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## Monitoring oestrus and ovulation in brushtail possums

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### ABSTRACT

The time of ovulation following removal of pouch young (RPY) was determined in 240 possums by repeated laparoscopic observation of the ovaries, and in a subset of these animals (n=20) also from changes in vaginal mucus. In a separate experiment (n=18), the time of oestrus was estimated from an influx of epithelial cells and leucocytes in urine, and confirmed by mating and hormonal data.

A preovulatory follicle was observed at laparoscopy in all animals that ovulated (n=138), but not in any that did not (n=102). An influx of neutrophils in vaginal mucus occurred in 12/20 possums, including 4 that failed to ovulate. An increase in epithelial cells in urine was observed in 9/12 possums that exhibited an LH surge. However, this change occurred after the time of the preovulatory LH surge in 6 of those animals and peak cell numbers varied widely between all animals.

These studies confirm that although changes in the cell content of mucus and urine can retrospectively define stage of the oestrous cycle, they are of little value for predicting impending oestrus, the LH surge or ovulation.

**Keywords:** brushtail possum; oestrus; ovulation; vaginal smears; urine; laparoscopy.

### INTRODUCTION

Many studies of reproductive physiology of possums, especially those investigating potential biocontrol strategies, require the collection of tissues at known stages of the oestrous cycle. To achieve this, a reliable method of identifying oestrus or ovulation is needed. The traditional method of synchronising oestrus and ovulation in marsupials is removal of pouch young (RPY), with the day of oestrus then being estimated from changes in cellular components of vaginal mucus or urine (Pilton and Sharman, 1962; Tynedale-Biscoe and Renfree, 1987). However, laparoscopic studies involving direct observation of the ovaries to accurately determine the time of ovulation have shown that this method can be very imprecise in possums (Crawford *et al.*, 1997; 1998). In this study, we monitored the time of oestrus and/or ovulation in possums following RPY, using either laparoscopy, mating or hormone data to confirm reproductive status and related this to cellular changes in mucus or urine.

### MATERIALS AND METHODS

#### Animals and management

The possums included in the study were all wild-caught adult females (liveweight > 2.0 kg) that were maintained in a group housing system (McLeod *et al.*, 1997) at either the AgResearch Invermay (Experiment 1) or AgResearch Wallaceville (Experiment 2) possum facilities. All animals were housed under conditions of natural daylength and temperature and were fed an *ab libitum* diet of fresh fruit, bread and cereal-based pellets, as well as selective browsing of *Pinus radiata* branches. Fresh water was always available.

AgResearch Animal Ethics Committees under the Animal Protection (Codes of Ethical Conduct) Regulations 1987 approved all experimental procedures.

#### Experimental design

##### Experiment 1

This is a compilation of data from a series of investigations in which the incidence and timing of ovulation was determined in groups of female possums following removal of their pouch young (N = 240) by repeated laparoscopic observations of their ovaries. All animals were group-housed (6-15 animals/pen) over the period of laparoscopic examination, either as single-sex groups in isolation from males or in the presence of males (ratio 2-4 females/male). We have previously shown that the presence of males does not affect the timing of ovulation following RPY, although it has a significant influence on the proportion of females that do ovulate (Crawford *et al.*, 1998). Within experimental groups, all females had their pouch young removed on the same day and these were euthanased immediately by intraperitoneal injection of barbiturate (Euthal; 1-4ml, Delta Veterinary Laboratories Ltd, Hornsby, NSW, Australia).

In a subset of 20 animals, vaginal smears were collected daily for 20 days from the time of pouch young removal, following the procedure of Curlewis *et al.*, (1985), as described by Crawford *et al.*, (1997). Pro-oestrus was identified by a marked increase in the number of cornified epithelial cells present in the smear and the first day of oestrus was defined as the day on which there was a large influx of neutrophils within the vaginal mucus (Pilton and Sharman, 1962).

##### Experiment 2

For this experiment urine samples (1-4ml) were collected daily for 20 days, from female possums (n=18)

housed in the presence of males (ratio of 3:1 females to males). Changes in vaginal cytology were monitored from cell types found in the samples; namely epithelial cells, keratinised epithelial cells and neutrophils. Moreover, in some cases sperm cells could be identified and used to confirm when mating had occurred. For this, 20µl of urine was placed in a hemocytometer and using a phase contrast microscope, the different cell types were identified and counted. The time of pro-oestrus was estimated as described in Experiment 1 above.

One week after the monitoring of vaginal cytology had begun, all possums were fitted with indwelling jugular catheters (Curlewis *et al.*, 1985) and daily blood samples were taken for the remainder of the experiment. Plasma was stored at -20°C until assayed. Two days after blood sampling began, pouch young were removed from females where present (n=14). All females were euthanased by i.v. injection of barbiturate (Euthal) at completion of the experimental period and the ovaries examined to confirm reproductive status.

Possums were determined to have ovulated if a corpus luteum or new ovulation site was present at the time of slaughter and/or there was evidence of a LH surge (e.g. plasma LH concentration > 2ng/ml).

### Laparoscopy

Each animal underwent a maximum of eight laparoscopic examinations, carried out at one to four day intervals following RPY, with the frequency of laparoscopy being increased around the expected time of ovulation which is indicated by the emergence and subsequent vascularisation of a preovulatory follicle. The laparoscopy procedure is described in Crawford *et al.*, (1997). In brief, anaesthesia was induced and maintained by halothane inhalation (Fluothane, ICI New Zealand Ltd, Lower Hutt), with local anaesthetic (lignocaine hypochloride, Techvet Laboratories Ltd, Auckland) being administered at the sites of incision. A 4 mm diameter endoscope (Karl Storz, Tuttlingen, Germany) was used for all laparoscopies. At completion of laparoscopic examination the incisions were closed with a single michel clip and dusted with antibiotic powder (Negasunt, Bayer New Zealand Ltd, Petone). The same incision sites were re-used for subsequent laparoscopies. The progressive development of preovulatory follicles and the time of ovulation were determined according to the criteria described by Crawford *et al.*, (1997), and with vascularisation of a preovulatory follicle indicative of impending ovulation, the frequency of laparoscopy was increased from four to one-day intervals.

