TECHNICAL ASPECTS OF CANINE OVULATION TIMING

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RATIONALE

The practice of ovulation timing has become increasingly useful to veterinarians who recognize its value in improving reproductive services. Accurate ovulation timing improves conception and facilitates breeding management. Popular stud dogs' owners commonly permit a limited number of breedings (usually 2), and may need to prioritize bitches based on ovulation timing. Owners of bitches need to make travel arrangements in advance, and usually wish to minimize time spent at the stud dog facility. Boarding of bitches in season can be abbreviated by recognition of the end of their fertile period. The use of extended, chilled, or frozen semen and the management of breedings using subfertile stud dogs require ovulation timing to optimize conception. Improved litter size occurs with properly timed breedings. Proper ovulation timing permits accurate evaluation of gestational length, important when managing parturition, and is essential in the evaluation of apparent bitch infertility.

Many elements of breeding management are now performed by motivated dog fanciers. Breeders may learn the technical skills associated with obtaining and evaluating vaginal cytologies from their veterinarians, and purchase inexpensive microscopes and staining systems. Some breeders have even invested in the equipment for obtaining and centrifuging blood samples, for evaluation with counter top semiquantitative progesterone kits. Others prepare serum samples and deliver them to commercial laboratories for quantitative progesterone assays. It is common practice for breeders to perform artificial insemination with fresh semen, but the use of extended chilled semen or frozen semen requires veterinary participation, according to AKC. Non veterinarians can freeze canine semen. Even so, eventually, professional input by a veterinarian or veterinary technician is sought for interpretation of these results.

CLINICAL REALITY

Clinical ovulation timing generates clients and income. This benefit may be offset by the time consuming nature of the breeder client, the technical skills required for performing proper ovulation timing, and the necessity of a solid understanding of canine reproductive physiology. If a veterinarian is not comfortable with canine reproductive physiology, they are unlikely to be enthusiastic about undertaking the practice of breeding management. Breeder clients typically request "emergency" ovulation timing appointments, which frequently turn into "emergency" artificial insemination appointments, often with semen evaluation required. It is logical that practices offering ovulation timing should offer expert artificial insemination as well. Client education is a large component of breeding management. Without proper practice management groundwork, ovulation timing and breeding management can become a chaotic component of a veterinary practice.

The investment in equipment for optimal ovulation timing is modest by practice standards today. Cotton tipped applicators, routine diff-quick stains, frosted glass slides, a light microscope, venipuncture equipment and a centrifuge for processing blood are likely already present in the small animal practice. In house kits are available from several companies for performing semiquantitative progesterone and luteinizing hormone assays. Commercial veterinary laboratories commonly provide quantitative progesterone assays with rapid turnaround (12-24 hours). Some veterinary theriogenologists advocate the use of human laboratories for quantitative progesterone assays, once validated for use in the dog. Rigid pediatric proctoscopes used for vaginoscopy are inexpensive by endoscopic standards and easy to maintain. The newer rigid cystourethrasopes used for trans cervical catheterization can be used for ovulation timing vaginoscopy as well, but are significantly more costly.

The investment in time a veterinarian makes in providing ovulation timing can be challenging. This is easily offset by training technical personnel to counsel breeders correctly about breeding management and details of the bitch and dog reproductive physiology. Prepared handouts are invaluable. After the initial consultation and examination, follow up visits for ovulation timing do not require office calls which could disrupt the normal busy flow of a practice catering to sick patients. Once a patient-doctor-client relationship has been established, bitches can be presented for ovulation timing appointments with technicians. It is perfectly reasonable to have a receptionist or technician receive the dog from the breeder, walk it into the treatment area for sample acquisition, and return it to the client within a matter of moments, without involvement of the veterinarian. Some breeder clients insist on staying with their dogs throughout any procedure, these clients can be taught to restrain their bitches properly for vaginal cytology, vaginoscopy, and/or venipuncture. Technicians can be taught to obtain and perform vaginal cytologies in a matter of minutes; these results can be relayed to the breeder client immediately. Vaginoscopy is a skill that is also readily taught to qualified technicians, with immediate interpretation possible. In house progesterone kits offer results in 15-30 minutes. The results of progesterone assays sent out to a commercial laboratory are subsequently relayed to breeder clients by telephone. Thus, the time investment by a busy veterinarian is minimal once proper technical training of staff has occurred and practice management has been structured. The fees charged for such services should be fair: they should cover the veterinary clinic costs adequately for technician time, laboratory costs and interpretive skills. However, they need to be affordable enough that breeder clients will return a bitch 3-6 times during

a cycle for completion of ovulation timing. An average ovulation timing in a California practice (vaginal cytologies, serial progesterone assays and vaginoscopies) costs approximately $300-$500.00. If the client and bitch have never been seen at the practice, an initial office call for physical examination and establishment of the patient-doctor-client relationship is necessary. It is of utmost importance that the breeder relay to the practice what type of breeding is being planned: natural, fresh artificial insemination, chilled extended semen that is shipped, or frozen semen, as arrangements for the latter two need to be made well in advance. Clinic costs can be contained by reducing unnecessary office calls, performing all vaginal cytologies in house, having technicians perform sample acquisition and run tests, and bartering with commercial veterinary laboratories on the cost of progesterone assays.

