

Advances in ovulation timing in the bitch

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In order to understand how ovulation timing (OVT) works, it is important to understand the basic physiology of the estrous cycle. Ovulation timing consists of vaginal cytology, vaginal speculum examination, progesterone and luteinizing hormone (LH) concentrations. Once an understanding of basic OVT is obtained, this knowledge can be used to assist with breeding management decision making.

Puberty and the estrous cycle

Puberty occurs on average at nine months of age, with a range of six to 14 months. Smaller breed bitches tend to attain puberty earlier than do large breed bitches. The bitch typically has one or two cycles/year; however there is significant individual variation within breeds and between breeds. Certain breeds have more frequent cycles (Akitas, German shepherd dogs, Cocker spaniels and Labrador retrievers may have a normal four month interestrus interval) and others less frequent (Basenjis and Tibetan mastiffs tend to have only one cycle/year). In general, a minimum interestrus interval of 4.5 – 5 months is considered necessary for normal fertility.

The estrous cycle consists of 4 phases: proestrus, estrus, diestrus and anestrus. Proestrus is when follicle development occurs. Estrogen concentrations begin to rise significantly early in this stage and peak near its end, when the follicles are mature. Luteinizing hormone is initially produced in small surges which result in thickening of the walls of small follicles present on the ovaries, resulting in a slow, but steady rise in progesterone. At the end of proestrus, a large pre-ovulatory surge in LH occurs, which results in ovulation of any mature follicles, which subsequently causes a rapid rise in progesterone concentrations. On average, proestrus lasts seven to nine days. Early in proestrus, the vulva begins to swell and bloody vulvar discharge begins, which is initially bright red in color. This discharge comes from the uterus as a result of leakage of red blood cells across capillary walls following exposure to estrogen. As proestrus continues the discharge lessens and becomes lighter and more straw-colored. Most intact males show minimal interest in the bitch early in proestrus but become more and more interested as this stage proceeds. The bitch is typically not receptive during early – mid proestrus but begins flagging and standing for the male towards the end of proestrus or in early estrus.

Estrus is the stage of the cycle associated with the fertile period (time when eggs are completing maturation or are awaiting fertilization) and sexual receptivity in the bitch. Receptive behavior results from the rapid change in the estrogen to progesterone ratio. Ovulation occurs over several hours (4 – 30) depending on the number of eggs ovulated. Canine eggs are ovulated in an immature state and require two days to mature before fertilization may occur. Once mature, the eggs are capable of being fertilized for four to days, provided sperm can gain access to them (see next section). Thus, the fertile period lasts six to eight days, beginning with ovulation. Estrus generally lasts seven to nine days. During estrus, the vulva softens but remains swollen. There is usually minimal to no vulvar discharge during this stage; although some bitches will have bloody vulvar discharge through estrus and on into diestrus. In most cases, intact male dogs are intensely interested in the bitch during this stage of the cycle. Most bitches flag readily and stand to be mated.

Diestrus is the stage of the cycle associated with progesterone production and pregnancy, if the bitch is successfully mated. It lasts 57 days in the pregnant bitch. If no fertilization occurs or the pregnancy fails, progesterone production can last anywhere from 45 – 72 days. Progesterone rises for seven to 21 days after ovulation. It then remains at a plateau for about 10 – 20 days after which it slowly begins to decline until the end of diestrus. Receptive behavior ends at the end of estrus or early diestrus and males quickly lose interest in the female in early diestrus. During diestrus when the bitch is not pregnant, vulvar swelling and discharge decreases and returns to its pre-proestrus condition. In the pregnant bitch, vulvar swelling initially decreases but then begins again in late pregnancy, along with relaxation of the pelvic ligaments and perivulvar area in preparation for delivery. In some bitches, there

will also be a small amount of clear – mucoid vulvar discharge during diestrus and pregnancy. This discharge normally has no odor or color.

Anestrus is the stage of the cycle associated with relative ovarian inactivity. Intact males are not interested in the bitch nor does the bitch display receptive behavior. There is no vulvar swelling or discharge.

Vaginal cytology and speculum examination

Epithelial cell types present on vaginal cytology (from deepest to most superficial) include basal, parabasal, intermediate, nucleated superficial (non-cornified) and anucleated superficial cells (cornified; Table 1). Other cell types include white blood cells (WBC), red blood cells (RBC), foam cells (clean up cells) and metestrual cells (epithelial cells eating a white blood cell). Bacteria, mucus and background debris are also seen at different stages of the cycle.

