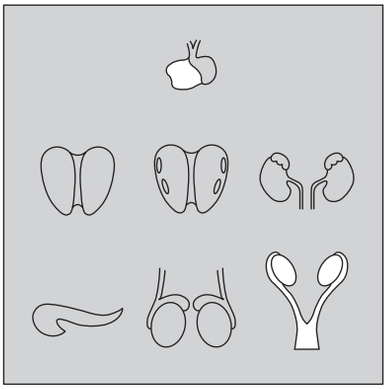

# 19

# OVARIAN CYCLE AND VAGINAL CYTOLOGY



## THE OVARIAN CYCLE

### Onset of Puberty

Breed has a significant effect on the timing of a dog's first estrus. Generally, bitches exhibit their first cycle several months after they achieve adult height and body weight. There is, however, considerable variation within a breed as well as between different breeds. The Beagle, for example, usually exhibits her first proestrus between 7 and 10 months of age. Even in a controlled laboratory environment, the first proestrus may occur as early as 6 months of age or as late as 13 months (Concannon, 1987). Thus it is reasonable to advise owners that some small breeds enter their first heat between 6 and 10 months of age. Although a large-breed bitch may also begin her first proestrus before 1 year of age, some normal large-breed dogs may not begin to cycle until 18 to 24 months of age. A good deal of individual and breed variation is reported. This natural variability, coupled with cycles referred to as *silent heats,* adds to a veterinarian's or owner's inability to predict the timing of a first season (estrus) (Concannon, 1980; Evans and White, 1988; Johnston, et al, 2001).

*Silent heat* is simply one that goes unnoticed by the owner because little vulvar swelling, bleeding, attraction of males, or behavioral change may be associated with estrus in a young bitch. The owner's experience, hair length of the dog (vulvar bleeding is easier to see in a short-haired dog), cleanliness of the dog (one that licks a great deal is more apt to hide bleeding), and presence of a male dog in the home are just a few of the variables that determine the ease with which an owner may notice estrus (Concannon, 1980). We tend not to begin to evaluate a female dog clinically for failure to experience an ovarian cycle until she is at least 2 years of age. (See Infertility, Chapter 24.) The ideal age for breeding is between 2 and 6 years. First breeding is recommended during the second or third estrus, *after* an owner has witnessed at least one complete normal ovarian cycle.

### Seasonality of the Ovarian Cycle

Bitches experience ovarian cycles throughout the year. Breeding seasons depend on both genetic and management factors. The preference for late winter and early spring breedings may stem from an evolutionary advantage in whelping litters at a time when the food supply is beginning to increase in association with improving climatic conditions. Owner preferences may also play a role. Certainly, whelping puppies in October provides a breeder with puppies to sell for an upcoming Christmas season. For many breeders, a second choice for whelping is late winter, in order to have puppies for sale in the spring. Seasonal patterns to ovarian cycles of the bitch should be distinguished from owner preferences. Dogs experience ovarian cycle activity, breed, and whelp litters in all months of the year, but this process does tend to have subtle peaks in the late winter/early spring as well as in autumn months.

### Intervals Between Ovarian Cycles

The "average" bitch begins proestrus approximately every 7 months. Thus, an ovarian cycle would begin at least once in every month of the year during a bitch's life if this schedule were maintained. There is a tendency to vary somewhat. The common *interestrus interval* (the period from the end of standing heat to the beginning of the following proestrus) is 5 to 11 months, with

the normal interestrus interval as short as 3.5 months and as long as 13 months (Concannon, 1987). Interestrus intervals more frequent than every 4 months, however, are often associated with infertility, and those greater than every 12 months *may* be associated with subfertility or infertility. Breed and individual variability can be striking. The German Shepherd dog, for example, is one breed that often cycles every 4 to 4.5 months and yet maintains fertility. Rather than relying on knowledge concerning individual breeds, we use the 5- to 11-month interval as normal. It also appears that most dogs 2 to 6 years of age are relatively consistent in cycle length as well as in the duration of each phase. The obvious exceptions to the 7-month average normal interestrus interval are the African dog breeds such as the Basenji. These dogs cycle once yearly. Litters of this breed are typically whelped in December, with January and November following as likely months of births.

As dogs advance beyond the optimal breeding age (approximately 7 years), various changes are likely to occur, including progressive lengthening in the duration of the interestrus interval, reduction in litter size, increases in congenital birth defects, and problems at parturition. In one study, mean interestrus intervals increased from approximately 240 days to more than 330 for Beagle bitches after they reached 8 years of age, but healthy dogs continue to experience ovarian cycles throughout life (Anderson and Simpson, 1973).

## Phases of the Ovarian Cycle

There are four phases of the canine ovarian cycle.

PROESTRUS. This is the period of heightened follicular activity preceding estrus when males are attracted to females but females refuse to breed.

ESTRUS. Estrus is the period during which the female allows the male to mount and breed. The term *estrus* is derived from the Greek word *oistros,* meaning "a vehement desire."

DIESTRUS. This is the period following mating. It is associated with corpora luteal activity. *Metestrus* refers to the period of corpora luteal activity as a distinct entity. In the dog, mating activity continues despite rising plasma progesterone concentrations. This progesterone is derived first from luteinized follicles and then from corpora lutea. Both estrus and diestrus are phases dominated by progesterone. In estrus the bitch breeds, whereas in diestrus she refuses to breed and is "physiologically pregnant." Thus, *diestrus* rather than *metestrus* is the term used as an aid in describing the unique sexual cycle of the bitch.

ANESTRUS. The time between the end of the luteal (progesterone) phase (diestrus) and the beginning of the following follicular phase (proestrus) is called *anestrus.* This has traditionally been described as a time of quiescence within the pituitary-ovarian axis. Clinically, this is a time of inactivity. Endocrinologically, hormonal activity continues.

## Proestrus

DURATION. Proestrus is usually, and most reliably, defined as beginning when vaginal bleeding is first seen and ending when the bitch allows a male dog to mount and breed. Additional criteria used in defining the onset and completion of proestrus include changes in the appearance of the vaginal mucosa viewed endoscopically (Fig. 19-1) or changes seen on exfoliative cytology of vaginal epithelial cells (Fig. 19-2). Less reliable criteria used in describing the onset of proestrus include enlargement of the vulva, attraction of males, and changes in behavior toward males (Fig. 19-3).

The length of time from the onset of proestrus to the time of first breeding is usually 6 to 11 days, with an average of 9 days. However, variations within "normal" can be extreme (i.e., as brief as 2 or 3 days to as prolonged as 25 days) (Fig. 19-4). These extremes in normal duration of proestrus can be confusing, and their significance is discussed in greater detail in association with the evaluation of female infertility (Chapter 24).

CLINICAL SIGNS. Early proestrus behavior includes attraction of males, in part due to pheromone secretion (Goodwin et al, 1979). An increase in playful/teasing activity with discouragement of any mounting attempt by a male is common (Jeffcoate, 1998). This unwillingness to breed may involve antisexual growling, moving away, baring the teeth, and snapping. The bitch may also keep her tail tight against the perineum, between the rear legs and covering the vulva. This initial behavior pattern gradually changes as proestrus progresses. The female usually becomes more receptive, as demonstrated by actually seeking and playing with males. The response by the bitch to male mounting attempts progressively becomes more restrained, as she prevents mounting by retreating, crouching, or lying down. In late proestrus, the behavior of the bitch may be described as passive, and she may sit quietly when mounted, with or without intermittent displays of tail deviation and lordosis.

Proestrus is typically but not always associated with varying quantities of bloody vaginal discharge. Vaginal bleeding begins as diapedesis of erythrocytes through the endometrium and subepithelially as capillaries rupture within the endometrium. This blood seeps through a slightly relaxed cervix and enters the vaginal vault. Small numbers of erythrocytes may also originate from the vaginal mucosa, since erythrocytes have been observed in vaginal smears obtained from proestrus bitches that had been previously hysterectomized (Johnston et al, 2001). Rapid changes in thickness and development of the vaginal mucosa and the endometrium are responses to follicular estrogen secretion. The volume of bleeding and bloody vaginal discharge vary from bitch to bitch.

Bitches that keep themselves clean through licking may present a difficult challenge in detecting proestrus. Short-haired dogs without tails (e.g., German Short-Haired Pointers) can be contrasted with long-haired dogs with flowing tails (e.g., Newfoundlands). Obviously, vaginal bleeding associated with proestrus is easier

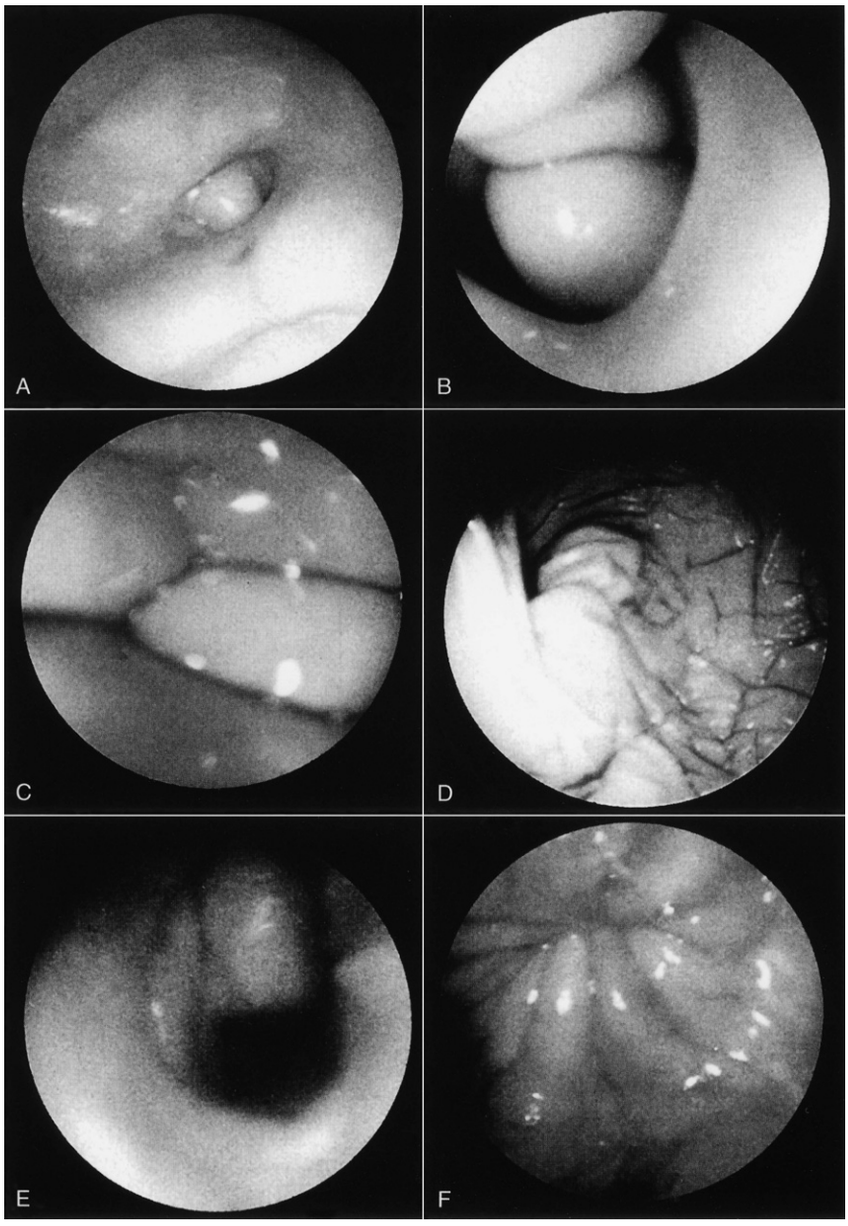


**FIGURE 19-1.** Photographs of the canine vagina as viewed during endoscopy. *A,* Cranial vagina during anestrus. In view is the round caudal tubercle of the dorsal median fold in center of field. *B,* Cranial vagina during early proestrus. Slightly oblique view of the dorsal median fold. Mucous membranes develop new folds and become edematous during proestrus. *C,* Cranial vagina during early proestrus. Note edematous folds of mucous membranes. *D,* Cranial vagina during late estrus (oblique view). Mucous membranes appear angulated during estrus. By the end of estrus, all folds are in their most shrunken and angular state. *E,* Cranial vagina as viewed on the first day of diestrus (first day of postestrus refusal). *F,* Cranial vagina showing the fully formed "rosette" that can be viewed with endoscopy during diestrus.

to detect in some breeds than in others. Occasionally, a grayish mucoid-like vaginal discharge is observed before actual bleeding or swelling of the vulva. Classically, dogs cease vaginal bleeding as proestrus proceeds into estrus. In these bitches the bloody discharge fades and becomes transparent to straw-colored. However, changes in color of the vaginal discharge are inconsistent, with some bitches showing bloody discharge throughout proestrus, estrus, and into diestrus, whereas others bleed little or only at the beginning of proestrus.

THE VULVA. The vulva slowly enlarges throughout proestrus due to fluid accumulation. This edematous and turgid vulva impedes intromission by a male. As proestrus proceeds into estrus, however, the vulva softens dramatically, eliminating this obstacle.

