Introduction

There is considerable variation in the time of ovulation in relation to the onset of vulval swelling and serosanguinous discharge of early proestrus. This is often not understood by dog breeders and they frequently impose standard mating regimes upon their bitches. These regimes usually involve a plan for breeding at a predetermined or defined number of days after the onset of vulval "bleeding", i.e., serosanguinous discharge. As a result of this, many bitches are often mated at an inappropriate time, and this constitutes the commonest cause of apparent infertility in bitches. However, there is often a need to schedule the times of planned matings several days ahead of time to accommodate the transport of the bitch to the locality of the intended stud dog. Breeding management as a veterinary service is often limited to advising on when to transport the bitch for breeding, given the constraints of personal schedules and possibly limited availability of the intended stud dogs.

There are several investigative methods for identifying the optimal mating time, including measurement of plasma hormone concentrations, examination of exfoliated vaginal cells, and vaginal endoscopy. Failure to detect the time of ovulation and achieve a pregnancy during a particular ovarian cycle can be a significant problem in this species. Dogs are mono-estrous, and although the inter-estrus interval averages about 7 months (i.e., 31 weeks) it is very variable, ranging in normal cycling bitches from 4.5 to 13 months or longer.

The literature on which this review is based is listed in the References section [1-11]. The following topics are covered in the review.

Reproductive Physiology
The Fertilization Period
The Fertile Period
Assessing the Optimal Time for Breeding
Clinical Assessments
The Unreliability of Relying on a Predetermined Day
Variable Onset and Expression of Estrus Behavior (Acceptance of Male)
Vulval Softening
Measurement of Plasma LH or Progesterone
Progesterone ELISA Assay Kits for In-Clinic Use
Vaginal Cytology
Vaginal Endoscopy
Ovarian Ultrasound Examination
Examination of Cervico-Vaginal Secretion
Review of Normal Sequence of Events
Caveat: Normal Estrus Versus False Estrus
Conclusion

Reproductive Physiology

The oocytes of the bitch are ovulated in an immature state, as primary oocytes, and they cannot be fertilized immediately. Fertilization can only occur following maturation of the primary oocyte, i.e., extrusion of its first polar body and completion of the first meiotic division to form the secondary oocyte. While these maturation events are accomplished prior to ovulation in most other species, they are not completed until at least 48 hours after ovulation in the dog. The same is true in the fox.

Ovulation is caused by a surge in plasma luteinizing hormone (LH) concentrations. In dogs, ovulation occurs two days after the surge in LH, and oocytes remain viable within the reproductive tract for a further four or five days before beginning to undergo degeneration. The behavioral periods of proestrus and estrus are defined based on ready acceptance of the male, and follow one upon the other. The transition from pro-estrus to estrus behavior may be rapid or slow, and may occur shortly before, concurrent with, or even some days after the pre-ovulatory surge in LH.

In the accompanying figure (Fig. 1), behavioral (i.e., clinical) proestrus and estrus are indicated as the components of the "heat"
period. The individual periods of proestrus and estrus have been redefined in endocrinological terms, i.e., with "endocrine proestrus" ending at the time of the preovulatory LH surge, and with "endocrine estrus" beginning about the time of the LH surge (Fig. 1). The transition from proestrus behavior to estrous behavior often occurs at the time of the LH surge; the mean time is about 1 day after the LH surge, and the normal range is from 3 days before to 5 days after the LH surge.

One very useful way to view the cycle during late proestrus and estrus is to consider the "fertilization period" and the "fertile period".

Fertilization in the bitch requires some definition. Herein we consider fertilization to mean fusion of the male pronucleus (from the sperm) with the female pronucleus of the mature secondary oocyte to form the 1-cell zygote. However, sperm can penetrate the immature primary oocyte any time after ovulation. In these cases, the timecourse of the formation of the male pronucleus has not been well defined. Likewise, in these cases, the time from subsequent maturation of the oocyte (by extrusion of the first polar body) until completion of meiosis (extrusion of the second polar body) and fusion of the pronuclei is not known. When oocyte maturation occurs (2 days after ovulation) without sperm penetration, the oocytes remain in a second meiotic arrest (metaphase II); the completion of meiosis and extrusion of the second polar body does not occur until sperm penetration does occur, as in other species.

The Fertilization Period

The fertilization period of the bitch is the time when viable oocytes are available in the uterine tubes and are sufficiently mature as secondary oocytes to be fertilized by spermatozoa. Under typical circumstances in the majority of bitches it extends from four days after the preovulatory surge of LH until about seven days after the LH surge (i.e. from two days after ovulation until about five days after ovulation). In the extreme (Fig. 1), it can extend to day 8 or 9 (and even to day 10) after the LH surge, albeit with reduced fertility. Fertility usually declines very rapidly beginning 7 days after the LH surge, as oocytes undergo degeneration and the cervix closes over a 1 to 2 day period. The termination of the fertilization period may be primarily due to degeneration of oocytes or to closure of the cervix and prevention of sperm entering the reproductive tract in sufficient numbers. Both phenomena contribute significantly to the rapid decline in fertility. Natural matings only rarely result in pregnancy when they occur on days 8, 9 and 10 after the LH surge, and they do so with decreasing frequency, and involve reduced litter sizes. But, in groups of bitches that underwent intrauterine insemination at 8, 9 and 10 days after the LH surge [11,12], pregnancy rates were higher (60, 60 and 20% respectively), compared to natural matings on those days (all 0%). Therefore, optimization of the chances for a bitch to become pregnant requires that she be bred or inseminated during the fertilization period at a time when it overlaps the period of peak fertility (Fig. 1), preferably days 4 to 7 after the LH surge.

