Ovarian ultrasonography and follow-up of estrus in the bitch and queen

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Dr. Malandain graduated from the National Veterinary School of Lyon (France). After one year at the Reproduction Department where she wrote her master's thesis on cat breeding, she joined the National Veterinary School of Alfort, Breeding and Sport Medicine Unit (UMES) with the task of developing the cat breeding sector.
Elise Malandain spent four years conducting clinical trials on reproduction as well as parasitic and respiratory diseases in catteries. Recipient of a Master of Science in the physiology of reproduction, her research is focused on monitoring estrus in queens and developing artificial insemination for breeders. She joined the Royal Canin scientific communications team in 2003. Dr. Malandain is also Vice President of the Société Française de Félinotechnie, an association of cat breeders and veterinarians.

Follow-up of estrus in bitches

Introduction

Timing the day of ovulation as accurately as possible is considered by most authors as one of the most important factors in determining when to inseminate bitches (See the paper by Autumn Davidson in this issue p13). This is especially important when using frozen semen, due to the relatively short survival of frozen/thawed semen in the female genital tract after artificial insemination. In this respect, many different techniques and plans for breeding have been tested by veterinarians over the past 20 years. Very recently, ovarian ultrasound examination in the bitch has been tested by some authors as a tool to diagnose ovulation in bitches.

Ovulation timing in the bitch is not always an easy event to predict. It is important to remember that the oocytes of the bitch are ovulated in an immature

state two days after the LH peak, and they need to mature at least 48 hours before being able to be fertilized. Recently, it has been demonstrated that canine oocytes cannot be penetrated by sperm when they are still immature (1). Therefore, it is essential to be as accurate as possible to detect the day of ovulation.

A distinction has to be drawn between the difficulty in predicting ovulation and the period of maximum fertility. Thus, the period during which a mating or insemination can result in pregnancy, can sometimes be as long as 5 days before ovulation until 5 days after ovulation. This is particularly so if the semen of the sire is of good quality, and therefore remains alive after deposition in the genital tract of the bitch and able to fertilize oocytes for a long period of time (2). Note, however, that when inseminating a bitch with frozen or chilled semen, which may have a shorter life *in vivo*, it is recommended to perform it at the optimal time of fertilization which occurs between 2 to 4 days after ovulation, when the oocytes are fully mature and have not yet undergone degeneration.

Classical techniques used to detect the timing of ovulation in the bitch

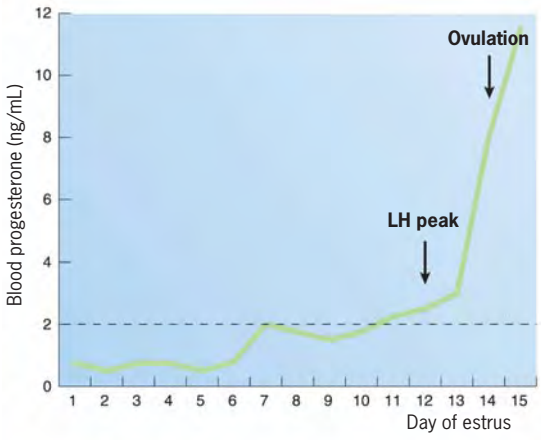
None of the **clinical assessments**, such as vulval edema, the quantity and aspect of the vulval discharge (more or less hemorrhagic), the Amantea sign (turning the tail aside when the veterinarian touches the perinal region) or the acceptance to be mounted by the male, are precise enough to detect the occurrence and the day of ovulation (2).

Furthermore, it is well known that there is no reliability on a predetermined ovulation day, and consequently, a predetermined mating date. Some bitches may ovulate as early as day 5 of estrus period, and others as late as day 30. In the same bitch, it has been shown that significant variations of the day of ovulation may occur among successive estral periods in around 44% of the bitches (3).

Vaginal cytology cannot be used to detect ovulation prospectively. At the end of the heat period, the "onset of vaginal metestrus", when there is a sudden increase in intermediate cells and parabasal cells, occurs around 5 days after ovulation. Therefore, this technique only helps to detect ovulation retrospectively.

Vaginal endoscopy is performed by some authors to determine the "fertile period", but once again, with this method, which also relies on expensive equipment, it is impossible to be accurate in timing the exact day of ovulation.

