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Use of Commercial Luteinizing Hormone and Progesterone Assay Kits in Canine Breeding Management (24-May-2001)

M. V. Root Kustritz

Department of Small Animal Clinical Sciences, College of Veterinary Medicine, University of Minnesota, St. Paul, MN, USA.

Summary

Accurate timing of ovulation permits optimal breeding management in bitches. Correlation between physical and behavioral changes during estrus and time of ovulation is poor. Measurement of serum progesterone concentration is the preferred technique for assessment of day of ovulation in estrous bitches; use of in-house and commercial assays is described.

Endocrinology of Ovulation in the Bitch

The estrous cycle of the bitch consists of four stages: proestrus, estrus or standing heat, diestrus, and anestrus. In proestrus, the serum estrogen concentration is elevated. The increased estrogen causes overt physical changes characteristic of heat in the bitch, including vulvar swelling and exudation of serosanguinous discharge through the vagina, and increased cornification of the vaginal epithelium. Serum estrogen concentration falls abruptly late in proestrus, stimulating a surge in serum luteinizing hormone (LH) concentration and subsequent ovulation [1]. Peak serum LH concentration, of 7 to 50 ng/ml, lasts 24 to 40 hours before returning to basal levels [1-3]. Ovulation occurs 2.0 to 0.1 days after the LH peak [4,5].

Serum progesterone concentration begins to rise from basal levels coincident with the fall in serum estrogen concentration and the LH peak, due to preovulatory luteinization of follicular cells [1,6]. Serum progesterone concentration on the day of the LH peak, about 2 days prior to ovulation, is 2.0 to 2.9 ng/ml [2,3,7]. Serum progesterone concentration on ovulation day is 4 to 10 ng/ml [1,7].

Decline in serum estrogen concentration and rise in serum progesterone concentration signals onset of behavioral estrus, or standing heat, in the bitch [1,3]. The average bitch may ovulate anywhere from 2 days before to 6 days after onset of behavioral estrus [3,8]. Estrus is characterized cytologically by complete cornification of vaginal epithelial cells with greater than 50 % of those cells apparently anuclear.

Six days after ovulation (8.0 to 0.3 days after the LH peak), the bitch enters cytologic diestrus, characterized behaviorally by cessation in standing behavior and cytologically by an abrupt return to non-cornified epithelium [9].

Ovulation Timing in the Bitch

Methods for ovulation timing in the bitch include assessment of behavioral or physical changes in the bitch, changes in the vaginal fluid, assessment of cytologic or vaginoscopic changes of the vaginal epithelium, and hormone assays.

Behavioral or Physical Changes in the Bitch - Bitches may ovulate anywhere from 2 days before to 6 days after onset of standing heat [3,8]. Dominant bitches may never exhibit standing heat, and submissive bitches may allow mounting by a male even when not in estrus. Physical changes described in bitches include decreased vulvar turgidity at the onset of estrus, decreased vulvar width at the time of the LH surge, and a change in color of the vaginal discharge from serosanguinous to straw colored at the onset of standing heat [10,11]. Behavioral and physical changes in the bitch during estrus are variable, and are poorly correlated with ovulation.

Changes in the Vaginal Fluid - Attempts have been made to measure concentration of progesterone in vaginal fluid. Although progesterone concentration does rise in vaginal fluid coincident with elevation in serum progesterone concentration, correlation is poor [12,13]. Vaginal fluid pH has been reported to be decreased at onset of estrus, as has electrical resistance of vaginal secretions, and concentration of glucose in vaginal fluid has been reported to increase at time of estrus onset, but clinical usefulness of these changes is limited [11,13]. Arborization (ferning) of vaginal fluid allowed to air dry on a glass slide is maximal 2.3 to 1.2 days after the LH peak in dogs; in one study, breeding by time alone (10 and 12 days after estrus onset) yielded a pregnancy rate of only 78 %, while breeding around the time of maximal ferning of vaginal fluid yielded a pregnancy rate of 92 % [14].

Vaginal Cytology and Vaginocopy - Vaginal cornification increases gradually in proestrus. Complete cornification generally

occurs before the LH peak [15]. Estrus, or standing heat, is defined cytologically as complete cornification with greater than 50 % of the cells appearing anuclear. There is poor correlation between onset of cytologic estrus and ovulation. The onset of cytologic diestrus, characterized as an abrupt return to complete non-cornification, occurs 6 days after ovulation, allowing retrospective assessment of ovulation day [9].