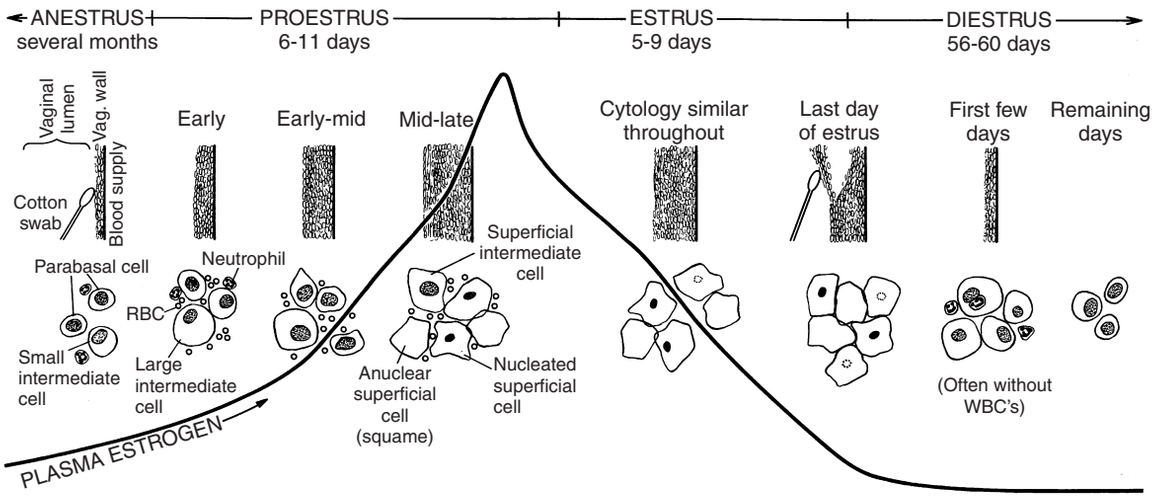


**FIGURE 19-2.** Illustration of the changes in vaginal wall thickness, vaginal cytology, and relative plasma estrogen concentrations in an average bitch experiencing an estrous cycle. Note that near the last day of estrus, rafts of vaginal cells are sloughed.

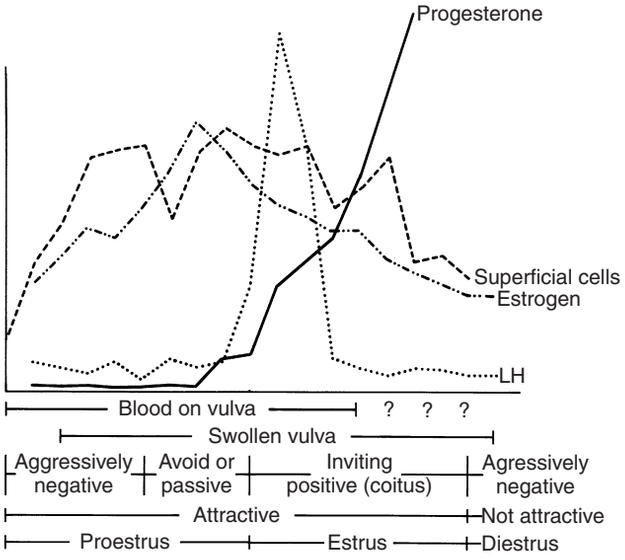


**FIGURE 19-3.** Diagrammatic illustration of the behavior changes, hormonal fluctuations, and vaginal cytology of a bitch experiencing proestrus and estrus.

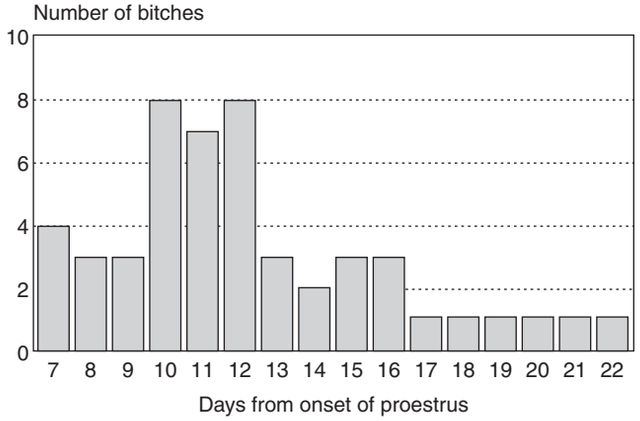


**FIGURE 19-4.** Predicted time of ovulation (or approximate onset of behavioral estrus) for 50 bitches, based on serum progesterone concentrations. More than 20 breeds were represented. (From Johnston SD, et al, 2001, with permission.)

HORMONAL CHANGES AND OVARIAN ANATOMY. The bitch in proestrus is under the influence of estrogen. This estrogen is synthesized and secreted by developing ovarian follicles. Proestrus is the phase of estrogen dominance in the bitch (see Fig. 19-2). FSH and LH pulses are released in concordance in late anestrus, and progression from anestrus into proestrus is associated with an increase in the secretion of FSH with a concomitant rise in LH secretion (Kooistra and Okkens, 2000). Follicles that develop at a time coinciding with gonadotropin stimulation mature and attain the capacity for estrogen synthesis and secretion. The secreted estrogen results in vaginal discharge, attraction of males, and uterine preparation for pregnancy. The potent effects of estrogen can be demonstrated in the ovariohysterectomized female, which can easily be brought into a classic proestrus state (without vaginal bleeding) by administration of estrogens (Concannon, 1987). Estrogen alone usually does not result in breeding activity. Breeding in the dog is typically associated with a serum combination of decreasing estrogen and increasing progesterone concentrations. Estrogen alone usually does not cause behavioral estrus (Leedy, 1988).

The appearance of the ovaries has been monitored during proestrus with ultrasonography. Ovaries of bitches in anestrus can be visualized as structures with an echogenicity equal to or slightly greater than that of the renal cortex. Follicles appear as focal hypoechoic

to anechoic rounded structures. Ovaries are easier to identify as follicular development progresses. Ovarian size increases throughout proestrus (England and Allen, 1989a, 1989b; Wallace et al, 1992).

The dramatically rising estrogen concentrations in venous plasma during proestrus correlate with dramatic changes in the uterus, the vaginal mucosa, and the vulva, as well as with follicular secretion and behavior patterns in the bitch. Circulating estrogen concentrations during anestrus are usually between 5 and 15 pg/ml. Proestrus is associated with an increase in estrogen (estradiol) concentration above 15 pg/ml. Early proestrus is usually associated with estradiol concentrations above 25 pg/ml, and late proestrus is associated with concentrations that may be in excess of 60 to 70 pg/ml. The peak in plasma estradiol concentration is achieved 24 to 48 hours *before* the termination of proestrus, that is, preceding estrus (standing heat).

*Decreasing* plasma estradiol concentrations stimulate the onset of estrus. During the subsequent 5 to 20 days, plasma estradiol concentrations taper progressively to basal levels. Therefore, proestrus is a phase of rising and then stable serum estrogen concentrations, and estrus is a phase of decreasing serum estrogen concentrations. "Basal" serum estrogen concentrations are observed in anestrus and in diestrus, although variations within individual dogs may be observed (Olson et al, 1982). Concentrations of testosterone increase in the serum of bitches during late proestrus, reaching concentrations of 0.3 to 1.0 ng/ml at the time of the LH surge that occurs in early estrus (Olson et al, 1984).

Progesterone concentrations throughout all but the last 24 to 72 hours of proestrus are low (basal: <0.5 ng/ml). The end of proestrus and the beginning of estrus are associated with a plasma progesterone concentration that rises above a critical plateau (1.0 ng/ml) while estrogen concentrations are decreasing. Serum progesterone concentrations of >1.0 ng/ml are required for induction of the behavior changes typical of estrus and maintenance of pregnancy. The progesterone is secreted by *follicles* progressively more *luteinized,* prior to ovulation and development of corpora lutea. Thus follicles begin to evolve from estrogen-producing "factories" in early and mid-proestrus to "factories" producing primarily estrogen and small amounts of progesterone in late proestrus. Progressively more progesterone and less estrogen are synthesized as the bitch progresses through estrus.

ANATOMY OF THE UTERUS, OVIDUCTS, AND MAMMARY GLANDS. Several "estrogen-target" tissues are affected by increases in serum estrogen concentrations during proestrus. These include growth of mammary gland ducts and tubules, proliferation of oviductal fimbria, thickening of oviducts, elongation of uterine horns, thickening of endometrium, increased myometrial sensitivity, enlargement of the cervix, elongation and edema of the vagina, proliferation of the vaginal wall, and synthesis of hepatic steroid-metabolizing enzymes (Concannon and DiGregorio, 1986; Concannon, 1987). The preparation for implantation in the endometrium includes a remarkable increase in wall thickness and glandular activity, changes that are associated with bleeding. This uterine hemorrhage is the primary source of vaginal bleeding associated with proestrus and, in some bitches, estrus.

VAGINAL ANATOMY AND ENDOSCOPY. Increasing estrogen concentrations thicken the vaginal wall, which protects the bitch from the traumatic effects of breeding. The vaginal lining in anestrus is only a few cell layers thick and is relatively fragile. The basal or germinal cell layer is orderly, and the less orderly overlying cells are situated in rather close proximity to the blood supply present below the germinal layer. The increasing estrogen concentrations associated with proestrus cause rapid multiplication in the number of cell layers lining the vaginal vault, resulting in a wall 20 to 30 layers thick by the end of proestrus (Johnston et al, 2001). Thus intromission of the penis into the estrogen-primed vaginal vault is not harmful to the female.

This increased number of cell layers moves the cells lining the lumen further from their blood supply, causing the death of those cells. These dead cells function as a less sensitive and less fragile tissue. Fragility decreases not only because of increased cell layers, but also because of the development of keratin precursors within these cells. This nuclear material is similar to that found in fingernails. Thickening of the vaginal mucosa can be easily recognized with cytology (see Fig. 19-2).

Thickening in the vaginal mucosa, easily demonstrated with cytology, may also be detected with vaginoscopy. The procedure is simple and well tolerated by nonsedated bitches that have normal vaginovestibular anatomy, but does require some practice and experience. Before inserting any vaginoscope, one should first perform a digital examination of the vaginal vault to confirm that no stricture is present (see Chapter 25). The mid- to anterior vagina can be visualized using a rigid pediatric proctoscope or flexible endoscope. The less expensive proctoscope is an excellent tool for this purpose. Otoscopes are completely inadequate for viewing anything but the "vestibule" of the vagina. The vaginal mucosa is flat in anestrus because relatively few cell layers are present (see Fig. 19-1).

During proestrus, the mucosa appears markedly rounded, edematous, smooth, and shiny (Jeffcoate, 1988). Vaginal folds are pink and billow out into the lumen as a result of fluid retention, resulting in an inability to visualize the lumen (Goodman, 2001). The decreasing estrogen and increasing progesterone concentrations associated with the final 1 to 3 days of proestrus (or the first days of estrus in some bitches) cause edema in the vaginal mucosa to subside, and the stretched luminal surface becomes progressively wrinkled (see Fig. 19-1). This is referred to as *crenulation.* Initial vaginal crenulation, observed as this subtle wrinkling of the mucosa, appears within 24 hours of the preovulatory LH surge (Lindsay and Concannon, 1986; Concannon, 1987; Jeffcoate, 1998).

The LH surge, in turn, precedes the onset of ovulation by approximately 24 to 48 hours. Breeding

should begin at this time. The mucosa becomes progressively more crenulated, the lumen more obvious, and the vaginal folds more flattened as the edema diminishes. This wrinkling is most obvious during the fertile period 4 to 7 days after the LH surge. By diestrus the mucosa is flat and variegated. Further, because the protective thickened layers of epithelium have disappeared, the mucosa once again becomes fragile and superficial hemorrhage may be associated with vaginoscopy (Goodman, 2001). With practice and observation of individual variation in this process, vaginoscopy becomes a useful adjunct when attempting to best time a breeding.

## Classification of Vaginal Cells

GENERAL BACKGROUND. Changes in the vaginal mucosa due to increases in serum estrogen concentration during proestrus and estrus are reflected in the appearance of exfoliated vaginal epithelial cells. These alterations are believed to be due solely to increases in circulating estrogen concentrations. Therefore vaginal cytology can serve as a crude but reliable indirect estrogen assay.

Nomenclature of vaginal cells is based on cell morphology. Older literature refers to keratinized versus nonkeratinized cells and/or to cornified versus noncornified cells. Some species actually develop a hard keratin lining of the vaginal tract as a result of increasing circulating estrogen concentrations. The bitch, however, does not develop a "cornified" vaginal lining, which explains the different nomenclature used in describing alterations in canine vaginal cells due to estrogen influences. Different cell types represent stages of cell death. As healthy round vaginal cells die, they become larger and more irregular in shape. The nuclei within vaginal epithelial cells also undergo changes that reflect cell death: the nucleus becomes progressively smaller, then pyknotic, before eventually disintegrating, leaving a nuclear "ghost" and then an anuclear cell (see Fig. 19-2).

PARABASAL CELLS. Parabasal cells are the healthiest and smallest of the vaginal cells. They are round or slightly oval and have a large vesiculated nucleus and relatively small amounts of cytoplasm. They usually stain well with Wright-Giemsa stain or some of the rapid stains (Diff-Quik, American Scientific Products, McGraw, IL). These cells are exfoliated from near the germinal cell layer, close to the underlying blood supply (Fig. 19-5).

INTERMEDIATE CELLS. Intermediate cells vary in size from slightly larger than parabasal cells to twice their size. These cells have smooth, oval to rounded irregular borders and a nucleus that is vesiculated but generally smaller than those found in parabasal cells (Fig. 19-6). This change in morphology reflects the first step in cell death: cells appear larger, have relatively larger amounts of cytoplasm, and have smaller nuclei. For descriptive purposes they are classified as *small intermediate cells* and *large intermediate cells*.