In early proestrus, the deeper cell layers predominate because the vaginal wall is thin. White blood cells, RBCs, bacteria, mucus and background debris are present in larger amounts. As proestrus progresses, the vaginal walls thicken as a result of increasing estrogen concentrations. Superficial cells become more predominant, initially with nuclei and eventually becoming more and more anucleated. As proestrus continues, WBCs disappear and RBCs reduce in numbers. Bacterial numbers, mucus and background debris diminish to negligible amounts by the end of proestrus. The vaginal mucosa is pink and swollen (edematous) or billowy in appearance during early proestrus. As proestrus continues, it becomes more dehydrated and paler (whiter) in color. This dehydration is termed crenulation and it is most pronounced when anucleated superficial cells predominate at the end of proestrus and continues to be visible throughout estrus.

In estrus, the predominant cell type is the anucleated superficial cell. Typically there are 70 - 90% anucleated superficial cells during estrus. The vaginal mucosa is white in color and there is marked crenulation or wrinkling present throughout this stage of the cycle. Ovulation cannot be determined using cytology and speculum examination alone.

There is an abrupt transition in cell types between estrus and diestrus. On day 1 of diestrus, the number of nucleated epithelial cells increases dramatically. This occurs as a result of the sloughing of the thick layer of cornified cells and exposure of the deeper layers of the wall of the vagina. Intermediate, parabasal cells and WBCs return during the first few days of diestrus. The WBCs help with removal of dead sperm and bacteria from the vagina following breeding. Two new cell types become evident during early diestrus: metestrual cells and foam cells. The amount of background debris and mucus increases during diestrus. The vaginal mucosa attains a mottled appearance (blotchy pink and red) and becomes flatter.

During anestrus, the vaginal wall is thin, so the deep layers of cells begin to predominate again. Mostly basal and parabasal cells are present. There are few WBCs present and no foam or metestrual cells. Moderate to heavy mucus and background debris are present throughout anestrus. The vaginal mucosa is a solid dark red color and smooth.

Sample handling

Samples for progesterone should be obtained on the fasted bitch (at least six to eight hours) to prevent fat in the bloodstream from interfering with results. Atraumatic blood draws (using the jugular veins) and care with placing the blood sample directly into the tube, rather than using the vacuum pressure in the tube to draw the blood in, will prevent red blood cell rupture from occurring which also may affect hormone concentrations. Use of plain glass red top tubes are recommended. Blood should be allowed to clot for 15 - 30 minutes at room temperature and then the tube should be centrifuged for 5 - 10 minutes. The serum should be removed promptly and placed in another plain glass red top tube. If the blood will be tested within 48 hours it should be refrigerated. If it will be longer, then it should be frozen. Serum separator tubes should be avoided; serum should not be left sitting on top of the red blood cells after centrifugation for more than 10 – 15 minutes; nor should clotted blood be submitted without

centrifugation and removal of serum. Blood obtained for LH samples should be handled similarly but should be frozen until assayed.

Tests available

Progesterone concentrations may be evaluated numerically (quantitatively) or in broad ranges (qualitatively with in-house kits). Using the same laboratory and testing procedure is recommended during a single cycle. Switching between laboratories or types of tests can result in small to large differences in hormone concentrations which may affect interpretation of results especially around the time of ovulation. Most veterinary reference laboratories offer once or twice daily progesterone testing, providing quick turnaround for analysis.

Three in-house progesterone test kits are currently available in the US. These are all subjective tests. Results from these test kits are more variable than traditional quantitative testing. Current tests available are: Target® by Biometallics, Corp, Princeton, NJ; Ovucheck Premate® by Biovet, St. Anthony, MN; and K9 Proges-Check® by Endocrine Technologies, Newark, CA. Accuracy of these tests varies, with the Target® kit displaying the most comparable and consistent results compared to laboratory values. These tests give ranges of progesterone based on colorimetric changes – for example < 1 ng/ml; 1 – 2.5 ng/ml; 2.5 – 5 ng/ml; or > 5 ng/ml for the Target® kit. Subjective assessment may result in different observers recording different results, so consistency is critical if these kits will be used for breeding management.

