, Phillips T, Onclin K. Evaluation of the “Minitube SpermVision Computer-Based Automated System” for dog semen analysis. *Theriogenology* 2006;66:671 [Abstract].
- [9] World Health Organization (WHO). WHO laboratory manual for the examination of human semen and sperm-cervical mucus interaction. New York: Cambridge University Press; 1999.
- [10] Leary FJ. Clinical significance of hematospermia. *Mayo Clin Proc* 1974;49:815–7.
- [11] Bartlett DJ. Studies on dog semen. I. Morphological characteristics. *J Reprod Fertil* 1962;3:173–89.

- [12] Chatterjee SN, Sharma RN, Kar AB. Semen characteristics of normal and vasectomized dogs. *Indian J Exp Biol* 1976;14:411–4.
- [13] Daiwadnya CB, Huker VB, Sonawane SA. Studies on evaluation of dog semen. *Livest Advisor* 1995;20:34–7.
- [14] Threlfall WR. Semen collection and evaluation. In: Root Kustritz MV, editor. *The practical veterinarian: small animal theriogenology*. St. Louis: Butterworth-Heinemann; 2003. p. 97–123.
- [15] Boucher JH, Foote RH, Kirk RW. The evaluation of semen quality in the dog and the effects of frequency of ejaculation upon semen quality, libido, and depletion of sperm reserves. *Cornell Vet* 1958;48:67–86.
- [16] England GCW. Semen quality in dogs and the influence of a short-interval second ejaculation. *Theriogenology* 1999;52:981–6.
- [17] Pasqualotto FF, Sobreiro BP, Hallak J, et al. Sperm concentration and normal sperm morphology decrease and follicle-stimulating hormone level increases with age. *BJU Int* 2005;96:1087–91.
- [18] Ellington J, Scarlett J, Meyers-Wallen V, et al. Computer-assisted sperm analysis of canine spermatozoa motility measurements. *Theriogenology* 1993;40:725–33.
- [19] Agarwal A, Sharma RK, Nelson DR. New semen quality scores developed by principal component analysis of semen characteristics. *J Androl* 2003;24.
- [20] Gunzel-Apel AR, Gunther C, Terhaer P, Bader H. Computer-assisted analysis of motility, velocity and linearity of dog spermatozoa. *J Reprod Fertil* 1993;47(Suppl.):271–8.
- [21] Nunez-Martinez I, Moran JM, Pena FJ. A three-step statistical procedure to identify sperm kinematic subpopulations in canine ejaculates: changes after cryopreservation. *Reprod Domest Anim* 2006;41:408–15.
- [22] Rigau T, Fane M, Ballester J, et al. Effects of glucose and fructose on motility patterns of dog spermatozoa from fresh ejaculates. *Theriogenology* 2001;56:801–15.
- [23] Rijsselaere T, Van Soom A, Maes O, De Kruif A. Use of the sperm quality analyzer (SQA) for the assessment of dog sperm quality. *Reprod Domest Anim* 2002;37:158–63.
- [24] Rijsselaere T, Van Soom A, Maes O, De Kruif A. Effect of technical settings on canine semen motility parameters measured by the Hamilton-Thorne analyzer. *Theriogenology* 2003;60:1553–68.
- [25] Schafer-Somi S, Aurich C. Use of a new computer-assisted sperm analyzer for the assessment of motility and viability of dog spermatozoa and evaluation of four different semen extenders for predilution. *Anim Reprod Sci*; in press.
- [26] Walker JS, Winet H, Freund M. A comparison of subjective and objective sperm motility evaluation. *J Androl* 1982;3:184–92.
- [27] Gunzel AR, Syvari K, Krause D. Morphological examination of dog semen. *Deutsche Tierarzt Woch* 1985;92:13–5.
- [28] Harasymowycz J, Ball L, Seidel GE. Evaluation of bovine spermatozoal morphologic features after staining or fixation. *Am J Vet Res* 1976;37:1053–7.
- [29] Johnson C, Jacobs J, Walker R. Morphology-stain induced spermatozoal abnormalities. In: *Proceedings of the society for theriogenology*; 1991. p. 238.
- [30] Root Kustritz MV, Olson PN, Johnston SO, Root TK. The effects of stains and investigators on assessment of morphology of canine spermatozoa. *J Am Anim Hosp Assoc* 1998;34:348–52.
- [31] Salisbury GW, Willett EL, Seligman J. The effect of the method of making semen smears upon the number of morphologically abnormal spermatozoa. *J Anim Sci* 1942;1:199–205.
