AUSTRALIAN VETERINARY JOURNAL
AUSTRALIA'S PREMIER VETERINARY SCIENCE TEXT
SMALL ANIMALS

INVITED REVIEW

Evidence-based practice in canine artificial insemination

CJ Joonè[a,b]*

A number of methods are currently used to predict the optimal date of insemination of the breeding bitch, particularly with the use of frozen–thawed canine semen which has a far shorter lifespan than fresh semen. Aside from confirming cytological oestrus, vaginal cytology is of no assistance in predicting the most fertile day(s) in a bitch; however, a neglected avenue of research suggests that vaginal cytology may be of great importance in confirming the days of optimal fertility retrospectively. Similarly, vaginoscopy provides clues as to the stage of a bitch's cycle but is inadequate as a sole determinant of her most fertile days. Nevertheless, vaginoscopy is useful to identify very late oestrus and the onset of dioestrus, as well as Stage I of labour (cervical dilatation). Due to variations in the rate at which circulating progesterone concentrations rise in individual bitches, the reliability of circulating progesterone concentrations for determining the optimal day(s) of insemination with frozen–thawed semen decreases as values rise. Moreover, progesterone assay results can vary widely due to extrinsic factors such as the time of blood sampling, sample storage conditions and the assay employed. Finally, this review investigates evidence surrounding various insemination routes and suggests that well-performed vaginal insemination, even with frozen–thawed semen, may be an acceptable approach for cases where transcervical insemination is impractical.

Keywords dog; progesterone; surgical insemination; transcervical insemination; vaginal cytology; vaginal insemination; vaginoscopy

Abbreviations AI, artificial insemination; CLIA, chemiluminescence immunoassay; LH, luteinizing hormone; RIAs, radioimmunoassays; SCI, superficial cell index; TCI, transcervical insemination

Aust Vet J 2024;102:377–384 doi: 10.1111/avj.13336

Frozen semen has become an important component of clinical reproduction practice in the dog since pups were first produced from frozen–thawed semen over 60 years ago.[1] Over time, our understanding of canine reproductive endocrinology has advanced, hormone assays have become more accessible, and alternative insemination techniques have emerged. Yet, pregnancy rates with the use of frozen semen remain, in general, lower than that achieved with fresh semen.[2–4]

*Corresponding author.
[a]College of Public Health, Medical and Veterinary Sciences, James Cook University, Townsville, Queensland 4811, Australia; carolynne.joone@jcu.edu.au
[b]North Queensland Reproduction, Townsville, Queensland 4817, Australia

Today, Australian veterinarians are under increasing pressure to move away from surgical artificial insemination (AI),[5] with Australian Veterinary Association policy calling for the prohibition of canine surgical AI in Australia and surgical AI being currently banned in Norway, Sweden and the United Kingdom.[6] The route of insemination is, however, just one of many factors involved in creating a successful breeding attempt.

The aim of this review is to critically evaluate key studies that have shaped our understanding of reproductive physiology and breeding practices in this species.

Monitoring the oestrous cycle of the bitch

A number of tools are used to monitor the oestrous cycle in the breeding bitch in order to optimise pregnancy rates. Of these, the most widely accessible and clinically useful are, arguably, vaginal cytology, vaginoscopy and the measurement of circulating concentrations of progesterone. Measurement of circulating luteinizing hormone (LH) concentrations and behavioural acceptance of the male dog are included for completeness.

Vaginal cytology

The use of vaginal cytology to monitor the oestrous cycle of the bitch is well-established.[7] Under the influence of oestrogen, cells of the vaginal epithelium proliferate, differentiate and finally, exfoliate.[8] Through this process, cells are transformed from the basal, parabasal and intermediate cell stages, with their living, active nuclei, into large and angular superficial cells, with small and inactive, karyorrhectic (fragmented) or absent nuclei. The superficial cell index (SCI) refers to the proportion of superficial epithelial cells in relation to the total number of epithelial cells observed.[7] Cytological oestrus is associated with an SCI consistently at or near 100%. As oestrus progresses, subtle changes in vaginal cytology may be observed, such as a clearing of background debris and, in late oestrus, thickening of the smear with clumping or 'rafting' of superficial cells. Nevertheless, vaginal cytology cannot precisely indicate the start of ovulation or the onset of the fertilization period in the bitch and is, therefore, of no value in determining the optimum time for a bitch to be bred, other than in confirming the stage of cytological oestrus.[2,9]

While vaginal cytology may be insufficient for the prospective timing of inseminations, this tool is indeed clinically valuable as a means of retrospectively confirming the fertilization period. Holst and Phemister[10] identified the first day of cytological dioestrus, termed 'D1', as that day on which the SCI on vaginal cytology dropped by at least 20% in comparison to the previous day, with at least 10% of

epithelial cells identified as basal, parabasal or small intermediate cells. In practice, the transition from oestrus to dioestrus is distinct and abrupt.[11] In their classical study, Holst and Phemister[10] collected samples for vaginal cytology by gently aspirating a small volume of saline instilled into the caudal vagina. This study found that conception rates exceeded 95% in bitches mated once between 3 and 10 days before D1. Conception rates decreased markedly in bitches bred earlier than D-10, or later than D-3 (D-2: 80%, n = 6; D-1: 40%, n = 5; D1: 40%, n = 5; D2: 20%, n = 6). Similarly, Badinand et al.,[12] by alternating frozen semen inseminations using dogs of morphologically distinct breeds, showed that fertilisation occurred over either one (n = 2) or two (n = 4) consecutive days, spanning the period D-1 to D-4. Moreover, closer inspection of their data shows that 82% (23/28) of conceptions occurred on Days D-2 and D-3, with the remainder occurring on Days D-1 and D-4. Typically, a bitch will enter dioestrus 8 days (range 6–10 days) after the LH surge.[13] Further research on the fertility of days before cytological dioestrus is sorely lacking.

