

Part 5: Preparation, Reading and Reporting of Vaginal Smears

1. Introduction

- 1.1 Vaginal smears may be taken to provide information on estrous cyclicity at various intervals during a study. This guidance document provides recommendations for best practice for preparing, reading and reporting these vaginal smears.

2. Background

- 2.1 The cells lining the vagina of the female rat respond to the levels of circulating hormones and can provide a valuable marker of the stage of preparation of the ovary and uterus for mating and ovulation.
- 2.2 Vaginal smears taken on consecutive days over a period of time can provide detailed information on the oestrous cycle. The normal oestrous cycle in the rat usually follows a 4-day pattern and the varying characteristics of the cells in the smear allow the days of the cycle to be classified relative to the predicted time of ovulation. Ovulation occurs at approximately midnight after the pro-oestrous stage, when the females become receptive to the male. The classic stages of the rat oestrous cycle can be designated as oestrus (E), metoestrus (M), di-oestrus (D) and pro-oestrus (P). The stages of the vaginal cell cycle will normally correlate with changes in the female reproductive organs e.g. ovary and uterus.
- 2.3 These notes describe practical techniques which have been found to be successful in determining the daily stages of the cycle, in categorising the cycles and in assessing effects of treatment upon the cycles. The stage of the cycle on the day of sacrifice should be readily and reliably determined if these techniques are used.

3. Environmental effects on oestrous cycles

- 3.1 Rodent oestrous cycles are very sensitive to changes in the light: dark cycle. 12:12 hours is the standard cycle and laboratories running a 14:10 light: dark system could expect to see a different ratio in the number of 4 and 5-day cycles. Longer periods of light within each 24 hours may result in irregular, lengthened cycles and if rats are exposed to continuous light they will effectively cease cycling and show extended periods of vaginal oestrus.
- 3.2 Evidence for the influence of male and female pheromones on the oestrous cycle is less conclusive in the rat than in many other mammals. Rats are generally much less sensitive than mice and under normal laboratory conditions there is little evidence of the synchronisation of cycles with cage-mates, that would normally be apparent in the females of many other mammalian species living in close proximity. Similarly, there is no clear evidence of cycle changes after pairing, as seen in the “Whitten effect” in mice, although it has been reported that regular cycles are better maintained long-term in female rats if males are kept in the same room. For the purposes of TG 407 this is of little importance because the males would normally be in the same room throughout, and the duration of the study is relatively short.

4. Time of smearing

- 4.1 Although the oestrous cycle of the rat can be broadly divided into 4 daily parts, the actual duration of the various stages, as indicated by the vaginal smears, can vary between approximately 12 and 36 hours and therefore the probability of seeing particular stages on the expected days will vary according to the time of day the smears are taken (see Table 1). Transitional stages, i.e. showing characteristics of two consecutive stages e.g. M-D or D-P, are not uncommon, particularly if smears are taken very early or late in the day. Some short stages, especially pro-oestrus, can appear to be ‘missed’ if smears are taken

early in the morning because, for some females, the stage of pro-oestrus does not appear until mid-morning; a smear taken earlier than this will therefore still show the previous day's stage (di-oestrus).

- 4.2 To minimise the incidence of these transitional and 'missed' stages leading to inappropriate interpretation, it is important to take smears at approximately the same time of day each day. For vaginal smears taken for oestrous cycle evaluations prior to pairing on reproductive toxicology studies, this would normally be between 10:00 and 13:00 (with a light: dark cycle of 12:12 and lights on at 06:00 GMT). For cycle stage assessments on the TG 407 studies, smears will probably need to be taken somewhat earlier than 10:00 because of the need to send females for necropsy on the last day of smearing. Target smearing times of between approximately 09:00 and 11:00 are therefore suggested, although it is the consistency from day to day that is more important than the specific time.

5. Smearing techniques

- 5.1 The two most commonly used methods of obtaining vaginal cell samples are:

- a) Lavage or washing with saline or water from a pipette.
- b) Swab or cotton bud (moistened with saline or water).