REPRODUCTIVE PHYSIOLOGY

Sound knowledge of the bitch reproductive cycle is essential, as individual bitches will have interesting variations of normal, be presented at variable times during their estrous cycle for evaluation, and sometimes exhibit pathologic variations in cycles. Each of these scenarios requires veterinary interpretation. Controversies in ovulation timing arise in interpretation of the normal events of the bitch reproductive cycle, as they relate to the optimal way to predict or identify the fertile period. The normal canine reproductive cycle can be categorized into 4 phases, each having characteristic behavioral, physical and endocrinologic patterns. Considerable variation exists within the normal range of events in the reproductive cycle. The clinician must differentiate between bitches with normal estrous cycles but unexpected patterns and those with true abnormalities. Detection of individual variation within the normal range of events in a fertile bitch can be crucial to providing effective counseling concerning breeding management. Evaluation of the estrous cycle for true abnormalities is an important part of the workup of the apparently infertile bitch.

The interestrous interval is normally 4 to 13 months, with 7 months the average. The anestrus phase of the estrous cycle is marked by uterine involution and endometrial repair. The normal bitch is not attractive or receptive to male dogs. No overt vulvar discharge is present, and the vulva is small. Vaginal cytology is predominated by small parabasal cells, with occasional neutrophils and small numbers of mixed bacteria. The endoscopic appearance of vaginal mucosal folds is flat, thin and red. The physiologic controls terminating anestrus are still under investigation, but involve the spontaneous deterioration of luteal function and the decline of prolactin secretion. It has been shown that the administration of dopamine agonists shortens the interestrous interval, which in some instances is associated with inhibition of prolactin release (prolactin is luteotrophic). The natural termination of anestrus is induced by an increase in the pulsatile gonadotropin-releasing hormone (GnRH) induced secretion of pituitary gonadotropins, follicle stimulating hormone (FSH) and luteinizing hormone (LH). Hypothalamic GnRH secretion is itself pulsatile, its intermittent secretion a physiologic requirement of gonadotropin release. Mean levels of FSH are moderately elevated, and those of LH slightly elevated during anestrus. At late anestrus, the pulsatile release of FSH increases, causing proestrous folliculogenesis. Estrogen levels are basal (2-10 pg/mL) and progesterone levels at nadir (<1 ng/mL) at late anestrus. Anestrous normally lasts from 1 to 6 months.

During proestrus, the bitch becomes attractive to male dogs, but is still not receptive to breeding, although she may become more playful. A serosanguinous to hemorrhagic vulvar discharge of uterine origin is present, and the vulva is mildly enlarged. Vaginal cytology show a progressive shift from small parabasal cells to small and large intermediate cells, superficial-intermediate cells, and finally superficial (cornified) epithelial cells, reflecting the degree of estrogen influence. Red blood cells are usually, but not invariably present. The vaginal mucosal folds appear edematous, pink and round. FSH and LH levels are low during most of proestrus, rising during the preovulatory surge. Estrogen rises from basal anestrus levels (2-10 pg/mL) to peak levels (50-100 pg/mL) at late proestrus, while progesterone remains basal (<1 ng/mL) until rising at the LH surge (2-4 ng/mL). Proestrus lasts from 3 days to 3 weeks, with a 9 day average. The follicular phase of the ovarian cycle coincides with proestrus and very early estrus.

During estrus, the normal bitch displays receptive or passive behavior, enabling breeding. This behavior correlates with decreasing estrogen levels and increasing progesterone levels. Serosanguinous to hemorrhagic vulvar discharge may diminish to variable degrees. Vulvar edema tends to be maximal. Vaginal cytology remains predominated by superficial cells; red blood cells tend to decrease but may persist throughout. Vaginal mucosal folds become progressively wrinkled, or crenulated in conjunction with ovulation and oocyte maturation. Estrogen levels reduce markedly after the LH peak to variable levels, while progesterone levels steadily increase (usually 4-10 ng/mL at ovulation), marking the luteal phase of the ovarian cycle. Estrus lasts 3 days to 3 weeks, with an average of 9 days. Estrous behavior may precede or follow the LH surge, its duration is variable, and may not coincide precisely with the fertile period. Ovulation of primary oocytes occurs 2 days after the LH surge; oocyte maturation occurs 2-3 days later, the lifespan of secondary oocytes is 2-3 days.