- [32] Christensen P, Stryhn H, Hansen C. Discrepancies in the determination of sperm concentration using Burkert-Turk, Thoma and Makler counting chambers. *Theriogenology* 2005;63:992–1003.
- [33] Oettle EE, Soley JT. Sperm abnormalities in the dog: a light and electron microscopic study. *Vet Med Rev* 1988;59:28–70.
- [34] Santos NR, Krekeler N, Schramme-Jossen A, Volkmann DH. The knobbed acrosome defect in four closely related dogs. *Theriogenology* 2006;66:1626–8.
- [35] Chenoweth PJ. Genetic sperm defects. *Theriogenology* 2005;64:457–68.
- [36] Jouannet P, Ducot B, Feneux D, Spira A. Male factors and the likelihood of pregnancy in infertile couples. 1. Study of sperm characteristics. *Int J Androl* 1988;11:379–94.
- [37] Aziz N, Buchan I, Taylor C, et al. The sperm deformity index: a reliable predictor of the outcome of oocyte fertilization in vitro. *Fertil Steril* 1996;66:1000–8.
- [38] Oettle EE. Sperm morphology and fertility in the dog. *J Reprod Fertil* 1993;47(Suppl.):257–60.
- [39] Rijsselaere T, Van Soom A, Hoflack G, et al. Automated sperm morphometry and morphology analysis of canine semen by the Hamilton-Thorne analyzer. *Theriogenology* 2004;62:1292–306.
- [40] Power JH. A study of canine semen: physical factors which may effect the electrolyte concentration in the ejaculate. *Irish Vet J* 1963;17:226–31.
- [41] Olar IT, Amann RP, Pickett BW. Relationship among testicular size, daily production and output of spermatozoa, and extra-gonadal spermatozoal reserves of the dog. *Biol Reprod* 1983;29:1114–20.
- [42] Foote RH, Boucher JH. A comparison of several photoelectric procedures for estimating sperm concentration in dog semen. *Am J Vet Res* 1964;25:558–60.
- [43] Kuster C. Sperm concentration determination between hemacytometer and CASA systems: why they can be different. *Theriogenology* 2005;64:614–7.
- [44] Eljarah AH, Chandler JE, Chenevert J, et al. Determining bull semen concentration: a novel design for CASA. *Theriogenology* 2005;64:799 [Abstract].
- [45] Makler A, Fisher M, Lissak A. A new method for rapid determination of sperm concentration in bull and ram semen. *Theriogenology* 1984;21:543–54.
- [46] Root Kustritz MV, Kilty C, Vollmer M. Spermatocrit as a measure of concentration of canine spermatozoa. *Theriogenology* 2006;66:670 [Abstract].
- [47] Meyers-Wallen VN. Clinical approach to infertile male dogs with sperm in the ejaculate. *Vet Clin NA* 1991;21:609–32.
- [48] Root Kustritz MV, Johnston SD, Olson PN, Lindeman CJ. Relationship between inflammatory cytology of canine seminal fluid and significant aerobic, anaerobic, or mycoplasma cultures of canine seminal fluid: 95 cases (1987–2000). *Theriogenology* 2005;64:1333–9.
- [49] Rijsselaere T, Van Soom A, Tanghe S, et al. New techniques for the assessment of canine semen quality: a review. *Theriogenology* 2005;64:706–19.
- [50] Inamassu A, Vechi E, Lopes MD. Investigation of the viability of the hypoosmotic test and the relationship with some spermatoc characteristics. *Rev Bras Reprod Anim* 1999;23:302–4.
- [51] Pinto CRF, Wrench N, Schramme A. Simplified hypo-osmotic testing of canine spermatozoa. *Theriogenology* 2005;64:811 [Abstract].
- [52] Rodriguez-Gil JE, Montserrat A, Rigau T. Effects of hypoosmotic incubation on acrosome and tail structure on canine spermatozoa. *Theriogenology* 1994;42:815–29.

- [53] Kurni-Diaka J, Badtram G. Effect of storage on sperm membrane integrity and other functional characteristics of canine spermatozoa: in vitro bioassay for canine semen. *Theriogenology* 1994;41:1355–66.
- [54] England GCW, Plummer JM. Hypo-osmotic swelling of dog spermatozoa. *J Reprod Fertil* 1993;47(Suppl.):261–70.
- [55] De Souza FF, Martins MIM, Lopes MD. Vasectomy effect on canine seminal plasma biochemical components and their correlation with seminal parameters. *Theriogenology* 2006;66:1621–5.
- [56] Martikainen P, Sannikka E, Suominen J, Santti R. Glucose content as a parameter of semen quality. *Arch Androl* 1980;5:337–43.
