

Bouvier Health Foundation Education Series

In March 2014, the University of California, Davis, School of Veterinary Medicine, through its Theriogenology Foundation, inaugurated a one-day Canine Reproduction Conference. Among the many topics covered, this edition of the Dirty Beard Quarterly shares one focusing on Ovulation Timing - presented by noted Reproductive Researcher, Bruce W Christensen, DVM, MS. We all know that reproductive timing is a combination of science, technology & a little luck from Mother Nature and it is hoped that the information presented will spark interest and encourage your further investigation.



Ovulation Timing: The Art and Science

Bruce W. Christensen, DVM, MS

Diplomate, American College of Theriogenologists

Assistant Professor, University of California, Davis

The simplest, least invasive, most economical diagnostic tool for determining the stage of the estrous cycle in a bitch, and surely the most widely utilized, is the examination of the bitch's vaginal cytology. The types and appearance of individual cells varies between the stages of the estrous cycle and therefore cytology can be reliably used to determine which stage is represented in the cytological smear from a particular patient. Every cytological change appreciated in a canine vaginal smear is due to the effects of estrogens. Understanding the stages and the role of estrogen during each stage, therefore, is paramount to interpreting a vaginal smear. The stages of the estrous cycle in the bitch (and their usual lengths) include anestrus (2-10 months, though most bitches are between 4 and 6 month), proestrus (3-21 days), estrus (3-21 days), metestrus (10-14 days, note that this stage is sometimes lumped with diestrus), and diestrus (about 50-55 days, if you exclude metestrus as a separate stage).

Anestrus

Anestrus is the stage during which the reproductive tract is relatively quiescent, compared to the other stages of the cycle. While important, low-level endocrinological activity does continue throughout anestrus, overall estrogen levels are quite low, as would be expected in light of much reduced follicular activity in the ovaries (1). The vaginal mucosa is quite thin during this stage of the cycle, perhaps 1-2 cells thick from the basement membrane to the vaginal lumen (2,3). The epithelial cells live a normal cellular life span and by the time they degenerate they have fragile membranes and are easily misshapen and broken. As a result, the epithelial cells noted on a vaginal smear from a bitch in anestrus are mostly parabasal cells, with a few intermediate cells. The cells are often misshapen, oblong, or broken with only the nucleus clearly visible and some cytoplasmic streaming surrounding these "naked" nuclei. Red blood cells are absent to rare. Neutrophils are only occasionally identified and should not have signs of inflammation. Mucus is present, as are some bacteria.

Proestrus

Proestrus is the stage of the cycle often first recognized by the owners of the dogs due to the accompanying clinical signs of a soft, swollen vulva and a serosanguinous vulvar discharge. Breeders will often start counting the days and start breeding around 9 days after the discharge is first detected. This correlates with the fact that the *average* length of proestrus in the bitch is around 9 days. Breeding management may be mishandled, however, in bitches with short or long proestrus stages, and this happens with some frequency (2). Close clinical monitoring of these cases to determine the actual end

of proestrus and further to determine important events during estrus (discussed below) will result in better timing of breeding and higher pregnancy rates.

During proestrus, follicular growth is noted on the ovaries. Granulosa and thecal cells in the follicles begin producing greater concentration of estrogens. Estrogen has a mitogenic effect on vaginal epithelial cells, causing them to divide in quick succession (3). Within some days, the formerly very thin tissue layer of vaginal mucosa increases from about 10-20 cells thick to more than 300-400 cells. This increased distance between the vaginal lumen and the basement membrane, where the capillaries reside, results in dramatic changes in the epithelial cells closest to the lumen. The cells in the lumen, far from their blood supply, are not able to rid themselves of metabolic waste products. These waste products build up, instead, inside the cytoplasm, resulting in greatly increased cytoplasmic volume. This is the reason for the morphological difference notes between parabasal and intermediate-type epithelial cells. These intermediate cells are the predominant cell type of proestrus.