During diestrus, the normal bitch becomes refractory to breeding, with diminishing attraction of male dogs. Vulvar discharge diminishes and edema slowly resolves. Vaginal cytology is abruptly altered by the reappearance of parabasal epithelial cells and frequently neutrophils. The appearance of vaginal mucosal folds becomes flattened and flaccid. Estrogen levels are variably low, and progesterone levels steadily rise to a peak of 15-80 ng/mL, before progressively declining in late diestrus. Progesterone secretion is dependent on both pituitary LH and prolactin secretion. Proliferation of the endometrium and quiescence of the myometrium occur under the influence of elevated progesterone levels. Diestrus usually lasts 2-3 months in the absence of pregnancy. Parturition terminates pregnancy 64-66 days after the LH peak. Prolactin levels increase in a reciprocal fashion to falling progesterone

levels at the termination of diestrus or gestation, reaching much higher levels in the pregnant state. Mammary ductal and glandular tissues increase in response to prolactin levels.

IMPORTANT HORMONES

Estrogen

Increased estrogen causes an increased turnover rate of vaginal epithelial cells, resulting in the progressive cornification seen on vaginal cytology, and thickening of the vaginal wall in preparation for breeding. Also seen is progressive edema of the vaginal mucosa, which can be visualized with endoscopic examination. Estrogen assays are performed by RIA by many commercial laboratories. However, the information given is of little value for ovulation timing, since peak estrogen levels are variable from bitch to bitch, and even relative changes do not correlate to ovulation or the fertile period. Estrogen is best assessed by serial vaginal cytologies and vaginoscopy. Estrogen levels do not indicate the fertile period since ovulation is triggered by the LH surge, not an estrogen peak. Examination of the cells on the surface of the vaginal epithelium will give information about the stage of the estrous cycle. Proper technique is important so that the cells obtained are representative of the hormonal changes occurring. The sample should be collected from the cranial vagina, since cells from the clitoral fossa, vestibule and/or caudal vagina are not as indicative of the stage of the cycle. Under the influence of rising estrogen levels, the number of layers making up the vaginal epithelium increases dramatically, presumably to provide protection to the mucosa during copulation. Therefore, as estrogen rises during proestrus, the maturation rate of the epithelial cells increases, as does the number of keratinized, cornified epithelial cells seen on a vaginal smear. Full cornification continues throughout estrus, until the "diestrual shift" that occurs 7 to 10 days after the LH surge, signifying the first day of diestrus. The vaginal smear then changes abruptly from full cornification to 40-60% immature (parabasal and intermediate) cells over a 24-36 hour period. If vaginal cytology is performed until the diestrual shift is observed, a retrospective analysis of the LH surge, ovulation and the fertile period can be obtained, and calculation of the due date is possible (56-58 days from the diestrual shift).

Luteinizing Hormone (LH)

At the end of the follicular phase of the estrous cycle, a marked increase in LH over usual baseline values occurs over a 24-48 hour period, followed by a return to baseline values. This surge in LH is thought to take place in response to the decline in the estrogen: progesterone ratio that occurs as estrogen levels decrease and progesterone rises. The LH surge triggers ovulation and thus makes it the central endocrinological event in the reproductive cycle of the bitch, with all events following being consistent between bitches. Therefore, daily serial measurement of LH to identify the exact date of the LH surge is the most accurate diagnostic tool for timing breedings. Affordable semiquantitative in house kits are available for measuring serum LH levels in the dog, identifying the pre-ovulatory LH surge and thus, the time of ovulation and the true fertile period. This testing is the most accurate means of ovulation timing, and thus should be considered a “gold standard”. Samples must be drawn daily, at about the same time, for LH testing, since the LH surge may have a duration of only 24 hours in many bitches, and could be missed if one day was skipped. The commercially available LH kits can be subject to variable operator interpretation, thus the same person should run the tests if possible.

Progesterone

Progesterone levels begin to rise at approximately the time of the LH surge (prior to ovulation). Rising progesterone acts synergistically with declining estrogen to reduce edema of the vulva and vagina, which can be appreciated on vaginoscopic exam. Other observable clinical signs are minimal. Serial blood samples performed every 2 days may be used to identify the initial rise in progesterone (usually >2 ng/mL) which indicates that the LH surge has occurred. Progesterone can be assayed by RIA or chemiluminescence at most veterinary commercial laboratories. Several in house semiquantitative kits are also available. By examination of the range and overlap of progesterone values at different points in female reproductive physiology, it becomes clear that no one absolute value of progesterone correlates to any particular stage of the cycle. Progesterone varies at the point of the LH surge from 0.8-3.0ng/ml, from 1.0-8.0 ng/mL at ovulation, and from 4.0 to >20.0 ng/mL during the fertile period. However, if accurate serial quantitative progesterone assays are obtained, the LH surge may be estimated as the day a distinct increase in progesterone level is seen. While this will not be as accurate as actual identification of the LH surge by use of an LH assay, estimation by progesterone results is still very useful, and is often more widely available and convenient. When timing breedings using semi-quantitative in-clinic progesterone assays, only a range of progesterone is obtained, which makes it difficult to accurately identify the day of the initial rise in progesterone or the true fertile period. Technical problems with these kits have occurred. Therefore, these assays should only be used for routine breedings where a wider margin of error is acceptable. A safe rule of thumb to follow is that when testing indicates progesterone has risen above 2 ng/mL, breeding should begin. Optimal ovulation timing should utilize quantitative progesterone assays from commercial laboratories, the cost difference is minimal. Regardless of which assay is used, an additional test should always be performed 2-4 days after the first rise is detected to indicate that the cycle has progressed as expected, a functional corpus luteum has been formed and ovulation has occurred. When doing progesterone testing for ovulation timing, it is important to remember that at best, detection of changes in progesterone will give an estimation of the LH surge and the fertile period, and thus is not as accurate as actual identification of the LH surge with an LH assay.