### Hormone Analyses

Plasma concentrations of LH were measured by heterologous radioimmunoassay using the procedure previously described for the possum (Moore *et al.*, 1997). Within this study, the limit of detection was 0.15 ng/ml plasma and intra- and inter-assay coefficients of variation were both < 10%.

### Statistical Analyses

Comparison of liveweight between animals that ovulated and those that did not was made by Students t-test.

## RESULTS

### Experiment 1

Of the 240 possums that underwent laparoscopic observations from the time of pouch young removal, 132 ovulated within the period of monitoring and 108 did not. There was no correlation in mean liveweight at the time of RPY between those animals that ovulated ( $2.48 \pm 0.05$  kg) and those that did not ( $2.44 \pm 0.09$  kg). At the time of RPY, numerous small (1-3 mm) healthy and atretic follicles were present on the surface of the ovaries and in all animals, the uteri and the vaginal cul-de-sac were small and pale in colour. In more than 70% of the animals examined by laparoscopy, 1-4 of the healthy follicles increased in diameter over the following 4-8 days to reach a diameter of about 2.5-4.0 mm. This development was coincident with a modest increase in size and in vascularisation of the cul-de-sac. In all those animals that subsequently ovulated, a single healthy follicle became predominant, increasing in size to approximately 5-6mm diameter and protruding from the surface of the ovary. The emergence of these preovulatory follicles, which were easily identifiable, occurred within 12 days of RPY. Further development of preovulatory follicles included progressive vascularisation of the surface. In a proportion of the possums that ovulated, a concentrated area of vascularisation was observed to develop at the apical pole of the preovulatory follicle within one day of the time of ovulation.

In the subset of animals from which vaginal smears were taken from the day of RPY, 11 ovulated and 9 did not. Mucus samples indicative of pro-oestrus were observed in 8/11 possums that ovulated at a mean time of 2 days (range 1 - 4 days) before ovulation. Two of the nine animals that failed to ovulate exhibited pro-oestrus type smears for a period of 2 days or more.

### Experiment 2

Fifteen possums cycled during the course of this experiment including 4 possums that did not have pouch young removed. There was evidence of an LH surge in 12 possums and the mean time from RPY to the LH surge was  $9.3 \pm 0.6$  days (mean  $\pm$  s.e.m.). However, in only 3 animals did an increase in epithelial cells occur before the day of the LH surge. In 6 animals there was an increase in cell numbers the day after the LH surge and in the remaining 3 animals no increase in cell numbers was noted at all. The number of cells that constituted an 'increase' was highly variable (range 40 - 4000).

Of the 3 possums that did not cycle, 2 had a large follicle (~5mm diameter) present on one ovary and enlarged reproductive tracts on the day of slaughter (13 days after RPY), suggesting these follicles were oestrogen active. The other possum had no follicles greater than 1mm diameter present on its ovaries and its reproductive tract was small.

## DISCUSSION

These studies confirm previous reports that a proportion of possums fail to ovulate following RPY, irrespective of the stage of the breeding season at which the procedure is carried out (Crawford *et al.*, 1997:1998). The physiological basis for failure of ovulation following RPY remains to be determined, although it is clear that isolation from males increases the incidence of anovulation (Crawford *et al.*, 1998). Interestingly, the initial ovarian response to RPY including the development of medium-sized follicles often occurs in animals that subsequently fail to ovulate. The fact that the development of medium-sized follicles is accompanied by an increase in size and vascularisation of reproductive tract tissues strongly suggests that these follicles secrete oestrogen (Crawford *et al.*, 1999).

Repeated laparoscopic observation of the ovaries following RPY has proved to be a very reliable method of monitoring the incidence of ovulation, and to a limited extent, of predicting the time of ovulation (Crawford *et al.*, 1997). However, laparoscopy is an invasive surgical technique requiring anaesthesia, skilled personnel and specialised equipment. Obviously, a non-invasive method of monitoring oestrus and/or ovulation in possums would be of considerable advantage. Monitoring changes in the cellular components of vaginal mucus over the oestrous cycle has been used successfully as a non-invasive method of determining the time of oestrus in several eutherian species, especially rodents (Mandl, 1951). This approach has also been used in marsupials (Pilton and Sharman, 1962) and the present study confirms that changes in numbers of epithelial cells and of leucocytes within vaginal mucus do occur in response to physiological and hormonal events of the oestrous cycle in the brushtail possum. However, the number of cells observed within a mucus sample at any given stage of the cycle can vary by 10 to 50-fold between individual possums. As a consequence, in some animals it may be necessary to monitor changes in cell numbers over several oestrous cycles before the time of oestrus can be identified with confidence.

In summary, oestrus and ovulation are poorly synchronised in possums following RPY and can occur anywhere between 6 and 18 days later. Furthermore, there is a significant incidence of anovulation following RPY, even when females are housed in the presence of males. Methods that are based on monitoring changes in cellular components of vaginal mucus or urine can identify the time of oestrus with some accuracy when these changes are assessed retrospectively. However, this negates their usefulness when the requirement is to predict the time of oestrus, the preovulatory LH surge or ovulation. Although laparoscopy can be used to reliably monitor preovulatory follicle development and ovulation, this is a very invasive and time-consuming procedure. The need for a simple, non-invasive method of predicting ovulation in the possum remains to be met.

## ACKNOWLEDGEMENTS

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