Vaginal cytology as a tool is simple, quick and cheap to perform, although sample collection technique and interpretation can differ between operators.[11] However, the detection of D1 seems to be more repeatable across time and between operators. Three decades ago, Badinand et al.[12] found that the interval between D1 and whelping was 56–60 days (n = 6). Similarly, Holst and Phemister[10] found that the average day of whelping was Day 56.9 of dioestrus (n = 93; standard deviation [SD] = 1.61). Recently, De Cramer and Nöthling[14] identified stage one of labour, on average, on Day 56.7 of dioestrus (n = 242; SD = 0.96). Similarly, 21 bitches whelped 57.3 (SD 0.9) days after the onset of cytological dioestrus.[15] These studies showed that the onset of cytological dioestrus was a more precise tool for predicting the date of parturition than either acceptance of the male dog[10] or thresholds in circulating concentrations of progesterone[12,14] or a rise in LH.[12] In the study by De Cramer and Nöthling,[14] vaginal cytology samples were collected from the cranial end of the vagina using a moistened, sterile cotton bud fixed to an extension handle, inserted through a vaginal speculum, and gently rotated against a vaginal fold. Badinand et al.[12] do not describe their method. It is postulated that the vagina (referred to as the 'cranial vagina' by some authors) is more sensitive to oestrogen than the vestibulum (referred to as the 'caudal vagina' by some authors).[16,17] Notably, these structures have differing embryological origins: the paramesonephric ducts (vagina) and the urogenital sinus (vestibulum).[18] Recently, a comparison of vaginal cytology using a cotton swab versus the less invasive vulvar stamp smear demonstrated significant differences between these two methods,[19] although others consider the two methods comparable.[7] Given the gravity surrounding the accurate prediction of whelping dates, particularly for bitches at high risk of obstetrical complications, further research is required to establish the reliability of D1 determination by different operators using similar and differing sampling techniques. It is the author's preference to correlate vaginal cytology, sampled from the cranial end of the vagina, with vaginoscopy to confirm the transition into dioestrus.

Interestingly, the term 'cornified' in reference to vaginal epithelial cells is, strictly speaking, only appropriate when Schorr trichrome stain or a derivative is used, as this stain distinguishes keratinised from non-keratinised cells. Other staining methods that do not stain keratin precursors, such as Wright's, Giemsa and Diff-Quik, require cells to be identified based on morphological features alone. The proportion of cornified epithelial cells denoted as the Eosinophilic Index (EI; number of acidophilic cells/total number of epithelial cells), varies over the oestrous cycle in unison with the SCI.[20,21]

Vaginoscopy

Vaginoscopy as a tool for monitoring the oestrous cycle of the bitch was first described in 1983.[22] Using endoscopy, Lindsay[22] noted distinct changes in the appearance of the vaginal mucosal folds as bitches progressed through various phases of the oestrous cycle. Briefly, the folds of the cranial vagina appear large and oedematous during pro-oestrus (Figure 1A), show signs of shrinkage or wrinkling around the time of the LH surge (Figure 1B), and become distinctly pale with sharply angular folds from mid- to late-oestrus, around the time of the fertilization period (Figure 1C). Dioestrus and anoestrus are indistinguishable on vaginoscopy; both are identified by small, pink folds, often in a rosette pattern (Figure 1E).[22] Although endoscopic systems for vaginoscopy are currently commercially available, a more simple and cost-effective technique is the use of a sterile, clear, glass or perspex cylinder as a vaginal speculum. The speculum should measure 220–250 mm in length, with an external and internal diameter of 15 and 11 mm respectively, and should have smooth, rounded edges for atraumatic use. The folds of the vaginal mucosa should be illuminated by a good-quality, cold light source.[23] Larger and smaller speculums are useful for giant breeds and prepartum monitoring, and small breeds, respectively. The speculum is inserted vertically through parted vulval lips, keeping in contact with dorsal tissues to avoid the clitoral fossa and urethral opening on the ventral aspect. Once resistance is met, immediately ventral to the anus, the speculum is redirected horizontally and carefully inserted into the vagina with a gentle twisting motion. In the author's opinion, no insufflation is necessary or desired, as this may distort the clinical assessment through a flattening effect.[22]

In most cases, no lubricant is used to enable the clinician to judge the dryness of the vaginal mucosa. During mid to late oestrus, the vaginal mucosa is typically dry and 'tacky' against the twisting motion of the speculum, becoming distinctly moist as dioestrus approaches (Day D-1 onwards).

In the author's experience, most maiden and virtually all multiparous bitches tolerate vaginoscopy, particularly during standing oestrus. Great care should be taken at the vestibulovaginal junction, particularly in bitches that have never mated or whelped naturally, as vestibulovaginal strictures and bands will impede the progress of the speculum and, where possible, should be resolved prior to further attempts at vaginoscopy.

Although moderately subjective and notwithstanding some natural variation between bitches, vaginoscopy is a useful tool in canine reproduction practice, providing an instant assessment of a bitch's progress through the oestrous cycle. Lindsay[22] found that bitches' vaginal folds first became distinctly angular 2–3 days after the LH peak. Similarly, Jeffcoate and England[24] found that the vaginal folds showed shrinkage with angulation from 2.1 days after the LH peak, on average. Similarly, angular vaginal folds were observed in Beagle

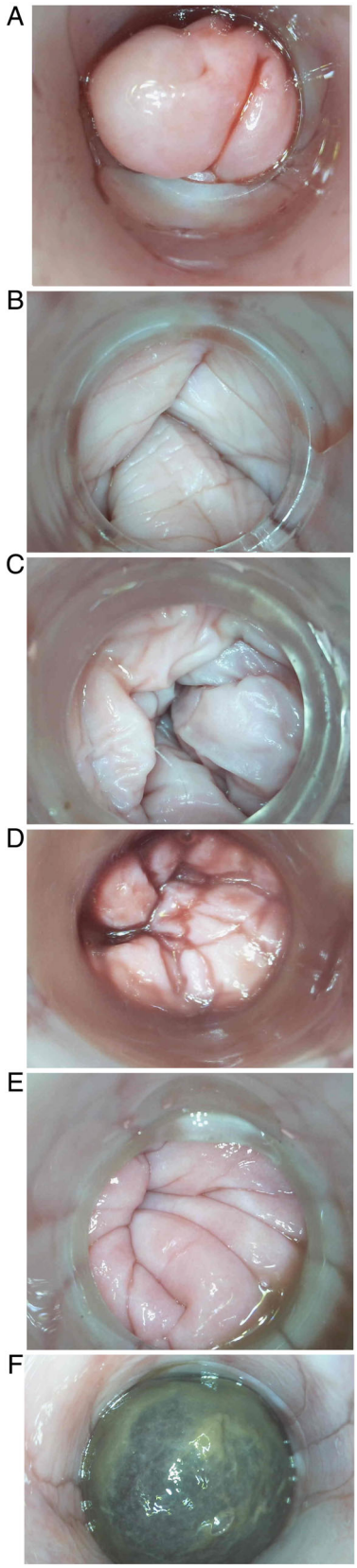

Figure 1. Legend on next column.