- 5.2 Each of these methods has advantages and disadvantages in terms of the expertise and time required to take the samples, and to read the resulting smears. The decision on which method to use will necessarily be left to individual laboratories, according to the skills, experience and preferences of staff, but for the short-term smearing period on TG 407 studies **the pipette technique is recommended**.

5.3 Pipette smear technique

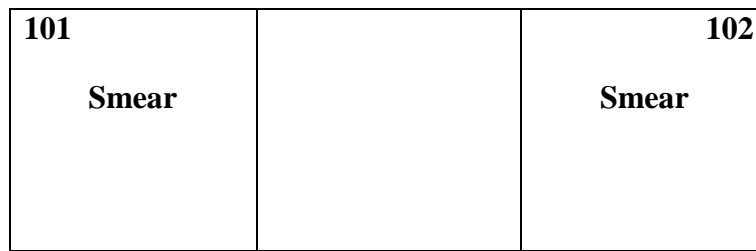
This method consists of flushing cells from the vaginal lining by introducing a small amount of fluid into the vagina using a pipette and placing one or two drops of the resulting cell suspension onto a slide. This technique produces good quality cell samples and there should be no risk of inducing pseudopregnancy (see sections 5.6, 6.8 and 7.6), but the smears do need to be examined shortly after being taken (unless fixed and stained). Staining of vaginal smears is time consuming and is considered unnecessary, once familiarity with the appearance of the cell types has been acquired. Staining is not described in this guide. Disposable pipettes made of soft plastic with an internal tip bore of approximately 1.5 mm are recommended. Glass Pasteur pipettes with rubber teats and rounded tips may be used repeatedly but these pipettes require thorough cleaning between animals to prevent sample contamination.

- 5.4 A small amount (approximately 0.2 ml) of saline or distilled water is drawn up into the pipette tip. The rat is held around the thorax, ventral surface uppermost, with one hand whilst the hand holding the pipette is used to restrain the tail, to provide additional support and help prevent the animal struggling. The tip of the pipette is pushed gently into the entrance of the vagina to a depth of 2-5 mm and the fluid is flushed into the vagina and back up into the pipette two or three times by gently squeezing and releasing the bulb of the pipette. If the fluid is seen to be 'cloudy' after the first flushing the subsequent flushings are unnecessary.

- 5.5 A small amount of the cell suspension is then expelled onto a labelled glass slide. Slides should be labelled with the female identification numbers; the date and study number may also be shown but as the smears are discarded immediately after reading this is not usually considered necessary as long as the tray holding the slides is identified appropriately. The tip of the pipette should be rested on the slide at an angle when placing the sample on the slide, to maximise control of the volume and prevent contamination from splashes, which may occur if drops of the sample are expelled from a height. Up to two smears may be placed on a slide, one at each end (Figure 1). The drops of cell suspension can be examined without further preparation but the use of cover slips is recommended because they ensure

the smears are of a uniform depth, making it easier to focus with the microscope, and help prevent the smears coalescing during movement or transport.

Figure 1. Slide with two pipette smears and cover slips



5.6 Swab smear technique

This method consists of inserting a moistened cotton bud swab into vagina, gently removing the cells from the vaginal lumen and walls and transferring the cells to a glass slide. It is the quickest method of smearing and the smears retain their original appearance indefinitely. There is a risk of causing pseudopregnancy if the cervix is stimulated during oestrus, although this risk is very low if good technique is used. Suitable wooden swab sticks with small cotton wool tips (approximately 5 mm wide and 12 mm long) are readily and cheaply available from biological suppliers.