The Fertile Period

The fertile period is the time during which a mating or insemination can result in a pregnancy. This period includes not only the fertilization period, but also the preceding few days, due to the fact that dog sperm can remain fertile for several days within the female reproductive tract. Sperm may survive in the tract for up 5 or 6 days before ovulation and the opportunity to penetrate a recently ovulated singular oocytes, and then form a viable male pronucleus and result in fertilization 7 or 8 days after semen deposition. The fertile period can be considered to extend from three days before the preovulatory LH surge until 7 days after the pre-ovulatory LH surge, and may be even longer when using stud dogs with exceptional semen quality or bitches in which the oocytes may survive another day or two beyond the norm. Importantly, for many stud dogs, their sperm may survive no longer than 1 or 2 days in the female tract. Matings earlier than the day of the LH surge have reduced pregnancy rates, suggesting that in most cases sperm are not capable of penetrating oocytes after 2 days in the female tract. The timing of the fertility period and fertilization period is summarized in Table 1, below.

<table>
<thead>
<tr>
<th>Table 1. Timing of periods of fertility, of oocyte maturation, and of oocyte fertilization by previously deposited sperm in relation to the day of the LH surge and day of ovulation in the domestic bitch.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Period</strong></td>
</tr>
<tr>
<td>---------------------------------------------------------------</td>
</tr>
<tr>
<td>Period of potential fertility, the &quot;Fertile Period&quot;</td>
</tr>
<tr>
<td>Period of reduced fertility with early matings</td>
</tr>
<tr>
<td>Period of peak fertility within the fertile period in bitches of high fertility (natural matings)</td>
</tr>
</tbody>
</table>
Assessing the Optimal Time for Breeding

The period of peak fertility for natural mating in highly fertile experimental animals was determined empirically, and it ranges from the day of the LH surge until six days after the LH surge. That is, from 2 days before ovulation until 4 days after ovulation. There was no difference in pregnancy rates or litter sizes following single matings on any of those days. Matings earlier or later result in lower pregnancy rates, and late-mating when successful typically results in smaller litters. The optimal time for breeding in an individual bitch is early in the fertilization period, on LH-days 4 to 5, or even slightly in advance of this time, on LH-day 3, so as to allow for capacitation of spermatozoa within the female reproductive tract. When multiple breedings are possible, the first should be 1 or 2 days earlier, to compensate for the possibility that the estimated times of the LH surge and ovulation were somewhat in error and "late". Two breedings, two days apart, can compensate for a 2-day error in estimating the timing of events. Determination of the optimal time to breed can be made by timing the LH surge, and by direct clinical examination methods that can reasonably estimate the time of ovulation or indicate the onset of the fertile period or the fertilization period. When the use of cryo-preserved semen is contemplated, insemination should be performed only during the fertilization period, to increase the chance of success. Thus, if the time of the pre-ovulatory LH surge can be reasonably estimated, then breedings or insemination times can be optimized to the following periods (Table 2).

| Table 2. Suggested days of cycle for insemination of bitches depending on the type of breeding management being conducted. |
|---|---|---|
| **Type of Breeding Management** | **Days to Breed or Inseminate** |
| Natural mating or fresh semen AI | 3 to 6 days after the LH surge (i.e., 1 to 4 days after ovulation) |
| Chilled fresh semen | 4 to 6 days after LH surge (i.e., 2 to 4 days after ovulation) |
| Frozen-thawed semen | 5 to 6 days after the LH surge (i.e., 3 to 4 days after ovulation) |

These times for mating can be estimated based upon clinical assessments including serial vulval palpation, vaginal cytology, and vaginal endoscopy, or upon measurements of hormone changes during proestrus and estrus, especially the preovulatory rise in concentrations of progesterone in serially collected plasma or serum samples. Accurate timing of peri-ovulatory events during breeding management also permits accurate determination of the time to best perform pregnancy testing by ultrasound or palpation, and of the likely date of parturition 64 to 66 days after the LH surge.