SUPERFICIAL CELLS. Superficial cells are dead cells that line the vaginal lumen of bitches in estrus. They are the largest cells identified on vaginal cytology. These cells have sharp, flat, angular cytoplasmic borders and small, pyknotic, fading nuclei or no nuclei (Fig. 19-7).

SUPERFICIAL-INTERMEDIATE CELLS. These are vaginal cells that appear to have relatively healthy vesiculated nuclei but also have the angular, sharp, flat cytoplasmic border typical of superficial cells (Olson et al, 1984b; Olson, 1989) (Fig. 19-8). The superficial-intermediate cell provides evidence for potent estrogen effect on the vaginal lining, but not quite the full effect. Full estrogen effect is associated with superficial cells and *anuclear squames*. Vaginal exfoliative cytology may not progress beyond the presence of superficial-intermediate cells at the peak of estrogen effect. No study has demonstrated associations between failure to develop superficial cells on vaginal cytology and subsequent breeding or fertility problems.

ANUCLEAR SQUAMES. These large, dead, irregular vaginal cells with no nuclei represent the end of a process that began with healthy round parabasal cells. This cell death is caused by the thickened vaginal lining. As the vaginal wall thickens in response to increases in serum estrogen concentrations, from several cell layers to 20 to 30 cell layers, those cells lining the lumen become far removed from their blood supply and their death is inevitable (see Fig. 19-2). These cells are also called *anuclear superficial cells*. They are large cells with flat, angular borders (Fig. 19-9). These are the cells that have also been called "fully cornified" or "fully keratinized" cells.

METESTRUM CELLS. Metestrum cells are usually large intermediate vaginal cells that appear to have one or more neutrophils contained within their cytoplasm (Fig. 19-10). As described below, metestrum cells are usually seen in the vaginal smear from a bitch in early diestrus or one with vaginitis. Rarely, such cells are observed in early proestrus.

FOAM CELLS. Foam cells are parabasal and intermediate cells that have obvious cytoplasmic vacuoles. These cells may be associated with diestrus and anestrus.

## Vaginal Cytology in Proestrus

EARLY PROESTRUS. The vaginal smear from a bitch in early proestrus is similar to that from a bitch in anestrus, with one difference: the presence of blood within the vagina derived primarily from a rapidly developing endometrium (see Fig. 19-2). In addition to varying numbers of red blood cells, the vaginal smear usually contains numerous parabasal, small, and large intermediate vaginal epithelial cells. Neutrophils are common in varying numbers, and bacteria may be present in small to large numbers (Fig. 19-11; Table 19-1). The background of these smears is often granular or "dirty" in appearance, owing to the presence of viscous cervical and vaginal secretions that appear to take a slight amount of stain.

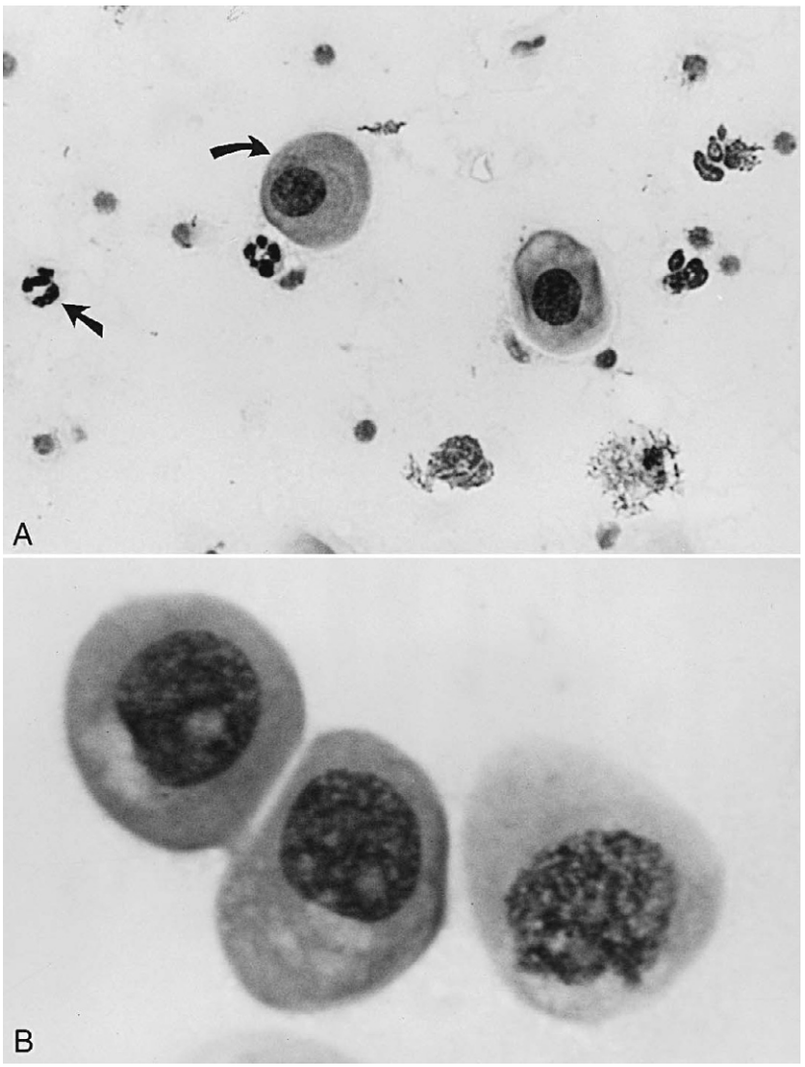


**FIGURE 19-5.** *A,* Parabasal cells *(curved arrow)* and neutrophils *(straight arrow)* on vaginal cytology. *B,* Parabasal/small intermediate cells on vaginal cytology.

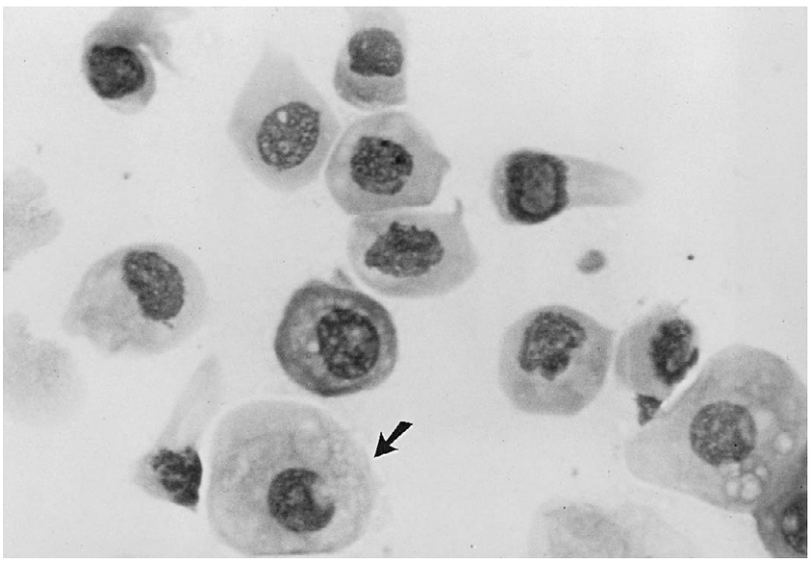

**FIGURE 19-6.** Intermediate cells on vaginal cytology; *arrow* points out one excellent example of a large intermediate cell.

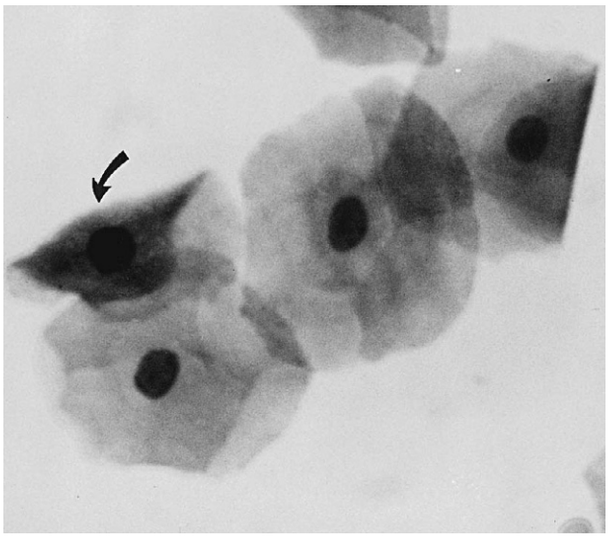

**FIGURE 19-7.** Superficial vaginal epithelial cells with angular perimeter borders and pyknotic nuclei. The variable density of the cell is caused by folding over *(arrow).*

MID-PROESTRUS. The first evidence of continued estrogen effect on the reproductive tract is visualized in the vaginal cytology. This includes (1) disappearance of neutrophils, (2) appearance of the red blood cells, and (3) progressively increasing percentage of superficial cells replacing the smaller parabasal and intermediate cells. White blood cells are believed to enter the vaginal lumen through the mucosal wall. With the dramatic thickening in the wall caused by estrogen, neutrophils are unable to penetrate this barrier and enter the lumen. Neutrophils are not normally visualized in vaginal smears from bitches between mid-proestrus and the start of diestrus Fig. 19-12).

LATE PROESTRUS. In late proestrus, the vaginal smear contains no neutrophils, the presence of red blood cells is variable, and the background is clear. It is important to remember that some bitches have a bloody vaginal discharge for only a few days into proestrus (if at all), most bleed throughout proestrus, some bleed throughout proestrus and estrus, and, finally, a small percentage exhibit vaginal bleeding in proestrus that continues into diestrus. Recognizing this variation in vaginal bleeding among healthy bitches prevents unnecessary worry.

More than 80% of exfoliated vaginal cells are "superficial," with either vesiculated nuclei, pyknotic nuclei, or no nucleus (anuclear superficial cells; Fig. 19-13). It is not possible to distinguish late proestrus from estrus with vaginal cytology. To the contrary, vaginal cytology from bitches in the final 1 to 8 days of proestrus is the same as that from bitches in estrus (see Table 19-1). In addition, variations in duration of proestrus (2 days to 3 weeks) in normal bitches may contribute to owner confusion when attempting to evaluate the ovarian cycle.

## Estrus

The estrus phase encompasses the time during which the bitch allows a male to mount and breed. The first day that the female allows breeding (standing heat) is the start of estrus, and this phase ends when she no longer accepts the male. The other criterion defining the end to estrus is based on vaginal cytology (see page 764).

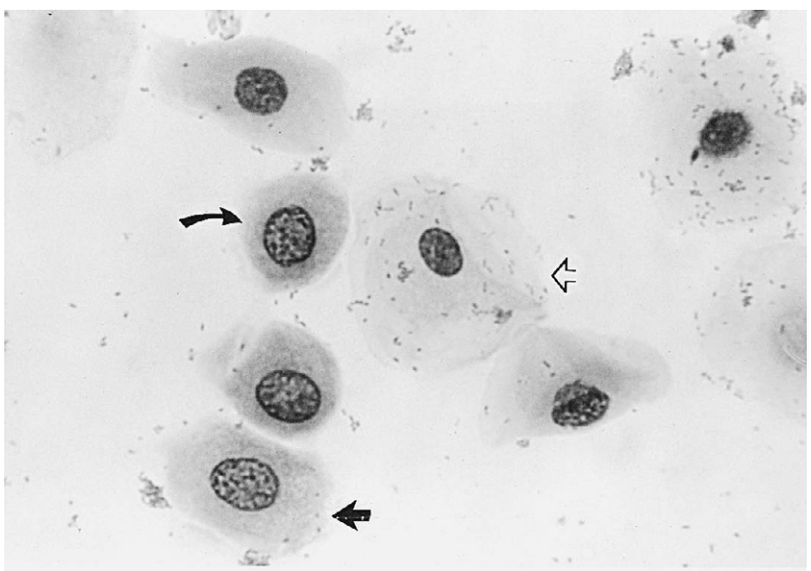

**FIGURE 19-8.** Vaginal cytology illustrating a small intermediate cell *(curved arrow)* and two different superficial-intermediate cells, one small *(closed arrow)* and one large *(open arrow).* Bacteria in background are not considered abnormal.

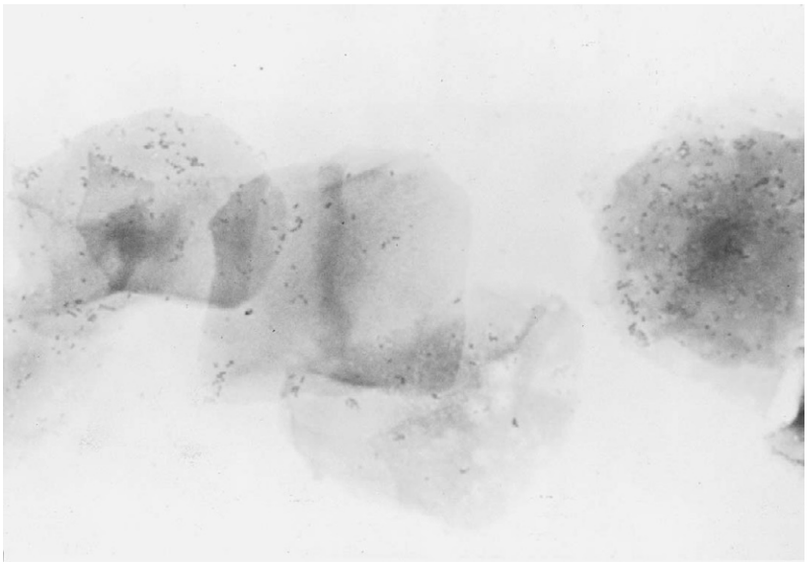

**FIGURE 19-9.** Vaginal cytology revealing anuclear superficial cells (squames). Note the angular perimeter to the cells and the light staining characteristics. Bacteria are often seen in normal bitches.