- 5.7 The cotton wool tip is moistened slightly by dipping into a jar of saline or distilled water and sharply flicking off any surplus. The rat is held around the thorax, ventral surface uppermost, whilst providing lumbar support as far as possible. Holding the swab stick close to the smearing tip gives maximum control and lightly gripping the tail with the same hand gives further support and minimises movement of the rat. The tip of the swab stick is inserted carefully into the rat's vagina to a depth of approximately 1.0 cm, with a rotating action of the swab and at an angle of about 45° to the animal's body. The rotating action of the swab stick is continued, in the same direction, as the swab is removed. The aim is to achieve a single, brief, in-and-out action, so that any cervical stimulation is minimal and is insufficient to induce a pseudopregnant response. Good animal handling skills are important because a struggling animal may push up onto the swab stick, which is undesirable in terms of welfare and the risk of causing pseudopregnancy.
- 5.8 With the swab held almost horizontally, to ensure cells from the full length of the vagina are transferred, the tip is rolled gently onto a clean, pre-labelled glass slide, below the relevant animal number. The swab stick is discarded, a new one being used for each animal.
- 5.9 Slides are pre-labelled with the study number, animal numbers and date, using a permanent marker, and are placed on a suitable tray. Slides can be set up on an animal number basis, adding new dated smears to the same slide(s) for each female, or on a date basis, with one set of slides for all females each day. The latter method is considered most convenient and it also minimises the risk of misplacing smears e.g. placing smears under the wrong date. Up to five smears can be placed on each slide. For gang-housed animals, it is usually convenient to have one slide for each cage of four or five females (Figure 2).

Figure 2. Slide labelled ready for swab smearing

Smears are applied vertically below each animal number

	101	102	103	104	105
Study No. Date					

- 5.10 It is important to avoid getting too much fluid on the slide (usually resulting from too much pressure being applied when rolling the swab onto the slide) and to avoid urine contamination as these factors can mask the cells and make reading very difficult.
- 5.11 On completion, all slides should be checked for the presence of the required number of smears. This is particularly important if smears are not going to be examined the same day. Care should be taken in case a 'missing' smear was actually placed on top of another female's smear by mistake; if in doubt, all females on the slide should be smeared again. Slides should be covered with paper until boxed for permanent storage.

6. Identification of vaginal cytology - smear reading

- 6.1 A stereoscopic microscope with widefield eyepieces and no mechanical stage is the best equipment for rapid and accurate smear reading, using a magnification of between 80X and 100X, but a standard laboratory microscope can also be used at a similar magnification. Care should be taken to examine the whole slide as cell types (particularly leucocytes on swab smears) and cell numbers can vary in different areas.
- 6.2 The descriptions and classification in this section relate specifically to the appearance of smears taken by pipette lavage. Although the same cells are present in the vagina whatever smearing techniques is used, swab smears often have fewer leucocytes at metoestrus and di-oestrus and a low but variable incidence of superficial cornified cells at all stages of the cycle. This makes swab smears somewhat more difficult to read compared with pipette smears.
- 6.3 The four basic stages of the cycle are oestrus, metoestrus, di-oestrus and pro-oestrus, abbreviated to E, M, D & P respectively. These stages can be recognised by the presence, absence or proportional numbers of epithelial cells (two types), cornified (keratinised) cells and leucocytes. Occasionally mucus is also seen, especially in acyclic females. The normal 4-day cycle will be in the sequence E, M, D, P, E, M, D, P, E etc.
- 6.4 There are three main cell types seen in vaginal smears from the rat:

Cornified (keratinised) cells - large, angular and irregularly shaped, mostly non-nucleated when mature (as seen at oestrus).

Epithelial cells - not quite as large as the cornified cells and much more rounded in shape. Those seen at pro-oestrus are mostly nucleated and with a granular appearance; those seen at metoestrus tend to be non-nucleated and less granular.

Leucocytes - very small, round cells. Nuclei not usually evident at the low magnifications used

It is the balance of proportions between these cell types that permits the classification of the stage of the cycle between successive ovulations.

6.5 The four stages do not all last for the same length of time and, depending on the time of smearing, transitional smears are often seen i.e. showing characteristics of two consecutive days of the cycle e.g. M→D or D→P (recorded as MD or DP respectively).