Clinical Assessments

Successful use of clinical assessments in breeding management requires an appreciation of the normal transition from proestrus to estrus, the misinformed views about the day of the cycle held by many dog owners and breeders, and the normal changes in sex behavior. Also needed is an understanding of the limitations as well as the value of available techniques - vaginal cytology, degree of vulval tumescence, and vaginoscopic examinations, and even of serum progesterone assays - in estimating the day of ovulation and subsequent period in which fertilization can occur. The major features in the parameters of interest at four selected times in the early part of the cycle are indicated in Table 3.
Table 3. Status of Various Clinical Parameters in Normal Fertile Bitches at Selected Times Before and After the Preovulatory LH Surge

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Day 7 before LH surge</th>
<th>Day of LH Surge</th>
<th>Days 4 - 5 after LH surge, when oocytes mature</th>
<th>Days 8 - 11 after LH surge</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vaginal cytology cornification index</td>
<td>30 % to 100%</td>
<td>80% to 100%(a)</td>
<td>80% to 100%(a)</td>
<td>0 to 80%</td>
</tr>
<tr>
<td>Leucocytes in smear</td>
<td>Few</td>
<td>None or few (b)</td>
<td>None or few (b)</td>
<td>Many</td>
</tr>
<tr>
<td>Vulval turgor</td>
<td>Progressively increasing</td>
<td>Peak or just decreased</td>
<td>Obviously decreased</td>
<td>Vulva soft but still enlarged</td>
</tr>
<tr>
<td>Vaginal Mucosal Folds</td>
<td>Smooth, round, white, edematous</td>
<td>White, slight shrinking and wrinkling</td>
<td>White, grossly wrinkled and angulated</td>
<td>White and pink or red, blotchy, nearly flat</td>
</tr>
<tr>
<td>Serum progesterone</td>
<td>&lt; 0.5 ng/ml</td>
<td>0.9 - 3.0 ng/ml</td>
<td>3.5 - 12 ng/ml</td>
<td>8 - 25 ng/ml</td>
</tr>
<tr>
<td>Serosanguinous discharge or erythrocytes in vaginal smear</td>
<td>+</td>
<td>+</td>
<td>+/-</td>
<td>+/-</td>
</tr>
<tr>
<td>Reproductive status</td>
<td>Infertile</td>
<td>Period of peak fertility onset, 2 days before ovulation</td>
<td>Period of fertilization onset, 2 - 3 days after ovulation</td>
<td>Infertile or Rarely fertile</td>
</tr>
</tbody>
</table>

(a) typically 95 - 100% cornification by 2 to 8 days before LH surge
(b) typically none, as leukocytes are expected to be absent in smear from 4 - 10 days before the surge until 7 - 10 days after LH surge.

The Unreliability of Relying on a Predetermined Day

Many breeders rely upon counting the number of days from the onset of proestrus, and believe that bitches always ovulate a defined number of days from the onset of this event. This is not the case, for any breed. The duration of proestrus is variable among bitches. While the "average bitch" may ovulate 12 days after the onset of proestrus (and should therefore be mated on day 14 and 16), some bitches ovulate as early as day 5, and others as late as day 30 after the onset of proestrus. Therefore, mating on the 12th and 14th day, which is common breeding practice, often fails to result in conception. The term "heat" is not particularly helpful, but is commonly used to refer to the combined periods of proestrus and estrus. The physiological and endocrinological "Day 0" of the cycle is the day of the preovulatory LH surge, the event that triggers the ovulation of ovarian follicles 2 days later. It may or may not coincide closely with a distinct transition from proestrus to estrus behavior.

Variable Onset and Expression of Estrus Behavior (acceptance of male)

In some domestic species the onset of the behavioral signs of estrus can be used to determine the optimal time for breeding. In the bitch however there is often a poor correlation between endocrine events and behavioral events. Studies on laboratory-kept bitches suggested that the onset of standing estrus occurred, on average, at about the same time as the LH surge in some trials and at one day after the LH surge in others. Using these data, 3 or 4 days after the onset of standing estrus would be a suitable time for mating in many bitches. However, in many bitches the behavioral events have been shown to correlate poorly with the underlying hormonal events, and this method therefore has little value, unless breedings are continued until later in estrus and unless there are examinations to determine that in fact the bitch does not need be bred earlier than her first display of full estrus behavior. A further complication is the fact that the onset of estrus behavior may be rapid and very obvious, and in other cases may be slow, intermittent, and unclear for several days. Even in bitches in which the time of the onset of estrus appears to be clear and rather distinct, this can occur as early as 3 days before ovulation and as late as 5 days after ovulation, in the extreme, in otherwise normal fertile bitches. In one family of whippets, the onset of behavioral estrus was routinely 1 to 2 days before the end of the fertile period, based on vaginal cytology and vaginoscopy (F. Lindsay, personal communication, 1990). It is also important to consider that in preparing for certain planned breedings, a teaser male dog is often not readily available to test the bitch for estrus. And, when one is available, testing for estrus can result in an unwanted breeding to the wrong male. Finally, bitches may be receptive to some males and not others at the same time during estrus, and a failure of the bitch to "stand" for a particular male may be related to the male and not to the reproductive status of the bitch. When breeding management relies solely on the receptivity of the bitch, she should be mated as early as possible (to cover the possibility that estrus onset is late in relation to ovulation) and every other day for 3 consecutive matings (to cover the possibility that estrus onset was early in relation to ovulation).

