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Diagnostic tests in canine andrology - What do they really tell us about fertility?

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ABSTRACT

Dog breeders often require breeding soundness evaluations which include andrological examinations of the genital organs, hormone measurements, and semen analyses. During the past decades, a considerable number of research results have been published, allowing diagnoses of specific andrological conditions and fertility assessment.

For specific examinations, however, no standard procedures have been defined and for some parameters different reference ranges have been published. Therefore, examination results from different facilities are difficult to compare and profound conclusions regarding health and fertility of a male dog are not always possible.

Conventional semen examination, however, is still useful in identifying deviations or no deviations from normality, especially if confounding factors are taken into account and if the exam is repeated in case of inconclusive findings. A standardization of examination procedures and reference ranges would help to harmonize the exchange of examination results and interpretation of the findings.

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1. Introduction

Since health of the genital organs and quality of the semen are directly related to the ability to produce offspring, only healthy animals in excellent body condition should be used for breeding [1–3]. Impaired fertility may lead to no or lower number of puppies, emotional disappointments of breeders and potential puppy buyers and substantial financial losses for breeders [4–6].

A breeding soundness evaluation (BSE) can be performed for evaluating the health of genital organs and breeding potential of dogs [7]. Therefore, breeders often require BSE which include andrological examinations of the genital organs, in some cases measurements of hormones, and semen analysis [8,9]. Proper examination techniques and interpretation of the findings are in turn essential skills for small animal practitioners who work with breeders [10]. This is especially true in the context of semen transfer, preservation by chilling or freezing, or investigation in cases of suspected subfertility or infertility [11,12].

2. Fertility, subfertility and infertility

The result of a breeding soundness evaluation should give reliable insights into the breeding potential of a dog. However, different definitions and explanations of terms such as fertility, fecundity, subfertility, and infertility are being used in human and veterinary literature [13]. Therefore, formal definitions for these conditions are important for appropriate determination of reproductive function and disorders [14], because different meanings may be misleading or even misused [13]. In human reproduction, only recently many terms have been defined or revised [13].

The term fertility of a mammal means the capability to produce offspring through reproduction following the onset of sexual maturity [15]. In human medicine, fertility has also been referred to as the circumstances in which livebirths occur as the term is often used by demographs in the context of prediction of the development of a population in a specific area [16]. The fertility rate is the average number of offspring born by an individual during its lifetime [15]. Furthermore, the biological theoretical ability and

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capacity for reproduction, i. e. the biological potential of the number of offspring during a lifetime, has been referred to as fecundity [17].

In livestock, for example for cow herds, the term reproductive performance is often used in the context of fertility [18,19]. Fertility is a critically important factor in cattle production because it directly relates to the ability to produce the offspring necessary to offset costs in production systems [20]. Sub-fertile bulls delay conception, prolong the calving season, reduce calf weaning weights and increase female culls [21]. Common measures of reproductive performance are conception rate, pregnancy rate, services per conception, and more management related parameters such as estrus detection rate, days to first service, days to conception, and calving interval [22]. Bull fertility can also be measured by the percentage of cycling females exposed to the bull and impregnated during a specific time period [23]. Since in livestock, fertility on the herd level is much more relevant in terms of economy than the fertility of a single animal, these measures are used to monitor fertility management. These measures, in turn, cannot be applied to single animals. For intensively used stud dogs it may be possible to calculate a proportion of the number of pregnant divided by the number of mated female dogs. But a lot of confounders such as day in relation to ovulation, number and frequency of matings, and other would need to be taken into account.

Infertility in humans has been defined as the failure to achieve a pregnancy after 12 months or more of regular unprotected sexual intercourse [13,14]. In the male reproductive system, infertility is most commonly caused by problems in the ejection of semen, absence or low numbers of sperm, abnormal morphology or impaired motility of the sperm [15,24].

Subfertility generally describes any form of reduced fertility with prolonged time of unwanted non-conception in humans [25]. It has been suggested that the term infertility may be used synonymously with sterility, both describing only sporadically occurring spontaneous pregnancies [25]. Since these definitions are widely overlapping, a consensus of human reproduction specialists has been obtained. Subfertility should be used interchangeably with infertility because subfertility does not define a different or less severe fertility status than infertility, nor is subfertility a condition that exists before infertility is diagnosed [13].