- [57] De Souza FF, Martins MIM, Fernandes CDS, et al. Heparin-binding proteins of canine seminal plasma. *Theriogenology* 2006;66:1606–9.
- [58] De Souza FF. Electrophoretic characterization of protein profile and biochemical analysis of canine seminal plasma. Thesis. UNESP, Brazil; 2003.
- [59] Bellin ME, Oyarzo IN, Hawkins HE, et al. Fertility-associated antigen on bull sperm indicates fertility potential. *J Anim Sci* 1998;76:2032–9.
- [60] England GCW, Allen WE. Evaluation of cellulase acetate/nitrate filters for measuring the motility of dog spermatozoa. *J Reprod Fertil* 1990;88:369–74.
- [61] Hanunadeh ME, Kuhnen A, Amer AS, et al. Comparison of sperm preparation methods: effect on chromatin and morphology recovery rates and their consequences on the clinical outcome after in vitro fertilization embryo transfer. *Int J Androl* 2001;24:360–8.
- [62] Mogas T, Rigau T, Piedrafita J, et al. Effect of column filtration upon the quality parameters of fresh dog semen. *Theriogenology* 1998;50:1171–89.
- [63] Cunha ICN, Lopes MD. Centrifugation effects on canine semen quality. *Rev Bras Reprod Anim* 1999;23:306–8.
- [64] Hishinuma M, Sekine I. Separation of canine epididymal spermatozoa by Percoll gradient centrifugation. *Theriogenology* 2004;61:365–72.
- [65] Lopate C. Personal communication, 2 January 2007.
- [66] Garcia-Macias V, Martinez-Pastor F, Alvarez M, et al. Assessment of chromatin status (SCSA TM) in epididymal and ejaculated sperm in Iberian red deer, ram and domestic dog. *Theriogenology* 2006;66:1921–30.
- [67] Nunez-Martinez I, Moran JM, Pena FJ. Do computer-assisted, morphometric-derived sperm characteristics reflect DNA status in canine spermatozoa? *Reprod Domest Anim* 2005;40:537–43.
- [68] Schubert CL, Seager SWJ. Semen collection and evaluation for the assessment of fertility parameters in the male Dalmatian. *Canine Pract* 1991;16:17–21.
- [69] Seager SWJ, Schubert CL. Semen collection and evaluation for the clinical assessment of fertility parameters in the male Rottweiler. *Canine Pract* 1996;21:30–4.
- [70] Wildt DE, Baas EJ, Chakraborty PK, et al. Influence of inbreeding on reproductive performance, ejaculate quality and testicular volume in the dog. *Theriogenology* 1982;17:445–52.
- [71] Taha MS, Noakes DE, Allen WE. The effect of frequency of ejaculation on seminal characteristics and libido in the Beagle dog. *J Small Anim Pract* 1983;24:309–15.
- [72] Schafer S, Holzmann A, Arbeiter K. The influence of frequent semen collection on the semen quality of Beagle dogs. *Tierarztl Prax* 1996;24:385–90.
- [73] Kawakami E, Hori T, Tsutsui T. Changes in semen quality and in vitro sperm capacitation during various frequencies of semen collection in dogs with both asthenozoospermia and teratozoospermia. *J Vet Med Sci* 1998;60:607–14.
- [74] Heywood R, Sortwell RJ. Semen evaluation in the Beagle dog. *J Small Anim Pract* 1971;12:343–6.
- [75] Kuroda H, Hiroe K. Studies on the metabolism of dog spermatozoa. I. Seasonal variation in semen quality and aerobic metabolism of spermatozoa. *Jpn J Anim Reprod* 1972;17:89–98.
- [76] Taha MB, Noakes DE, Allen WE. The effect of season of the year on the characteristics and composition of dog semen. *J Small Anim Pract* 1981;22:77–84.
- [77] Takeishi M, Iwaki T, Ando Y., et al. Studies on reproduction in the dog. VII. Seasonal characters of semen. *Bull Coli Ag Vet Med Nihon Univ* 1975;32:224–31.
- [78] Wong WT, Dhaliwal OK. Observations on semen quality of dogs in the tropics. *Vet Rec* 1985;116:313–4.
- [79] Mickelsen WD. Maximizing fertility in stud dogs with poor semen quality. In: *Proceedings of the society for theriogenology*; 1991. p. 244–6.
- [80] Eilts BE, Paccamonti DL, Pinto C. Artificial insemination in the dog. In: Root Kustritz MV, editor. *The practical veterinarian: small animal theriogenology*. St. Louis: Butterworth-Heinemann; 2003. p. 61–95.