Vulval Softening

One clinical assessment that may be useful in monitoring the bitch is the timing of vulval softening. During proestrus the reproductive tract becomes edematous and the vulva and perineal tissues become enlarged and increasingly turgid in response to increasing estrogen (Fig. 2 and Fig. 3). At the time of the LH surge there is a change in serum estrogen from very high concentrations to progressively lower concentrations, combined with a concomitant rise in progesterone concentrations. With this hormonal change there is a reduction in edema of the reproductive tract with the consequence that a distinct softening of the vulva...
occurs. It can be monitored by subjective estimation of the extent of vulval turgor once or twice daily by gentle palpation of the vulva and perineum.

**Measurement of LH or Progesterone Concentrations in Plasma or Serum**

**LH Assays** - Measurement of the peripheral plasma concentration of LH is a reliable and accurate method for determining the optimum time to mate. In most countries there is no readily available commercial assay for canine serum LH, and at present measurement requires radioimmunoassay. This method is time-consuming, expensive and there is frequently a delay in obtaining the results, because samples are assayed in batches in service laboratories. Should LH concentration be measured, critical matings or insemination can be planned between four and six days after the LH surge. At least one ELIZA assay kit for measuring LH in canine serum has been marketed for ovulation timing (Status-LH, Synbiotics), as reviewed by Root-Kustritz (2001), and has been used successfully. One concern is that it must be used daily to detect the day of the LH surge.

**Plasma Progesterone and Progesterone Assays** - There is a significant preovulatory luteinization in the bitch during and following the LH surge as there is in many rodent and primate species. Plasma progesterone concentration begins to increase rapidly from baseline approximately 2 days before ovulation, during the LH surge (Fig. 1). This rapid increase is distinct and detectable, whereas the very slow, minor rise in progesterone reported to occur over the previous week is more subtle and typically near or below the limit of detection for most assays. Serial monitoring of plasma progesterone concentrations therefore allows anticipation of ovulation by about 1 to 2 days, and if continued allows confirmation of ovulation and detection of the fertilization period. Since the initial rise in progesterone is progressive, it is only necessary to collect blood samples every second or even third day, unlike the daily regime required to detect the LH surge. However, the less frequent the measurement, the less accurate the estimation of the times of LH surge, ovulation, and onset and termination of the fertilization period. Daily assays can determine the day of ovulation within 1 or 2 days in most cases, and should be used for very critical breedings and inseminations. Assuming that breedings will occur twice, on either two consecutive days or two days apart, one can establish planned breedings based on the following considerations. Critical breedings or inseminations should be planned between four and six days after the plasma progesterone concentration exceeds, or likely exceeded, 2.0 ng/ml (6.5 nmol/L) - the concentration typically observed at the time of the LH surge or on the following day. Some reports suggest that breeding should preferably commence one day after values exceed 8 to 10.0 ng/ml (25.0 to 32.0 nmol/L), which are typically seen at the beginning of the fertilization period. Progesterone may be measured most accurately by radioimmunoassay (RIA), quantitative enzyme-linked immunosorbent assay (ELISA), or immuno-chemiluminescence assay. Many veterinary diagnostic laboratories have a turn-around time of 1 day for these accurate assays. RIA of LH in daily serum samples can pinpoint the day of the LH surge in the majority of dogs, and be accurate within 1 day in over 90% of cases. Several semi-quantitative progesterone ELISA test kits have become commercially available for use in the clinic, and they can accurately identify the day of the LH surge within 1 or 2 days in most cases.

**Progesterone ELISA Assay Kits for In-Clinic Use**

These kits allow progesterone concentrations to be assessed either qualitatively (using microwell or microcup methods) or semi-quantitatively. Results are usually obtainable within 45 to 60 minutes of sample collection. Measurement of plasma progesterone using ELISA’s have found wide clinical acceptance and have produced a significant increase in pregnancy rate. They should be used following the manufacturer’s recommendation. Several commercial, in-clinic progesterone assay kits marketed for ovulation timing in dogs have been available in recent years, include the following. Most are modifications of kits marketed for milk progesterone assays used in the dairy industry.
- Premate (Vetoquinol, Europe; Camelot Farms, College Station Texas, USA);
- K9-Proges-Check (Endocrine Technologies, Inc., Newark CA, USA);
- Status-Pro (Synbiotics, San Diego, CA USA);
- Target Canine Ovulation Test Kit (Biometallics, Princeton, NJ, USA).
Details about the use of such assays are described elsewhere (Root-Kustritz, 2001).