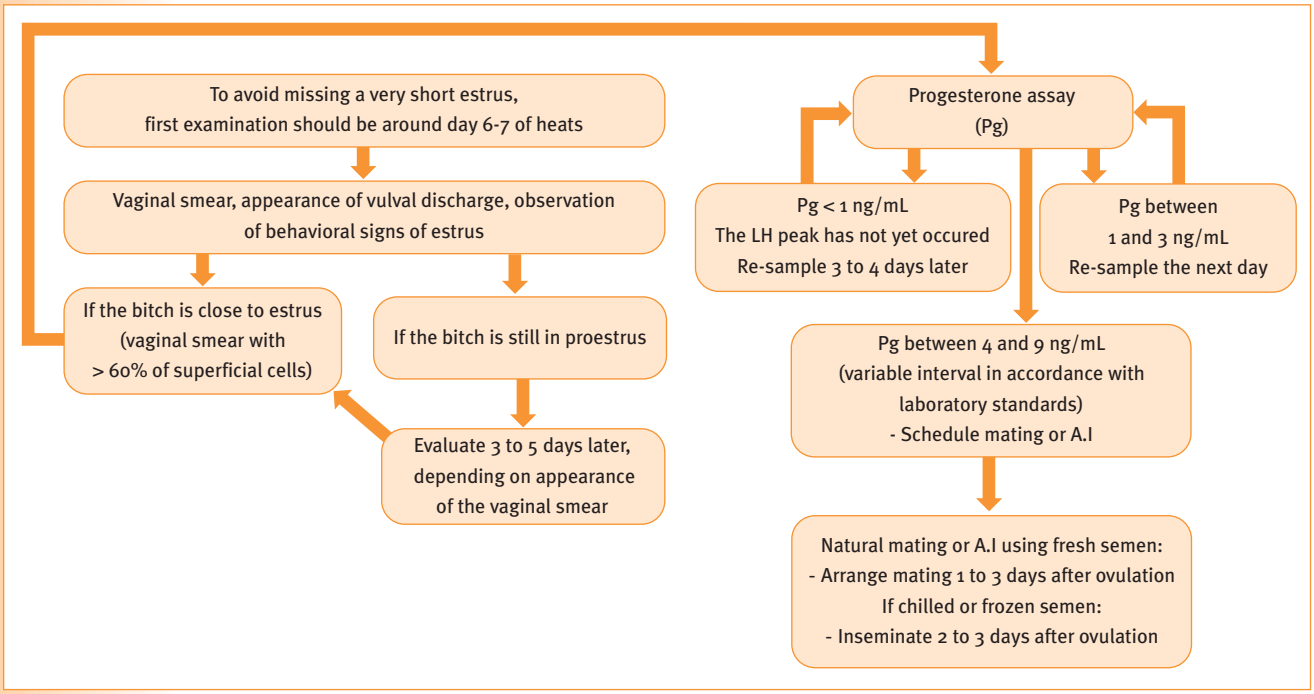
Figure 1.
Determination of LH peak through progesterone assay.
Assaying progesterone to detect the LH peak can lead to mistakes. In the case of this bitch, one might have thought that the LH peak was reached on day 7 of estrus (progesterone assayed at 1.91 ng/L), when in fact this actually occurred around day 12 and ovulation on day 14 of estrus.



Hormonal assays are therefore commonly used by veterinarians as an aid to predicting ovulation. LH assays are ideal in theory, but timing the LH peak requires at least two blood samples per day every day, and, in many countries, no commercial assays for canine LH are available. Researchers willing to perform LH assays therefore have to rely on expensive and time consuming radio-immunoassay tests. It has been suggested that using progesterone assays to estimate the day of the preovulatory LH peak may be useful. England and Concannon (2) have suggested that the plasma progesterone exceeds 2.0 ng/mL (6.5 nmol/L) at the time of the LH surge or on the following day. However, unpublished personal data suggest to us that, at least in some breeds (e.g. German Shepherd), relying only on this early pre-ovulatory increase in plasma progesterone may not be adequate to estimate the day of ovulation **(Figure 1)**.

Accordingly, we advise continually assaying progesterone until it reaches a value considered to indicate with certainty that ovulation has occurred. According to Arbeiter (4), a reliable identification of mating time in bitches requires monitoring of rising progesterone concentrations up to at least 32.0 nmol/L (11 ng/mL). Recently, it has been demonstrated that the progesterone plasma level at the time of ovulation, whatever the

Figure 2.
Detection of the fertile period in a bitch: decision tree.



breed, is fairly constant (5). Therefore, progesterone assays appear as one of the most reliable techniques to assess ovulation in the bitch.

In practice most clinicians use a combination of vaginal cytology and progesterone assays to optimize the cost/benefit **(Figure 2)**.