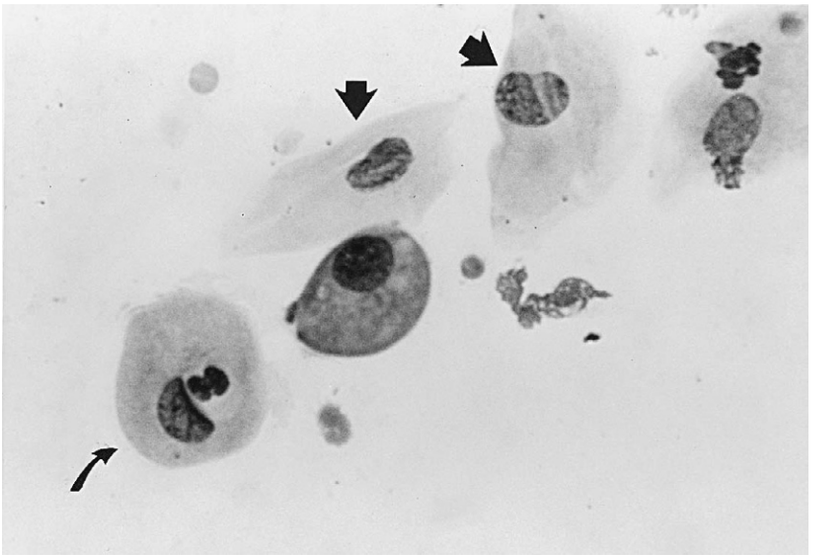

**FIGURE 19-10.** Vaginal cytology revealing one vaginal epithelial cell that contains a neutrophil within its cytoplasm (metestrum cell; *curved arrow*). Also note the superficial-intermediate cells *(straight arrows)*.

**TABLE 19–1 CYTOLOGIC STAGING OF ESTROUS CYCLE**

| Stage | Red Blood Cells | Neutrophils | Bacteria | Epithelial Cells |
|---|---|---|---|---|
| Early proestrus | Usually | Often | Many | Parabasal, intermediate, superficial intermediates, few superficial |
| Late proestrus | Usually | Few or none | Many | Superficial intermediates, superficial |
| Estrus | Present, may be decreased in number<br>May be absent | None | Many | >80–90% superficial |
| Diestrus | Usually none | Few to many | May be clearing | Parabasal and intermediate predominate; dramatic decrease in superficial cells |

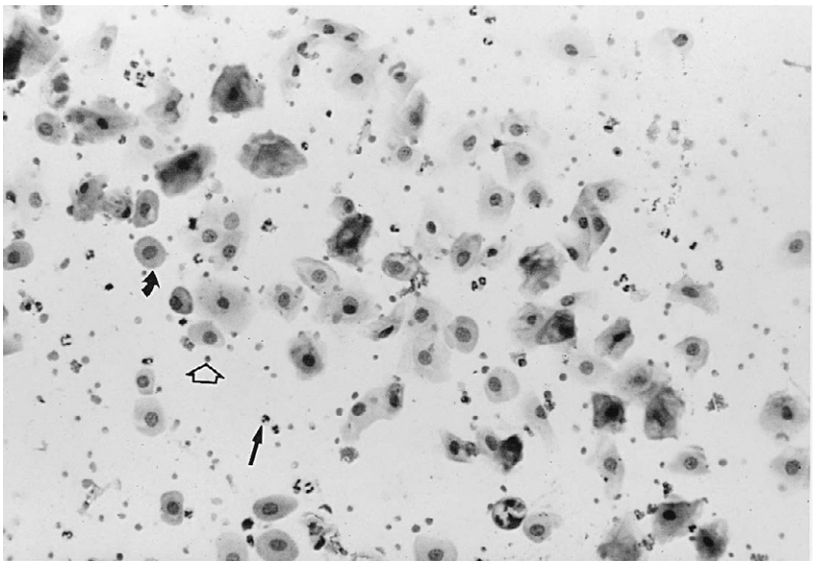

**FIGURE 19-11.** Vaginal cytology (at scanning microscope power) revealing neutrophils *(straight arrow),* red blood cells *(open arrow),* and vaginal epithelial cells that are primarily of the intermediate type *(curved arrow).* This smear is typical of early proestrus.

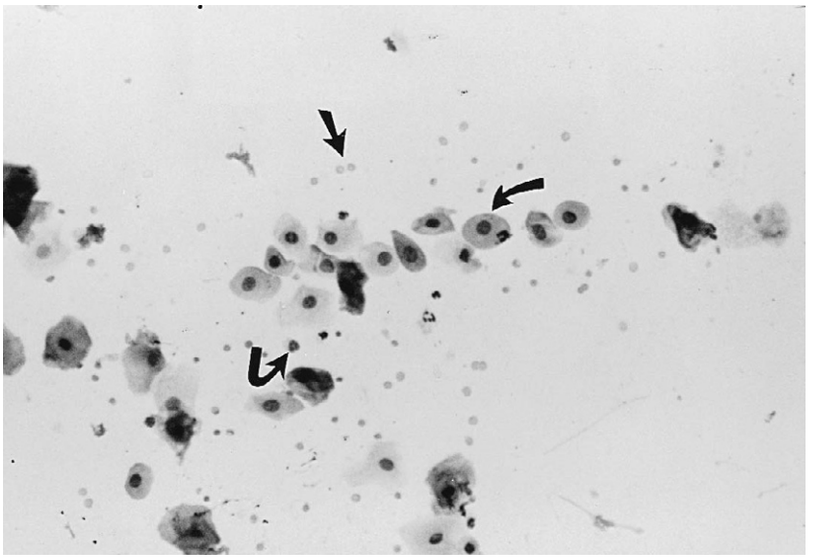

**FIGURE 19-12.** Vaginal cytology (at scanning microscope power) revealing red blood cells *(straight arrow)* and intermediate-type vaginal epithelial cells *(curved arrow).* Presence of few neutrophils *(hair-pin arrow)* makes this smear suggestive of mid-proestrus.

HORMONAL CHANGES. Serum estrogen concentrations peak 1 or 2 days *before* the onset of estrus (see Fig. 19-1). Typically, bitches usually begin to exhibit signs of standing heat only when circulating estrogen concentrations, that have progressively increased, begin to decrease. Decreasing serum estrogen concentrations reflect the final maturation process of ovarian follicles, several days before ovulation. Further demonstrating the importance of declining estrogen concentrations, ovariectomized bitches given estrogen parenterally exhibited behavioral signs of proestrus within 3 days, but not estrus. Of nine ovariectomized bitches given estrogen for 9 days, three exhibited estrus behavior 1 to 2 days before the last estrogen injection, whereas the majority exhibited estrus only *after* the last injection; in other words, their behavior was enhanced by estrogen concentrations that were decreasing (Concannon, 1987).

Ovarian follicular cells begin synthesizing progesterone in excess of that required to serve as precursors for estrogen synthesis within days of beginning proestrus. In concert with declining estrogen levels, later in proestrus and immediately preceding the onset of estrus, additional follicular cells "luteinize" and secrete

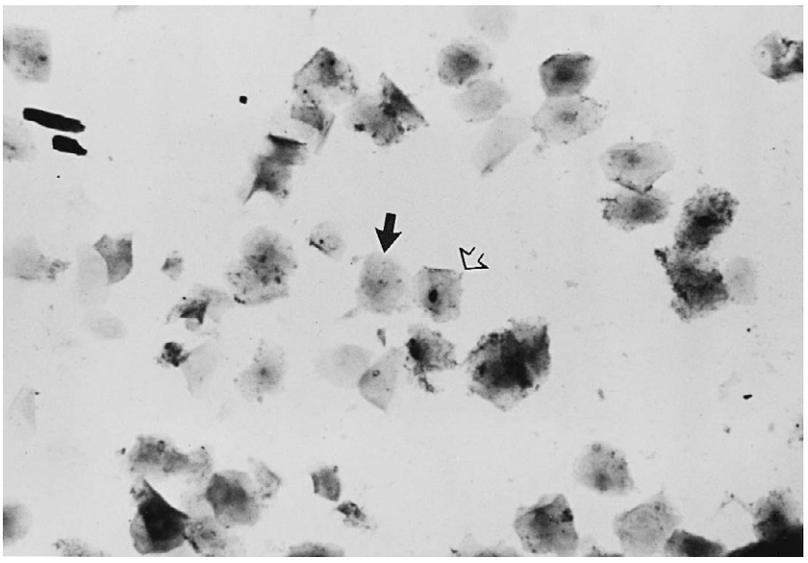

**FIGURE 19-13.** Vaginal cytology (at scanning microscope power) revealing both nucleated *(open arrow)* and anuclear *(closed arrow)* superficial cells. This smear is typical of late proestrus or estrus.

progressively greater amounts of progesterone. The combination of increasing serum progesterone concentrations and decreasing serum estrogen concentrations in the final days of proestrus stimulates two major events. The first is the change in behavior: the bitch that is passively resistant to breeding in late proestrus is transformed into one that actively seeks breeding in estrus. The second and equally important event stimulated by the decreasing serum estrogen and rising serum progesterone concentrations is the strong positive feedback to the hypothalamus and pituitary (Fig. 19-14), resulting in secretion of FSH and, most importantly, LH at the beginning of estrus.

Serum progesterone rises above basal concentrations *before* the LH surge. In other words, follicular cells capable of synthesizing and secreting progesterone are functioning before the development of corpora lutea. These cells cause the initial rise in progesterone concentration associated with the last day of proestrus and then the beginning of standing heat. This rise in progesterone concentration (see Fig. 19-3) enhances the intensity and duration of behavioral estrus. Ovariectomized bitches given progesterone-containing implants 6 hours following 9 days of estrogen injections had a 9-day mean duration of estrus behavior. In contrast, bitches that never received exogenous progesterone averaged only 5 days of estrus behavior following discontinuation of parenteral estrogen (Concannon, 1987). The combination of decreasing serum estrogen concentrations and increasing serum

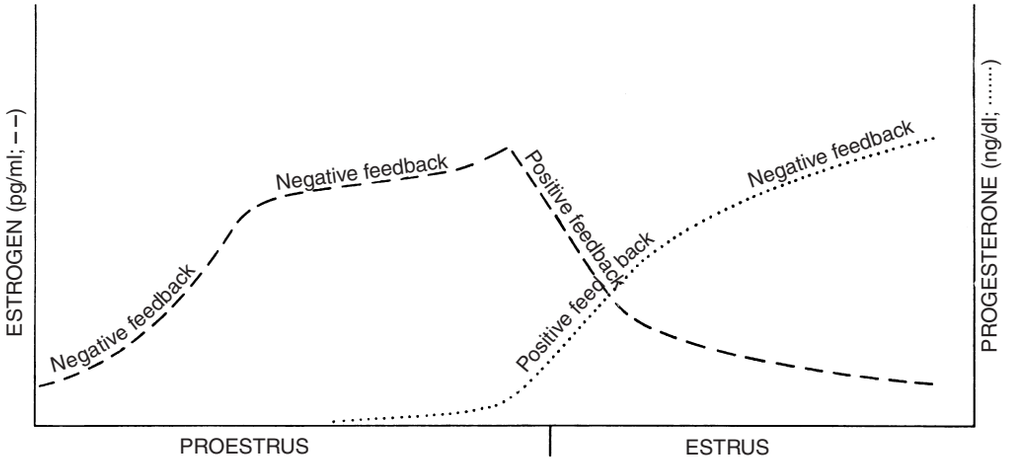


**FIGURE 19-14.** Diagrammatic illustration of the negative and positive feedback associated with estrogen and progesterone on the pituitary and hypothalamus by a normal bitch as she progresses through proestrus and estrus.

progesterone concentrations may be necessary for exhibition of maximal estrus behavior in most bitches (Olson et al, 1984c).

Declining estradiol concentrations with increasing progesterone concentrations are also thought to initiate the LH surge in the bitch (Concannon, 1987). The LH surge initiates ovulation within 24 to 48 hours, after which corpora lutea form. Progesterone concentrations steadily rise in the circulation during these first days of estrus. With the development of functioning corpora lutea, progesterone concentrations continue to increase further for a period of 1 to 3 weeks.

Thus, hormonally, estrus is a period of progressively decreasing estrogen concentrations, progressively increasing progesterone concentrations, and a brief (12- to 24-hour duration) burst of LH release (see Fig. 19-3). At the end of proestrus, the serum progesterone concentration usually rises to levels of 0.5 to 1.0 ng/ml from anestrus concentrations of less than 0.5 ng/ml. In contrast, approximately 24 to 48 hours before the preovulatory LH surge, the serum progesterone concentration rises above 1.0 ng/ml, reaching concentrations of 2 to 4 ng/ml. At the time of ovulation 2 days later, the serum progesterone concentration is typically in the range of 4 to 10 ng/ml. All this progesterone has been secreted *before* the development of corpora lutea, structures that are not identifiable until several days after ovulation.