bitches, monitored on alternate days, from an average of 6.9 days (SD 2.1 days, n = 18) prior to D1.[13] In a later study, angular vaginal folds were first observed in 24 German Shepherd bitches from 8 to 2 days prior to D1.[25]

Interestingly, the day before the onset of dioestrus (D-1) is particularly distinct on vaginoscopy. On this day, a rounding out and moistening of the vaginal folds is detected,[26] typically with a reddish-brown, pungent discharge, and caking of the now-sloughing vaginal epithelium along the internal aspect of the speculum when withdrawn (Figure 1D and Supplementary Video). Nöthling et al.[25] demonstrated this feature in a study where inseminations all ceased on D-1, identified through vaginoscopy. The ability to identify D-1 has important clinical relevance. Given evidence of decreasing fertility by D-1 as discussed previously, a breeding bitch found to be at this stage should be inseminated or mated urgently, if she is yet to be bred. An assay for progesterone concentration at this point would only cause unnecessary delay.

Vaginoscopy has an additional, extremely useful application in the late pregnant bitch. Once a late pregnant bitch enters Stage I of parturition, observed as dilatation of the cervix on vaginoscopy (Figure 1F), parturition is imminent and Caesarean section is considered safe.[27] At this point, the circulating progesterone concentration for most bitches would be expected to be below 6 nmol/L.[28] However, recent research has demonstrated variations in circulating progesterone levels in late pregnant bitches, including a surge in progesterone concentration at the time that cervical dilatation was first observed in two out of 25 bitches, possibly related to a rise in cortisol levels as parturition approached.[23] In these cases, vaginoscopy would be an essential tool to avoid inappropriately delaying Caesarean section in a bitch predisposed to dystocia, based on an atypical serum progesterone reading.

In summary, vaginoscopy is useful for verifying, loosely, that a bitch is in mid- to late-oestrus, but, perhaps more importantly, to identify bitches in D-1 and, in conjunction with vaginal cytology, the start of dioestrus (D1)—the end of the fertile period in a bitch. Moreover, vaginoscopy should be considered indispensable for monitoring late-term pregnant bitches.

Measurement of circulating concentrations of progesterone

The use of circulating progesterone concentrations to inform breeding practices in the bitch has evolved substantially over the last 40 years. Initially, radioimmunoassays (RIAs) for progesterone concentration were limited to research institutions and considered impractical for practitioners due to long turn-around times.[29] Today,

FIGURE 1 Photographs of the view through a vaginal speculum, demonstrating (A) large, oedematous vaginal folds typical of pro-oestrus, (B) shrinking, rounded vaginal folds typical of early oestrus, (C) pale, angular vaginal folds typical of late oestrus, (D) angular to rounded vaginal folds with a reddish-brown discharge and caking of vaginal epithelium along the internal aspect of the speculum, typical of D-1, and (E) small, pink vaginal folds typical of dioestrus and anoestrus. The vaginal speculum used in these images has an internal diameter of approximately 16 mm. (F) Stage 1 labour in a parturient bitch. Vaginoisopy showing protrusion of foetal membranes.

in-house progesterone assays return results within as little as 12 min.[30]

Early research focused on the absolute concentrations of progesterone at the time of successful versus unsuccessful inseminations with frozen semen. Of 14 bitches inseminated once with frozen–thawed semen, the mean plasma progesterone concentration at the time of insemination was not statistically different between nine bitches that went on to whelp (47.1 nmol/L, SD 10.176 nmol/L) versus five bitches that did not (38.5 nmol/L, SD 15.9 nmol/L). Similarly, in 22 bitches inseminated twice with frozen semen, the mean plasma progesterone concentration at the time of the first insemination was no different between pregnant (41.3 nmol/L, SD 19.1 nmol/l; n = 15) and non-pregnant (31.5 nmol/L, SD 24.2 nmol/L; n = 7) bitches. Mean plasma progesterone concentrations were significantly higher in pregnant bitches (65.2 nmol/L, SD 21.9), than non-pregnant bitches (44.8 nmol/L, SD 30.8), at the time of a second insemination, but again varied widely (SD > 10 nmol/L).[31]

Linde-Forsberg and Forsberg[2] compared plasma progesterone concentrations at the time of one to four daily, repeat inseminations with frozen semen, in 62 bitches. The highest recorded progesterone concentrations at the time of insemination ranged from 11.4 to >76.5 nmol/L in the 26 bitches subsequently diagnosed as pregnant, and from 0 to >76.5 nmol/L in the 36 bitches that later showed no evidence of conception. Similarly, in six bitches inseminated daily using semen from morphologically distinct males, circulating progesterone concentrations on the day of successful inseminations with frozen semen varied from 28.6 to 82.7 nmol/L.[12] The authors of the latter study note: 'The absolute value of progesterone concentration [at the time of, or shortly before, insemination] did not seem appropriate for determination of AI time because the increase in plasma progesterone was highly variable between bitches',[12] a notion echoed more recently.[32]

By this time, our understanding of the physiology behind the bitch's oestrous cycle had improved considerably, through the seminal work of Patrick Concannon and colleagues. Through frequent blood sampling for the assay of LH and progesterone, and the macroscopic and microscopic examination of ovaries excised at serial timepoints after the LH surge, Concannon et al.[33] showed that ovulation (of immature oocytes) in the bitch likely occurs over the period 38–50 h after the LH peak. Furthermore, the mean concentration of progesterone was 8.1 nmol/L (SD 2.3 nmol/L) at the time of the LH surge and 17.3 nmol/L (SD 7.2 nmol/L) around the time of ovulation, 48 h after the LH surge.[33] Other studies report mean circulating progesterone values, all measured using RIA, of 3.8 nmol/L,[24] 4 nmol/L,[34] 5.1 nmol/L,[35] 9.8 nmol/L[36] and 14.0 nmol/L[37] on the day of the LH peak. More recently, Nöthling and De Cramer[38] found that, for most bitches, the LH surge occurred on the day, or the day before, progesterone levels first exceeded 6.06 nmol/L measured by RIA, or 5.41 nmol/L measured by chemiluminescence immunoassay (CLIA; Immulyte1000).