In dogs, infertility has been defined as conception failure of at least three [26] or four [7] matings with different bitches. While definitions for aspermia, azoospermia [27] and oligozoospermia [28] in dogs are available, a clear definition of subfertility has not been published.

3. Breeding soundness evaluation

The principle of a BSE is that features predictive of good or poor breeding or fertilizing potential are assessed [9]. Fertility is relying on the proper functioning of the reproductive system, its anatomy and physiology of organs, including the hormonal regulation of the hypothalamus-pituitary-testicles axis (Fig. 1) [15]. Before further examinations, a throughout anamnesis including the reproductive history, medications and stays in different countries or regions in the world needs to be documented [6].

3.1. Morphological and hormonal examinations

The morphological examination includes an evaluation of size, shape, symmetry, consistency, and position of the sexual organs [29]. The findings may indicate normal function or lack of hormonal activation or enlargement because of pathological conditions. Several specific diagnostic tests for clinical examinations have been developed in combination with reference intervals for sizes and

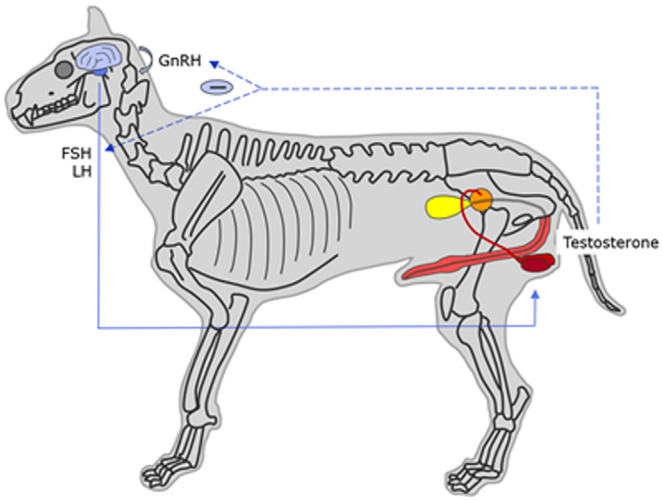

Fig. 1. Genital organs and hormonal regulation in the male dog.

volume of the testicles [30,31], testicular tissue consistency [32] or appearance of genital organs in diagnostic imaging [33]. Low testosterone concentrations, for example may lead to small and soft testicles and a decline of the volume and function of the prostate. However, despite the wide recommendation for examinations of breeding dogs, there have hardly been comprehensive studies evaluating differences in examination outcomes between fertile and infertile dogs [7] and potential confounders.

Over the last years, several advanced ultrasound technologies have been developed which provide additional information of the examined organs. However, their clinical application in the field of small animal reproduction is still limited [34]. Sensitivity and specificity for the detection of specific conditions or diseases have not widely been subject of research until now [34]. It has been shown that Doppler ultrasound parameters resistance index (RI) and pulsatility index (PI) may be potential markers of seminal quality in dogs [35], but these parameters did not differ between infertile and fertile dogs [7]. In addition, testicular artery RI and PI were not predictive of future total sperm output or proportions of live normal sperm in dogs [36]. It has been suggested that future research projects should focus on standardization of the used techniques, determination of thresholds to discriminate between fertile and infertile and on the predictive value of advanced ultrasound findings [34].

For the examination of the prostate, ultrasound is considered an excellent tool to detect any change of their homogeneous and echogenicity pattern of the gland. These features, however, are not pathognomonic of a specific disease [37,38]. For instance, the loss of normal ultrasonographic pattern and the increase in echogenicity can be observed in almost every prostatic disorder [39].

Pulsatile gonadotrophin-releasing hormone (GnRH) from the hypothalamus stimulates the pituitary secretion of both luteinizing hormone (LH) and follicle-stimulating hormone (FSH) and thus controls the hormonal and reproductive function of the gonads [40]. LH stimulates testosterone production from the Leydig cells while FSH stimulates the function of sertoli cells and sperm production [41,42]. Testosterone is responsible for sperm production, development of masculine characteristics, including sexual drive, secretory and cytologic activation in the prepuce and other organs, and influences muscle and bone mass, as well as fat distribution [42].