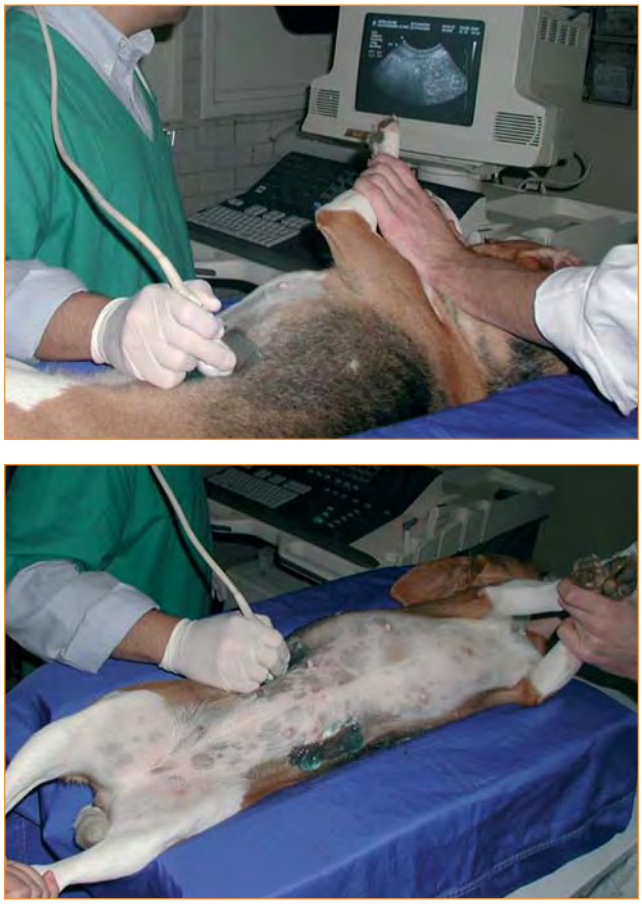
Ovulation detection using ovarian ultrasonography

The last technique to determine ovulation in the bitch, which is also the newest, is ovulation detection using ovarian ultrasound scanning. Unfortunately, ultrasound imaging of the ovaries of the bitch around ovulation are more difficult to analyze than in other species. Previous studies have shown that the ovarian follicles just before and just after ovulation look very similar (6), some follicles do not collapse at the time of ovulation (7, 8) and, furthermore, non-ovulated follicles often remain after ovulation (9). In consideration of these difficulties, it has been recommended that at least two daily examinations are made in order to determinate ovulation with accuracy (10). However, even when following a very precise protocol and frequent examinations, ovulation could only be diagnosed in 15.4 % (2 of 13) and 54.5 % (6 of 11) of the bitches (7, 11).

Modern ultrasound machines with high performances are readily available in veterinary clinics and it may be timely to reconsider the imaging of estrus using ultrasonography. For this purpose, a series of experiments were performed at the National Veterinary School of Alfort in France to investigate whether ovarian ultrasound examination could be a reliable and precise enough method to determinate ovulation in bitches. This work (5) was presented at the World Small Animal Reproduction Symposium in Sao Paulo, Brazil, in August 2004 and was awarded the title of "*best research report*".

In this study, the estrus period was followed using ultrasound and hormonal assays (progesterone, LH) in several bitches belonging to 36 different breeds. Bitches, aged between 9 months and 8 years, most of them belonging to private owners, were scanned during estrus in an attempt to detect ovulation by ultrasound. The day of ovulation was clearly detected in 91.7% (44 of 48) of the bitches. Four bitches, however, showed imprecise ovarian aspects around ovulation. Interestingly, they were all large breeds (two German and Belgian Shepherds, a Labrador Retriever and a Dogo Argentino).

In most bitches, the ovulation process appeared to be completed within 24 hours. In 14 of 41 bitches, it was even completed in less than 12 hours. No significant difference in the ovulation time was seen between the left and the right ovary. In this study, the progesterone



Figures 3.
Positioning the bitch for ovarian scanning.
The bitch can lie on a dorso-lateral left or right recumbency. Sometimes, the right ovary will be easier if the bitch is standing on the table.

level at the time of ovulation appeared to be fairly constant at 6.25 +/- 1.55 ng/mL (*Chemiluminescence assay, Progesterone II®, Roche diagnostics, Germany*).

Overall, in this study of the bitch, the detection of ovulation using ovarian ultrasound appeared to be the most accurate clinical method. Furthermore, since two daily examinations did not improve the detection of the day of ovulation, one daily ovarian scan was recommended to determinate a correct timing of ovulation.

Technique

High frequency linear or curvi-linear probes are recommended although most of the images of the ovaries, even in small breeds, were performed using 7.5 MHz probes. However, it was sometimes useful to confirm the slight changes in the ovarian aspects with higher frequencies, between 8 and 10 MHz.