6.6 Oestrus

Oestrous smears (Figure 3) consist entirely of cornified cells, in high numbers and usually forming clumps and sheets, often visible to the naked eye on swab smear slides. The cells are large (maximum size) and often non-nucleated. It is a long stage - starting during the preceding dark phase and lasting until at least early afternoon and is normally seen every 4th day, but can occur on two or more consecutive days e.g. in 5-day cycle - M, D, P, **E**, **E**, M. It should be noted that an oestrous (cornified) smear taken in the morning on the last day of the cycle is normally post-ovulatory and after the time of peak receptivity to the male. Both of these events would have occurred during the preceding night.

6.7 Metooestrus

Metooestrous smears (Figure 4) consist of large numbers of leucocytes and smaller numbers of mostly large, non-granular and non-nucleated epithelial cells. The leucocytes are often characteristically in contact with other cell types, forming small, tightly packed clumps of cells. It is normally seen only on the day after oestrus. It can be a short phase, appearing early, and so may be 'missed', resulting in two days of di-oestrus e.g. E, **D**, D, P, E. Occasionally the metooestrous characteristics can carry over into the next day, with a transitional (MD) smear on Day 2 of the cycle e.g. E, M, **MD**, P, E. The transition from oestrus to metooestrus/di-oestrus provides a marked change in smear appearance, and is an essential reference point for the calculation of cycle lengths because it confirms that the ovulatory point has passed, thereby demonstrating the completion of one cycle (oestrus) and beginning of the next cycle (metooestrus).

In the very early phase of metooestrus, cornified and large epithelial cells predominate, with smaller numbers of leucocytes, but this phase usually occurs in the late afternoon or evening on the day of oestrus and so is not seen in smears taken the following morning. Some literature sources define metooestrus strictly as this early transitional stage and, because it is not seen in a morning smear, then define the first two days of the cycle both as di-oestrus (D1 and D2), with no differentiation between these two days in terms of appearance. However, although the metooestrus seen in a smear taken the morning after oestrus (as described in the first paragraph) can be quite similar to the following day of di-oestrus, it is usually distinct and therefore the use of the broader definition of metooestrus is recommended. As it can be difficult to distinguish between the first two days of the cycle (M and D) it is sometimes useful to count the days from the E→M/D change to help define the stage of the cycle, particularly if attempting to correlate cycle stage with hormone levels or the histopathological examination of the reproductive organs.

6.8 Di-oestrus

Di-oestrous smears (Figure 5) consist mainly of leucocytes but with quite variable numbers of epithelial and small cornified cells. The cell numbers are low to moderate and the general appearance is similar to metooestrus but usually with far fewer cells and without the tightly packed clumps. Di-oestrus can be the most difficult stage to recognise because of the variability in the numbers and ratios of the cells. It is the longest stage, normally present throughout Day 2 (after metooestrus) but may also be present on Day 1 (after oestrus) and/or Day 3 (before oestrus) e.g. E, **D**, **D**, P, E or E, M, **D**, **D**, E. Transitional smears are quite common e.g. MD or DP. Di-oestrus is also the 'resting' stage of the cycle and females which become pseudopregnant and stop cycling (acyclic) will show consecutive days of di-oestrus, sometimes accompanied by swirls or strings of mucus (not seen in cycling di-oestrous smears).

6.9 Pro-oestrus

Pro-oestrous smears (Figure 6) are characterised by rounded, usually nucleated, epithelial cells, generally in low to moderate (occasionally high) numbers. It is a short stage, normally occurring the day before oestrus (Day 3 - M, D, P, E) but often not seen until mid-morning, so may appear to be missing if smears are taken early in the morning - M, D, D, E. Quite often it is seen as a transitional smear, in combination with preceding day of di-oestrus - M, D, DP, E. Some epithelial cells may show early stages of cornification i.e. becoming larger and more irregular in shape. A low incidence and degree of cornification at pro-oestrus is normal but if it is more marked then the smear may be recorded as PE, although still regarded as pro-oestrus for cycle staging purposes, provided the smear is seen the day before oestrus or three days after the last oestrus.