An important observation when assessing these studies is the increasing variability in progesterone concentrations as levels rise.[39,40] For example, in the study by Concannon et al.,[33] the SD rose from 2.3 nmol/L at the time of the LH peak to 7.2 nmol/L, 2 days later. More recently, Hollinshead and Hanlon[32] described progesterone profiles in 1420 oestrous cycles. On the day of the estimated LH surge, the authors report an SD of 1.9 nmol/L. Over subsequent days, the SD appears to increase progressively, reaching over 19 nmol/L by Day 7 after the LH surge.[32] In optimising fertility to frozen–thawed semen, some authors recommend inseminating 2 and/or 3 days after circulating progesterone levels first reach 16 nmol/L, a level that approximates to the start of ovulation.[41] However, given the increasing variation in progesterone concentrations as levels rise, Steckler et al.[39] investigated the success of insemination with frozen–thawed semen based on the day when circulating progesterone levels first increased above 6 nmol/L (measured by RIA), a level which approximates to the day of the LH surge. Using this approach, these authors achieved pregnancies in 12 out of 12 bitches, with 11%, 56% and 27% of available oocytes fertilised on Days 5, 6 and 7 after the initial progesterone rise above 6 nmol/L (measured by RIA), respectively.

Others argue that a rapid rise in circulating progesterone concentration, rather than an absolute value, should be used to indicate the LH surge for the planning of a breeding. Empirical evidence for this theory, in the form of a fertility trial, is yet to be published.[42] Moreover, a practical approach is to draw blood samples from bitches in oestrus every second day (at most, daily), with progesterone levels extrapolated to intervening days. Such an approach permits the estimation of when progesterone concentrations first exceeded a particular level. It could be argued that far more frequent blood sampling would be required to detect the onset of a sudden increase in progesterone concentrations.

There are a number of practical considerations to be kept in mind when using measurements of progesterone concentrations in canine blood samples for clinical decision-making.[43] One important aspect is sample handling. Although progesterone in canine blood samples appears to be fairly robust, particularly when collected into heparinised blood tubes,[44] refrigeration immediately after the collection was found to have a significant impact on progesterone levels in whole blood.[45] The recommendation, therefore, is to either separate serum by centrifugation promptly once clotted, or to hold whole blood samples at room temperature for at least 2 h before refrigeration.

Another interesting feature is the diurnal variation in circulating progesterone concentrations. In the study by Wildt et al.,[37] progesterone concentrations fluctuated considerably between samples taken in the mornings and afternoons, respectively. Thuróczy et al.[46] found that circulating progesterone concentrations in bitches are higher in the afternoons than the mornings until around the time of ovulation—after which, the reverse occurs. Furthermore, in other species, the impact of feeding on measured progesterone concentrations has been an area of research interest. In pigs, increased feed raised progesterone concentrations in veins closely associated with the ovaries, presumably through a luteotrophic effect.[47] However, increased feed decreases circulating concentrations of progesterone through more rapid hepatic clearance in pigs,[48,49] sheep[50,51] and dairy cattle.[52] Research into the effects of feeding on circulating progesterone concentrations in the dog is sorely lacking.

A further consideration is the variability within and between assays used to determine progesterone concentrations.[15] Historically, RIA

was considered gold standard for measuring progesterone concentrations in the bitch, however, this assay has largely been replaced by CLIA (IMMULITE) or electro-CLIA (e-CLIA; Elecsys) in reference laboratories. A number of studies have sought to compare different assays for the measurement of progesterone concentrations in the breeding bitch, with varying conclusions (reviewed by Conley et al.[43]). Kutzler et al.[40] found the CLIA to be comparable to RIA in measuring progesterone concentrations. However, Volkmann[45] found that, although highly correlated, progesterone concentrations measured by RIA were nearly 50% higher than those measured by CLIA. Since then, reports suggest that the IMMULITE2000 yields lower progesterone concentrations than its predecessor, the IMMULITE1000.[43] Recently, Nöthling et al.[30] compared a commercially-available in-house progesterone analyser to the CLIA. For practitioners faced with alternative assays, the author's suggestion is to consider running numerous comparisons, particularly between an in-house analyser and reference laboratory, in order to gain a better understanding of each assay's critical thresholds and reliability. An additional approach is to use the length of time between surmised LH surges and the onset of cytological dioestrus, which should be 8 days in most bitches, to inform the clinical application of an assay.[45]

In summary, the assessment of progesterone concentration in the breeding bitch should take into account various factors, including the time of sampling, sample handling, the progesterone assay employed and the potential impact of the animal's feeding regimen. To minimise the impact of these factors, a consistent approach to sampling is recommended. Such an approach should include a careful discussion, around appointment times and feeding practices, with the animal's owner or carer.

Measurement of circulating concentrations of LH

Given the central role of LH within the oestrous cycle of the bitch, one would assume that direct assay for this hormone would be superior to an indirect method, such as the measurement of progesterone concentration. Although anecdotally useful to some clinicians, the measurement of circulating LH has practical limitations. In the bitch, the preovulatory LH surge spans a period of between 1 and 3 days, although in some bitches LH may be elevated for less than 24 h.[35] No LH surge was detected in 2 out of 10 bitches, blood sampled once daily,[24] again suggesting that once daily sampling is insufficient. Moreover, to the author's knowledge, there is currently no substantial evidence that the measurement of LH in serum renders practically useful improvements to pregnancy rates in the dog, beyond that achieved through the measurement of circulating progesterone concentrations.

Acceptance of the male dog

Standing oestrus in the bitch is primed by prolonged exposure to increasing oestradiol levels, and is subsequently initiated by a relatively sharp decrease in the ratio of oestrogen to progesterone in circulation, typically associated with rising progesterone levels around the time of the LH surge.[53] Concannon et al.[53] reported that six of seven cycling bitches demonstrated standing oestrus 0–2 days after the LH surge, although one bitch showed oestrus 4 days prior to the LH surge. In contrast, Holst and Phemister[10] found that the onset of standing oestrus varied from 9 days prior to, to 5 days after the LH surge, and the day of first refusal of the male occurred from 3 days prior to, to 10 days after D1. This large variation in behavioural signs in relation to fixed events within the bitch's oestrous cycle, such as the LH surge or the onset of dioestrus, has been confirmed by others.[26] Sexual reflexes, such as deflection of the tail and lifting of the vulva upon perineal stimulation, are likely to be similarly unreliable, but nevertheless form part of the dataset gathered during oestrous monitoring of the bitch.