**Vaginal Cytology**

Serial collection, staining and microscopic examination of exfoliated vaginal epithelial cells is a simple method for monitoring the stage of the estrous cycle. Vaginal cells may be collected using either a saline-moistened cotton swab gently wiped over the surface of the vaginal mucosa, or by aspiration of the vaginal cavity using a plastic catheter. When using the former method it is important not to allow contact of the swab with the vestibule, since collection of these cells can give variable if not erroneous results. Swabs should be introduced and removed using a small speculum or guard [3]. Once collected, cells are placed onto a glass microscope slide by lightly rolling the cotton swab, or by application of the aspirated fluid that is then spread into a thin film.

The smear can be stained using either a simple modified Wrights-Giemsa stain (Harleco’s Diff Quik, EM Science, Gibbstown, NJ, USA; Merck, Darmstadt, Europe) or a trichrome stain. The modified Wright’s stain is readily available and has the advantage that sample preparation takes less than a minute (Fig. 4a). Trichrome staining has the advantage of identification of keratinized cells based on an eosinophilic index, but the staining technique is laborious (Fig. 4b).

![Figure 4a. Transfer of material from vaginal swab to slide in preparation of staining the vaginal smear with a modified Wright’s Giemsa hematology staining set (Harleco Dif-Quik). The sequence includes 5 to 10 one-second dips in fixative, orange stain, blue stain and water rinse. - To view this image in full size go to the IVIS website at www.ivis.org. -](image)

![Figure 4b. Low power photomicrographs of vaginal smears obtained in late proestrus or early estrus, and stained with modified Wrights Giemsa hematology stain (left) or with a trichrome stain (right). The red staining of the trichome stained cells indicates that all these cells are cornified, yielding an eosinophilic index of 100%. - To view this image in full size go to the IVIS website at www.ivis.org. -](image)

During proestrus, peripheral plasma concentrations of estrogen are increased and cause thickening of the vaginal mucosa, and an increase in the number of cell layers. The mucosa changes from a low, cuboidal epithelium into a stratified, keratinized squamous epithelium. During this transition, the surface cells change in their shape, size and staining character, becoming larger, irregularly shaped, and flat (squamous) nucleated cells (“intermediate cells”), and ultimately becoming anuclear cornified squamous cells (“superficial cells”) [3]. The latter are characterized as having no nucleus visible, or a faint and/or small pyknotic nuclear-remnant. The relative proportions of different types of epithelial cell collected from the surface and viewed in vaginal smears during proestrus can be used as markers of the changes in the endocrine environment, i.e., rising estrogen concentrations (Fig. 5). Several indices of cornification and keratinization have been used; in general, the fertile period can be crudely predicted by calculating the percentage of epithelial cells that are superficial cells (with absent, faint or pyknotic nuclei) when using a modified Wright-Giemsa stain such as Diff-Quik. Mating should be attempted throughout the period when more than 80% of epithelial cells are "superficial" cells as this is typically coincident with the fertile period. However, there is great variation, and the percentage of cornified cells may surpass 80 to 90% and reach nearly 100% (Fig. 6a and Fig. 6b) as early as 9 days before ovulation or as late as 2 days before ovulation [4]. Therefore, changes in the vaginal cytology cannot be used to accurately time ovulation prospectively. Nevertheless, vaginal cytology permits monitoring of the normal progress of proestrus, and waiting for the 80% cornification value allows one to avoid unnecessary testing, transportation or matings until the proestrus rise in estrogen is nearly complete.

![Figure 5. Photomicrograph of a vaginal smear collected during proestrus, showing an increased numbers of epithelial cells including small intermediate, large intermediate and superficial cells. There are a few erythrocytes, which arise from estrogen-dependent uterine diapedesis and discharge into the vagina. There are no leukocytes because estrogen has stimulated sufficient vaginal epithelial hyperplasia to prevent the natural migration of leukocytes through the vaginal mucosa normally seen in anestrus, early proestrus and metestrus. Stain: Diff-Quik hematology stain. - To view this image in full size go to the IVIS website at www.ivis.org. -](image)

![Figure 6a. Photomicrograph of a vaginal smear collected during early estrus, containing an increased numbers of epithelial cells, all of which are "superficial" cells with either no nuclei, faint nuclei, or dense but pyknotic and small nuclei. There are numerous erythrocytes, which arise from estrogen-dependent uterine long-lasting diapedesis and discharge into the vagina. - To view this image in full size go to the IVIS website at www.ivis.org. -](image)
Vaginal cytology should be monitored during and following the period of peak cornification, and following breedings or inseminations, as a means to retrospectively estimate the timing of breeding in relation to the time of ovulation and the fertilization period, as explained below.