Estrogen causes uterine blood vessels to become highly permeable and, through diapedesis, to leak red blood cells into the uterine lumen (2). Estrogen also causes the cervix to relax and open. The red blood cells easily move from the uterus through the cervix and vagina, exiting the vulva as the serosanguinous discharge noted by the client. Red blood cells are a prominent feature of the proestral cytological vaginal smears.

The increased amount of blood in the vagina serves as an excellent growth media for bacteria present as normal fauna. Bacteria tend to greatly increase in number, and this signals the recruitment of neutrophils, which migrate into the vagina via diapedesis. Initially, in early proestrus, an increased number of neutrophils is often noted on vaginal cytological smears. Later in proestrus, however, the neutrophils disappear from the cytological preparations because they are no longer able to migrate into the vaginal lumen, due to the increased thickness of the vaginal epithelium, as described above.

Estrus

Eventually, the epithelial cells closer to the vaginal lumen will die as a result of distance from the capillaries and consequent lack of nutrient exchange. The nuclei of the cells will become pyknotic as the nuclear chromatin condenses. The nuclear membrane will dissolve and the chromatin will then dissipate, leaving an anuclear, "cornified," superficial cell. When the number of superficial cells increases above 80% of the total number of epithelial cells, the bitch is said to be in cytological estrus. This change does not always correlate exactly with behavioral estrus (when the bitch will stand to be bred) or endocrinological estrus (after the LH surge, discussed elsewhere in these proceedings).

Estrogen concentrations begin to drop at the end of proestrus and the beginning of estrus as some of the granulosa and thecal cells of the follicles luteinize prior to ovulation (4). Estrogen concentrations drop precipitously after ovulation when the remained of the follicular cells luteinize. This drop in estrogens results in a cessation of the increased cellular proliferation in the vaginal mucosa and the increased diapedesis of red blood cells into the uterus. Red blood cells are often still present in early estrus, but usually decrease in numbers and disappear by the end of estrus, in correlation with dropping estrogen concentrations. Neutrophils disappeared in mid to late proestrus and are not noted in estrus. Bacteria are usually still present during estrus. Mucus, while still present in proestrus, is rarely noted in estrus. This gives a "clean" appearance to the background of the slide during estrus.

Metestrus

With estrogen concentrations now again at baseline levels, and all consequent mitogenic support gone, the many cellular layers of the vaginal mucosa begin to slough off. The result is the return of intermediate cells as the prominent epithelial cell type noted on the vaginal smears.

Neutrophils are often seen in greatly increased numbers as all of the neutrophils that attempted to reach the lumen, but were unable due to physical distance, are now released into the lumen with the sloughing epithelial cells. A unique kind of cell, called the "metestral cell" is often present at this stage. The metestral cell is an intermediate cell engulfing a neutrophil. Red blood cells are rarely seen, as they usually disappeared during estrus. Bacteria are present, as always. The shift from estrus to metestrus sometimes happens within a day or two, whereas other times it may take many days to transition.

Diestrus

Metestrus gradually changes into diestrus, with fewer superficial and large intermediate cells. The predominant epithelial cell types become middle and small intermediate cells. Red blood cells are absent. Neutrophils gradually wane in numbers and by late diestrus are only occasionally noticed. Mucus returns to the background of the slide.

Summary

Serial use of vaginal cytology (every 2-3 days) as a diagnostic tool will allow the clinician to accurately track the stages of the estrous cycle in the bitch. More specific events, such as ovulation, the LH surge, and the fertile period, cannot be accurately determined by vaginal cytology alone. Diagnostic tools to better determine these events include vaginoscopy and serum progesterone assays, which are discussed elsewhere in these proceedings.

Vaginoscopy

While vaginal cytology is an inexpensive, accurate way to diagnose the stage of the estrous cycle of a bitch, use of cytology alone will not allow the clinician to determine the truly important events in the cycle: the LH surge, ovulation, and the fertile period. Use of other diagnostic tests, such as vaginoscopy or serum endocrine assays will aid in accurate determination of these events.

Vaginoscopy involves the use of a device such as a human pediatric proctoscope to visualize the caudal vagina of a bitch. The vaginal mucosa changes dramatically in color and texture as the bitch progresses from anestrus through proestrus and estrus. Changes noted initially are due to the rise in estrogens, but later changes are due to the rise in progesterone.