Insemination method

Assuming that a bitch demonstrates a clear and sustained rise in daily or alternate day serum progesterone concentrations, corresponding to what could be considered a 'normal' ovulatory cycle,[32] and the date of insemination has been carefully selected, the next consideration is the method of insemination.

Intrauterine versus vaginal insemination of frozen semen

In one of the first reports on the insemination of bitches with frozen semen, Seager et al.[54] achieved an annual whelping rate of up to 66% (1972; n = 38) using vaginal insemination. In comparison, AI with fresh semen that year achieved a whelping rate of 65%, while 78% of naturally-mated bitches delivered pups. The volume of frozen–thawed semen varied from 3 to 9 mL, and the number of motile spermatozoa ranged from 150 to 700 × 10^6. Most inseminations were performed on Days 10 and 12 of oestrus, where Day 1 was the first day that a serosanguinous vulval discharge was detected; bitches were checked for discharge three times per week. Frozen–thawed and fresh semen doses were inseminated using a simple pipette and syringe, with the bitch's hindquarters elevated to an angle of 60° during the insemination and for 5 min thereafter.

In a later study focused on improving the semen freezing process, Platz and Seager[1] achieved litters from 12 of 13 (92%) beagle bitches inseminated vaginally with frozen semen. Bitches were inseminated at 48-h intervals, starting on Day 10 or 11 from the start of pro-oestral vulval discharge. Details on the number of sperm inseminated are not specified. The average litter size in this group was 6.6 pups.

In contrast, Norwegian researchers reported poor success with frozen semen inseminated vaginally, but satisfactory conception rates with intrauterine (surgical) inseminations. In order to overcome this perceived need for anaesthesia and laparotomy with the use of frozen semen, Andersen[55] developed a novel insemination technique using a device now commonly known as the Norwegian pipette. The Norwegian pipette consists of an outer plastic speculum, used to deliver an inner, metal catheter to the level of the cervix. The cervix is manipulated, via abdominal palpation, into a horizontal position and gently passed over the tip of the metal catheter. In this way, semen can be inseminated directly into the body of the uterus in the conscious, standing bitch, using relatively simple equipment. The external orifice of the cervix is reported to feel cartilaginous, or 'crispy', when in contact when the metal catheter.[9] Two to three inseminations, consisting of 75–140 million progressively motile spermatozoa, achieved pregnancies in 10 of 11 bitches.[55]

It should be noted that much progress in the monitoring of the bitch's oestrous cycle and the timing of inseminations has been made

in the decades since these early reports were published. In these studies, the timing of inseminations was based primarily on the number of days since the start of a bloody vulval discharge, in conjunction with evaluation of vaginal cytology, the appearance of the external genitalia and colour of the vulval discharge, and behavioural cues.[55,56]

In the mid 1980's, Farstad[57] reported a whelping rate of 25% with vaginal insemination and 84% with transcervical insemination, using the Norwegian catheter. This report may have helped to solidify the notion that intrauterine insemination is far superior to vaginal insemination with the use of frozen semen in the bitch,[3,58,59] despite the data being based on the use of fresh semen, not frozen semen. A pregnancy rate of 25% for fresh semen is extremely poor, and was thought to be due to backflow of semen into the insemination device.[57] Later, Farstad and Andersen Berg[31] reported an overall conception rate of 67% using frozen–thawed semen inseminated transcervically using the Norwegian pipette (n = 36).

In a subsequent study, Fontbonne and Badinand[60] achieved a pregnancy rate of 53% with frozen–thawed semen inseminated vaginally using an insemination pipette with a balloon cuff, designed to mimic the *bulbus glandis* of the dog (Osiris device; n = 38), and a pregnancy rate of 74% with frozen–thawed semen inseminated transcervically using the Norwegian pipette (n = 19). For vaginal inseminations, an average of 192 million progressively motile sperm were inseminated, whereas an average of 132 million progressively motile sperm were inseminated transcervically. The authors conclude with the recommendation that intrauterine insemination is required for frozen semen, despite acknowledging that the differences reported were below traditionally accepted levels of statistical significance, and that improvements in their practice likely occurred over time, alongside a transition from vaginal to transcervical inseminations. Theret et al.[61] and Tsumagari et al.[62] supported the use of a balloon catheter for the insemination of frozen–thawed semen, both reporting pregnancy rates of 80% in 5 and 20 bitches, respectively.

Interestingly, Nöthling[13] had noticed that the rate of progressive movement of frozen–thawed canine spermatozoa appeared to increase following exposure to autologous prostatic fluid. Twenty beagle bitches were inseminated into the cranial vagina with 100 million progressively motile frozen–thawed spermatozoa, daily over mid- to late-oestrus. Of 10 bitches, for which 7–10 mL of autologous, sperm-free prostatic fluid had been added to each inseminate, all 10 conceived with an average litter size of 5.2 pups. The control group, which was managed in an identical manner but without the addition of prostatic fluid to each inseminate, showed a pregnancy rate of 60% and average litter size of 2.4 pups.[63] It should be noted that daily inseminations were performed in order to remove the effect of improper timing of insemination on conception rates, and not to increase the overall semen dose per se. Frozen–thawed spermatozoa are thought to survive less than 24 h in the reproductive tract of the bitch, an assumption based on in vitro studies.[12,25,64,65] If so, successive doses of semen in this study should have had no cumulative impact. In a follow-up study using mainly Beagles and German Shepherds, a pregnancy rate of 97% was achieved in bitches where autologous or homologous prostatic fluid had been added to frozen–thawed semen prior to vaginal insemination (n = 30), and 60% in the group that received no prostatic fluid added to inseminates.[65]

The vaginal insemination of frozen semen (without prostatic fluid) was also compared to intrauterine insemination (performed laparoscopically) by Silva et al.[64] Using inseminate volumes of 5 mL (1 mL semen with 4 mL extender) and 2 mL (1 mL semen with 1 mL extender) for vaginal AI or intrauterine AI respectively, an identical pregnancy rate of 60% was achieved in both groups (n = 10 per group). The number of motile spermatozoa inseminated for both routes appears to be around 120 million. Inseminations were performed 3 and 5 days after a bitch's plasma progesterone concentration first reached a value between 4.77 and 6.35 nmol/L. Although the sample size was small, the site of semen deposition in this study had no effect on pregnancy rates.