CLINICAL PROTOCOL

Breeder clients should be advised to notify the clinic when they first notice a bitch for which timing is planned is in season, based on vaginal discharge or vulvar swelling/attraction to males. Even the most astute owner can fail to notice the true onset of proestrus for a few days. Early proestrus should be documented by vaginal cytology (less than 50 % cornification/superficial cells). A baseline progesterone level might be informative if the true onset of the cycle is unknown (usually 0-1 ng/mL). Vaginal cytology should be performed every 2-4 days until a significant progression in cornification is seen, usually above 70% superficial cells. At that point, serial hormonal assaying should begin. For routine breedings, progesterone testing may be done every other day, until a rise in progesterone above 2 ng/mL is identified. The day of the initial rise in progesterone above 2 ng/mL is identified as day "zero". Breedings are advised on days 2, 4 and 6.

When increased accuracy of ovulation timing is necessary (e.g., frozen or chilled semen breedings, infertility cases, breedings with subfertile stud dogs), daily LH testing is recommended. Once the LH surge is identified, breeding days may be planned. The day of the LH surge is also day "zero". It is useful to perform vaginal cytology every 2-3 days until full cornification (>90% superficial cells) occurs. This maximal cornification usually occurs prior to the fertile period is reached and continues until the onset of diestrus, which is usually a few days after the end of the fertile period. Vaginal cytology may be continued until the diestral shift is identified, which gives a retrospective evaluation of the breeding just completed. In addition, at least one progesterone assay should be performed after the LH surge/ initial rise in progesterone is identified to document that levels continue to rise. This illustrates sustained corpus luteum function and strongly suggests that an ovulatory cycle has occurred. Extended chilled breedings should occur on days 4 and 6, or 3 and 5 after day "zero". Which two days are chosen can depend upon overnight shipping possibilities and the involved clients' schedules. Frozen semen breedings should occur day 5 or 6 after day "zero".

If client economics dictate minimal testing, serum can be batched on a daily basis and quantitative progesterone tests performed as advised above. When the initial rise in progesterone is identified, the batched serum can be specifically evaluated for the day of the LH surge, confirming identification of day "zero". Vaginoscopy may be performed throughout the cycle as an adjunct to vaginal cytology and hormonal assays, especially when evaluating an unusual cycle. Behavior and other observations should also be made at each examination, but less weight should be put on these parameters. The clinician should keep in mind that the most accurate ovulating timing occurs when information from several tests is pooled (vaginal cytologies, vaginoscopy, and progesterone or LH tests).

MISCELLANEOUS TECHNIQUES

Ultrasonography may be used to identify ovulation in the bitch. Early attempts were discouraging; the small size of the ovaries and their similarity to close structures make them difficult to visualize. However, recent reports have identified ovulation as occurring when a detectable decrease in the number of follicles is seen during serial imaging. The data has shown a close correlation to the ovulation time established by LH and progesterone levels. The measurement of glucose in vaginal secretions has been used as a crude guideline for timing breedings by many dog owners. Increased glucose has been identified in vaginal secretions as an inconsistent finding; it is thought to be a result of insulin antagonism that occurs due to altered hormone concentrations at the time of the progesterone rise. This finding is not reliable, however, and so is not recommended for ovulation timing. Measurement of electrical conductivity of vaginal mucus is used routinely to time breedings in foxes and has been studied in several other species, including the dog. It was found that electrical resistance increases as estrus approaches and then plateaus at a maximal level for several days, which has been proposed is a result of rising estrogen levels. While it appeared that ovulation occurred at some point during this period of maximum electrical resistance, it has not been shown to be correlated to the LH surge or the fertile period and so cannot be recommended for accurate ovulation timing.