Breeders owning expensive pure-bred bitches undergoing regular shows or competitions are understandably reluctant to permit shaving of abdominal hair to facilitate ovarian scanning for the detection of ovulation. In our experience clipping appears unnecessary. Even in long-haired breeds (e.g. Afghans, Golden Retrievers) simply applying a large quantity of ultrasound gel on the region that has to be scanned, appears to be, most of the time, sufficient to get good images of the ovaries. In order to perform the ovarian ultrasound examination, we recommend the animals are laying in a dorso-lateral right or left position **(Figures 3)**, in order to scan, respectively, the left and the right ovaries. Occasionally the presence of intestines near the right ovary interfere with imaging and it may be useful to repeat the scan with the bitch standing.

The ovaries in the bitch are generally located in front of the 3rd or the 4th lumbar processes. They can be found caudo-laterally from the distal part of the kidneys. Since the right kidney is more cranial than the left kidney, the right ovary is located cranial to the left kidney. The best method is to start with the left ovary, which is easier to find. With the probe, the veterinarian has to detect the kidney, and then scan the caudo-lateral region of the kidney, remembering that the ovary has a very superficial location under the skin. The ovarian cortex appears less echoic - "darker" - than the renal cortex.

The ovaries may be more difficult to find in giant breeds, or in obese bitches. Some breeds (e.g. Shar-Pei, Chow-Chow, Newfoundland) have a thick skin that makes it difficult to get good images when performing any abdominal ultrasonography.

During the anestrus period, the ovaries are not always easy to find. They have a small size, and appear somehow heterogenous, especially in post-pubertal bitches, where remnants of anterior *corpora lutea* can be visualized.

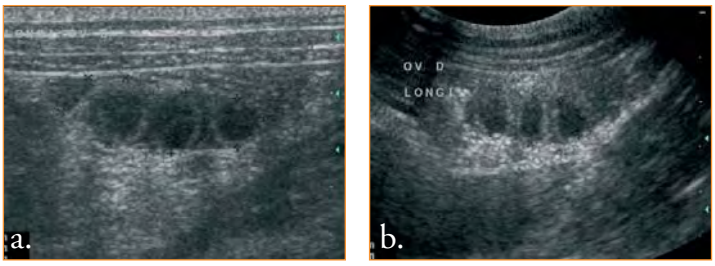
In proestrus bitches, the shape of the ovaries are easier to visualize. The ovaries are often found in a more caudo-ventral position from the kidneys. They contain several small circular anechoic follicles, surrounded by a thin echoic wall, less than 1 mm. At the end of proestrus, during the pre-ovulatory period, the size of the ovaries increase and, due to the large amount of anechoic fluid within the follicles, they become easy to visualize. At this stage, there is a pre-luteinization of the follicles, which secretes small amount of progesterone. Ultrasonographically, the follicular walls become thicker, around 1 mm of width. Depending on the size of breed,

the maximum size of the pre-ovulatory follicles varies between 6 mm to 9 mm. These pre-ovulatory follicles are usually wide circular anechoic structures, however, when numerous within the same ovary, they sometimes appear flattened and packed together **(Figures 4)**. We have unpublished data which suggest that using ultrasound we underestimate the number of follicles.

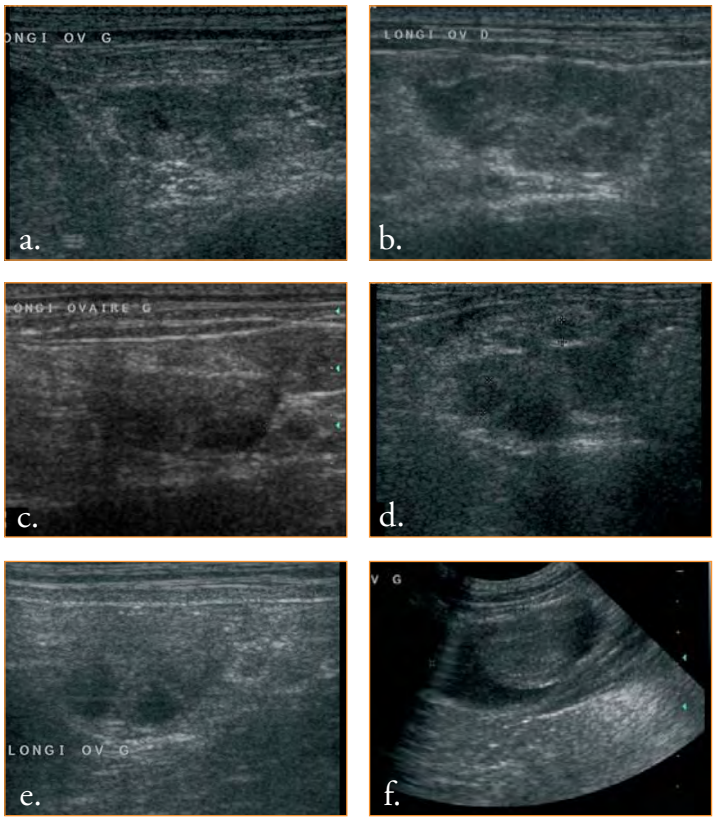
In some cases, at the time of ovulation, a complete disappearance of the follicular cavities can be visualized. In this study this was seen in only 37.5% (18 of 48) of the bitches. However, in 58.3 % (28 of 48) of the bitches, some intra-ovarian hypoechoic structures persist. These intra-ovarian structures always show a very different aspect than the pre-ovulatory follicles, being smaller and irregular (not circular) in shape **(Figures 5)**. Furthermore, in 45.9% (22 of 48) of the bitches, we identified apparent non-ovulated follicles, that remain round and anechoic up to 3 days after ovulation in the same ovary. This high percentage of non-ovulated follicles is in accordance with the work of Wallace *et al.* (9) who reported this phenomenon in 7 of 10 bitches. Care must be taken not to wait for the totality of the follicles to ovulate to time ovulation, as it will not happen in a large number of bitches. Finally, in 39.6 % (19 of 48) of the bitches, some liquid was visualized between the ovary and the ovarian bursa in the hours following ovulation. It is probably the intra-follicular fluid that accumulates after the follicular opening at ovulation.