In the mid- to late 1990's, with the use of frozen semen in dogs expanding, pregnancy results reported to the Swedish Kennel Club were relatively poor, at 55% for fresh semen inseminated vaginally (n = 468) and 39% for frozen semen inseminated transcervically (Norwegian pipette; n = 59).[66] Even when the data was corrected for stage of oestrous cycle on the day of insemination and semen quality, success with frozen semen remained around 50%. Combining data from the United States and the Swedish University of Agricultural Sciences, Linde-Forsberg et al.[67] later reported whelping rates of >70% with the use of frozen semen with a post-thaw motility greater than 40%. In this study, the Norwegian pipette achieved a pregnancy rate of 84.4% and average litter size of 5.4 pups (n = 167). In comparison, endoscopic intrauterine insemination achieved a pregnancy rate of 57.9% and mean litter size of 6.0 pups (n = 19), while vaginal inseminations achieved a pregnancy rate of 58.9% and average litter size of 4.0 pups. The latter echoes results reported previously for vaginal insemination of frozen semen without the addition of prostatic fluid.[13,63,64]

In order to establish whether the improved pregnancy rates achieved with vaginal insemination of frozen–thawed semen combined with prostatic fluid[63,65] was due to intrinsic properties of canine prostatic fluid or simply the increased volume inseminated, a follow-up study compared the fertility of frozen–thawed semen inseminated with prostatic fluid and frozen–thawed semen inseminated with an isotonic buffered solution. Approximately 50 million progressively motile sperm were inseminated daily over the period during which the vaginal folds appeared angular on vaginoscopy until D-1, in a total inseminate volume of 7 mL. An overall pregnancy rate of 75% was achieved in this study. Although less pregnancies were achieved in the group of bitches inseminated with prostatic fluid than with isotonic buffer (8/12 vs 10/12), bitches in the former group produced a total of 76 conceptuses with a ratio of conceptus: corpus luteum of 0.58, in comparison to 45 conceptuses with a ratio of conceptus: corpus luteum of 0.39 in the latter group. Statistical analysis of the data supported the conclusion that neither the total number of inseminations nor the total number of spermatozoa inseminated influenced conception rates.[25]

Thomassen et al.[41] reported on pregnancy rates using frozen semen in a variety of different breeds in Norway. Intrauterine insemination using the Norwegian pipette achieved a pregnancy rate of 75% (N = 665) while insemination into the cranial vagina, in those bitches where cervical catheterisation was not possible and again using the Norwegian

pipette, achieved a pregnancy rate of 10% (n = 20). The authors provide no details comparing the bitches, insemination timing or semen quality between the two routes of insemination, apart from briefly noting that very large or obese bitches are more likely to be difficult or impossible to catheterise with this method. These results are reminiscent of those reported by Farstad,[57] where a pregnancy rate of only 25% was achieved for fresh semen deposited into the cranial vagina using the Norwegian pipette.

More recently, Rota et al.[68] inseminated 10 bitches with 100 million motile spermatozoa (without the addition of prostatic fluid), twice, on Days 4 and 5 after circulating progesterone concentrations first rose to between 3.2 and 6.4 nmol/L (approximating to the LH peak). All bitches were inseminated into the cranial vagina using a simple plastic pipette, and all 10 conceived, with a mean litter size of 4.4 (range 1–10).

Interestingly, the impact of seminal fluid on fertility is an area of growing research focus, in both animals and humans.[69] Extracellular vesicles, secreted by all cells including the prostatic epithelium, consist of a lipid membrane enclosing proteins, peptides, lipids and DNA or RNA fragments, ready for delivery to a receiving cell.[70] Current research aims to unravel the potential link between these vesicles and the fertility-enhancing properties of canine prostatic fluid (F Hollinshead personal communication).

Surgical versus endoscopic intrauterine insemination of frozen semen

Only two studies have compared the insemination of frozen semen via laparotomy (surgical AI), with the now widely-accepted method of endoscopically-guided transcervical insemination (TCI). Hollinshead and Hanlon[4] found that the type of AI (surgical [n = 36] vs TCI [n = 609]) had no effect on either pregnancy rate or litter size. In contrast, Mason and Rous[71] reported pregnancy rates of 45% and 65% with surgical AI (n = 40) and TCI (n = 78), respectively.

Conclusion

Surgical AI as a veterinary procedure is currently under threat in Australia, with the widening availability of a less invasive alternative, namely TCI. Although more welfare friendly, TCI is not without its limitations, which include high start-up costs and a steep and ongoing learning curve. As such, TCI is best suited to practices that will perform the technique regularly. Nevertheless, general practitioners are encouraged to keep in mind that a carefully performed vaginal insemination, with the pipette placed within the vaginal fornix (confirmed by palpation of the cervix immediately cranial or dorsal to the tip of the pipette), may yield respectable pregnancy rates with frozen–thawed semen. Perhaps more importantly, the optimal date of insemination should be carefully selected using a range of monitoring techniques and an evidence-based approach. With the use of frozen–thawed semen, no insemination method can compensate for incorrect timing.

Conflicts of interest and sources of funding

The authors declare no conflicts of interest or sources of funding for the work presented here.

Acknowledgment

Open access publishing facilitated by James Cook University, as part of the Wiley - James Cook University agreement via the Council of Australian University Librarians.

