Hormonal evidence for induced ovulation in *Monodelphis domestica*

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Summary. Breeding activity in the South American grey opossum, *Monodelphis domestica*, is stimulated by the presence of males. This study presents the first analysis of changes in concentrations of plasma progesterone during pregnant and nonpregnant cycles.

In Expt 1, females were paired with either intact or vasectomized males to stimulate a reproductive cycle, or were isolated from males. Within 4–8 days of pairing, females showed marked changes in the size of the urogenital opening (vulva), which were paralleled by similar changes in body weight ($11.4 \pm 2\%$; mean $\pm$ SEM; $n = 13$). There was a second increase and decrease in body weight ($21.7 \pm 3.3\%$; $n = 13$) in pregnant and nonpregnant cycles during the luteal phase of the cycle. Changes in concentrations of plasma progesterone, determined from samples collected three times a week, were similar in pregnant and nonpregnant cycles. Plasma progesterone concentration, which was increased for 15–18 days, showed two distinct peaks. The first peak, $3\text{--}4.5 \text{ ng ml}^{-1}$, occurred immediately after the decrease in vulval swelling, and the second peak ($1\text{--}2 \text{ ng ml}^{-1}$) occurred 8–12 days later. Concentrations of plasma progesterone were low before parturition occurred. Females isolated from males for 35 days showed no changes in body weight or vulval swelling and concentrations of plasma progesterone remained undetectable.

In Expt 2, concentrations of luteinizing hormone (LH) in plasma were determined twice a day for the first few days after pairing. In three of five females an LH peak was detected coincident with the maximal swelling of the vulva.

Keywords: progesterone; pregnancy; oestrous cycle; luteinizing hormone; grey opossum; marsupial; induced ovulation

Introduction

In several eutherian species, the presence of a male, of male-related pheromones or of both (Whitten, 1966; Milligan, 1980) stimulates oestrus and ovulation in females (Whitten, 1956; Hughes, 1964; Carter *et al.*, 1980). In most marsupials, ovulation occurs spontaneously (Tyndale-Biscoe & Renfree, 1987); the phenomenon of male-induced oestrus and ovulation in females has been reported in only two species, the South American grey, short-tailed opossum (*Monodelphis domestica*), a member of the most ancient marsupial family, the Didelphidae (Fadem *et al.*, 1982; VandeBerg, 1983), and an Australian marsupial, a small ratkangaroo, the brushtail bettong, *Bettongia penicillata*, of the superfamily Macropodoidea (Smith, 1992; Hinds & Smith, 1992). In several other marsupials, including the woolly opossum, *Caluromys philander*, although ovulation will occur spontaneously, the frequency and synchrony of oestrus can be enhanced by the presence of males (Perret & Ben M'Barek, 1991).

In *M. domestica*, stimulation of oestrus and ovulation is primarily influenced by social and olfactory cues (Fadem *et al.*, 1982; VandeBerg, 1983; Fadem, 1985; Fadem & Rayve, 1985; Fadem, 1986, 1987). Unlike the woolly opossum, in which isolated females continue to undergo infrequent oestrous cycles (Perret & Ben M'Barek, 1991), female *M. domestica* isolated from males for long periods reportedly become sterile (Maliniak & Taft, 1981). However, breeding activity can be stimulated in *M. domestica* by the introduction of a male (Fadem *et al.*, 1982), with sensory contact alone effectively inducing oestrus in 60% of females (VandeBerg, 1983; Fadem, 1985). Females are also stimulated when moved to cages previously occupied by intact males (Fadem, 1987; 1989). Nevertheless, it is not only the presence of a male, but also, more particularly, the introduction of a new male, that stimulates breeding (Fadem *et al.*, 1982; VandeBerg, 1983; Fadem, 1985; Fadem & Rayve, 1985). After the introduction of a new male, a female enters oestrus and most conceptions occur within 9 days; births occur 19–24 days after the initial pairing, indicating a gestation period of 14–15 days (Fadem, 1985; VandeBerg, 1983).

Fadem (1989) observed differences in oestradiol concentrations in plasma collected from females on two occasions, before and during pheromonal stimulation. However, sequential changes in any of the reproductive hormones during an oestrous cycle or pregnancy have not been described. The present study determined concentrations of progesterone in plasma from females paired with intact or vasectomized males and in females isolated from males. Consistent changes in vulval appearance and body weight were considered in relation to changes in concentrations of progesterone or LH in plasma.