Vaginoscopy can be used to differentiate the edematous, pillowy, rose pink vaginal mucosa of proestrus from the dehydrated, sharp-edged, blanched mucosa of estrus [11]. Again, correlation between estrus onset and ovulation is variable.

Hormone Assay - Hormone assay is the most accurate measurement of ovulation time in bitches. The two hormones commonly assayed for breeding management are LH and progesterone.

Luteinizing hormone is a protein hormone that can be measured in an in-house assay (Status-LH, Synbiotics, San Diego CA). The test is stored at room temperature and has a relatively short shelf-life. The kit may not be accurate if used beyond the expiration date, or if the foil packet containing the test device and pipette is opened well before the test is to be run. The preferred sample is serum. The sample should not be lipemic or hemolyzed. The test should be run the same day the serum is collected. If that is not possible, the serum should be refrigerated, not frozen, until the test can be run. (Fig. 1, Fig. 2 and Fig. 3).



Figure 1. The Status-LH test kit for measurement of luteinizing hormone in canine serum (Synbiotics, San Diego CA). - To view this image in full size go to the IVIS website at www.ivis.org . -



Figure 2. Components of the Status-LH test kit for measurement of luteinizing hormone in canine serum (Synbiotics, San Diego CA). The foil packet containing the test device and pipette must not be opened until just before running the test. - To view this image in full size go to the IVIS website at

www.ivis.org . -



Figure 3. Final result of a test for luteinizing hormone using the Status-LH test kit (Synbiotics, San Diego CA). The pink line is the control, indicating that the test is complete and was run correctly. The absence of a similar pink line beneath the control marker, next to the letter T on the test device, is indicative of a concentration of LH in the test sample of less than 1 ng/ml. - To view this image in full

size go to the IVIS website at www.ivis.org . -

The semi-quantitative enzyme linked immunosorbent assay (ELISA) for determining the LH peak yields a result of low (less than 1 ng/ml) or high (greater than 1 ng/ml). The ELISA test has been shown to yield results consistent with an accurate radioimmunoassay (RIA) [16]. Because duration of the LH peak averages about one day in bitches, samples must be drawn daily, at about the same time of day. The manufacturer recommends that determination of the LH peak with this test be verified by measurement of serum progesterone, described below. Because of the test's short shelf-life, necessity of daily testing, and the requirement of assessing progesterone to verify accuracy, the author rarely uses this assay in practice. Measurement of progesterone by quantitative assays, such as RIA or chemiluminescence, allow prediction of ovulation using the following scheme (Table 1). Turn-around time for assay results can be as little as 24 hours with some veterinary diagnostic laboratories.

Table 1. Correlation of serum progesterone concentration with significant reproductive events in the bitch. The margin of error is estimated to be about 1 days.	
Serum Progesterone (ng/ml)	Event
Less than 1.0	Anestrus or proestrus
1.0 - 1.9	Ovulation minus 3 days □ recommend recheck
2.0 - 2.9	Ovulation minus 2 days
3.0 - 3.9	Ovulation minus 1 day
4.0 - 10.0	Ovulation day
-	Ovulation plus 2 days is optimal breeding day
Greater than 10.0 with cornified vaginal smear	Ovulation plus 1 to 5 days

Measurement of progesterone by in-house, semi-quantitative ELISA progesterone tests can also be used for estimating the time of ovulation in the bitch. The primary advantage of the in-house progesterone assays is their short turn-around time; results may be available as quickly as 20 minutes after the blood sample is drawn. The main disadvantages are increased labor and decreased accuracy [17]. The approach to sampling time and intervals varies based on whether samples are out-sourced for commercial RIA or are assayed by in-house ELISA (Table 2).

Table 2. Clinical scheme for ovulation timing in the bitch with assay of serum progesterone concentration using quantitative and semi-quantitative methods.	
Type of Progesterone Assay Used	Clinical Management for Ovulation Timing
Commercial assay (radioimmunoassay [RIA] or chemiluminescence) - Quantitative result	Blood should be drawn for measurement of progesterone concentration in serum beginning when the bitch has about 70 % cornification of vaginal epithelial cells. Samples are drawn and re-evaluated every 3 - 4 days until a concentration in the predictable range is reached (2 - 10 ng/ml; see Table 1).
In-house assay (enzyme linked immunosorbent assay [ELISA]) - Semi-quantitative result	Blood should be drawn for measurement of progesterone concentration in serum beginning about the fourth day after proestrus onset. Samples are drawn and evaluated daily until a high concentration (greater than 4 to 10 ng/ml) is reached. The first day the assay detects a value in the high range is considered to be ovulation day (see Table 1).