Serum progesterone in the bitch first begins to rise in the pre-ovulatory period as some of the granulosa and thecal cells luteinize prior to ovulation. After ovulation, serum progesterone concentrations rise even more precipitously as the remainder of follicular cells luteinize. The concentration of serum progesterone at certain stages and the rate of the rise is relatively consistent between animals so that monitoring the pattern of serum progesterone concentrations in a particular individual can be predictive of specific physiological events.

Anestrus

Vaginoscopic examination of a bitch in anestrus shows the vaginal mucosa to be pink and smooth. The only fold evident is the dorsomedial fold, which is an anatomic relic of embryological development and always present. The deep pink color comes from the close proximity of the capillaries since the vaginal mucosa is very thin during this stage.

Serum progesterone concentrations are at baseline values during anestrus (< 1.0 ng/mL). The ovaries are relatively quiescent with no luteal activity.

Proestrus

Estrogen concentrations begin to rise at the end of anestrus and throughout proestrus. Rising estrogen concentrations have a vasoconstrictive effect on the blood vessels in the reproductive tract, leading to edema of the vagina and vulva (3). Vaginoscopic examination during early proestrus shows longitudinal folds, termed "primary folds," throughout the vagina. The mucosa is still a light pink color, but becoming more pale. In late proestrus, the edema continues to build and transverse folds become evident on top of the longitudinal folds. These folds are termed "secondary folds" (5). The mucosa at this point has become even more pale.

Serum progesterone concentrations begin to rise as proestrus progresses and some of the granulosa and thecal cells undergo preovulatory luteinization (6). The concentrations will rise around or slightly above 1.0 ng/mL and remain in that range for usually a few days (7). Eventually, the serum progesterone concentrations will rise above 2.0 ng/mL. This usually coincides with the peak of the LH surge in the bitch and is regarded by theriogenologists as Day 0 (2,8). Indeed, the rise in progesterone and a concurrent drop in serum estrogen concentrations signal the LH surge. This is regarded to be the endocrinological boundary between proestrus and estrus.

Estrus

As progesterone concentrations rise and estrogen concentrations fall, the vasoconstrictive effects of estrogen are released. The edema resolves and leaves behind a very wrinkled, pale mucosa. These wrinkles are called "crenulations." The mucosa is very sensitive during this stage and responds to the touch of the vaginoscope with hyperemia, producing an effect known as the "rosette sign" because of the similarity to a rose.

Progesterone concentrations continue to rise and reach a range of 4.0 to 6.0 ng/mL around the time of ovulation (Day 2) (2,4). The bitch ovulates a primary oocyte and requires another 2 days before that primary oocyte matures to a secondary oocyte and is fertile and ready to fuse with a sperm cell (9). Progesterone concentrations during these two days continue to rise and are usually around 10.0 ng/mL at the beginning of the fertile period (Day 4) (10). The fertile period continues for another 2 days, at which time it ends because increasing progesterone concentrations result in closure of the cervix (11). The serum progesterone concentrations at the end of the fertile period (Day 6) are usually around 22 ng/mL (11).

It should be kept in mind that these notes reflect what values that are often seen when managing the normal, average cycles of bitches. Individual variation does occur and is not always abnormal. In addition to biological variation, abnormalities do occur and include silent heats, split heats, and anovulatory cycles.

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The Second Annual Canine Breeder Excellence Seminar was held this past March, with proceeds from this valuable symposium benefiting the work done by the Theriogenology Foundation of the University of California, Davis, School of Veterinary Medicine. This year's conference again included a presentation by Dr. Christensen, as well as, Dr. Joshua Stern, Chief Investigator of a research group responsible for the discovery of a gene mutation for Sub-Aortic Stenosis in Newfoundlands. Look to future editions of the BHF Education Series for presentations from this, as well as the August 2015 Bi-annual AKC/CHF Parent Breed Club Health Conference...and remember, it's through the sharing of knowledge that the lives of our canine companions will be enhanced.