Numerical testing may be performed using radioimmunoassay (RIA), chemiluminescence testing (CLIA), enzyme-linked fluorescent testing (ELFA), or enzyme-linked immunosorbent assay (ELISA). Most reference laboratories are using CLIA testing at this time. Reproductive practices that have in-house numerical testing machines are usually CLIA, ELFA, or ELISA. As long as there is good internal quality control and consistency, these in-house numerical tests are very accurate. Again, using the same testing procedure for all the samples is critical to accurate interpretation of results.

Most LH testing is done using a qualitative in-house assay called Witness LH® (Synbiotics Corp, Kansas City, MO). It provides a positive or negative test value (either >1 ng/ml or < 1 ng/ml) with a turnaround time of 20 minutes. Luteinizing hormone testing at the laboratory often has unacceptable turnaround times (2+ days) and most laboratories use the same in-house kit as described above. When using this test on a routine basis, clinics become good at interpreting the difference between weak and strong positive tests, which is important since the LH surge can be < 24 hours in length making the presence of a clear strong positive not possible in some cases.

Ovulation timing

Generally speaking, the bitch should be examined for the first time between days 5 and 8 of the cycle. If the onset of the cycle is accurately noted and the bitch has a normal cycle, this will be early enough to catch the LH surge if needed. It is important to remember that in some cases, the early signs of the estrous cycle may be missed so the bitch may be much further along in her cycle than believed. If the bitch will be bred with frozen semen it is more important to start earlier in the cycle than if she is being bred naturally, since determination of the LH surge is significantly more important when using frozen semen. Bitches are typically examined every one to three days until ovulation is confirmed.

If one is comfortable reading vaginal cytology slides and performing speculum examinations, the need to begin submitting progesterone samples can be delayed until mid-late proestrus is reached. Bitches that have slides indicative of early – mid proestrus may have blood samples drawn and the serum frozen and stored in order to be able to backtrack to evaluate progesterone or LH concentrations if future tests indicate. Vaginal cytology is typically performed every other day. Progesterone concentrations should be performed regularly once vaginal cytology indicates 50% superficial cells and 20% anucleated superficial cells. As the bitch approaches ovulation, every other day to daily sampling may be required depending on the type of breeding being performed and access to laboratory services. It is good practice to always obtain at least one vaginal cytology slide, on the day of presentation, when bitches are presented for ovulation timing in case the initial progesterone concentration is very elevated – in which

case the cytology is necessary to determine if the bitch is still within her fertile window or if she has already entered diestrus. Regular vaginal cytology and speculum examination can decrease the number of progesterone tests needed and will also alert the veterinarian to abnormalities of the cycle, days to weeks before progesterone will become suspicious. Vaginal cytology and speculum examination are indicators of estrogen concentrations and really do not give any clue to what progesterone concentrations may be and vice versa. So while cytology and speculum examination may seem unnecessary when progesterone testing is being performed, it is actually quite helpful.

If assessing only progesterone concentrations, every other day sampling is adequate. If LH concentrations are being determined as well, daily sampling is required because the LH surge is short (average – 18 - 24 hours) and is easily missed with every other day testing. In some practices, only LH testing is performed, and inseminations planned based on the LH surge. This is inadequate for bitches that do not ovulate or have an abbreviated LH surge, so use of LH and progesterone or progesterone alone is more likely to provide accurate breeding management than LH alone.

When bitches have normal cycles, progesterone concentrations are correlated to key hormonal events (Table 2). Ovulation begins after the bitch's progesterone reaches at least 4 ng/ml (between 4 – 10 ng/ml). Ovulation is confirmed by a rise in progesterone of AT LEAST 3 ng/ml in 24 hours. Some bitches rise 3 ng/ml in 24 hours while others jump by 10 or more ng/ml in 24 hours. The amount of the rise has to do with number of follicles ovulated, the size of the follicles and the amount of progesterone-producing tissue (luteal tissue) present. Increases of less than 3 ng/ml/day are not confirmatory for ovulation and additional samples should be obtained in 1 – 2 days.

Failing to confirm ovulation is the most common mistake in ovulation timing. Following a bitch to the 4 – 5 ng/ml stage and then not obtaining another sample to confirm ovulation may result in conception failure, small litter size and makes determination of an accurate due date more difficult.