6.10 Although it should be possible to easily recognise the majority of smears as typical examples of E, M, D or P, or a transition between two of these stages, there will inevitably be some smears that are difficult to classify, especially with some types of treatment-related changes. Whilst a reasonable effort should be made to classify smears by a cycle stage, it is sometimes better to record just the cell types for a genuinely atypical smear, rather than ‘forcing’ a particular stage classification onto a smear. This would create an artificially normal impression for that particular smear and could result in an abnormal sequence of stages being recorded, if an incorrect stage has been assigned. The relative importance of the occasional atypical smear is best judged retrospectively, when all smears have been examined, but this can only be achieved if the original smear recording reflects the unusual appearance. Smear assessments can be recorded according to the following degrees of precision:

- a) Single stage (E, M, D or P) - if within the normal range of appearance.
- b) Transitional stage (e.g. MD or DP) - if characteristic of both stages.
- c) Single or transitional stage with additional comment - if recognisable but not typical e.g. D + Corn (D but with more cornified cells than normal).
- d) Cell types only - if not sufficiently normal to classify as a stage.

TABLE 1. Vaginal smear cell types and numbers during the oestrous cycle of the rat¹

Stage	Typical cell numbers					Reliability*
	Leucocytes	Nucleated epithelials	Cornified	Non-nucleated epithelials	Total	
Oestrus	-	-/+	+++	+/-	+++	+++
Metoestrus	+++	-	-/+	+	+++	++
Di-oestrus	++	+/-	+/-	+/-	+/>++	+++
Pro-oestrus	-	++	-/+	-	+/>++	++

* Probability of stage being seen on expected day in smears taken between 08:00 and 10:00 from females in room with 12:12 light: dark cycle and lights on at 06:00 GMT

- None of very few

+ Low

++ Moderate

+++ High

¹ Based on pipette smears; swab smears often show fewer leucocytes and more cornified at M and D than indicated in this table

7. Smear sequences and cycle characteristics

7.1 Many of the possible smear sequences have already been described in the individual cycle stage sections. The following details show the relationship between the most commonly seen smear sequences and specific cycle lengths and cycle types. Although the short smearing period used will not allow a detailed analysis of cycle length in these studies, an understanding of normal and abnormal cycles in the rat is important for even the most basic assessment of the vaginal smear data.

7.2 The vast majority of rats show regular 4-day cycles from soon after vaginal opening until at least 6 months old, although a small but significant proportion will have occasional 5-day cycles amongst these 4-day cycles. A very small number will have regular 5-day cycles. As rats get older the incidence of 5-day cycles usually increases; then the occasional longer (e.g. 6 or 7-day) cycles begin to occur and some females may show periods of extended oestrus. Eventually the cycles disappear and oestrus only occurs at irregular and extended intervals (typically 12 to 16 days), between which the females show di-oestrous smears and are considered to be acyclic.

7.3 In the following cycles, ovulation and (if in pairing) mating would normally occur during the night preceding the day of oestrus, or the final day of oestrus if two or more consecutive days of vaginal oestrus occur in a cycle (as in 5-day cycle). The main exception to this is when females are in extended or continuous oestrus, where ovulation does not normally occur except, sometimes, if mating takes place.