Figure 1: Proper technique for obtaining a vaginal cytology sample

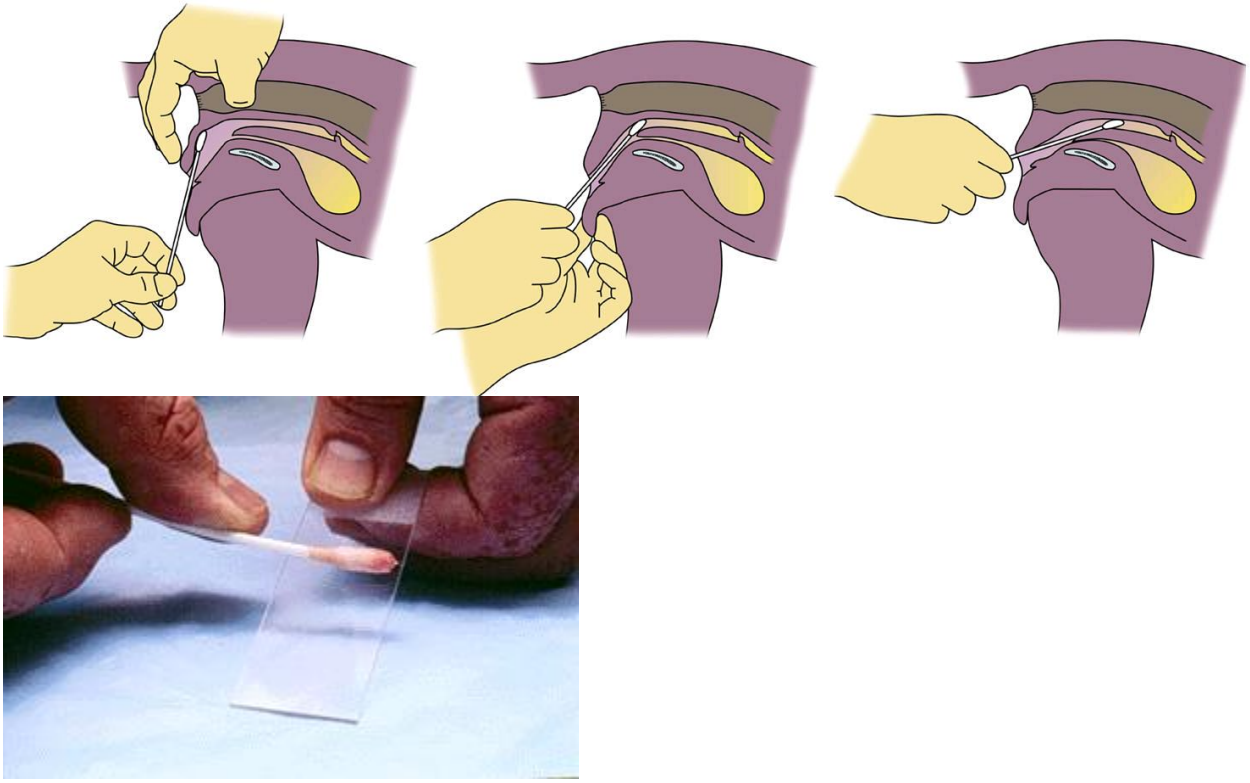


Figure 2: Cells of the estrous cycle

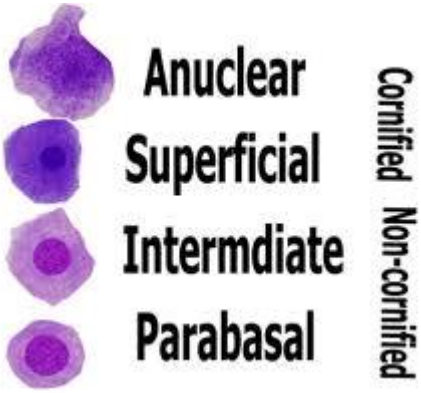


Figure 3: Parabasal cell

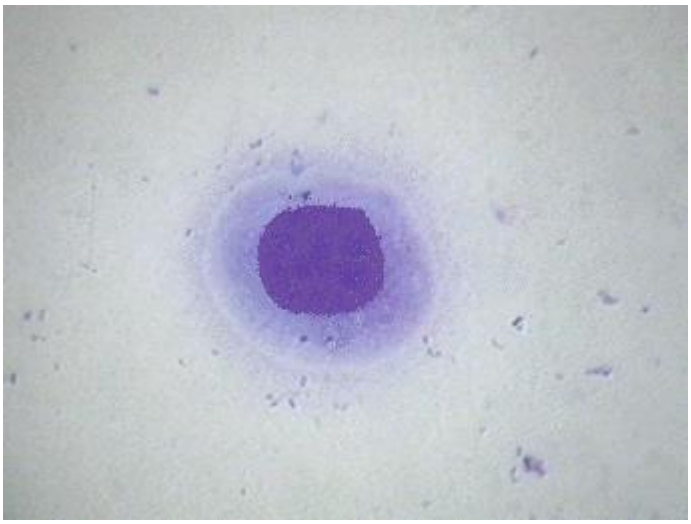
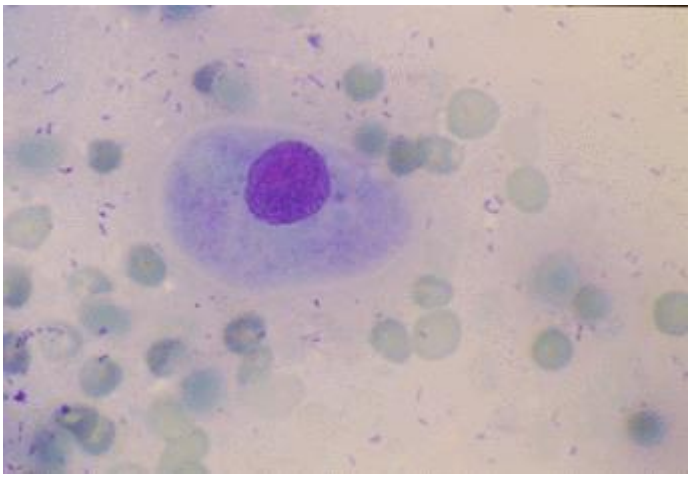