At the end of the fertilization period, plasma estrogen has declined to low levels over the previous week, and the plasma progesterone concentrations continue to increase to high values. As a result much of the vaginal epithelium sloughs off and is lost. The number of cell layers decreases, deeper cells are uncovered and the percentage of large irregularly shaped cornified and anuclear cells decreases. Polymorphonuclear leukocytes are absent from the vaginal smear during the fertile period because the thickened mucosa is a barrier to their migration to the surface. They reappear, often in large numbers, at the end of the fertilization period due to desquamation of the mucosa as plasma progesterone concentrations are high and estrogen is reduced (Fig. 7). At this time, the vaginal smear shows an increase in small and large intermediate sized epithelial cells, parabasal cells, and polymorphonuclear leukocytes. This time has been referred to as the "end of vaginal estrus", "onset of vaginal metestrus", or "onset of diestrus" [3]. Natural mating or vaginal insemination is rarely fertile when performed after a bitch has reached this stage, typically at 7 - 8 days after the LH surge (and 5 - 8 days after ovulation). However, such "late" matings have been reported to result in pregnancies and small litters in some instances. Counting backwards from the end of vaginal estrus to the days of breeding or insemination can estimate retrospectively whether or not they occurred during the period of fertilization. Ideally, inseminations would have occurred 2 to 4 days before the end of vaginal estrus. In one study the greatest success with frozen semen AI was when insemination was performed at 3 days prior to the onset of metestrus, i.e. diestrus. The index of superficial cells with faint or pycnotic nuclei declines dramatically over the next several days, and parabasal cells become the predominant epithelial cell (Fig. 8). The smear then progressively changes rapidly to become more like the smear of the anestrous bitch.

Vaginal cytology is clearly an easy and useful technique, although in rare cases polymorphonuclear leukocytes may be found throughout the fertile period and reported in some instances peak values of only 60% anuclear cells may be reached. In the latter cases, it is important to recognize when peak cornification is occurring, even though all exfoliated cells are not anuclear.

**Vaginal Endoscopy**

Vaginal endoscopy (vaginoscopy) is the examination of the surface of the vaginal mucosa, usually using a rigid endoscope with fiber optic capability (Fig. 9). Flexible scopes can also be used. Vaginoscopy can also be performed by direct visualization without magnification, using a rigid, human pediatric proctoscope. The procedure is well tolerated in the non-sedated, standing bitch. The examination can take as little as 2 minutes, and with experience valuable information may be collected quickly and with minimal expense. Vaginoscopic assessment for breeding management is based upon observation of the mucosal fold contours and profiles, the color of the mucosa and of any fluid present, and changes in these during proestrus and estrus. During anoestrus the vaginal mucosa is relatively flat, dry and red in appearance [4]. This represents a very thin, fragile and friable mucosa, easily traumatized by manipulation or instrumentation.
At the onset of proestrus the mucosal folds are greatly enlarged, edematous, and pink or pink/white in color, with serosanguinous fluid in crevices formed by the folds. These changes are due to thickening of the mucosal epithelium, and edema accumulation within the submucosa, both of which are effects produced by rising estrogen concentrations at this time (Fig. 10). The serosanguinous fluid is of uterine origin and leaks through the cervix. The mucosa surface itself become progressively less pink and typically white or cream-white in color, because the thickened mucosa hides from view the underlying capillaries that had been visible during anestrus and early proestrus. In very late proestrus or early estrus, at approximately the same time as the LH surge, there begins a progressive shrinking of the folds that is accompanied by pallor. These effects are the result of an abrupt withdrawal of the "water-retaining" edematous effect of estrogen. Estrogen concentrations decline rapidly during and following the LH surge. Subsequently, over the next several days, mucosal shrinkage occurs and causes minor and then gross wrinkling of the mucosal folds that by day 3 or 4 after the LH surge become distinctly angulated while retaining dense cream to white color (Fig. 11). The wrinkling has also been referred to as crenulation of the mucosal folds [4].

The onset of the peak fertile period can thus be detected by observing the onset of mucosal shrinkage without excessive angulations, whilst gross shrinkage of entire mucosal folds with obvious angulation is characteristic of the fertilization period. Matings or inseminations should be planned to coincide with the fertilization period, about four days after first detecting mucosal shrinkage, and at the onset of the period of obvious angulation of mucosal folds. The termination of the fertilization period can be detected by observing a decline or cessation of mucosal shrinkage, a flattening and thinning of the mucosa and, related to the sloughing of much of the superficial layers of epithelial cells, the development of a mucosal surface that has been described as blotched or variegated [4]. Some areas are white and still thick, while other areas becoming reddish and rather thin (Fig. 12). This occurs simultaneously with the onset of vaginal metestrus or diestrus, as defined by vaginal cytology.

Ovarian Ultrasound Examination
It has been clearly demonstrated that the ovaries of a bitch can be identified using real-time diagnostic B-mode ultrasound. With careful and repeated examination it is possible to monitor follicular growth and to detect the time of ovulation. In general
however, ovulation is difficult to identify because follicles do not collapse, echogenicity changes are not always consistent, and new corpora lutea also have central fluid filled cavities similar to those of the follicles. In ultrasonograms, ovarian antral follicles appear as black, anechoic spheres that increase in size during proestrus [1]. They are about 2 - 3 mm in diameter in early to mid proestrus, about 5 mm in diameter in late proestrus, and reach maximal size of 7 to 10 mm between the day of the LH surge and the day of ovulation (Fig. 13). For experimental studies of canine reproduction, the time of ovulation can be determined during daily sonographic examinations by the detection of an apparent decrease in the number of large anechoic follicles, or their complete disappearance, due to an increase in echogenicity and/or a related apparent decrease in follicle size (Fig. 14). This decrease or absence of anechoic structures on the ovary persists for 1 to 3 days around the time of ovulation [1]. This is often followed by the reappearance of anechoic structures in the ovaries. These are the early developing corpora lutea which contain central fluid for several days to 2 weeks. However, the technique appears to have little clinical application at the present time.