Serum progesterone concentrations continue to increase throughout estrus and then several weeks into diestrus. Serum estrogen (estradiol) concentrations, which reach levels of 70 pg/ml and higher 1 to 3 days before the onset of estrus, progressively decrease throughout estrus. Estrogen concentrations greater than anestrus levels, even decreasing, maintain the various effects outlined in the description of proestrus. When the serum estrogen concentration decreases below basal levels of 15 pg/ml, estrus ends. This is reflected in the behavior of the bitch, vaginal cytology, vaginal wall thickness, vulvar size, and attraction of males.

*Clinically,* knowledge of plasma progesterone concentrations and how they change in a normal bitch progressing from proestrus into estrus can be extremely valuable. Several in-hospital ELISA kits are now available to estimate progesterone concentration as less than 1 ng/ml, 2 to 5 ng/ml, and greater than 5 ng/ml (Synbiotics Corp., San Diego, CA). Commercial laboratories are also beginning to provide results quickly (<24 hours) for this purpose. Establishing a plasma progesterone concentration less than 1 ng/ml in early-to-mid proestrus thereafter allows a veterinarian to obtain blood every 1 to 3 days in order to reliably detect the progesterone rise associated with the onset of estrus. The more frequent the sampling, the more precise the veterinarian can be in determining the day that progesterone begins to increase. From this knowledge, one can recommend breeding dates or predict whelping dates (Hegstad and Johnston, 1989; Cain, 1991; Manothaiudom et al, 1995).

It has been demonstrated that the normal bitch has increasing serum testosterone levels during proestrus. Testosterone reaches maximal concentrations near the time of both the preovulatory surge of LH and behavioral receptivity. After this time, testosterone levels decline (Olson et al, 1984a). Whether testosterone contributes directly to behavioral estrus or to the surge of LH remains speculative. It is possible that testosterone is merely derived from progesterone during steroidogenesis. Aromatization of androgens to estrogens in the central nervous system (CNS) may be one contributory component initiating estrus behavior in the bitch (Olson et al, 1984a). CNS metabolism of androgens to estradiol could be one mechanism regulating the release of LH in the bitch, as has been postulated in the male (Worgul et al, 1981; Olson et al, 1984a).

OVARIES AND UTERUS. The hormonal changes described are reflected in the anatomic alterations that occur simultaneously within the ovary. Early estrus (standing heat) is associated with the final maturation process of developing follicles. Estrogen synthesis begins to wane and progesterone synthesis simultaneously increases. Ovulation, spontaneous in the bitch, occurs 24 to 72 hours following the LH surge. The number of ova released for future fertilization depends somewhat on the breed of the bitch. Smaller breeds, with their small litter size, ovulate fewer ova (2 to 10) than do larger breeds, which may ovulate 5 to 15 ova. Ovarian weight is greatest immediately before ovulation.

The enlarged ovaries with follicles may be visualized by means of abdominal ultrasonography. Apparent ovulation is characterized by a decrease in the number of follicles that can be visualized from one day to the next. The ovaries have an oval shape that becomes rounded after ovulation. At some time after ovulation, the ovaries have cystic, anechoic structures that are not distinguishable from follicles, demonstrating the value of a series of abdominal ultrasound studies. Structures identified after ovulation may represent nonovulatory follicles, corpora hemorrhagica, fluid-filled corpora lutea, or cystic luteinized follicles. Ovulation can be accurately identified using ultrasonography (England and Allen, 1989a, 1989b; Wallace et al, 1992).

All ovulatory follicles are thought to rupture within 12 to 96 hours (Jeffcoate, 1998). Because this process is not perfect, ova not released at precisely the same time are in similar stages of development, ensuring that embryonic development of all fetuses progresses similarly (Johnston et al, 1982) (see Chapter 20). This is an example of the exquisitely sensitive and synchronous nature of the delicate balance between falling estrogen concentrations, rising progesterone concentrations, the LH surge, and ovulation. Follicles that are not mature enough to ovulate following the LH surge undergo atresia, providing evidence against the possibility of ova being present for fertilization for more than 4 days.

Ruptured follicles luteinize rapidly. These rupture sites undergo reorganization, with development of mature corpora lutea capable of sustaining progesterone synthesis and secretion for 2 months and longer. The corpora lutea are bright salmon pink in color for

approximately 10 days following ovulation and are easily recognized structures on the surface of the ovary.

The number of ova present in the ovaries of a newborn bitch has been estimated at 700,000. At puberty this number is reduced to 250,000; at 5 years of age, 30,000; and at 10 years, only a few hundred. Fertility appears to decline progressively once a bitch has reached 7 years of age or older. However, a corollary to the menopause of women is not typically seen in the bitch.

To review the timing of major events during the "average" standing heat, one must correlate changing hormone concentrations, follicular maturation, ovulation, corpora luteal development, and the behavior pattern of the bitch. Fig. 19-15 demonstrates that the "average" bitch first exhibits "standing" behavior as estrogen concentrations decline and progesterone concentrations rise (day 1). Day 2 is the day of the LH surge, and day 3 is associated with the final maturation process of the follicles and continued progesterone secretion by luteinized follicular cells. Days 4 through 7 are the time during which ovulation takes place. Days 5 to 9 include time for maturation of the primary oocytes to secondary oocytes, which can then be fertilized. Days 4 through 9 are the days of fertilization, and day 10 is the first day of diestrus.

Throughout this period, the uterus continues to prepare for implantation. Bleeding from the uterine microvasculature has usually but not always diminished or stopped, and glandular development with increased vascularity is nearing completion. The uterus may actually become palpable on a careful abdominal examination owing to its increase in size and thickness.

DURATION. The duration of estrus is usually 5 to 9 days. However, similar to proestrus, the length of this phase may vary dramatically among normal dogs. Estrus may be as brief as 1 to 2 days or as prolonged as 18 to 20 days. Individual bitches are usually consistent from cycle to cycle within 2 to 6 years of age. However, variations within and among breeds make it difficult to predict the length of proestrus or estrus in any one dog.

CLINICAL SIGNS. The behavior changes in the female entering standing heat are those of reflexive receptivity to mounting and attempts at copulation by the male. These bitches may crouch and elevate the perineum toward the male. Any pressure placed on or near their lower back causes the tail to be held off to one side and an obvious tensing of the rear legs to support the weight of a mounting male. The bitch may attract males over long distances owing to the presence of potent pheromones. Typically, the vulva has progressed through the turgid phase associated with proestrus and becomes soft and flaccid, no longer the difficult barrier for a male to penetrate. The vaginal discharge is often a straw-colored or pink fluid. Less commonly, it continues to be obviously hemorrhagic.

Occasionally the vaginal discharge may contain enough glucose to register positive on urine test strips. This may be caused by rising progesterone concentrations that result in carbohydrate intolerance via insulin resistance (Ryan and Enns, 1988). The vaginal discharge during estrus is derived from uterine extracellular fluid, but such testing is not considered reliable.

The bitch in standing heat may be passive and accepting of a male or may actively approach a male as if to arouse his interest. It has been thought that the bitch breeds only with a dominant male and repels submissive males. This is a good reason to recommend delivery of the female to the home of the stud, where he is more likely to be comfortable and dominant. The female placed on the stud's territory is more likely to be submissive and receptive.

VAGINAL CYTOLOGY. Throughout standing heat the vaginal cytology remains relatively constant (see Fig. 19-2). No features on vaginal cytology identify the day

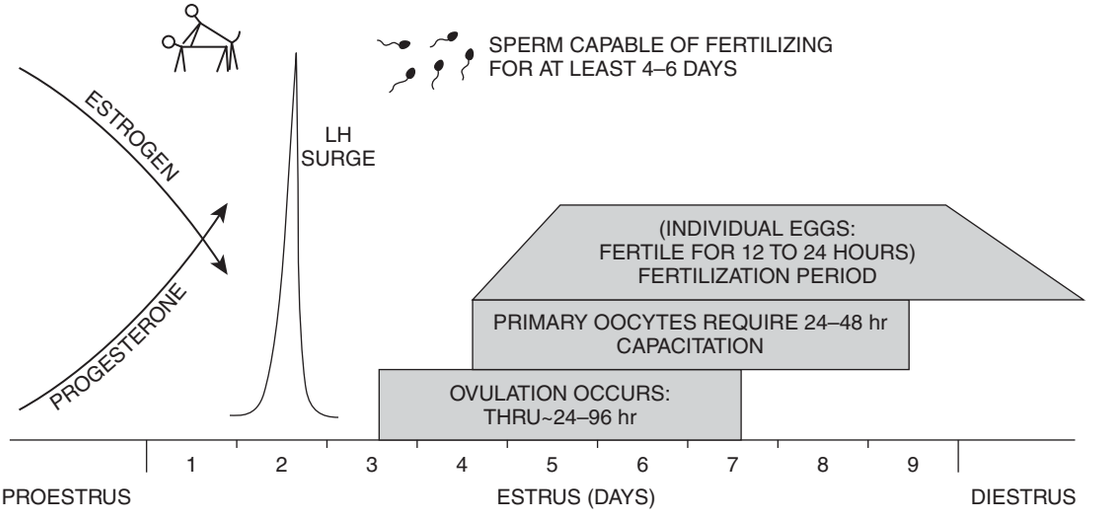


**FIGURE 19-15.** Illustration of the hormonal changes and sequence of events concerning the timing of ovulation and fertilization of ova in the average bitch.

of the LH peak, ovulation, or the timing of fertilization. Rather, the exfoliative vaginal cytology appears to be a reflection of serum estrogen concentrations, which continue to be greater than basal concentrations, even though they are declining toward basal levels. Superficial cells and anuclear squames account for greater than 80% of the total vaginal cells, often reaching 100%. No neutrophils are seen on cytology throughout this phase. Red blood cells may or may not be present. The background of the slide is clear of the granular material often seen in proestrus (Fig. 19-16).

The percentage of superficial vaginal epithelial cells has been described as progressively increasing throughout proestrus, with intermediate and parabasal cells simultaneously disappearing. With the onset of standing heat and the day of the LH surge, virtually 100% of the vaginal epithelial cells have been described as anuclear superficial cells (anuclear squames). These anuclear squames are then present for the duration of standing heat and continue to represent 100% of the vaginal epithelial cells.

Most researchers, however, have not found such a simple and predictable pattern to the vaginal exfoliative cytology in normal bitches. Vaginal epithelial cells at the beginning of proestrus are associated with 40% to 60% intermediate cells, and mid-proestrus with 40% to 60% superficial cells. Greater than 60% to 80% superficial cells (pyknotic nucleated and/or anuclear

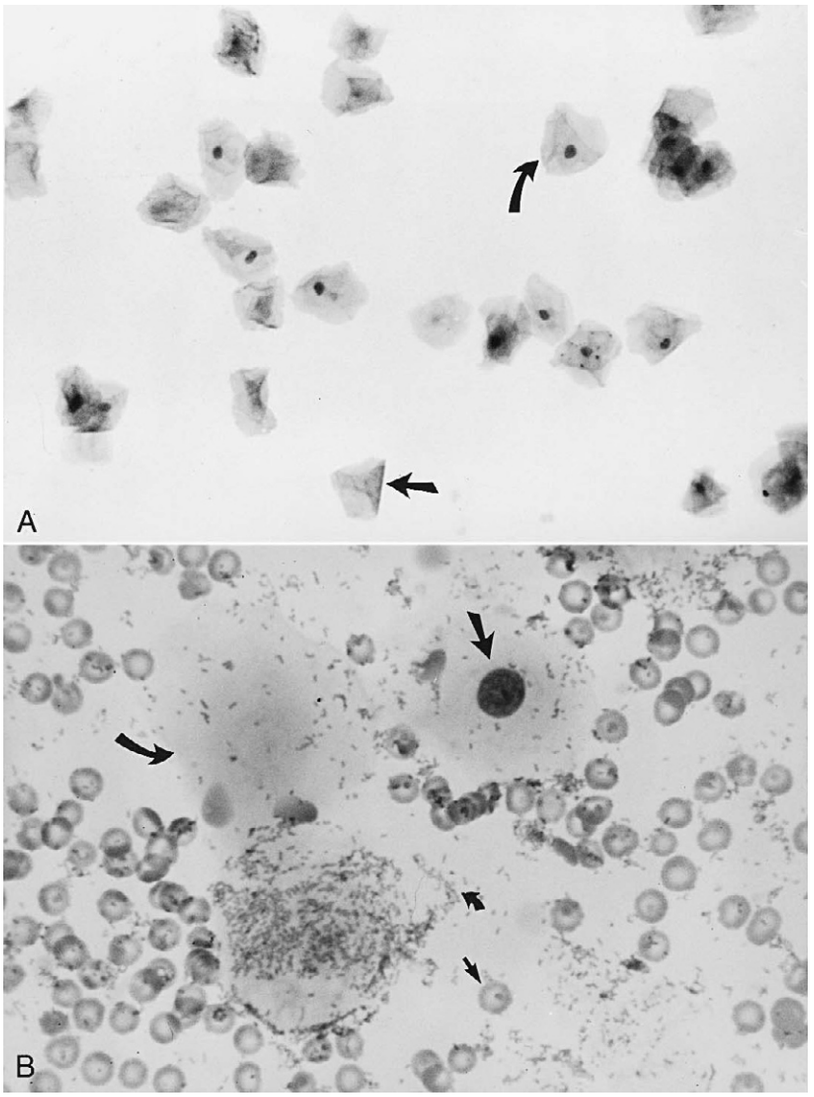


**FIGURE 19-16.** *A,* Vaginal cytology (at scanning microscope power) revealing nucleated *(curved arrow)* and anuclear superficial *(straight arrow)* vaginal cells, no red blood cells or neutrophils, and a clear background; this finding is classic for estrus in the bitch. *B,* Vaginal cytology at 40× revealing nucleated *(straight arrow)* and anuclear *(curved arrow)* superficial vaginal cells surrounded by large numbers of red blood cells *(small straight arrow)* and bacteria *(small curved arrow).* The Beagle bitch with this smear was also in estrus, bred on that day only, and had a litter of 11 healthy puppies.

squames) are consistently seen during the final 1 to 6 days of proestrus. More important, no further change in the percentage of superficial cells (with or without nuclei) is consistently noted from 3 to 4 days before the start of standing heat until the first day of diestrus (see Fig. 19-2). It is extremely valuable to remember that studies have demonstrated that the first day when 80% to 90% of vaginal epithelial cells are superficial in type ranged from 6 days before to 4 days after the LH surge (Olson, 1989).