Ovulation timing and type of insemination planned

Expected spermatozoal longevity

Fresh semen can be expected to survive at least 48 hours in the bitch and may live significantly longer depending on the dog (up to 11 – 14 days in some cases). Fresh, chilled semen can be expected to survive at least 24 hours in the bitch and may live significantly longer depending on the dog. Frozen semen has a relatively short lifespan after thawing, on average 8 - 12 hours.

Type of hormone analysis recommended

Natural breeding. Use of qualitative progesterone testing alone is typically adequate with natural breeding, although use of numerical progesterone assays are ideal. Testing every 2 – 3 days once the cytology is predominantly superficial cells is usually adequate. If using numerical progesterone testing, ovulation should be confirmed by repeating the test 1 – 2 days after reaching 4 – 5 ng/ml.

Vaginal artificial insemination (VAI). Progesterone testing (without LH testing) is usually adequate for VAI with either fresh or chilled semen. When using shipped, chilled semen, numerical progesterone testing is strongly recommended while when using fresh semen, either numerical or in house tests may be used. Ovulation should be confirmed when using numerical testing by repeating the test 1 – 2 days after reaching 4 – 5 ng/ml.

Transcervical and surgical insemination (TCI/SAI). Progesterone testing alone is sufficient for fresh or fresh, chilled semen, while when using frozen semen the addition of LH testing will help pinpoint the optimal day(s) for breeding. Ovulation should always be confirmed prior to inseminating with frozen semen and always prior to anesthetizing the bitch for SAI.

Type of insemination recommended

Natural breeding is recommended for fertile bitches and dogs with normal semen quality.

Vaginal AI can be performed using fresh or fresh chilled semen and is recommended for breeders who do not want to risk injury or transmission of venereal disease to the bitch or dog during natural

breeding, for inexperienced or nervous dogs or bitches when natural breeding is not successful, for bitches with vaginal anatomic defects (strictures or septums), for size discrepancies between dogs and bitches and for shipped, chilled semen that is of adequate quantity and quality that it can be used vaginally.

Transcervical insemination can be performed using fresh, fresh chilled or frozen semen and is recommended for fresh semen when semen quality is poor or sperm numbers are low in order to introduce an acceptable breeding dose into the uterus. It is recommended if the bitch owner only wants to do one insemination of chilled semen and anesthesia is not desired, although two breedings are usually recommended. It is recommended for large and giant breed bitches to ensure an adequate breeding dose reaches the uterus. When using frozen semen, two breeding doses inseminated 24 hours apart is ideal.

Surgical insemination can be performed using fresh, fresh chilled or frozen semen and is recommended for all the same reasons as TCI (except that anesthesia is required). It is often performed with frozen semen when there is only a single breeding dose available or if the frozen semen is of average or less than average quality. Surgical AI is also preferable for bitches with uterine pathology (subacute endometritis and/or cystic endometrial hyperplasia). There is less contamination with SAI than any other type of insemination and it also affords the ability to evaluate the uterus and manually rupture any cysts present in the uterus prior to insemination.

Breeding management

Natural breeding

Breeding should commence one or two days after the bitch ovulates. The bitch should be bred every other day for up to three matings or until she is no longer receptive.

Vaginal insemination

The first insemination is performed two days after ovulation. The insemination is repeated in two days.

Transcervical insemination

If using fresh or fresh chilled semen with two inseminations planned, inseminations are ideally performed two and four days after ovulation. If one insemination is planned, insemination on day 2, 3 or 4 after ovulation is equally ideal. When using frozen semen, two inseminations are preferable to one. Breedings should be planned on days 5 and/or 6 after the LH surge or days 3 and/or 4 after ovulation.

Surgical insemination

When using fresh or fresh chilled semen, surgical AI is can be performed on day 2, 3 or 4 after ovulation. If semen longevity is poor, waiting to day 3 or 4 after ovulation is preferable. When using frozen semen, SAI on day 3 or 4 after ovulation (days 5 or 6 after LH surge) is recommended.

The art of ovulation timing

When the bitches read the textbook, breeding management is fairly straight forward. It is in the cases where the bitches prove to us that they cannot read, that ovulation timing becomes more art than science.

Common situations where ovulation timing does not go according to expectations

Short proestrus. In these situations the vaginal cytology and speculum examination lag behind hormonal changes resulting in progesterone concentrations being significantly higher than expected. Bitches are commonly not receptive during the fertile period when this occurs resulting in poor pregnancy rates when bred based on behavior alone.