Figure 4: Intermediate cells



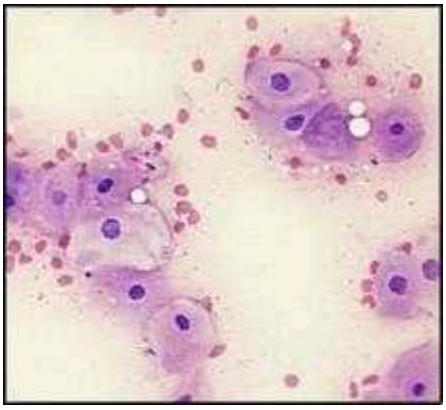
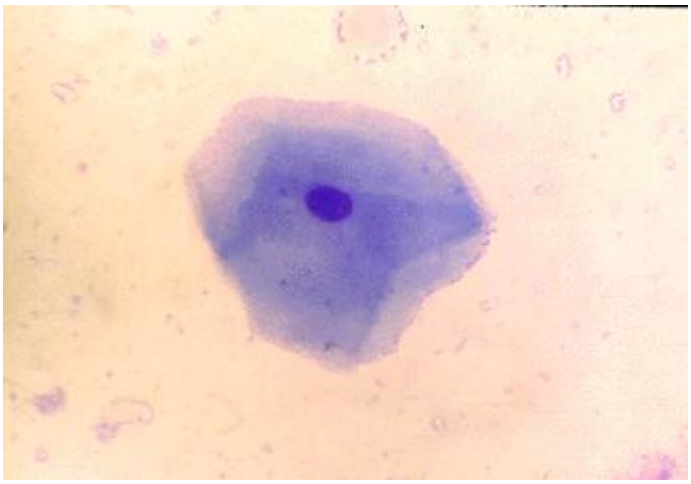
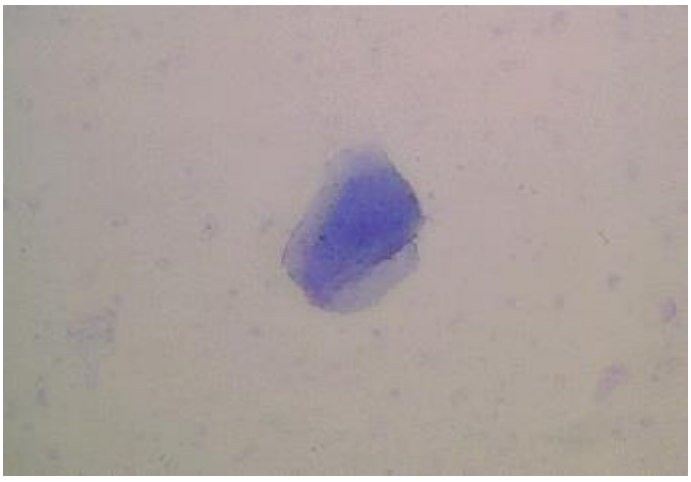


Figure 5: Superficial cells





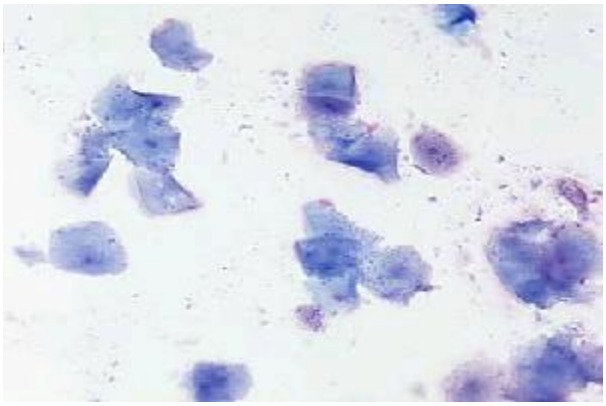
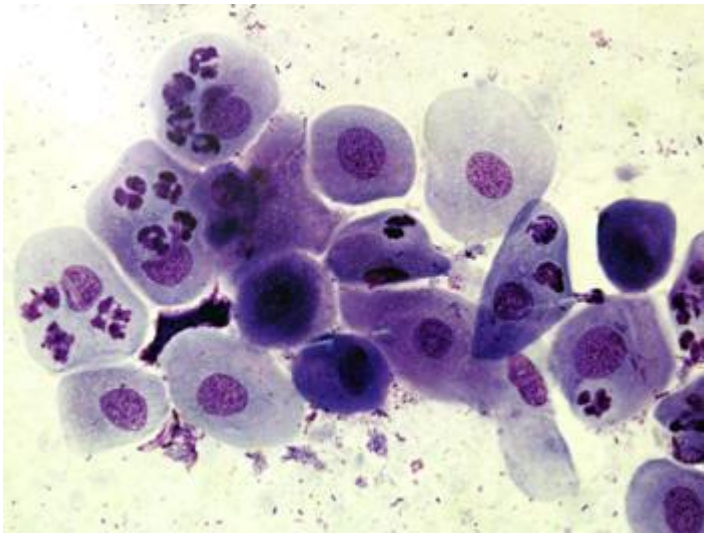