Unpredictable but minor fluctuations are noted both in the percentage of superficial cells and in the presence of nuclei throughout estrus (see Table 19-1). Throughout the final days of proestrus and the entire period of standing heat the percentage of superficial cells typically does not fall below 60%, usually remaining between 90% and 100%. The presence or absence of pyknotic nuclei within these superficial cells fails to correlate consistently with alterations in plasma hormone concentrations or with the presence of follicles versus corpora lutea within the ovaries.

Vaginal cytology is a satisfactory means of estimating plasma estrogen concentrations. The effect of increased estrogen concentrations on vaginal wall thickness persists despite declining toward the basal levels typical of early diestrus. Perhaps the dramatic change in vaginal cytology noted on the first day of diestrus correlates best with the first or second day that plasma estrogen concentrations decrease to basal levels.

VAGINAL ENDOSCOPY. The switch from estrogen to progesterone secretion by pre-ovulatory follicles reduces vaginal mucosal vascularity and edema, reflected by a marked change in the appearance of the vaginal mucosa (Concannon, 1987). The luminal surface of the vagina then becomes progressively more wrinkled, or "crenulated." This effect is often visible to the trained individual within 24 hours of the surge in pituitary LH that is associated with the onset of estrus (see Fig. 19-1).

Maximal crenulation, seen as development of angulated folds of vaginal mucosa with sharp edges, is noted to occur in the interval between ovulation and oocyte maturation, 2 to 5 days later. This process translates into a time period beginning as early as 24 hours before the LH surge and continuing as long as 4 to 9 days after the surge. At the approximate time of the end of estrus, the vaginal mucosa again becomes flaccid with a patchy white and red surface (see Fig. 19-1). Thus vaginal endoscopy, as well as vaginal cytology, behavior of the bitch, and monitoring of plasma progesterone concentrations, can be a useful tool in determining breeding dates. This is especially true when only one or two breedings are allowed by the owner of the male or when using fresh extended or frozen semen (Lindsay and Concannon, 1986; Jeffcoate, 1998).

## Diestrus

DEFINITION. *Diestrus* is defined as the phase of progesterone dominance following estrus. Diestrus begins with the cessation of standing heat (estrus) and ends when blood progesterone concentrations return to basal levels (<1.0 ng/ml). An alternative definition for the onset of diestrus is the day that a dramatic "shift" is observed on vaginal cytology: from a phase of 80% to 100% superficial cells (estrus) to one of 80% to 100% parabasal and intermediate cells (diestrus).

HORMONAL CHANGES. Progesterone concentrations in the plasma rise above basal concentrations (>0.5 ng/ml) at the end of proestrus to levels greater than 1.0 to 2.0 ng/ml at the onset of estrus. The progesterone rise contributing to the onset of estrus is derived from luteinized cells within follicles. After ovulation, corpora lutea develop on the ovaries within the ruptured follicular cavities, resulting in a cell population capable of synthesizing and secreting progesterone during the projected period of pregnancy. The zenith in progesterone synthesis from these corpora lutea is usually achieved 20 to 30 days after ovulation. This maximal rate of secretion occurs approximately 2 to 3 weeks after the beginning of diestrus (Johnston, 1980). A transient plateau in progesterone concentration persists for an additional 1 to 2 weeks. The progesterone concentrations at that time are dramatically higher than basal concentrations, usually in the range of 15 to 90 ng/ml (Concannon et al, 1989).

Statistically, pregnant bitches have higher progesterone concentrations than nonpregnant bitches, beginning several weeks after the onset of diestrus. Individual variation, however, precludes the use of progesterone assays for pregnancy diagnosis. This is extremely important. All nonpregnant healthy bitches that have progressed through standing heat (estrus) are "pseudopregnant" in the sense that they have functioning corpora lutea despite lack of pregnancy (i.e., a pregnancy recognition system does not exist in the bitch). Therefore corpora lutea function throughout a normal gestational period regardless of the presence or absence of a fetus (or fetuses). In fact, the corpora lutea of nonpregnant bitches have a longer functioning life expectancy than the corpora lutea of pregnant bitches (Johnston et al, 2001).

Once the plateau period in diestrual plasma progesterone concentration has passed, a prolonged decline in luteal function follows. The luteal phase ends abruptly in the pregnant bitch (approximately 65 days after fertilization) as part of the onset of parturition. However, the luteal phase slowly wanes in the nonpregnant bitch, often lasting 10 to 30 days longer than observed in pregnant bitches. The cause for the decline in luteal function, as well as its inevitable cessation in function, is not well understood. Corpora lutea represent functioning endocrine glands that have an inherent, rather brief lifespan. The function of declining corpora lutea is thought to cease abruptly with the initiation of parturition owing to the action of prostaglandins. The destructive effects of prostaglandins act on degenerated corpora lutea but fail to have a similar luteolytic effect in younger, healthier corpora lutea. Prostaglandins may be the sole luteolytic factor in nonpregnant as well as pregnant bitches.

Several studies have been completed in an effort to better understand the hormonal regulation of the cyclic corpus luteum in the dog. Since progesterone profiles are similar for pregnant, nonmated, and hysterectomized bitches during diestrus, it is unlikely that the uterus or uterine prostaglandins play a vital role in the physiologic maintenance or regression of corpora lutea (Olson et al, 1989; Jonhston et al, 2001). LH may be luteotrophic, and function of the canine corpus luteum may depend on basal LH secretion during the initial phase of the ovarian cycle (Concannon, 1980). Others believe that LH is not as important a factor in luteal function and that prolactin is the important luteotrophic factor for the second half of the luteal phase (Okkens et al, 1985a, 1985b, 1986, 1990; Concannon, 1987; Schaefers-Okkens, 1988).

It has been demonstrated that the pulsatile secretion pattern of growth hormone (GH) changes during the luteal phase, with higher serum basal concentrations and fewer pulses. It was hypothesized that this is caused by a partial suppression of pituitary GH release by progesterone-induced GH production in the mammary glands. This mammary GH may promote the physiological proliferation and differentiation of mammary gland tissue during the luteal phase of the bitch by local autocrine/paracrine effects. In addition, progesterone-induced mammary GH production may exert endocrine effects such as hyperplastic changes in the uterine epithelium and insulin resistance (Kooistra et al, 2000).

Estrogen concentrations early in diestrus are usually at basal levels (i.e., similar to anestrus concentrations). Corpora lutea initially synthesize only progesterone, but during the last week or two of gestation, estrogen concentrations have been shown to rise subtly (Concannon, 1987). Perhaps this slight increase in estrogen synthesis and secretion occurs in concert with falling progesterone concentrations as a component of the complex interactions that lead up to parturition. In any event, these small concentrations of estrogen do not cause attraction of males or any of the other obvious alterations associated with proestrus. It is not known whether the nonpregnant bitch experiences similar changes in estrogen concentration.

Secretion of LH and FSH from the pituitary during diestrus is thought to be episodic but of minimal importance. Mean LH concentrations are slightly increased during late diestrus, but the significance of this rise remains speculative. Prolactin is one of the key hormones of diestrus. Prolactin concentrations are low during anestrus, proestrus, and estrus. As progesterone concentrations decline in the latter half of diestrus, prolactin concentrations increase. An inverse relationship appears to exist between serum concentrations of progesterone and prolactin immediately prior to parturition as well as during pregnant and nonpregnant diestrus. Mammary enlargement and secretory activity during diestrus are presumed to be initiated and continued by prolactin (De Coster et al, 1983; Concannon, 1986).

Unlike concentrations of serum progesterone, which are similar among pregnant, nonmated, and hysterectomized bitches, serum immunoreactive relaxin concentrations differ between pregnant and nonpregnant dogs (Steinetz et al, 1989; Buff et al, 2000). Serum immunoreactive relaxin concentrations are <0.25 ng/ml in diestrus bitches that are not pregnant, but increase to maximum concentrations (>3.0 ng/ml) after about 6 to 7 weeks of gestation in pregnant bitches. While progesterone production is entirely of ovarian origin, relaxin production is primarily of placental origin (Tsutsui and Stewart, 1991).

OVARY AND UTERUS. Corpora lutea are located on the surface of the ovaries throughout diestrus. The uterus responds to increases in progesterone concentration by maintaining the glandular structure and vascularity required for pregnancy regardless of whether the bitch has mated. Maximum nonpregnant uterine size is seen 20 to 30 days following the onset of standing heat, a time coinciding with the highest progesterone concentrations. Earlier in diestrus, differences between the pregnant and nonpregnant uterus are insignificant. Once implantation occurs 17 to 21 days following fertilization, spherical fetal units become palpable.

With degeneration of corpora lutea and cessation of progesterone secretion, diestrus ends and the uterus undergoes a period of repair. This period of uterine involution requires 1 to 3 months in the bitch and may represent one of the factors that accounts for the relatively long interestrus period in normal bitches.

DURATION. Diestrus is the phase of progesterone dominance (increased plasma progesterone concentrations). The duration averages 56 to 58 days in pregnancy and 60 to 100 days in the nonpregnant bitch. Corpora lutea cease to function sooner in pregnancy than in nonpregnancy, presumably owing to the effects of prostaglandins. The fetus stimulates synthesis and secretion of prostaglandins, a process that has not been documented in nonpregnant bitches.

CLINICAL SIGNS. Diestrus begins when the "receptive" bitch abruptly refuses to breed. She may also no longer attract males. The vulva returns to a normal or anestrual size and is no longer enlarged or flaccid. Basically, no clinical difference is apparent between an anestrus bitch and one that is in diestrus and not pregnant. No obvious method has been found to distinguish a pregnant bitch early in diestrus (first 7 to 10 days) from one that is not pregnant.

VAGINAL CYTOLOGY. Vaginal cytology obtained from a bitch entering diestrus is *clearly different* from that of a bitch in early, mid-, or late estrus. The smears obtained during the final days of standing heat are no different on vaginal cytology from those obtained in early estrus (see Fig. 19-2). These smears contain more than 80% superficial cells, no neutrophils, and a clear background. Within a 24- to 48-hour period at the end of estrus, the percentage of superficial cells falls to approximately 20%, with the majority of cells being intermediate and/or parabasal cells (Fig. 19-17). This represents an abrupt and obvious change in the vaginal cytology (see Table 19-1).

Occasionally, sheets or rafts of vaginal epithelial cells are seen on vaginal cytologic smears, just preceding the onset of diestrus (see Fig. 19-17, *B*). This phenomenon

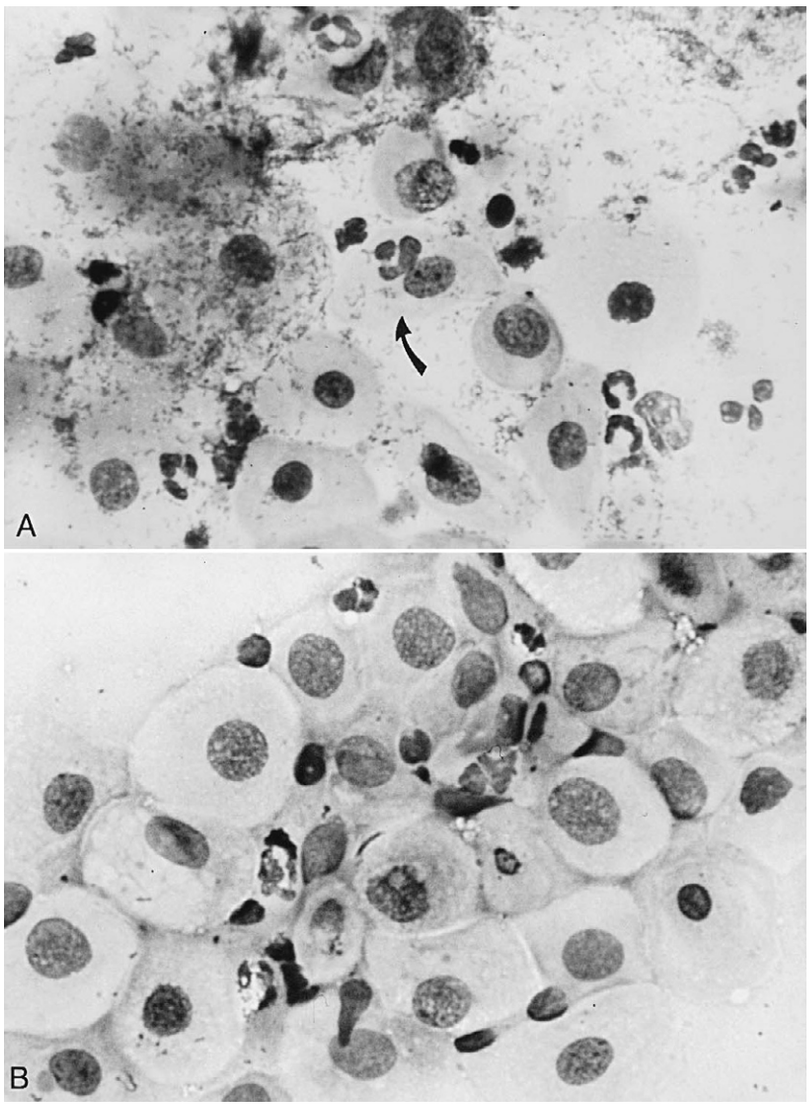


**FIGURE 19-17.** *A,* Vaginal cytology at 40× revealing bacteria, intermediate cells, neutrophils, and a metestrum cell *(arrow)* suggestive of diestrus. *B,* Vaginal cytology at 40× revealing sheets of large intermediate vaginal epithelial cells and a few neutrophils. Rafts of cells like this are sometimes seen on or around the first day of diestrus.

represents sloughing of large numbers of cells at once. Neutrophils may occasionally reappear, and the background may contain large amounts of debris. However, the dramatic change in the microscopic appearance of individual vaginal epithelial cells (from superficial cells to intermediate and parabasal cells) is usually the first indicator that diestrus has begun. The presence or absence of neutrophils is not a reliable criterion. The behavior of the stud or the bitch is even less reliable than the cytology (Olson, 1989).