Long proestrus. Some bitches have an extended proestrus making progesterone testing quite prolonged. Owners may become frustrated with the number of tests required due to high costs. Bitches progressing slowly through proestrus may only rise at a rate of 0.1 – 0.2 ng/ml per day. Bitches are typically not receptive during their fertile period when this occurs resulting in poor pregnancy rates when breeding is based on behavior alone.

Split heat. Bitches may progress through proestrus and enter early estrus, but never rise above an ovulatory threshold. Eventually they drop back to baseline and vaginal cytology returns to proestrus or anestrus. After 7 – 21 days, they enter proestrus again. The second cycle is typically fertile and bitches should be bred after ovulation is confirmed. The use of vaginal cytology will help determine when progesterone samples should be obtained and can help determine that a split heat is occurring.

Anovulatory cycles There are cases where the bitch never ovulates and may be due ovarian cysts, abnormal follicular development, abnormal hormone production of endogenous LH, or in bitches with chromosome defects.

Bitches that do not test positive on in-house testing. Occasionally when using in-house kits, bitches do not have normal colorimetric changes associated with the rise in progesterone. These bitches will seemingly remain at baseline while vaginal cytology and speculum examinations progress normally. In bitches where colorimetric change is not occurring as expected, numerical progesterone should be submitted to ascertain if this is the situation.

Stalling. Some bitches have progesterone rise to a certain level (2 – 5 ng/ml, or higher) and then fail to progress for several days. If one bases breeding management on a ‘magic number’ (usually 5 ng/ml) then ovulation may be missed by several days. Confirming ovulation has occurred is critical to good breeding management.

Backtracking. Some bitches will rise to a certain level and then drop significantly lower for a day or two. Care should be taken that this is not a case of laboratory error (ask for validation when values are not as expected) or poor sample handling.

Rapid rise. Once bitches reach mid – late proestrus, they tend to rise at a rate of 0.5 – 2 ng/ml per day. Once ovulation occurs, progesterone typically rises at a rate of 3 – 4 ng/ml/day. In some cases, bitches may rise at rates of more than 5 - 10 ng/ml per day. This rapid rise is normal and does not change the successful window for fertilization.

Accurate use of ovulation timing allows precise breeding management and increases pregnancy rates and litter size. The most common cause of infertility in the canine is poor breeding management. Breeders often depend on their intact males to determine when to breed bitches but this can result in bitches being bred at an inappropriate time. If pregnancy rates are low with appropriate breeding management, other causes of infertility should be evaluated. Interpretation of vaginal cytology, speculum examination, progesterone and LH concentrations takes considerable experience. Whelping dates can be used to help determine if the day that ovulation was estimated at the time of breeding was accurate. Ovulation timing can be fine tuned by use of retrospective analysis.

References

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Table 1. Cell types present on vaginal cytology slides during the estrous cycle of the bitch

	Basal Cells	Parabasal Cells	Intermediate Cells	Superficial Cells	Anucleated Superficial Cells	RBC	WBC	Foam and Metestruual Cells	Mucus and Background Debris
Early Proestrus	++	++	++	+/-	-	+++	+	-	++
Mid Proestrus	-	+/-	+	+	+/-	++	-	-	+
Late Proestrus	-	-	+/-	++	++	+	-	-	+/-
Estrus	-	-	-	+/-	+++	+/-	-	-	-
Diestrus	+	++	++	+/-	-	+/-	+	+	+
Anestrus	++	+	+/-	-	-	-	+/-	-	+

Table 2. Hormonal concentrations associated with key events

Anestrus or early Proestrus	0 – 1.0 ng/ml	0 – 3.14 nmol/L
LH surge (2 days prior to ovulation)	1.1 – 1.9 ng/ml	4.08 – 5.97 nmol/L
Progesterone surge (1 day prior to ovulation)	2.0 – 3.9 ng/ml	6.28 – 12.25 nmol/L
Ovulation	4.0 – 10 ng/ml	12.56 – 31.4 nmol/L
Oocytes mature and available for fertilization	10.0 – 34.0+ ng/ml	31.4 - 106.76+ nmol/L

Conversion to SI units - 1 ng/ml = 3.14 nmol/L