Several in-house assays for measurement of progesterone in canine serum are commercially available. Three commercially available assays are described.

The K9 Proges-Check test is a semi-quantitative ELISA kit (Endocrine Technologies, Inc., Newark CA). The kit is stored at refrigerator temperature, and brought to room temperature before use. Either serum or heparin plasma may be used for test samples, but samples must not be hemolyzed, lipemic, or icteric. Serum can be either refrigerated or frozen, if necessary, before running the test but the test sample should be at room temperature when the test is run. The three controls yield colors of dark blue, indicative of 0 ng/ml of progesterone; lighter blue, indicative of 2 ng/ml of progesterone; and lightest blue, indicative of greater than 10 ng/ml of progesterone. The test sample's color is compared with the controls and is read as 2 ng/ml or less of progesterone if the intensity is near or equal to the medium to dark blue control colors, between 2 and 10 ng/ml of progesterone is the intensity is less than or equal to the lighter blue control, and greater than 10 ng/ml of progesterone is the test sample intensity is lighter than the lightest blue control. The manufacturer makes no recommendation regarding timing of breeding at these various concentrations (Fig. 4, Fig. 5 and Fig. 6).



Figure 4. The K9 Proges-Check test kit for measurement of progesterone in canine serum (Endocrine Technologies, Inc., Newark CA). - To view this image in full size go to the IVIS website at www.ivis.org . -



Figure 5. Components of the K9 Proges-Check test kit for measurement of progesterone in canine serum (Endocrine Technologies, Inc., Newark CA). The test is run in clear test wells with three controls. - To view this image in full size go to the IVIS website at www.ivis.org . -



Figure 6. Final results of a test for progesterone in canine serum using the K9 Proges-Check test kit for measurement of progesterone in canine serum (Endocrine Technologies, Inc., Newark CA). The well to the left (darkest blue) is equivalent with 0 ng/ml of progesterone, the second well (lighter blue) with 2 ng/ml, and the third well (lightest blue) with 10 ng/ml. The unknown sample, on the far right, contained no progesterone. - To view this image in full size go to the IVIS website at www.ivis.org . -

The PreMate test is a semi-quantitative ELISA kit (Camelot Farms, College Station TX). The kit is stored at refrigerator

temperature, and brought to room temperature before use. One of the test components must be frozen if the kit is to be maintained for longer than three months, and also must be at room temperature before running the test. Either serum or heparinized plasma may be used for test samples. The two controls yield colors of dark pink, apparently indicative of less than 4 ng/ml of progesterone ("prior to ovulation", according to the manufacturer), and light pink, apparently indicative of greater than 4 ng/ml of progesterone ("ovulation day or later", according to the manufacturer). The test sample's color is compared with the controls and is read as "prior to ovulation" if the color is darker than the light pink sample, and "ovulation day or later" if the color is equal to or lighter than the light pink sample. The manufacturer recommends that dogs with samples indicative of "prior to ovulation" be rechecked in two days, and that dogs with samples indicative of "ovulation day or later" be bred immediately (Fig. 7, Fig. 8 and Fig. 9).



Figure 7. The PreMate test kit for measurement of progesterone in canine serum (Camelot Farms, College Station, TX). - To view this image in full size go to the IVIS website at www.ivis.org . -



Figure 8. Components of the PreMate test kit for measurement of progesterone in canine serum (Camelot Farms, College Station, TX). The test is run in clear test wells with two controls. - To view this image in full size go to the IVIS website at www.ivis.org . -



Figure 9. Final results of a test for progesterone in canine serum using the PreMate test kit for measurement of progesterone in canine serum (Camelot Farms, College Station, TX). The far left test well (dark pink) is equivalent with less than 4 ng/ml of progesterone, and the center well (light pink) with greater than 4 ng/ml of progesterone. The unknown sample, on the far right, contained no progesterone. - To view this image in full size go to the IVIS website at www.ivis.org . -

Status-Pro is a semi-quantitative ELISA kit (Synbiotics, San Diego CA). The kit is stored at refrigerator temperature, and brought to room temperature before use. Serum is the preferred test sample; samples must not be hemolyzed or lipemic. Serum can be either refrigerated or frozen, if necessary, before running the test but the test sample should be at room temperature when the test is run. The test is run in a test cup containing three wells that appear as dots within the cup. The presence of all three dots is indicative of less than 2 ng/ml of progesterone, the presence of two dots indicates 2 to 7 ng/ml, and the presence of one dot indicates greater than 7 ng/ml. There should always be at least one dot present; this is the control dot which is used to confirm proper performance of the test. The manufacturer recommends that breedings be performed several times, every second or third day, beginning when serum progesterone concentration reaches the "two dot" stage (2 to 7 ng/ml of progesterone); if chilled or frozen semen is to be used, the manufacturer strongly recommends concurrent testing with the Status-LH and Status-Pro kits. The Status-Pro test kit is under revision at the time of this writing.