7.4 4-day cycle

The classic cycle consists of the following sequence of smears:

	E	M	D	P	E	M
Cycle day	1	2	3	4	1	

But, as detailed in the individual cycle stage sections, some stages may appear to be ‘missed’ due to variation in the length of the stage, the time of smearing and between individual animals. The following sequences are therefore all possible and a low incidence should not necessarily be considered abnormal:

E **D** **D** **P** **E** **D** (D instead of M on Day 1)

E **M** **D** **D** **E** **M** (D instead of P on Day 3)

7.5 5-day cycle

A small proportion of females show 5-day cycles, either amongst other cycles of 4 days or, more rarely, repeatedly and consistently. This can be achieved by showing a 2nd day of any of the four stages but the two most common stages to be repeated are oestrus and di-oestrus:

	E	M	D	P	E	E	M
Cycle day	1	2	3	4	5	1	
	E	M	D	D	P	E	M
Cycle day	1	2	3	4	5	1	

In the first example, showing two consecutive days of oestrus, it is important to note that ovulation will only occur on the night preceding the 2nd day of oestrus. The first day of apparent oestrus is vaginal oestrus only; although the smear may appear identical to that seen on the 2nd day, ovulation will not occur and the female will not mate if in pairing with a male.

7.6 Acyclic

A small number of female rats will spontaneously show episodes, usually lasting 12 to 16 days, of repeated di-oestrous smears, when they are considered to be acyclic. Such episodes can also be triggered by stimulation of the cervix during smearing with swabs or mating with an infertile male, in which case the female will also be pseudopregnant. The repeated di-oestrous smears may appear similar to the normal, cycling di-oestrus but they often contain more leucocytes or mucus.

Typical acyclic smear sequence

E M D D D D D D D D D P E M

7.7 Irregular cycle length

Any cycle length of less than 4 days or more than 5 days (e.g. 3 or 7 days) is unusual and should therefore be considered as abnormal in the adult (but not aged) female rat. Long cycles (6 or 7 days) are generally more common than short cycles of less than 4 days. Such cycles may occur as isolated occurrences amongst regular 4 or 5-day cycles or on a more frequent basis.

7.8 Extended oestrus

Any episode of four or more consecutive days of vaginal oestrus is considered to be abnormal. Some females may show short 4 or 5-day episodes of extended oestrus whilst others may show permanent or continuous oestrus throughout the smearing period. The incidence will increase naturally with age, or as a result of exposure to continuous or extended periods of light in the animal room. The appearance of the oestrous smears may be similar to that seen during a normal 4 or 5-day cycle but the cells are often less cornified (PE appearance) or a few leucocytes may sometimes be present (EM appearance). These episodes of vaginal cornification do not reflect normal, ovulating days of oestrus, although mating will often occur on the first night of pairing, which may trigger ovulation.

8. Reporting and interpretation

- 8.1 The main aim of the termination / pre-termination smears is to establish the cycle stage on the day of sacrifice. Individual cycle lengths would normally be assessed by smearing for a minimum of approximately two weeks. A shorter smearing period (at least four days) would preclude any accurate determinations of individual cycle lengths, but gross effects on cycles may still be detected e.g. failure to show oestrus during 4/5-day smearing period would suggest females were acyclic, and vaginal oestrus occurring every day would obviously be abnormal.
- 8.2 It is recommended, therefore, that all individual smears are reviewed and reported, in addition to presenting the cycle stage on the day of sacrifice. Simple group total calculations and statistics can then be applied if there are obvious inter-group differences in the incidence of females showing the expected one or two days of vaginal oestrus during the smearing period. However, with a typical group size of only five females, little toxicological significance can be attached to inter-group differences in the incidence of females with normal cycles showing a particular stage on the day of sacrifice.
- 8.3 If an attempt is made to correlate the cycle stage shown in the vaginal smear with the histopathological examination of the reproductive organs of individual females on the day of sacrifice, it is important to remember the potential for the di-oestrous smear to be seen on the expected days of metoestrus or pro-oestrus, as explained earlier (Sections 6.8 and 7.4). This may account for some discrepancies between the two methods of cycle stage assessment.

9. Bibliography

These references provide useful background information to support many of the views and recommendations detailed in these guidance notes.