Another hormone which is nowadays commonly measured in blood serum is canine prostate specific esterase (CPSE) [43,44]. The concentration was reported to be higher in dogs suffering from

prostatic diseases [44]. The use of CPSE measurement has been suggested as an early marker for diseases of the prostate but different cut off levels, i.e. 50 ng/ml [45], 61 ng/ml [46] or 90 ng/ml [47] have been used in research. It has been shown, however, that in asymptomatic dogs increased CPSE concentrations were associated with ultrasonographic alterations and increased size of the prostate [45]. A study investigating potential confounders for CPSE concentrations showed that neither circadian rhythms nor transrectal palpation performed during the andrological examination do affect CPSE but ejaculation does. Therefore, a sexual rest of at least 24 h before sampling has been recommended [48]. Since, an accurate diagnosis of prostatic diseases is crucial and challenging because a variety of different disorders share the same clinical signs, several diagnostic tools need to be used [37].

Measurement of concentrations of FSH and LH in blood serum or plasma provides good insights into the hormonal regulation [49] but not all laboratories provide reliable quantitative tests for these parameters, yet. Even if for testosterone good assays are available, interpretation of the findings from a single sample may be misleading. Considerable natural fluctuations of up to 62% [50] need to be taken into account, so that in some cases repeated serum samples are necessary to get proper insights into testosterone secretion in the dog. An alternative approach and a common procedure is the GnRH stimulation test, which can be used to investigate the function of the pituitary-gonadal axis in mammals [42,51]. The test is mostly used in male animals with suspected infertility or to confirm the presence of testes or testicular remnants [52]. In male dogs, the test is usually performed by measuring serum testosterone before and 1 h after an intravenous (IV) administration of 0.1–100 μg/kg of GnRH (gonadorelin) [51].

Anti Muellerian Hormone, which is produced in sertoli cells and can be used to proof the presence of gonadal tissue [53], has been discussed as a potential fertility marker and has shown a sensitivity (86%) and specificity (63%) to predict canine semen quality in a preliminary research project [54]. Measurement of this hormone may also be used to identify subfertility oder previous treatment with GnRH analogs [55]. Elevated concentrations may be used as a biomarker for Sertoli cell tumors [56].

3.2. Timing of the examination

When planning a breeding soundness evaluation, several factors should be taken into account such as age [57,58], size of the animal, time from last semen collection and perhaps season of the year [11,58,59].

Spermatogenesis in the dog lasts around 56–63 days [60] but medical history should include also any diseases or medication that may have impaired spermatogenesis within the at least past six months before semen collection [10]. In terms of last semen collection, it has been shown, that a second sample, collected around 60 min after the first sample, had significantly lower values for the volume of the second fraction, the spermatozoal concentration and the total spermatozoal output [61]. However, a dual semen collection for freezing with a 1 h interval and combination of the two ejaculates led to a higher number of insemination doses without impairment of morphology and motility [62].

On the other hand it has been suggested that after prolonged sexual rest for more than 10 days, an ejaculate may contain a greater percentage of morphologically abnormal spermatozoa due to presence of aged spermatozoa from epididymal storage [11]. Some authors, therefore, suggest a semen collection or mating around 4–5 days before the planned semen collection to obtain a maximum sperm output [10].

An influence of season in the year may also play a role but remains controversial. Some studies have found that best semen quality with highest sperm count has been found in spring, and an increase of abnormal morphology in summer [63,64]. Another study found no influence of season [65].

3.3. Semen collection procedure

Semen collection can be performed from most dogs in the clinic setting with minimal standard equipment [10,66]. The most common collection method is by digital stimulation of the penis [67].

One of the most important factors for a successful collection seems to be sexual arousal of the dog. Under ideal conditions, this can be achieved with the presence of a bitch in estrus [68]. Unfortunately, the availability of teaser bitches and pheromones is frequently limited and clinicians are therefore forced to collect semen without sufficient arousal which may lead to lower semen volume and total sperm count [5].