From one day after ovulation, blood accumulates in young hemorrhagic *corpora lutea*. Their appearance is sometimes very similar to pre-ovulatory follicles **(Figures 6)**. Therefore, it is very important to make daily ovarian examinations at the pre-ovulatory period to detect ovulation. If no ultrasound is made on the day of ovulation, the veterinarian cannot be sure whether the bitch has ovulated or not.

In conclusion, ovulation can be accurately determined using ovarian ultrasonography in the bitch. However, this technique increases the accuracy of the determination in only around 10% of the cases compared with progesterone



Figures 4 (a and b).
Ultrasonographic appearance of the ovaries during the late proestrus (preovulatory) period in the bitch.
Due to the large amount of anechoic fluid within the follicles, they become easy to visualize; the follicular walls become thicker, around 1 mm in width. Depending on the size of breed, the size of the pre-ovulatory follicles varies between 6 to 9 mm.



Figures 5 (a to f).
Ultrasonographic appearance of the ovaries of the bitch at the time of ovulation.
A complete disappearance of the follicular cavities ("follicular collapses") can be visualized (photos a and b). However, in 50 % of the cases, some intra-ovarian hypoechoic structures persist (photos c and d). Often non-ovulated round follicles remain in the same ovary (photo e). In around 40% of the cases, some liquid is visualized between the ovary and the ovarian bursa in the hours following ovulation (photo f).

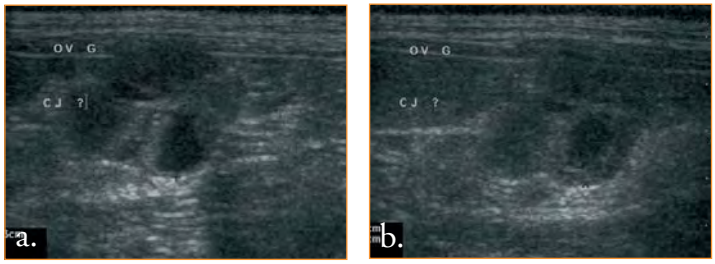


Figure 6 (a and b).
Ultrasonographic appearance of the ovaries in the bitch just after ovulation.
From one day after ovulation, blood accumulates in young hemorrhagic corpora lutea*, their appearance is sometimes very similar to pre-ovulatory follicles. Therefore, it is very important to make daily ovarian examinations at the pre-ovulatory period to detect ovulation.*

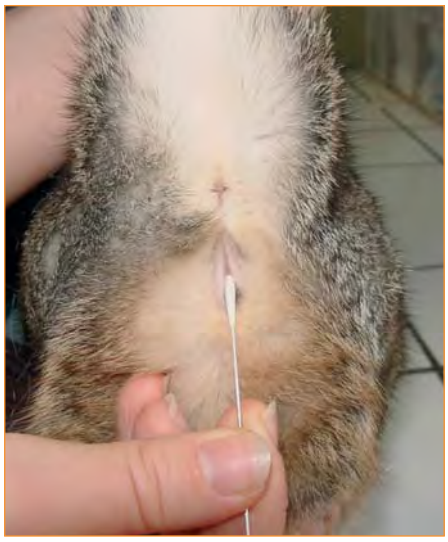


Figure 7. Obtaining a vaginal smear in a queen. *The swab is introduced dorsally into the vagina. It should not be inserted beyond the cotton tip.*

assays. Therefore, in our daily clinical practice, we now use this technique only when it is important to be as precise as possible in the detection of the day of ovulation. This technique is also used when artificial insemination with frozen or chilled semen has to be performed, or when we wish to check the occurrence and the qualitative aspects of ovulation in bitches that are presented for evaluation of infertility.