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Figures 3 - 6. Main stages of the oestrous cycle in the rat

Pipette lavage smears (unstained) with original microscope magnification of 100X

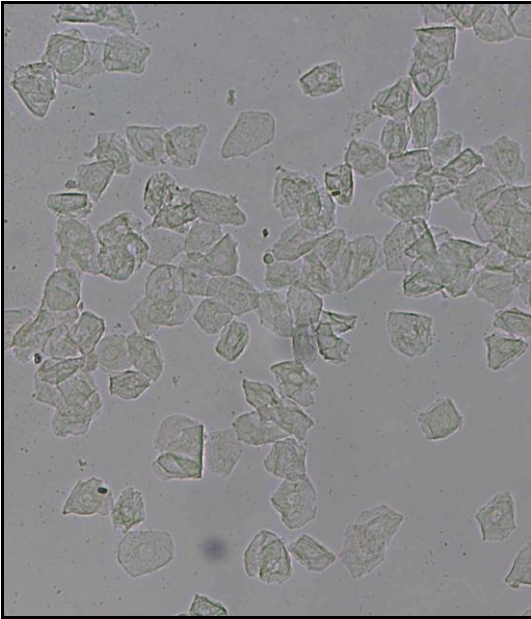


Figure 3 - Oestrus

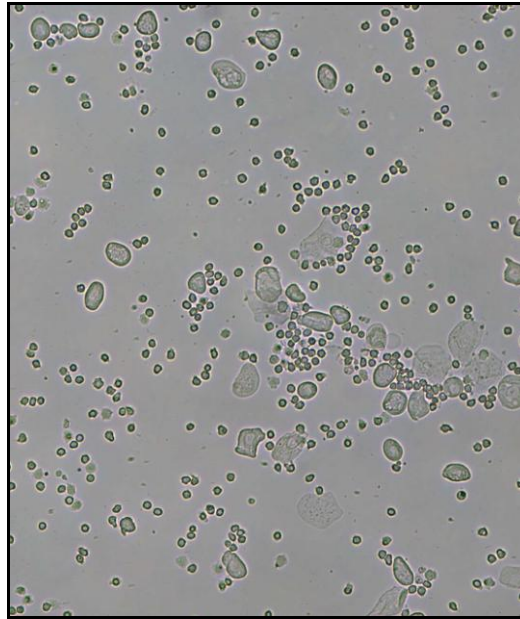


Figure 4 - Metoestrus (late)



Figure 5 - Di-oestrus

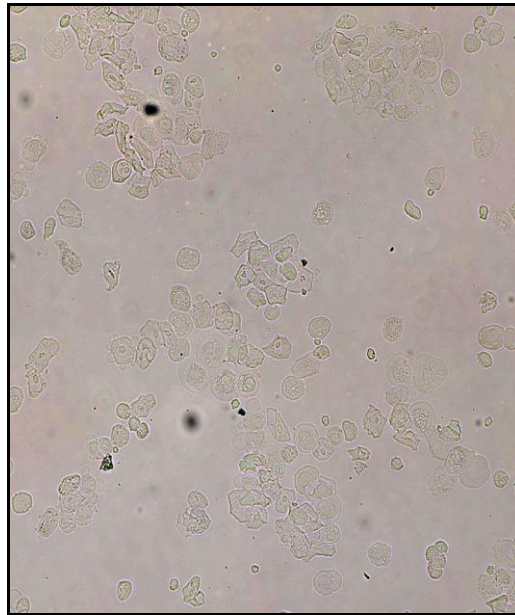


Figure 6 - Pro-oestrus

Figure 3 - Oestrus: large cornified cells in clumps. **Figure 4 - Metoestrus (late):** large numbers of leucocytes with smaller numbers of non-nucleated epithelial cells (note characteristic clumping together of two cell types centre-right). **Figure 5 - Di-oestrus:** mainly leucocytes but with small number of epithelial and cornified cells. **Figure 6 - Pro-oestrus:** epithelial cells - mostly rounded but some cells showing early stages of cornification of approaching oestrus.