Less effective are pheromones in the form of vaginal discharge from a bitch in estrus (preserved on a swab or other absorbing material). To the authors experience, a storage of swabs in the fridge leads to a further decline of effectiveness of vaginal specimens. Previous research has shown that administration of prostaglandin F2alpha [5] or short acting GnRH analogs [41,69] may improve the libido and the volume of collected semen in dogs.

It is also imperative that any distractions or procedures that would induce anxiety be eliminated or minimized since fear and pain will prohibit a dog from attaining a complete erection and ejaculating [68].

Other semen collection techniques are urethral catheterization and electro ejaculation. However, this procedure is much more invasive and may lead to urine contamination [70], which in turn may impair semen quality and fertility [71].

Obviously, it is important to consider sexual arousal and semen collection method when interpreting semen parameters. In some cases, it will be difficult to evaluate if low semen parameters are due to suboptimal collection or really represent the fertility of the dog.

3.4. Semen analysis

Semen analysis evaluates various sperm quality parameters with the aim to obtain indicators for male fertility [15]. The three main areas to consider during evaluation of canine semen quality are total sperm count, viability, assessed as motility, progressive motility, live/dead ratio, and acrosome membrane integrity; and morphology [24].

First, gross characteristics and sample volume are recorded [10]. Percentage and quality of motility are evaluated immediately [10] as these parameters may change over short time. Sperm evaluation methods that include conventional microscopic methods [72], computer-assisted sperm analyzers (CASA), and flow cytometric analysis, provide precise information related to sperm morphology and function [15]. Even if reference ranges for human semen are under debate the sperm concentration, motility and morphology are the three classical sperm parameters measured by laboratories worldwide [73].

Although semen analyses are routinely performed to evaluate reproductive potential in canines, only a few authors have attempted to relate semen characteristics to donor traits such as age, weight, and fertility [12,57,58]. Body weight, for example, plays a major role in terms of semen parameters we can expect from a male dog. It has been shown that the commonly used reference values should be adjusted, since values for ejaculate volume, total sperm output and testicular dimensions for dogs ≤5.0 kg bodyweight differed significantly from values of dogs with a bodyweight from 5.1 to 10.0 kg [30]. Maybe even breed specific reference ranges

would be helpful.

One study investigated the effect of sperm morphology on fertility based on conception rates. Conception rate (CR) is a measure derived from number of attempted breedings versus number of litters born [74]. The insemination of 42 female dogs led to a CR of 61% with semen >60% normal morphology (14 of 23 inseminated bitches) whereas the CR of dogs with <60% normal morphology was 13% (two of 15 inseminated bitches) [75].

In addition, specific stainings for morphometry [76] or tests such as the hypoosmotic swelling test or measurement of alkaline phosphatase [77] can be used. Undoubtedly, the findings of these examinations have a clinical relevance but giving clear prognostic estimations regarding fertility or expected conception rates remains difficult in many cases.

3.5. Standardization and quality control

Results of semen evaluation tests are influenced by sample collection technique and timing, concentration of spermatozoa in the sample, amount of time from semen collection to evaluation, temperature at which the sample was held, equipment used, and many other factors [11,78].

In addition, a common question encountered in reproductive biology is whether or not the measurement of a variable by two different methods, or by two different operators using the same method, or by one operator repeating the measurement at two different times, produces essentially the same result [79].

In that regard it is important to assess the repeatability and reproducibility of the measurement process of a diagnostic method [79,80]. Repeatability is defined as an agreement between two measurements on the same samples, whereas reproducibility means that two individuals are using the identical methodology on identical samples [79]. Only a few studies on canine andrology have addressed these issues [80,81].

It has been claimed by several authors that standardized protocols for canine semen evaluation including volume, semen color, percentage of progressively motile spermatozoa, percentage MNS, and concentration should be developed [11,80]. Furthermore, it has been suggested that strict quality control should be applied to correctly interpret the results [15].