**Figure 13.** Trans-abdominal ultrasonogram of a bitch showing an ovary with three anechoic, large follicles at 1 day before ovulation. The white cursors indicate the 6mm diameter of the follicle on the left. - To view this image in full size go to the IVIS website at www.ivis.org . -

**Figure 14.** Trans-abdominal ultrasonogram of a bitch showing an ovary at 1 day after ovulation, with no evidence of the distinct spherical anechoic structures present 1 to 2 days earlier. - To view this image in full size go to the IVIS website at www.ivis.org . -

**Examination of Cervico-Vaginal Secretion**

In a small number of bitches it has been shown that the electrical resistance of the vaginal secretions, as measured by readings from a commercial vaginal resistance probe, increases during proestrus and early estrus, and decreases during mid to late estrus. These changes might therefore be useful for indicating the fertile period. The technique has been poorly investigated in the domestic bitch, although it is used widely to detect the optimal time of insemination in fox vixen. Some workers have examined changes in the concentration of glucose within the vaginal discharge of the bitch, and this method is sometimes used by dog breeders for the prediction of mating time. However the technique has failed to stand up to scientific investigation, and appears to be almost useless. Crystallization or ferning of mucus collected from the anterior vagina and observed microscopically has been described in the bitch, and occurs after the peak in plasma estrogen concentration [6]. Assessment of the mucus, which originates from cervical glandular tissue, may be useful in combination with vaginal cytology for determining the optimal mating time [6].

**Review of Normal Sequence of Events**

Table 4 chronicles many of the events that can be identified by clinical examinations over time and their relation to the fertile period, ovulation and the fertilization period.

<table>
<thead>
<tr>
<th>Table 4. Typical sequence of events during canine proestrus and estrus, including periods of fertility for natural matings, and recognizing the considerable variation in the time of onset of estrus behavior.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Change in clinical, physiological or fertility status</strong></td>
</tr>
<tr>
<td>Increased serum estrogen concentrations begins</td>
</tr>
<tr>
<td>Onset of obvious signs of proestrus (vulvar swelling, serosanguinous discharge)</td>
</tr>
<tr>
<td>Increasing vaginal hyperplasia, cornification and edema</td>
</tr>
<tr>
<td>Increasing vulval swelling and turgor observed</td>
</tr>
<tr>
<td>Peak cornification index in vaginal smears attained</td>
</tr>
<tr>
<td>Maximal vaginal hyperplasia and cornification attained</td>
</tr>
<tr>
<td>Maximal serum estrogen concentrations and vulval turgor</td>
</tr>
<tr>
<td>Onset of potential fertility and &quot;the fertile period&quot;</td>
</tr>
<tr>
<td>Decreased serum estrogen</td>
</tr>
<tr>
<td>Major component of LH surge occurs.</td>
</tr>
<tr>
<td>Progesterone is increased to 0.9 to 3 ng/ml serum or plasma.</td>
</tr>
<tr>
<td>Onset of period of &quot;peak fertility&quot; for natural service in bitches of high fertility</td>
</tr>
<tr>
<td>Decreased vulval turgor can often be detected.</td>
</tr>
<tr>
<td>Decreased vaginal edema and onset of vaginal wrinkling.</td>
</tr>
<tr>
<td>Onset of estrus behavior (variable, range of Day -3 to + 7)</td>
</tr>
<tr>
<td>Day 0 to 1 on average, and in many bitches</td>
</tr>
<tr>
<td>Ovulation(s) occur.</td>
</tr>
<tr>
<td>Progesterone is increased to 2 to 8 ng/ml</td>
</tr>
<tr>
<td>Further decrease in vaginal edema, increased vaginal wrinkling</td>
</tr>
</tbody>
</table>
Maturation of oocytes in oviducts | Day 4 to 5
---|---
Onset of fertilization period | Day 4 to 5
Maximal vaginal wrinkling and angulation of vaginal folds | Day 3 - 5
Peak desquamation of cornified epithelial cells of vaginal mucosa | Day 4 to 6
Flattening of vaginal mucosal folds | Day 5 to 6
Progesterone increased to 8 - 20 ng/ml | Day 6
End of period of "peak fertility" | Day 6
Decreased cornification index, denuded patchy appearance of vaginal mucosa, first seen in most bitches | Day 7 to 10
End of period of fertilization and period of fertility | Day 7 to 10
Leucocytes influx into vagina and re-appear in vaginal smears | Day 7 to 10
End of "cytological or vaginal estrus", first day of metestrus/diestrus | Day 7 to 10
Cessation of serosanguinous discharge (very variable) | Variable
Termination of estrus behavior (about Day 9, on average) | Variable
Implantation | Day 21 to 22
Pregnancy ultrasound via fetal heartbeats | Day 25 to 28
Parturition | Day 64 to 66