Follow-up of estrus in queens: a different physiology, a different approach

Introduction

In queens, mating or comparable vaginal stimulus is generally needed to initiate the endocrine sequence and LH release, which result in ovulation. The interval between coital stimulation and ovulation has been reported to vary in cats, ranging from 25 to 50 hours, but it is commonly accepted that this interval is closer to 25-30 hours (12). Some authors suggest that the occurrence of ovulation may shorten the period of sexual receptivity. Other studies reveal no significant difference in estrus length between ovulating queens and unmated, non ovulating queens. In our experience, some queens can still be bred three or four days after ovulation even with a high progesterone level. The cessation of the sexual receptivity cannot be used as an accurate indication of ovulation.

Artificial insemination in queens is still in its infancy. Practical difficulties include low semen volume of the tom cat, choice of semen extender, anatomy of the queen and optimal method of insemination (intrauterine or intravaginal). Currently, most teams working with cats opt to inseminate the 3rd or 4th day of induced estrus, without any follow-up of estrus.

Few authors have studied the influence of the duration of estrus at the time of mating. Some authors consider that during the first two days of estrus, the queen is unable to ovulate if mated, perhaps due to a lack of estradiol impregnation. In practice it is not an important question for natural breeding because the cats are allowed to mate during the whole estrus. However, it does become relevant if artificial insemination is considered or if the queen is infertile.

Currents techniques available to follow estrus in the queen

Observation of sexual behavior

Some authors describe a period of proestrus including male attraction but without sexual receptivity. In fact, proestrus is observed in a minority of queens (13). Neither the vaginal cytology nor hormonal assay can easily differentiate proestrus and estrus. Estrus behavior in the queen includes persistent vocalization, rolling, rubbing, lordosis, tail deviation and repeated monotonous howling. During estrus, follicular maturation is associated with follicular estrogen secretion (>20 pg/mL). Unlike the bitch, progesterone levels stay basal during the follicular phase before ovulation.

Cytological evaluation of samples of exfoliated vaginal cells can be easily performed by introducing a dampened cotton-tipped swab into the vestibular and caudal vagina **(Figure 7)**. We advocate the use of a human urethral swab as it is much smaller than the one most commonly used for vaginal cytology of bitches. In our experience, queens tolerate the dampened swab well.

Some have advocated introducing some saline into the vagina before the swab is introduced but we have found that queens do not tolerate this well. The introduction of the swab must be done carefully, advancing horizontally, to avoid inducing ovulation. The staining methods are the same as in the bitch, but interpretation differs widely. Three phases of the queen cycle can be easily identified using vaginal cytology: estrus, anestrus, and interestrus/diestrus.

In contrast to the situation in the bitch, it is impossible to use vaginal cytology. The majority of the cells are cornified and acidophilic **(Figure 8)**, and no statistical significant variations are seen during estrus. However, in some queens (but not all) cellular clumps around the day

of maximal follicular maturation, and a sparse number of neutrophils at the end of estrus can be noted. In practice vaginal cytology in the cat may confirm that the queen is in estrus (sometimes a queen's behavior is not characteristic). Cytology will also confirm vaginitis.

Endocrinological methodology is not really feasible in the queen. The progesterone rise begins 24-48 hours after ovulation and therefore, this assay can only confirm it retrospectively, after the fertile period. As ovulation occurs at least 24 hours after mating, it is recommended to perform the progesterone assay at least 72 hours (better 96 hours) after the last mating for a meaningful result. Ovulation failure is associated with serum progesterone of less than 1 ng/mL. Conversely, high progesterone level 3 days after the last mating confirms ovulation. If ovulation failure is suspected, it is recommended to perform an assay before mating, to rule out a luteal cyst (or a progesterone secreting ovarian tumor). The colorimetric progesterone assays used in the bitch are efficient in the queen (14). However, feline estradiol assays are not routinely available, and are difficult to interpret. Finally, feline LH assay is difficult, only performed in research laboratories and needs several blood samples (15). However, this repeated blood sampling leads to stress, which compromises ovulation!

Ultrasonography in the queen

To date, the use of ultrasonography in queens has been limited by the small size of the ovarian follicles. However, the development of high resolution equipment makes the procedure feasible (16, 17). The techniques are similar to the ones described above, for bitches and the examination is usually well tolerated by queens.