Other than in humans [73], analysis of canine semen has not been standardized throughout the world. For human spermiologic examinations, a WHO manual provides step by step methods on how to perform a routine semen analysis, guidance on establishing internal and external quality control for these measures, and recommendations on more commonly used tests to assess sperm function [82]. These standardized methods allow accurate assessment of sperm quality, comparison among laboratories [73] and even pooling of data from across the globe for epidemiology studies on semen quality [83,84].

Another issue is documentation of andrological and spermatological findings. Several diagnostic procedures usually are accepted to belong to a complete andrological examination. However, no international standard requirements regarding essential tests or thresholds for test results have been defined. This becomes obvious when looking at protocols of andrological or spermatological examinations from different practices or clinics, especially those sent alongside chilled or frozen semen. Semen banks often use different protocols, which provide different data. At present, harmonized protocols for semen shipment are under development.

3.6. Semen and fertility

Although the traditional semen parameters like concentration, motility and morphology are often used to classify male fertility or infertility, it has become apparent that none of these have good predictability [24,85]. For example, in one study the cause of acquired infertility could not be identified in almost half of the dogs [26]. In the past it has been even suggested that semen analysis can only be reliably predictive of fertility if the semen quality is either very good or very bad or showing specific conditions such as presented in Figs. 2 and 3 [86].

The limitations of the diagnostic methods may include subjectivity, variability, the small number of spermatozoa analyzed, and poor correlation with fertilizing potential [72,86]. 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 [11].

Components of semen that really affect pregnancy rates have not been sufficiently identified in dogs [11,57]. Hence, sperm fertility parameters in male dogs are largely extrapolations of research and clinical observation from other species [57]. As been claimed, therefore, that routine semen analysis does not measure the fertilizing potential of spermatozoa and the complex changes that occur in the female reproductive tract before fertilization [73].

It has been shown, for example, that in human medicine, sperm number and morphology are associated with time to natural pregnancy, whereas sperm motility is less predictive [73] and its significance is sometimes misestimated [87]. Spermatozoa are mainly passively transported in the female genital tract [88], while their motility is, however, required for reaching the oocytes and during zona pellucida penetration [89,90].

Even more recent and elaborate investigation, for example of sperm DNA peroxidase, has found no differences between infertile and fertile dogs [85].

4. Discussion and conclusions

A throughout andrological examination and knowledge of normal parameters for canine semen should enable the practitioner to evaluate the results of these tests with confidence [10]. But as shown, several factors such as breed, age [1,77,91] and size of the animal [67,91], inbred status [92], time since last semen was collected, and season of the year [11] may affect findings and fertility to a widely unknown extent. In addition, semen collection is not always successful and semen analysis requires experience and specialized equipment, which is not available in all veterinary practices [54].

Some very good research has been published in the past years

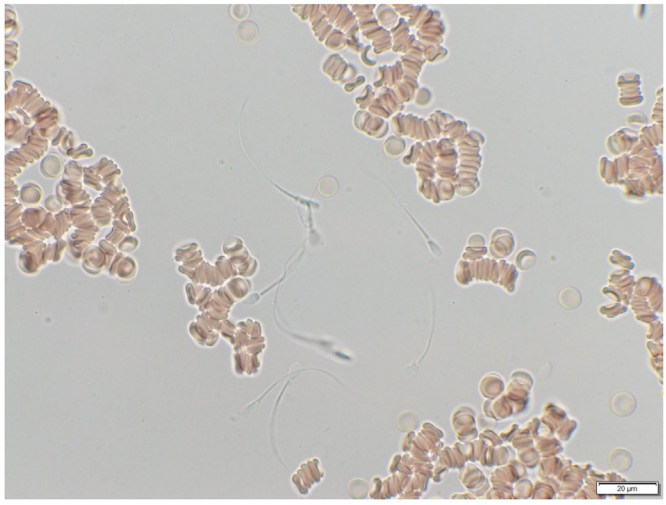

Fig. 2. Microscopic picture of canine haemospermia.