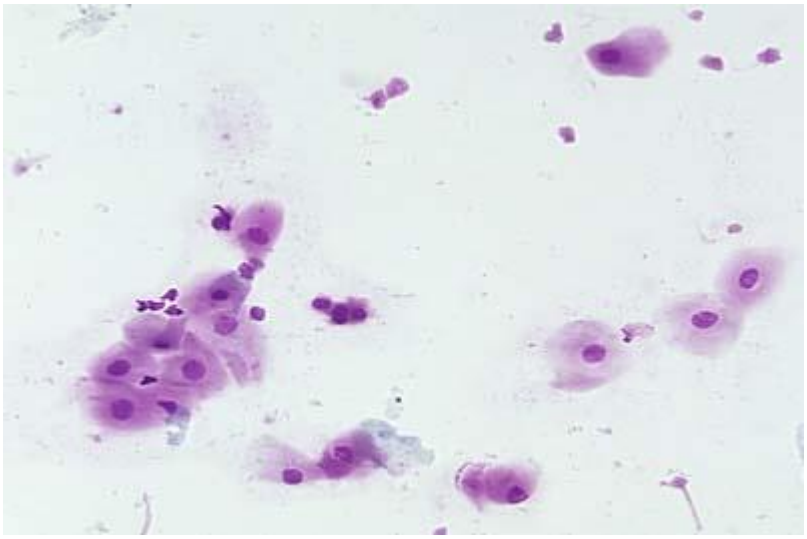
Figure 6: Parabasal cells and neutrophils



The estrous cycle

Anestrus

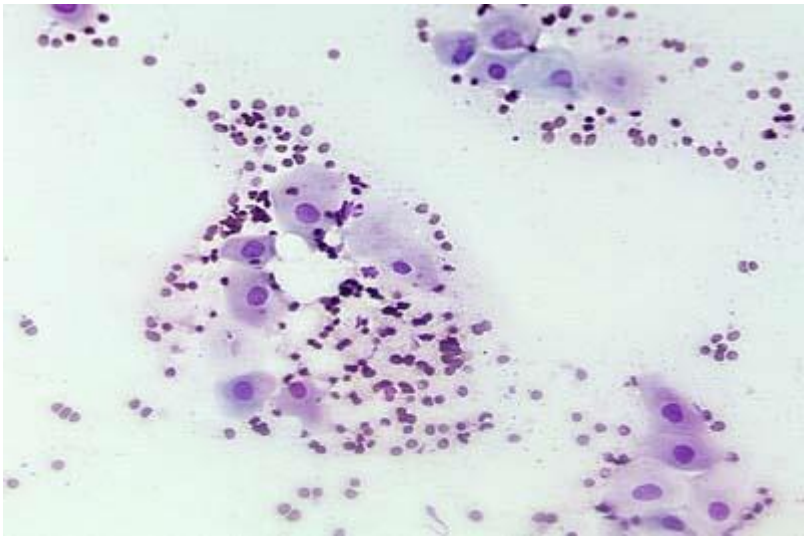
Intermediate and parabasal cells predominate in smears taken during anestrus. Superficial cells are absent or found in very small numbers. Neutrophils may also be present or absent.



Proestrus

Serum concentrations of estrogen rise during proestrus, leading to capillary breakage and leakage of red blood cells through uterine epithelium, as well as proliferation of the vaginal epithelium.

Examination of vaginal smears from early to late proestrus will reveal a gradual shift from intermediate and parabasal cells to superficial cells. Typically, red blood cells are present in large numbers and neutrophils are commonly observed. Large numbers of bacteria are also often present.