Occasionally, specific "metestrum" cells associated with the diestrus phase are seen. These cells have been used as an aid in attempting to distinguish early proestrus from the beginning of diestrus. In examining the vaginal smear of a bitch with an unclear history, one may not be certain if the smear being examined represents early or mid-proestrus or represents a bitch that has just completed standing heat. "Metestrum" and "foam" cells are supposedly seen only in diestrus. Metestrum cells (see Fig. 19-10) are vaginal epithelial cells that contain one or two neutrophils within their cytoplasm. Unfortunately, we have identified these cells in other phases of the cycle whenever a large number of neutrophils are present. When there is doubt, a series of smears obtained every 2 or 3 days almost always identifies the correct stage of any cycle. The bitch in proestrus has a progressively increasing percentage of superficial cells on vaginal cytology and exhibits behavioral changes. The bitch in diestrus does not exhibit such changes.

Following the initial few days of diestrus, vaginal cytology smears from bitches in diestrus resemble those of anestrus. White blood cells may or may not be present, red blood cells are absent or present in small numbers, and the epithelial cells typically consist of small intermediate cells plus parabasal cells. Exfoliative vaginal cytology from the normal bitch is similar in comparing diestrus with anestrus. Smears from anestrus to proestrus and proestrus to estrus tend to change relatively slowly. However, the appearance of vaginal epithelial cells from estrus into diestrus change abruptly. In studying infertility patients and in attempting to identify whelping dates, recognizing the first day of diestrus becomes critically important (see Chapter 24).

MAMMARY GLANDS. The increasing progesterone concentrations of standing heat initiate glandular development in mammary tissue. These changes usually become obvious to the owner as the bitch reaches the final trimester of diestrus. Prolactin concentrations, which rise during the final weeks of gestation, cause overt lactation in preparation for the newborn.

## Anestrus

DEFINITION. Anestrus is the phase of the female reproductive cycle in which the uterus involutes. Anestrus begins with whelping and ends with proestrus. The beginning of anestrus is not readily discernible in the nonpregnant bitch, in which no obvious demarcation between diestrus and anestrus is *clinically* detectable.

DURATION. Like the other phases of the ovarian cycle, anestrus varies in duration. This variation depends on breed, health, age, time of year, environment, and multiple other factors. The typical bitch begins proestrus every 7 months. Proestrus lasts 9 days; estrus, 7 to 9 days; diestrus, 58 days; and anestrus, 4.5 months. However, duration of these phases remains variable, in part because it is difficult to know when diestrus ends and anestrus begins in any nonpregnant bitch. The duration of diestrus progesterone secretion is likely to be the major factor determining the interval between nonfertile ovarian cycles. Shortening the luteal phase by either prostaglandin-induced luteolysis (Vickery and McRae, 1980) or prolonged administration of bromocriptine (Okkens et al, 1985b) reduced interestrus intervals by weeks to months (Concannon, 1987). In addition, the effect of pregnancy, the realization that interestrus periods are rarely totally constant, and other factors make predicting the onset of proestrus quite difficult.

CLINICAL SIGNS. No obvious clinical difference can be seen between the anestrus bitch and one that is not pregnant but in diestrus or one that has been ovariohysterectomized (spayed). In fact, sometimes one cannot tell whether a bitch has been spayed without assaying pituitary hormone (FSH and LH) concentrations. These gonadotrophs are dramatically elevated in the spayed bitch.

HORMONE CONCENTRATIONS. As with other species, sporadic bursts in LH secretion occur throughout anestrus in the dog. These transient abrupt increases in plasma LH appear to lead up to two major brief but potent secretory episodes. One LH peak immediately precedes the onset of proestrus, and one precedes or coincides with the onset of estrus and subsequent ovulation. Concentrations of serum FSH increase during anestrus, reaching levels in late anestrus that are as high as those present during the preovulatory FSH surge during estrus. It is hypothesized that these apparent paroxysmal pulses of pituitary secretion are not by chance. Rather, they likely represent a delicate mechanism necessary in recruiting follicles for the next cycle (Johnston et al, 2001). Once recruited into the preovulatory pool, these follicles selectively regulate pituitary gonadotroph secretion via negative feedback mechanisms, thereby affecting the subsequent cycle (see Fig. 19-3).

Estrogen concentrations fluctuate significantly throughout anestrus. Surges in estrogen concentration have been observed, and these are assumed to be derived from waves of follicle development that are subclinical in nature and probably short-lived. These follicles synthesize and secrete estrogen, causing minor increases in circulating estrogen concentrations. Because the follicles never fully mature, they regress, after brief periods of function, before ever developing luteinized cells that could synthesize progesterone. Serum estrogen concentrations decrease before the onset of proestrus (Olson et al, 1982). By contrast, progesterone remains at extremely low concentrations throughout anestrus.

It is not known what factor initiates proestrus and a new ovarian cycle. This is likely to be a result of complex interactions between environment, general health, ovarian status, uterine status, and age. This concept becomes clinically relevant in attempts to improve fertility through the parenteral administration of pituitary and/or ovarian hormones. The delicate coordination of events leading to ovulation is difficult to mimic.

THE UTERUS. The uterus during anestrus undergoes self-repair. It must reach a state of complete involution following the effects of pregnancy or pseudopregnancy (clinical or subclinical). Complete repair of the endometrium to a basal state requires approximately 120 days after serum progesterone concentrations return to basal levels in the nonpregnant cycle and after 140 days in a fertile cycle (Talwar et al, 1985; Johnston et al, 1985). Externally, the veterinarian detects little change in the palpable uterus once involution reduces the uterus to a size comparable with loops of bowel. At this point, further changes in uterine anatomy continue subclinically.

VAGINAL CYTOLOGY. The vaginal cytology of anestrus is relatively constant. One sees primarily parabasal and intermediate vaginal epithelial cells. Neutrophils may or may not be present, and red blood cells are absent. Bacteria may or may not be seen. When present, bacteria usually represent normal flora. The background appearance, after staining, may be clear or granular.

## VAGINAL EXFOLIATIVE CYTOLOGY

### Techniques or Methodology in Making Vaginal Smears

Various methods are recognized for obtaining exfoliated vaginal smears. All the techniques may succeed in providing necessary information. However, the criteria for choosing one method depends on several factors: the method (1) should be simple to perform and inexpensive, (2) should be applicable to dogs regardless of their size or temperament, (3) must not be painful, (4) should be successful regardless of the presence or absence of a vaginal discharge, and (5) should be able to be performed by owners after they have been given a brief lesson. A veterinarian should use and become familiar with one method for obtaining vaginal smears. The clinician can then become comfortable and confident in this one procedure. The cotton swab method described below is recommended because it best meets the suggested criteria.

### Cotton Swab Technique

The lips of the vulva are gently separated with one hand. The other hand holds a sterile, cotton-tipped applicator, 5 to 7 inches long. We often use the cotton swab found within a culturette tube (Culturette; Marion Scientific, Kansas City, MO). The cotton-tipped end of this swab is passed into the dorsal commissure of the vulva.

Initially, the cotton tip is gently pressed against the caudodorsal surface of the vaginal vault to avoid the clitoral fossa and then advanced in a craniodorsal direction, toward the vertebral column, until it passes over the ischial arch (Fig. 19-18). The swab is inserted at least the distance needed to reach the pelvic canal. The applicator is then rotated a complete revolution in each direction and withdrawn. The entire procedure should take only seconds and is rarely painful. A bitch may appear uncomfortable if there is no vaginal discharge. Therefore the cotton should be moistened with two to three drops of sterile saline to act as a lubricant if a discharge is not obvious. The clitoral fossa must be avoided because this is a small blind pocket and attempts to advance the swab against this surface would be painful. In addition, the cells of the clitoral fossa may be confused with superficial vaginal epithelial cells (Olson, 1989).

Once the cotton swab is withdrawn, the cotton tip is *rolled* gently from one end of a glass microscope slide to the other. There should be space on the slide for two or

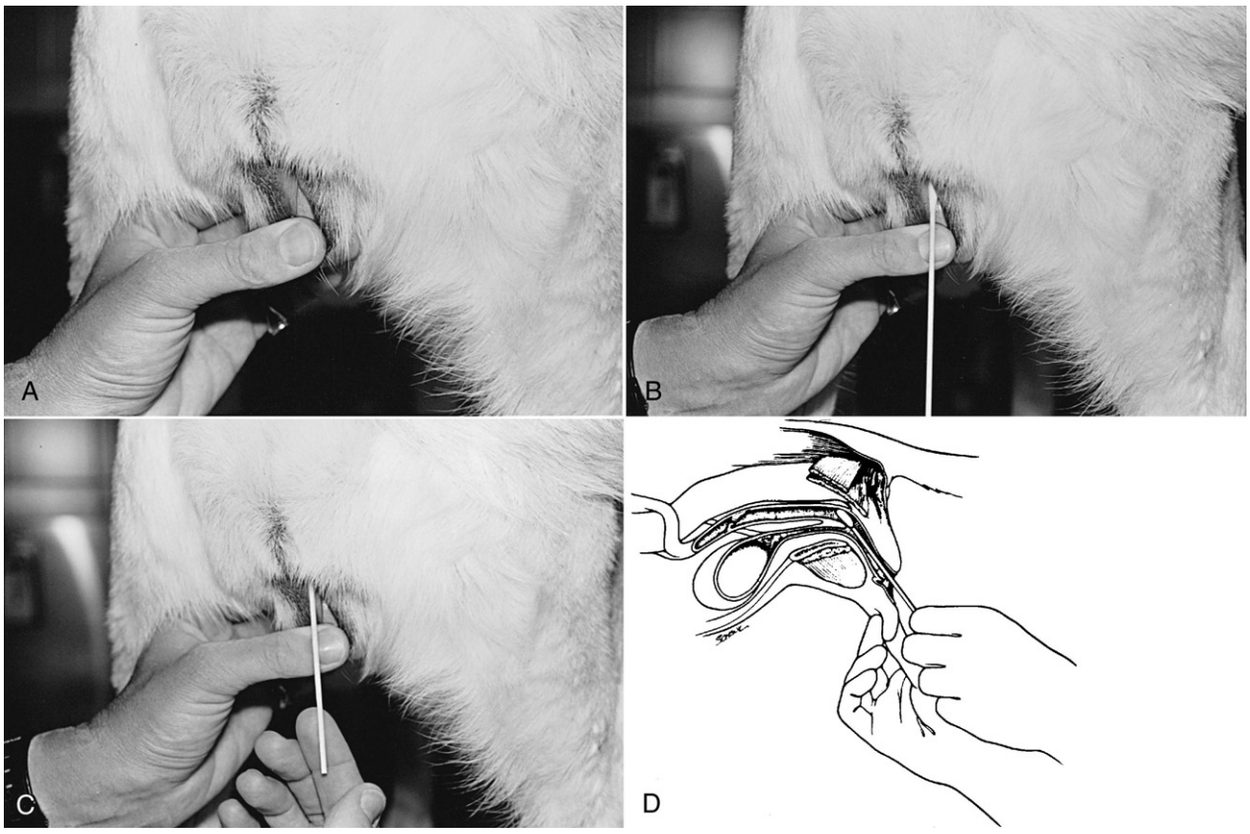


**FIGURE 19-18.** Obtaining a vaginal smear with a cotton swab. *A,* Spreading open the vulvar lips for insertion of the swab. *B,* The swab placed at the vulva, just before insertion into the vagina. *C,* The swab has been inserted into the vaginal vault. *D,* A diagram illustrating the location of the cotton-tipped swab. (*D* From Johnston SD: The Female. Vet Clin N Amer 11:543, 1981.)