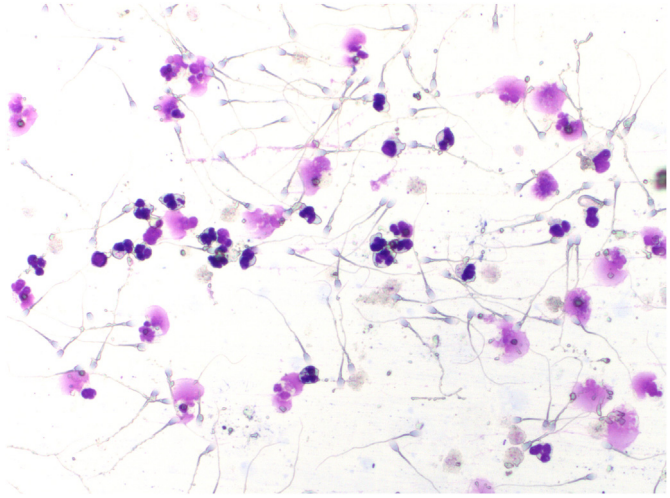

Fig. 3. Neutrophil granulocyts in the sperm rich fraction of canine ejaculate indicating inflammation in the genital tract.

about canine andrology but many publications suffer from low numbers of included animals and reporting deficits [80]. In some studies, semen was pooled and therefore it is not possible to analyze the individual variation of parameters [80,93]. Especially for the determination of reference ranges or cut of values, which are partly heterogenous or unproven in veterinary andrology, sufficient sample sizes are essential [94,95]. Reasons for this phenomenon may include limited access to research male dogs or privately owned dogs, limited number of female dogs that are bred or limited funding possibilities. Other reasons may be a limited budget or the rare occurrence of a genital disease [96].

In farm animals such as cattle, it is easier to assess fertility parameters such as pregnancy rates because plenty of cows can be inseminated with semen of the same bull, but also bovine fertility traits still vary considerably among studies and correlations between several spermatological parameters and fertility vary among studies [20].

Saying that the very small studies should not be done at all is probably an inappropriate message [94]. However, potentially practical alternatives such as a narrowing of the scope of a study, fixation of confounding factors to obtain parameters of lower variance, or using more standardized protocols which may allow pooling of study data could be implemented [94]. It appears important to build further research on strict and statistically well managed protocols [97].

Interestingly, we have been getting used to the fact that some thresholds are different in different countries. For example, in the US a number of 100 million motile sperms is considered one insemination dose whereas in Europe it is 150 million. Other authors have claimed that the recommended dose for insemination is 220–250 million normal spermatozoa per ejaculate [11]. However, it can be assumed that similar dogs should have similar minimal sperm counts. In addition, it has been shown that very small dogs have around 100 Million sperms in total, only [30], which implies that more appropriate thresholds for semen parameters should be developed, considering size and breed of dogs.

Nevertheless, in the light of new emerging technologies and markers [98] we can expect that diagnostic procedures and tests will shed new light into stud dog fertility in the near future. Regarding the possibilities of systems such as CASA, further research is required to determine which measurements are of clinical importance in canine andrology [91]. It is likely that there are not many single spermatological “on-off” parameters, but a multiparametric approach may help to reliably predict fertility [87].

Until then, we need to 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 impregnate a bitch up to 90% [11]. Conventional semen examination, however, is still useful in identifying deviations or no deviations from normality [12], especially if confounding factors are taken into account and if the exam is repeated in case of inconclusive findings. To the experience of the authors, most breeders are nevertheless reluctant to let do and pay for several semen collections.

The advent of the molecular era in animal breeding allows to learn more about the genetic complexity of male fertility and identification of genetic markers is already the basis for Marker-Assisted Selection of breeding bulls [99]. In bulls, genetic heritabilities for semen production and quality traits have been studied, mostly with a basis of 1000 animals or more [20]. If and when this will be possible in dogs, remains open.

The use of advanced laboratory tests to evaluate sperm parameters beyond the standard motility, morphology, and concentration will open investigation to more specific and sensitive fertility tests in canine reproduction [57]. Proper estimation and having an appreciation of the degree of uncertainty of dependent and independent variables are crucial for using predictions to make decisions, like it is done in bulls already [100].

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