The ovaries are found against or 3 cm behind the kidney, and they tend to move in the abdomen during examination. If the queen is in estrus before ovulation, the ovaries are easier to find. Ovarian follicles appear as anechoic spherical structures, from 1.5 to 4 mm in diameter, which may appear very small, but are visible **(Figure 9)**. If the queen is in anestrus or interestrus, the ovaries are more difficult to find, as they appear as homogenous structures of less than 2 cm length. In this situation the ultrasonographer can try to follow the uterine horns (identified as round structure in a transversal plane dorso-laterally to the bladder), proximally until the ovaries are reached.

During anovulatory cycles the follicular diameter progressively increases during the estrus period, reaching a diameter of 3.2 mm in average (minimum 2.6 mm; maximum 4.1 mm), with at least one follicle per queen being larger than 3.0 mm. The maximal follicular size is reached between the second and the sixth day of estrus (average 4.25 ± 1.5 d), with great variation between individuals, and cycles. During a normal cycle, some follicles remained small (around 2 mm) and are not consistently seen during examination.

During ovulatory cycles, the follicles suddenly disappear at the time of ovulation, and, sometimes, hypoechogenic structures (*corpora lutea*) may be seen in the days following **(Figure 10)**. In contrast to what may be seen in bitches, there is no reapparition of anechoic structure after ovulation, making the diagnosis easier.

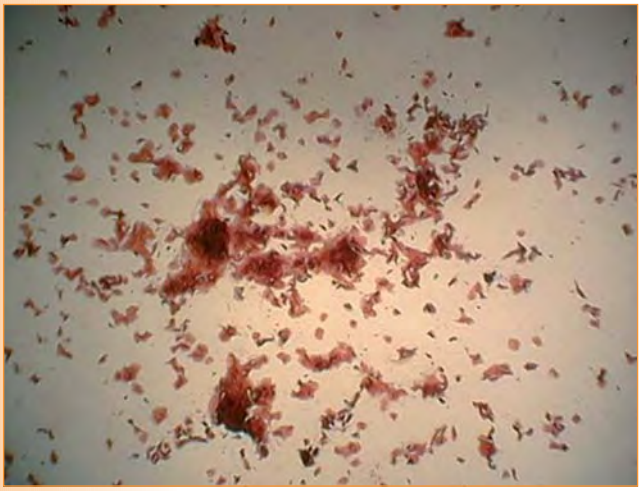


Figure 8.
Vaginal smear of a queen in estrus.
Usually, estrus is characterized by cornified cells tending to clump together.

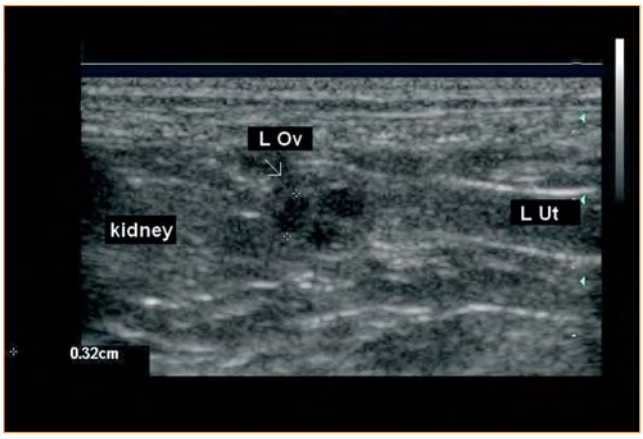
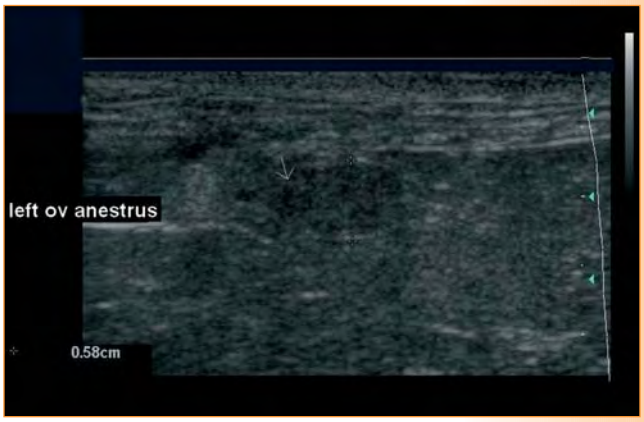


Figure 9.
Ultrasonographic examination of a queen during estrus.
During estrus, follicles can be detected on ovaries as round anechoic structures. Their size range from 0.1 mm to 0.4 mm.