**FIGURE 19-19.** Two stained glass slides after the cotton swab has been rolled across the surface to make two or three columns containing vaginal smear material.

three separate linear impressions (Fig. 19-19). The swab should not be pressed firmly, and the cotton should not be rubbed or smeared against the glass, because the result of either mistake is nondiagnostic material. Usually two slides are prepared using one or two cotton swabs. The slides with the exfoliated cells can be quickly air dried and then dipped once or twice in 95% to 100% methanol to prevent cellular deterioration or distortion. Such slides can be stained immediately or stored and stained at a later date (Olson et al, 1984a).

## Stains

CRITERIA FOR CHOOSING A STAIN. As with the techniques available for obtaining cells for vaginal exfoliative cytology, the staining of these smears is quite important. Several stains have been shown to be excellent. The stain chosen should be easy to use and inexpensive; it should also store well over a period of time and, ideally, provide a permanent slide that can be saved for years.

WRIGHT'S STAIN OR WRIGHT-GIEMSA STAIN. Wright's stain is methylene blue polychromated with sodium bicarbonate and heat, to which eosin is added. Giemsa stain consists of a combination of azure II dyes and eosin. Wright's stain is commonly used in staining peripheral blood smears. This stain is also used for vaginal cytology and provides easy-to-read slides with colors that are consistent, reproducible, and of excellent quality. The stain does require careful maintenance of the solutions, and the clinician must carefully follow the instructions regarding their make-up.

DIFF-QUIK. Diff-Quik (American Scientific Products, McGraw, IL) is the stain we recommend for routine vaginal smears performed by veterinary practitioners. It is a modified Wright-Giemsa stain that is easy to use; it reliably stains both vaginal epithelial cells and red blood cells and is inexpensive. Slides must be immersed in methanol and two staining solutions. It has been recommended that vaginal smears be immersed in the two staining solutions longer than the period normally required for staining peripheral blood smears (Olson et al, 1984b). Diff-Quik–stained slides can be stored for several days if reference to a series of slides is desired. If a permanent coverslip is used, the slides can be stored indefinitely. This is a stain that all veterinary hospitals can use.

NEW METHYLENE BLUE. Vaginal smears are stained by placing a drop of the new methylene blue solution on the slide, followed by a coverslip. Slides can be viewed immediately. The stain is excellent for vaginal cell morphology but does not stain red blood cells. For a quick and simple stain, it is excellent. This is usually the stain we recommend for use by breeders, and it has also been chosen by a number of veterinarians.

PAP AND TRICHROME STAINING. The staining method most commonly used in human vaginal cytology is that of Papanicolaou (PAP). The PAP and trichrome methods are polychromatic staining reactions designed to display the many variations of cellular morphology, showing degrees of cellular maturity and metabolic activity. The major advantage of these techniques is provision of good differentiation of the exfoliated cells; keratinized cells stain orange-red, whereas nonkeratinized cells stain blue-green. Other advantages include definition of nuclear detail and cytoplasmic transparency. Despite the benefits, these staining methods are not recommended because they are labor-intensive, complicated, and impractical (Thomas, 1987).

## Clinical Usefulness of Vaginal Cytology

MANAGEMENT OF NORMAL BREEDING. Vaginal cytology is one of the most commonly used diagnostic tools in clinical canine reproduction. This tool may be used in helping an owner to determine the proper time to breed a bitch. Observation of the behavior of a bitch with a stud is the most reliable method of learning when a bitch has entered standing heat (i.e., when the bitch is ready to breed, she will breed). However, numerous exceptions to this philosophy point out the value of vaginal cytology.

As described in the preceding section on phases of the ovarian cycle, vaginal cytology is an excellent aid in distinguishing between early proestrus, estrus, and diestrus. It is always wise to recommend that a minimum of two or three smears, taken over a period of 4 to 7 days, be examined. However, when the number of superficial cells makes up 80% or more of the vaginal epithelial cells, the bitch and stud should be brought

together. If mating does not take place within 1 or 2 days, a second smear should be obtained. If it is similar to the first smear and not suggestive of diestrus, another breeding or artificial insemination should be pursued. The presence of 80% or more superficial cells often precedes estrus by as many as 3 to 6 days. Vaginal cytology becomes an extremely important diagnostic aid whenever an infertility problem or abnormality in behavior is believed to exist.

Vaginal cytology, behavior of a bitch, and monitoring of her serum progesterone concentrations are of real value in breeding management. Knowing the precise day of a cycle, beginning with the first day of vulvar swelling or vaginal bleeding, is helpful but should not be relied on as the sole criterion of when a bitch should be bred. Some bitches are in standing heat with 80% or more superficial cells without ever having had an obvious proestrus, whereas others begin estrus as long as 21 days after the apparent start of proestrus. Only the *average* dog enters standing heat on the tenth day after beginning proestrus. Some bitches never stand for a male (i.e., do not display behavioral estrus). Such dogs are often fertile, and correct use of vaginal cytology aids in determining dates for artificial insemination.

An attempt at breeding should be pursued, naturally or artificially, *throughout* the period when 80% or more superficial cells are seen on vaginal cytology. This advice is more reliable once the serum progesterone of the bitch has been documented to exceed 1.0 ng/ml. After the vaginal smear is suggestive of estrus, 3 to 6 days may pass before natural breeding commences, because of the overlap in vaginal cytology appearance seen at the end of proestrus and throughout estrus. However, once natural breeding begins, it should be allowed to continue until the bitch refuses to breed. *This is the most important advice we provide to owners.* We recommend breeding dogs every second, third, or fourth day of estrus. More frequent intervals are suggested if this is the first breeding or if the bitch is known to have an abbreviated estrus. Less frequent breedings are suggested if she is known to have been in standing heat for 9 days or longer in previous ovarian cycles.

SHIPPING OR RECEIVING A BITCH. Bitches may be sent long distances for breeding. Bitches should be shipped before finding 80% or more superficial cells on the vaginal smear. The duration of proestrus can be difficult to predict, and shipping early is recommended. When a bitch is received, her behavior status can be assessed. If she is not standing for the stud, a vaginal smear and a serum progesterone should be evaluated. Both parameters should be monitored serially.

UNUSUAL CYCLES. Any bitch that appears to have normal reproductive cycles but never stands for the stud may be hormonally normal and fertile. Some of these bitches apparently fail to stand for a male because they are not exposed to the stud during their standing heat (i.e., standing heat is often unrecognized because it does not occur between days 9 and 12 of the cycle). For various reasons, standing heat may not be recognized or may not occur. Bitches that refuse natural breeding can be artificially inseminated (AI). AI should begin the first day superficial cells reach or exceed 80% of the total vaginal cells. AI should be continued on an alternate-day basis throughout this phase. Insemination should be discontinued only when diestrus is definitively recognized on assessment of vaginal cytology.

PREDICTING WHELPING DATES. Vaginal cytology is a superb tool in predicting the approximate date of whelping. The prediction is based on the knowledge that whelping occurs near day 57 of diestrus (Olson et al, 1983; Linde and Karlsson, 1984). Obtaining a series of vaginal smears from a bitch on a daily basis readily allows recognition of the first day of diestrus. This method is considerably more reliable than using breeding dates.

INFERTILITY PROBLEMS. Vaginal cytology is a crude reflection of plasma estrogen concentrations and, as such, a test of ovarian follicular function. Infertility cases should be evaluated with vaginal cytology and serum progesterone concentrations to determine whether the problem is one of mismanagement (incorrect timing), inadequate estrogen (poor follicular development), or some less common disorder.

FOLLICULAR CYSTS. By definition, an ovarian follicular cyst is one that synthesizes and secretes estrogen. A bitch with a follicular cyst appears to have a prolonged proestrus and/or prolonged estrus. The first evidence that a bitch has a follicular cyst includes her behavior of prolonged proestrus and/or estrus and a persistent bloody vaginal discharge (> 2 to 3 weeks). With or without these parameters, the bitch should have persistent evidence of proestrus/estrus on vaginal cytology. The next diagnostic aid used in establishing a diagnosis is abdominal ultrasonography.

VAGINITIS. The vaginal smear from a bitch with vaginitis usually contains a large number of healthy to degenerated neutrophils. Neutrophils with engulfed bacteria may be observed if a bacterial vaginitis is present. Neutrophils and bacteria are often seen in smears from normal bitches as well, but large numbers of neutrophils may be abnormal. Early diestrus may be transiently associated with large numbers of bacteria and neutrophils within the vaginal cytology. Persistent significant neutrophilia seen on vaginal cytology may be consistent with vaginitis.

VAGINAL TUMORS. Not surprisingly, tumors invading the vaginal vault exfoliate cells that may then be recognized on vaginal cytology. The tumors most frequently identified with vaginal cytology include transmissible venereal tumor (Fig. 19-20) and transitional cell carcinomas of the bladder, which may extend into the vagina via the urethra.

PYOMETRA AND ACUTE METRITIS. These two disorders are serious problems that usually cause systemic illness. These conditions are often associated with fever, clinical signs, and abnormalities on blood, urine, radiographic, or ultrasonographic evaluations. Therefore vaginal cytology is not the most valuable or reliable diagnostic aid when a bitch is being evaluated for one of these disorders. Diagnosis of uterine disease should *not* be made with vaginal smears. However, animals with open-cervix pyometra or metritis may have large

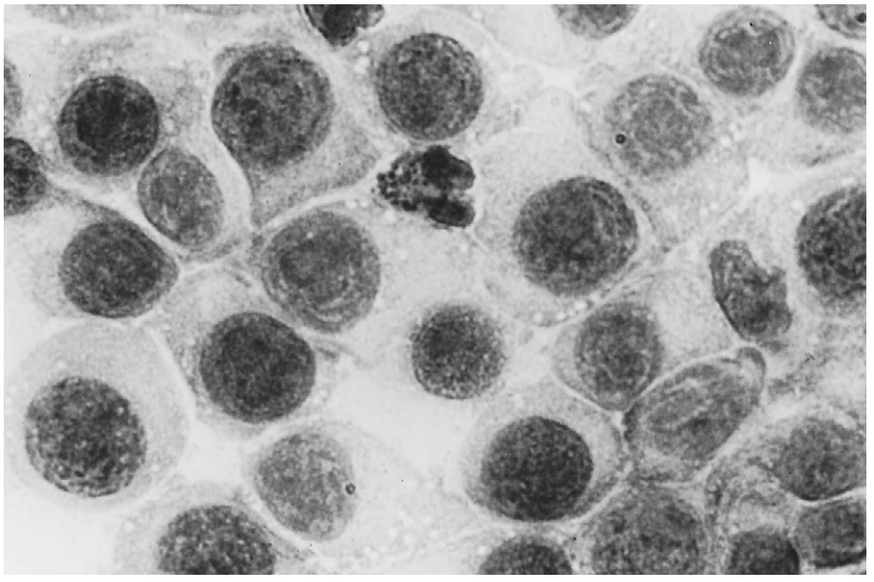

**FIGURE 19-20.** An impression smear obtained from a vaginal mass caused by a transmissible venereal tumor in a bitch.

numbers of degenerated neutrophils and, occasionally, vacuolated endometrial cells (Olson et al, 1984c).

DETERMINING WHETHER AN ABORTIFACIENT SHOULD BE ADMINISTERED TO A BITCH. An owner may not actually observe an unwanted breeding. However, if an unwanted breeding was likely, the finding of spermatozoa or the heads of spermatozoa on vaginal cytology confirms that breeding has occurred (Fig. 19-21). Lack of these findings does not eliminate the possibility that a breeding took place. In addition, if the vaginal smear is suggestive of proestrus, diestrus, or vaginitis, there is little reason for an owner to be concerned. (A complete discussion of mismating is presented in Chapter 22.)

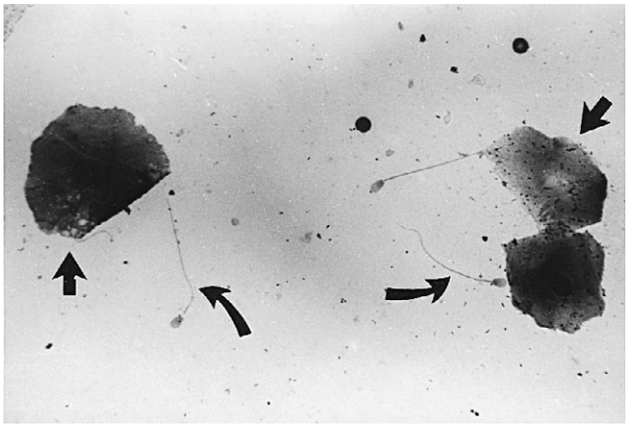

**FIGURE 19-21.** Vaginal smear from a bitch that had been bred to a normal male 12 hours earlier. Note the superficial vaginal epithelial cells *(straight arrows)* and the sperm *(curved arrows)*.

## Limitations of Vaginal Cytology

Vaginal cytology is an extremely useful tool in canine reproduction. Understanding the clinical applications of cytology increases the value of the procedure. It must be pointed out, however, that vaginal exfoliative cytology does not answer some common questions. Vaginal cytology cannot identify the day of ovulation or fertilization; therefore the "perfect" day for breeding cannot be determined from an evaluation of smears. Retrospectively, once the first day of diestrus is identified, ovulation can be assumed to have occurred approximately 6 days earlier. Vaginal cytology cannot be used for pregnancy diagnosis. Finally, vaginal cytology, although a valuable tool, is an imperfect substitute for owner observations of behavioral estrus. Observation of behavior plus a review of vaginal cytology is an excellent pairing of complementary assessments.

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