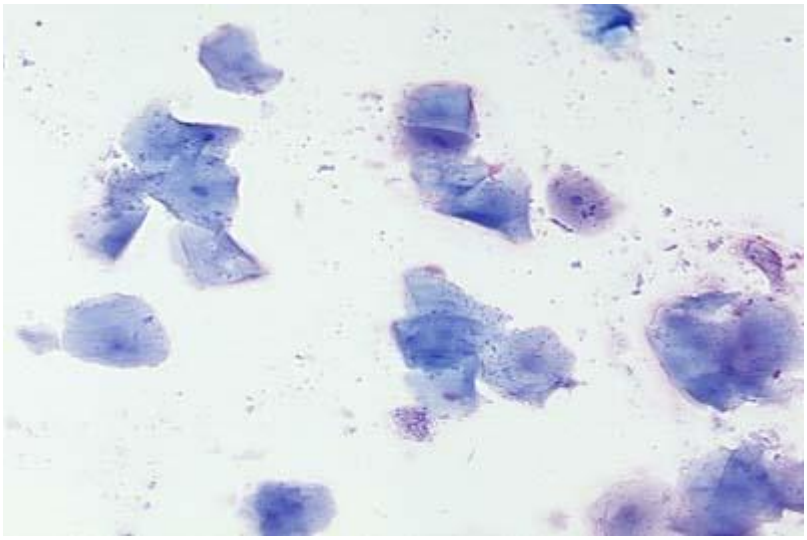


In some bitches, proestrus can persist for two to three weeks. In such cases, prolonged lack of receptivity may suggest the need to artificially inseminate or force-breed the animal. Examining

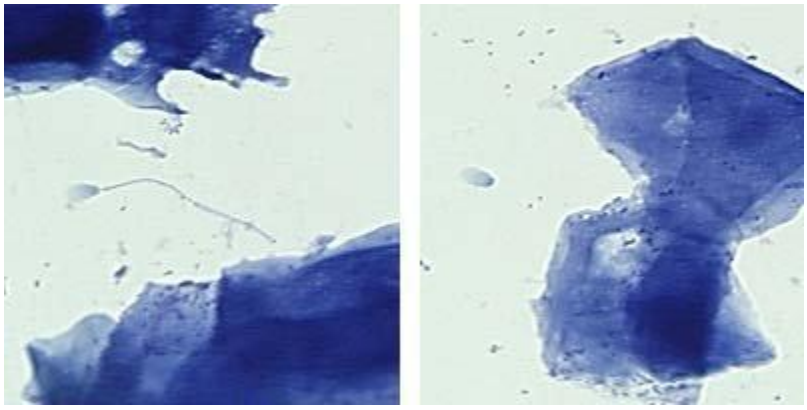
vaginal smears in such cases will alleviate such concerns - certainly, if more than a very small percentage of cells are parabasals and small intermediates, breeding is a waste of time.

Estrus

The defining characteristic of cytologic estrus is the predominance of superficial cells. Most, but not all, bitches will undergo full cornification, and the smear will reveal a monotonous pattern composed almost exclusively of anucleate superficial cells.

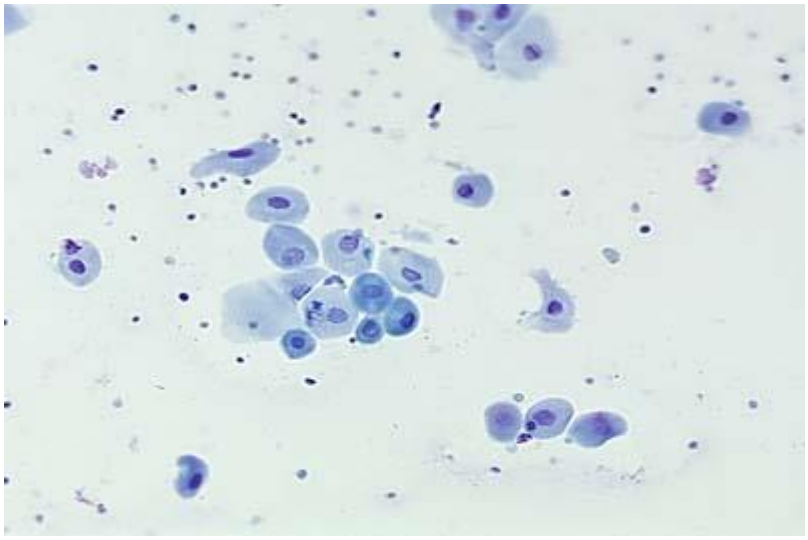


If the bitch has been bred within a day of preparing a vaginal smear, it is quite likely that sperm will be observed among the epithelial cells. Indeed, careful examination for sperm in a smear taken within a few hours of an alleged breeding is a fairly reliable means of confirming or denying such an incident. In the image below, an intact sperm (left panel) and a sperm head (right panel) are present next to superficial cells.



Diestrus

The onset of diestrus is marked by a precipitous decline in the number of superficial cells and reappearance of intermediate and parabasal cells. Most commonly, the cellular profile changes within a single day from essentially 100% superficial cells to less than 50% superficial cells.



The significance of identifying the onset of diestrus is that it is a considerably more accurate predictor of the time of ovulation, and hence gestation length, than sexual behavior.

Dogs ovulate 5-7 days prior to the onset of diestrus (7-9 days after the preovulatory LH surge), and hence, gestation length is usually 57 $\pm$ 1 day from the onset of diestrus day 1. The period of behavioral estrus is variable, and often extends up to several days before and/or after cytologic estrus. Gestation lengths calculated from the onset or cessation of receptivity are correspondingly inaccurate. The onset of diestrus also correlates well with loss of fertility, and breedings after the diestrus shift rarely result in conception.