**Caveat: Normal Estrus versus False Estrus**

As reviewed above, breeding management and efforts to estimate the time of ovulation typically involves:

1. Examination of vaginal smears collected every 1 to 3 days to monitor the extent, percent or index of vaginal epithelial cell cornification, and the presence versus absence of leucocytes in the smear.
2. Characterization of the extent of estrogen-induced vulval swelling and turgidity, and timing of the decrease in turgidity associated with the periovulatory decline in estrogen;
3. Where possible, vaginoscopic evaluations of the edema and hypertrophy caused by the rise in estrogen, and the timing and extent of the subsequent vaginal mucosal shrinkage and wrinkling caused by the decline in estrogen shortly before and after ovulation; and,
4. A consideration of the normally expected changes in the sexual behavior of bitches, including the timing and extent of sexual receptivity during a normal periovulatory period.
5. Where possible, measuring serum or plasma progesterone concentrations, and estimating the day of the LH surge as the time when progesterone most likely first exceeded 1 to 2 ng/ml.

However, it is important to appreciate that all the physical, clinical and behavioral changes expected in normal ovulatory cycles can be observed in bitches that experience a "false estrus", i.e. a failure to have an LH surge and thus a failure to ovulate following the peak in estrogen concentrations at the end of proestrus. This is the case because all the normal changes in the genitalia, vagina and behavior primarily reflect the normal rise and fall in estrogen. In "false estrus" the onset of estrus behavior may be slow and intermittent because there is no concomitant rise in progesterone to facilitate estrus behavior but, nonetheless, the sexual receptivity may be nearly normal after estrogen concentrations undergo a spontaneous decline for several days during atresia of the unovulated follicles. The only means to differentiate false estrus from a normal cycle is by determining whether or not there is a normal increase in progesterone after ovulation. And it is not a straightforward as one might expect. Often there is no increase in progesterone. But, in some instances these cases of "false estrus" will exhibit a transient but almost normal rise in progesterone for 1 to 2 days. This is followed by a decline in progesterone instead of the normal continuous increase in concentrations to near-peak levels over a period of 2 to 3 weeks. Thus, monitoring of progesterone until concentrations reach 10 ng/ml or more should be considered. Whether such aborted increases in progesterone are spontaneous, or occur in response to a release of LH in amounts inadequate to cause ovulation, is not known. Alternatively, the bitch should be examined for normally elevated progesterone levels at the time of pregnancy testing at 3 - 4 weeks after breeding in cases where the bitch fails to become present. False estrus is often followed by a return to proestrus within 1 to 10 weeks.

**Conclusion**

The majority of bitches presented for fertility investigation and breeding management are normal, healthy, fertile animals whose apparent infertility is related to a misunderstanding of reproductive physiology of the bitch. Many bitches are mated at inappropriate times. The accurate monitoring of each bitch during each estrous cycle enables estimation of the appropriate time for mating or insemination. In the bitch, oocytes mature 2 to 3 days after ovulation, and thus 4 to 5 days after the preovulatory LH surge. Mating(s) should be planned to occur during the "fertile period", 0 to 6 days after the LH surge, or preferably the "fertilization period" (4 to 6 days after the LH surge). These periods can be readily detected with considerable accuracy using measurement of plasma progesterone, assessment of vaginal cytology and vulval swelling, or vaginal endoscopy. Ideally, all these methods are used in conjunction with one another. In most bitches, it is sufficient to start monitoring from approximately 7 days after the onset of proestrus, if the latter is detected sufficiently early in its course. However, some owners may not notice the signs of proestrus until late in proestrus and close to the time of ovulation, and owner-education about what to expect and look for may
be important before the impending cycle. Thus, early monitoring for the occurrence of an 80% cornification index in the vaginal smear can prevent missing the fertile period in bitches that are first presented late in the course of proestrus. Following this strategy for scheduling natural matings or fresh semen inseminations during the "fertile period", preferably twice between 2 and 6 days after the LH surge, will result in a significant increase in pregnancy rate and litter size. When it is necessary to use semen of a sub-optimal quality, for example where there is male-factor infertility, or when semen has been cryopreserved, it is essential that mating or insemination occur during the "fertilization period", preferably at 5 - 6 days after the LH surge. Pregnancy rarely occurs following matings or vaginal inseminations that occur after the end of cytological estrus, although intra-uterine insemination has had some success during the first 2 or 3 days of metestrus, i.e. diestrus.

Accurate timing of periovulatory events during breeding management also permits accurate determination of the time to best perform pregnancy testing and can allow the accurate prediction of the day of parturition which occurs 64 to 66 days after the LH surge.

References