Even if the general pattern of follicular growth appears similar between queens, its timing relating to behavioral estrus is highly variable between individuals. On the first day of specific estrus behavior, follicles show very different sizes between queens. Neither behavioral observation, or vaginal cytology appear to be accurate methods, despite the observation of cellular clumps in the smears around the day of maximal follicular diameter. Moreover, the day at which the maximal diameter is observed after the onset of estrus behavior is different between queens. As some queens begin to exhibit estrus while their follicles enter atresia, the decision to inseminate systematically on the third day of estrus should be revized. ◆

Figure 10.
Ultrasonographic examination of a queen after estrus.
Ovulation is characterized by a sudden disappearance of anechoic structure. Corpora lutea *are difficult to find, and ovaries have often the same ultrasonographic appearance in both anestrus and diestrus.*



References

1. Reynaud K, Fontbonne A, Marseloo N *et al.* In vivo meiotic resumption, fertilization and early embryonic development in the bitch. *Reproduction* 2005; **130**: 193-201.

2. England GC and Concannon PW. Determination of the optimal breeding time in the bitch: basic considerations. In: Recent advances in small animal reproduction, International Veterinary Information Service, www.ivis.org, Ithaca, New York, USA, 2002.

3. Badinand F and Fontbonne A. Repeatability of events during successive oestrous periods within bitches: comparison between breeding results and clinical and hormonal data. *J Reprod Fertil, Suppl.* 1993; **47**: 548.

4. Arbeiter K. Anovulatory ovarian cycles in dogs. *J Reprod Fertil, Suppl.* 1993; **47**: 453-456.

5. Marseloo N, Fontbonne A, Bassu G, *et al.* Comparison of ovarian ultrasonography with hormonal parameters for the determination of the time of ovulation in the bitch. In: *Proceedings of the 5th International Symposium on Canine and Feline Reproduction* Sao Paulo, Brazil, 2004.

6. England GC, Yeager A and Concannon PW. Ultrasound imaging of the reproductive tract in the bitch. In: *Recent advances in Small Animal Reproduction*, International Veterinary Information Service, www.ivis.org, Ithaca, New York, USA, 2003.

7. Hayer P, Gunzel-Apel AR, Luerssen D, *et al.* Ultrasonographic monitoring of follicular development, ovulation and the early luteal phase in the bitch. *J Reprod Fertil, Suppl.* 1993; **47**: 93-100.

8. Yeager AE and Concannon PW. Ovaries. In: Green RW, ed. *Small Animal Ultrasound.* Philadelphia: Lippincott-Raven, 1996. pp. 293-303.

9. Wallace SS, Mahaffey MB, Miller DM, *et al.* Ultrasonographic appearance of the ovaries of dogs during the follicular and luteal phases of the estrous cycle. *Am J Vet Res* 1992; **53**: 209-15.

10. England GC, Yeager AE. Ultrasonographic appearance of the ovary and uterus of the bitch during oestrus, ovulation and early pregnancy. *J Reprod Fertil Suppl.* 1993; **47**: 107-17.

11. Hase M, Hori T, Kawakami E, *et al.* Plasma LH and progesterone levels before and after ovulation and observation of ovarian follicles by ultrasonographic diagnosis system in dogs. *J Vet Med Sci* 2000; **62**: 243-8.

12. Shille VM, Munro C, Farmer SW, *et al.* Ovarian and endocrine response in the cat after coitus. *J Reprod Fertil* 1983; **69**: 29-39.

13. Shille VM, Lundström KE, Stabenfeldt GH, *et al.* Follicular function in the domestic cat as determined by estradiol-17b concentrations in plasma: relation to estrous behavior and cornification of exfoliated epithelium. *Biol Reprod* 1979; **21**: 953-963.

14. Hammer JG. Use of a semi-quantitative canine progesterone test kit in the domestic cat. *J Am Anim Hosp Assoc* 1994; **30**: 50-53.

15. Banks DH and Stabenfeldt GH. Luteinizing hormone release in the cat in response to coitus on consecutive days of oestrus. *Biol Reprod* 1982; **26**: 603-611.

16. Gunzel-Apel AR, Kawauchi R, Nautrup CP, *et al.* Sonographic presentation of the physiologic ovarian function in the anovulatory and pseudopregnant cycle of the cat. Tierarztl Prax Ausg K Klientiere Heimtiere 1998; **26**: 275-83.

17. Malandain E, Rault D, Froment E, *et al.* Follow up of follicular growth in queens during non ovulatory estral phases in queens by ultrasonography and vaginal cytology. Proceedings 3rd European EVSSAR Congress; Liege, 2002.