Timing of Breeding

Bitches ovulate an immature oocyte that requires about two days to mature before it can be fertilized. Optimal breeding day with natural service, artificial insemination (AI) with fresh semen, or AI with chilled semen is two days after ovulation. For insemination with frozen/thawed spermatozoa, which have decreased viability and longevity, optimal breeding day is 3 to 4 days after ovulation for a single breeding. Pregnancy rate is increased in all techniques if the bitch is bred more than once in the fertile period [18].

References

1. Wildt DE, Panko WB, Chakraborty PK, et al. Relationship of serum estrone, estradiol-17beta and progesterone to LH, sexual behavior and time of ovulation in the bitch. *Biol Reprod* 1979 ;20:648-658.
2. Concannon PW, Hansel W, Visek WJ. The ovarian cycle of the bitch: Plasma estrogen, LH and progesterone. *Biol Reprod* 1975; 13:112-121.

3. 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.
4. Phemister RD, Holst PA, Spano JS, et al. Time of ovulation in the Beagle bitch. *Biol Reprod* 1973; 8:74-82.
5. Concannon PW, McCann JP, Temple M. Biology and endocrinology of ovulation, pregnancy and parturition in the dog. *J Rep Fert Suppl* 1989; 39:3-25.
6. Hadley JC. Total unconjugated oestrogen and progesterone concentrations in peripheral blood during the oestrous cycle of the dog. *J Rep Fert* 1975; 44:445-451.
7. Johnston SD, Root MV. Serum progesterone timing of ovulation in the bitch. In: *Proceedings of the Annu Meet Soc Theriogenology* 1995; 195-203.
8. Bouchard GF, Solorzano N, Concannon PW, et al. Determination of ovulation time in bitches based on teasing, vaginal cytology, and ELISA for progesterone. *Theriogenology* 1991; 35:603-611.
9. Holst PA, Phemister RD. Temporal sequence of events in the estrous cycle of the bitch. *Amer J Vet Res* 1975; 36:705-706.
10. Nishiyama T, Narita K, Tsumagari S, et al. Shrinkage in the horizontal dimensions of the vulva (vulvar shrinkage) as an indicator of standing heat in the Beagle. *J Amer Anim Hosp Assoc* 2000; 36:556-560.
11. Hewitt D, England G. Assessment of optimal mating time in the bitch. In *Practice* 2000; 22:24-33.
12. England GCW, Anderton DJ. Determination of progestogen concentrations in the vaginal fluid of bitches in oestrus. *Vet Rec* 1992; 130:143-144.
13. Schulz A, Gilles M, Lange A, et al. Evaluation of low invasive methods to determine the optimum time for mating in bitches. *Rep Dom Anim* 2000; 35:42-43. [Abstract].
14. England GCW. Vaginal cytology and cervicovaginal mucus arborisation in the breeding management of bitches. *J Sm Anim Prac* 1992; 33:577-582.
15. Mestre J, Wanke M, Sucheyre S. Exfoliate vaginal cytology and plasma concentrations of progesterone, luteinising hormone and oestradiol-17 β during oestrus in the bitch. *J Sm Anim Prac* 1990; 31:568-570.
16. Nishiyama T, Kinugasa T, Kimura T, et al. Determination of optimal time for mating by artificial insemination with chilled semen using luteinizing hormone surge as an indicator in Beagles. *J Amer Anim Hosp Assoc* 1999; 35:348-352.
17. Manothaiudom K, Johnston SD, Hegstad RL, et al. Evaluation of the Icagen-Target canine ovulation timing diagnostic test in detecting canine plasma progesterone concentrations. *J Amer Anim Hosp Assoc* 1995; 31:57-64.
18. VanHaften B, Dieleman SJ, Okkens AC, et al. Timing the mating of dogs on the basis of blood progesterone concentrations. *Vet Rec* 1989; 125:524-526.

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