References

1. Platz C, Seager S. Successful pregnancies with concentrated frozen canine semen. *Lab Anim Sci* 1977;27:1013–1016.
2. Linde-Forsberg C, Forsberg M. Fertility in dogs in relation to semen quality and the time and site of insemination with fresh and frozen semen. *J Reprod Fertil Suppl* 1989;39:299–310.
3. Burgess D, Mitchell K, Thomas P. Coeliotomy-assisted intrauterine insemination in dogs: a study of 238 inseminations. *Aust Vet J* 2012;90:283–290. https://doi.org/10.1111/j.1751-0813.2012.00958.x.
4. Hollinshead F, Hanlon D. Factors affecting the reproductive performance of bitches: a prospective cohort study involving 1203 inseminations with fresh and frozen semen. *Theriogenology* 2017;101:62–72.
5. Gunn A, Hyatt J, Annandale A et al. The animal welfare aspects of surgical artificial insemination in the canine. *Aust Vet J* 2021;99:137.
6. Quartuccio M, Biondi V, Liotta L et al. Legislative and ethical aspects on use of canine artificial insemination in the 21st century. *Ital J Anim Sci* 2020;19:630–643.
7. Post K. Canine vaginal cytology during the estrous cycle. *Can Vet J* 1985; 26:101.
8. Schutte A. Canine vaginal cytology—I technique and cytological morphology. *J Small Anim Pract* 1967;8:301–306.
9. Linde-Forsberg C. Achieving canine pregnancy by using frozen or chilled extended semen. *Vet Clin North Am Small Anim Pract* 1991;21:467–485.
10. Holst P, Phemister R. Onset of diestrus in the beagle bitch: definition and significance. *Am J Vet Res* 1974;35:401–406.
11. Groppetti D, Aralla M, Bronzo V et al. Periovulatory time in the bitch: what's new to know?: comparison between ovarian histology and clinical features. *Anim Reprod Sci* 2015;152:108–116.
12. Badinand F, Fontbonne A, Maurel M et al. Fertilization time in the bitch in relation to plasma concentration of oestradiol, progesterone and luteinizing hormone and vaginal smears. *J Reprod Fertil Suppl* 1993;47:63–67.
13. Nöthling JO. *The effect of the addition of autologous prostatic fluid on the fertility of frozen-thawed dog semen after intravaginal insemination*. Pretoria, University of Pretoria, 1995.
14. De Cramer K, Nöthling J. The precision of peri-oestrous predictors of the date of onset of parturition in the bitch. *Theriogenology* 2017;96:153–157.
15. Gloria A, Contri A, Carluccio A et al. Blood periovulatory progesterone quantification using different techniques in the dog. *Anim Reprod Sci* 2018;192:179–184.
16. Goodman M. Ovulation timing: concepts and controversies. *Vet Clin North Am Small Anim Pract* 2001;31:219–235.
17. Röttger K. *Untersuchungen zur Durchführung der exfoliativen Vaginalzytologie bei der Hündin: ein Beitrag zur Standardisierung dieser diagnostischen Methode* (English Summary). Giessen, Universitätsbibliothek Giessen, 2010.
18. Palm CA, Segev G, Shipov A et al. Treatment of a congenital imperforate vestibulovaginal junction and secondary hydrocolpos with endoscopic laser ablation in two dogs. *Top Companion Anim Med* 2021;45:100576.
19. Liu I-L, Lee C-H, Shih P-C et al. Accuracy and patient acceptance of vulvar stamp smear for estrous cycle evaluation in dogs. *Taiwan Vet J* 2020;46:95–99.
20. Wright PJ, Parry BW. Cytology of the canine reproductive system. *Vet Clin North Am Small Anim Pract* 1989;19:851–874.
21. Fontbonne A, Dutey A-L, Maenhoudt C et al. Validation of fast simplified Harris-Schorr staining technique for vaginal smears in the bitch. 8th International Symposium on Canine and Feline Reproduction, 22–25 June 2016.
22. Lindsay F. The normal endoscopic appearance of the caudal reproductive tract of the cyclic and non-cyclic bitch: post-uterine endoscopy. *J Small Anim Pract* 1983;24:1–15.
23. De Cramer KGM, Nöthling J. The precision of predicting the time of onset of parturition in the bitch using the level of progesterone in plasma during the preparturient period. *Theriogenology* 2018;107:211–218.
24. Jeffcoate I, England G. Urinary LH, plasma LH and progesterone and their clinical correlates in the periovulatory period of domestic bitches. *J Reprod Fertil Suppl* 1997;51:267–275.
25. Nöthling J, Shuttleworth R, De Haas K et al. Homologous prostatic fluid added to frozen–thawed dog spermatozoa prior to intravaginal insemination

of bitches resulted in better fertility than albumin-free TALP. *Theriogenology* 2005;64:975–991.
26. Jeffcoate I, Lindsay F. Ovulation detection and timing of insemination based on hormone concentrations, vaginal cytology and the endoscopic appearance of the vagina in domestic bitches. *J Reprod Fertil Suppl* 1989;39:277–287.
27. De Cramer KG, Nöthling JO. Towards scheduled pre-parturient caesarean sections in bitches. *Reprod Domest Anim* 2020;55:38–48.
28. Smith FO. Challenges in small animal parturition—timing elective and emergency cesarian sections. *Theriogenology* 2007;68:348–353.
29. Farstad W. The correlation between a cyclus coefficient based on cytological indices in the vaginal smear and circulating progesterone in oestrous bitches. *Reprod Domest Anim* 1984;19:211–217. https://doi.org/10.1111/j.1439-0531.1984.tb00972.x.
30. Nöthling JO, Joonè CJ, Hegarty E et al. Use of a point-of-care progesterone assay to predict onset of parturition in the bitch. *Front Vet Sci* 2022;9:9.
31. Farstad W, Andersen BK. Factors influencing the success rate of artificial insemination with frozen semen in the dog. *J Reprod Fertil Suppl* 1989;39:289–292.
32. Hollinshead F, Hanlon D. Normal progesterone profiles during estrus in the bitch: a prospective analysis of 1420 estrous cycles. *Theriogenology* 2019;125:37–42.
33. Concannon P, Hansel W, Mcentee K. Changes in LH, progesterone and sexual behavior associated with preovulatory luteinization in the bitch. *Biol Reprod* 1977;17:604–613.
34. Bysted B, Dieleman S, Hyttel P et al. Embryonic developmental stages in relation to the LH peak in dogs. *J Reprod Fertil Suppl* 2001;57:181–186.
35. Concannon P, Hansel W, Visek W. The ovarian cycle of the bitch: plasma estrogen, LH and progesterone. *Biol Reprod* 1975;13:112–121.
36. Bergeron LH, Nykamp SG, Brisson BA et al. An evaluation of B-mode and color Doppler ultrasonography for detecting periovulatory events in the bitch. *Theriogenology* 2013;79:274–283.
37. Wildt D, Panko W, Chakraborty P et al. Relationship of serum estrone, estradiol-17 β and progesterone to LH, sexual behavior and time of ovulation in the bitch. *Biol Reprod* 1979;20:648–658.
38. Nöthling JO, De Cramer KG. Comparison of progesterone assay by chemiluminescence or radioimmunoassay for clinical decision-making in canine reproduction. *J S Afr Vet Assoc* 2019;90:1–6.
39. Steckler D, Nöthling J, Harper C. Prediction of the optimal time for insemination using frozen-thawed semen in a multi-sire insemination trial in bitches. *Anim Reprod Sci* 2013;142:191–197.
40. Kutzler MA, Mohammed HO, Lamb SV et al. Accuracy of canine parturition date prediction from the initial rise in preovulatory progesterone concentration. *Theriogenology* 2003;60:1187–1196.
41. Thomassen R, Sanson G, Krogenaes A et al. Artificial insemination with frozen semen in dogs: a retrospective study of 10 years using a non-surgical approach. *Theriogenology* 2006;66:1645–1650.
42. De Gier J, Kooistra H, Djajadiningrat-Laanen S et al. Temporal relations between plasma concentrations of luteinizing hormone, follicle-stimulating hormone, estradiol-17β, progesterone, prolactin, and α-melanocyte-stimulating hormone during the follicular, ovulatory, and early luteal phase in the bitch. *Theriogenology* 2006;65:1346–1359.
43. Conley AJ, Gonzales KL, Erb HN et al. Progesterone analysis in canine breeding management. *Vet Clin North Am Small Anim Pract* 2023;53:931–949.
44. Tahir M, Thoumire S, Raffaelli M et al. Effect of blood handling conditions on progesterone assay results obtained by chemiluminescence in the bitch. *Domest Anim Endocrinol* 2013;45:141–144.
45. Volkmann DH. The effects of storage time and temperature and anticoagulant on laboratory measurements of canine blood progesterone concentrations. *Theriogenology* 2006;66:1583–1586.
46. Thuróczy J, Wölfling A, Tibold A et al. Effect of anticoagulants and sampling time on results of progesterone determination in canine blood samples. *Reprod Domest Anim* 2003;38:386–389.
47. Athorn RZ, Stott P, Bouwman E et al. Effect of feeding level on luteal function and progesterone concentration in the vena cava during early pregnancy in gilts. *Reprod Fertil De* 2013;25:531–538.
48. Miller H, Foxcroft G, Squires J et al. The effects of feed intake and body fatness on progesterone metabolism in ovariectomized gilts. *J Anim Sci* 1999;77:3253–3261.
49. Prime G, Symonds H. Influence of plane of nutrition on portal blood flow and the metabolic clearance rate of progesterone in ovariectomized gilts. *J Agric Sci* 1993;121:389–397.
50. Parr R, Davis I, Miles M et al. Liver blood flow and metabolic clearance rate of progesterone in sheep. *Res Vet Sci* 1993;55:311–316.
51. Williams A, Cumming I. Inverse relationship between concentration of progesterone and nutrition in ewes. *J Agric Sci* 1982;98:517–522.
52. Sangsritavong S, Combs D, Sartori R et al. High feed intake increases liver blood flow and metabolism of progesterone and estradiol-17β in dairy cattle. *J Dairy Sci* 2002;85:2831–2842.
53. Concannon P, Weigand N, Wilson S et al. Sexual behavior in ovariectomized bitches in response to estrogen and progesterone treatments. *Biol Reprod* 1979;20:799–809.
54. Seager S, Platz C, Fletcher W. Conception rates and related data using frozen dog semen. *Reproduction* 1975;45:189–192.
55. Andersen K. Insemination with frozen dog semen based on a new insemination technique. *Reprod Domest Anim* 1975;10:1–4.
56. Seager S. Progress on the use of frozen semen in the dog. *Vet Rec* 1973;92:6–10.
57. Farstad W. Bitch fertility after natural mating and after artificial insemination with fresh or frozen semen. *J Small Anim Pract* 1984;25:561–565.
58. Battista M, Parks J, Concannon P. Canine sperm post-thaw survival following freezing in straws or pellets using PIPES, lactose, TRIS or TEST extenders. XIth International Congress on Animal Reproduction and Artificial Insemination (Dublin), 26–30 June 1988.
59. Thomassen R, Farstad W. Artificial insemination in canids: a useful tool in breeding and conservation. *Theriogenology* 2009;71:190–199.
60. Fontbonne A, Badinand F. Canine artificial insemination with frozen semen: comparison of intravaginal and intrauterine deposition of semen. *J Reprod Fertil Suppl* 1993;47:325–327.
61. Theret M, Treize G, Dumon C. *Artificial insemination of the bitch, using the Osiris gun. Mod Vet Pract* 1987;68:229–230.
62. Tsumagari S, Ichikawa Y, Toriumi H et al. Optimal timing for canine artificial insemination with frozen semen and parentage testing by microsatellite markers in superfecundency. *J Vet Med Sci* 2003;65:1003–1005.
63. Nöthling JO, Volkmann DH. Effect of addition of autologous prostatic fluid on the fertility of frozen-thawed dog semen after intravaginal insemination. *J Reprod Fertil Suppl* 1993;47:329–333.
64. Silva L, Onclin K, Lejeune B et al. Comparisons of intravaginal and intrauterine insemination of bitches with fresh or frozen semen. *Vet Rec* 1996;138:154–157.
65. Nöthling J, Gerstenberg C, Volkmann D. Success with intravaginal insemination of frozen-thawed dog semen: a retrospective study. *J S Afr Vet Assoc* 1995;66:49–55.
66. Linde-Forsberg C, Forsberg M. Results of 527 controlled artificial inseminations in dogs. *J Reprod Fertil Suppl* 1993;47:313–323.
67. Linde-Forsberg C, Holst BS, Govette G. Comparison of fertility data from vaginal vs intrauterine insemination of frozen-thawed dog semen: a retrospective study. *Theriogenology* 1999;52:11–23.
68. Rota A, Milani C, Romagnoli S et al. Pregnancy and conception rate after two intravaginal inseminations with dog semen frozen either with 5% glycerol or 5% ethylene glycol. *Anim Reprod Sci* 2010;118:94–97.
69. Rodriguez-Martinez H, Martinez EA, Calvete JJ et al. Seminal plasma: relevant for fertility? *Int J Mol Sci* 2021;22:4368.
70. Nagashima J, Ferraz M, Songsasen N. Extracellular vesicles and assisted reproductive technology. *Clinical Theriogenology* 2020;12:89–101.
71. Mason S, Rous N. Comparison of endoscopic-assisted transcervical and laparotomy insemination with frozen-thawed dog semen: a retrospective clinical study. *Theriogenology* 2014;82:844–850.

Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site: http://onlinelibrary.wiley.com/doi/10.1111/avj.13336/suppinfo.

Supplementary Video 1 Video of the view through a vaginal speculum (internal diameter approximately 16 mm) in a bitch that is approaching dioestrus (D-1).

(Accepted for publication 1 April 2024)