Materials and Methods

Animals

The grey short-tailed opossum, *M. domestica*, is a South American didelphid marsupial from the Chaco, Cerrado and Caatinga regions of Brazil (Streilein, 1982). The adult female, which weighs approximately 70–120 g, reproduces successfully under suitable laboratory conditions throughout the year (Fadem *et al.*, 1982; VandeBerg, 1983). The colony of *M. domestica* at University of Southampton, UK, was established from eight breeding pairs kindly supplied by Mark Ferguson (University of Manchester, UK).

Most animals were held singly or in pairs for breeding in rat boxes made of polycarbonate or polypropylene and containing a deep layer of wood shavings. Generally, females were paired with males for 13 days after which they were separated and placed in plastic nesting boxes (19 × 11 × 7 cm) filled with newspaper strips and wood shavings. Animals were fed daily on a feline carnivore diet (Special Diet Services, Stepfield, Witham, Essex, UK) and pregnant animals were supplemented with Farex (Farley Health Products Ltd, Nottingham, UK). Water was freely available. The animal room was maintained at an ambient temperature of 27°C and a cycle of 14 h light:10 h dark.

Assessment of vulval swelling

Previous observations of females paired with males indicated that there were significant changes in the size of the vulva/urogenital opening soon after pairing was established. To assess these changes objectively, the swelling of the vulva was scored once or twice a day and given a rating from 1 to 5 (1, small, pale, inactive vulva; 5, enlarged, vascular vulva) (Fig. 1).

Vasectomy

Six male *M. domestica* were vasectomized using a procedure similar to that described for the eastern quoll, *Dasyurus viverrinus* (Hinds, 1989). Vasectomies were performed at least 2 weeks before the males were paired with any females used in this study.

Collection of blood samples

Animals were wrapped in an envelope of paper towelling, and placed in a purpose-built Perspex cage with the tail exposed. The lateral tail vein was punctured with a 26 g needle and blood (about 150–200 µl) collected into three or four 50 µl heparinized capillary tubes. The samples were transferred to microcentrifuge tubes and held on ice until they

Fig. 1. Changes in the size and appearance of the everted urogenital opening (vulva) of a female *Monodelphis domestica* which was activated by the presence of a male. (a) Rating 1, vulva small, pale and inactive; (b) rating 3, vulva enlarging, more vascular; (c) rating 5 (maximum size), vulva enlarged, oedematous and well vascularized.

were centrifuged for 3 min at 3500 *g*. The volume of plasma (25–100 μ l) was recorded, and the plasma was frozen and then freeze-dried. The lyophilized samples were sealed with parafilm, stored in a desiccator at room temperature and subsequently sent to Canberra for analysis.

Hormone assays

Progesterone. The radioimmunoassay originally described for the tammar wallaby, *Macropus eugenii* (Sernia *et al.*, 1980), has been validated for *M. domestica*. The lyophilized samples (25–100 μ l) were reconstituted with double distilled water, made up to the same volume (300 μ l) with assay buffer (0.2 mol sodium phosphate buffer l⁻¹, pH 7.0 in 0.9% saline plus 1.0% gelatin) and then extracted twice with 20 volumes of freshly redistilled *n*-hexane. Antiserum 9817, a gift from R. I. Cox (CSIRO Division of Animal Production, Sydney, Australia) was used at a final dilution of 1:224 000. Duplicate samples of plasma (10–50 μ l) were measured. The sensitivity of the assay was 10 pg per tube or 0.25 ng ml⁻¹ plasma, and the hexane solvent blank and charcoal-treated (steroid-free) plasma blank always measured less than these limits. After extraction with freshly redistilled *n*-hexane the recovery of [³H]progesterone from *M. domestica* plasma was $98.2 \pm 0.6\%$ (mean $\pm$ SD, $n = 6$). The recovery of unlabelled progesterone added to charcoal-treated plasma was within 7% of that expected, while the intra- and interassay coefficients of variation were 7.5% ($n = 12$) and 9.5% ($n = 7$), respectively. Serial dilutions (5–100 μ l) of two pools of plasma collected from females in late pregnancy were parallel to the progesterone standard curve. Pooled plasma (25–150 μ l) collected from adult males showed no displacement of label in the assay system. The addition of charcoal-treated plasma (250 μ l) had no effect on maximum binding (60% in both cases), and standard curves prepared in charcoal-treated plasma or buffer could be superimposed.

Luteinizing hormone. The heterologous radioimmunoassay for determining plasma LH in the tammar wallaby (Sutherland *et al.*, 1980) has been partially validated for *M. domestica* plasma, using antiserum GDN 15 (a gift from G. Niswender) and rat LH-I-6 as tracer and NIH-oLH-S-21 as standard. No pure *M. domestica* LH was available to test for parallelism with the ovine standard curve. However, serial dilutions of a pituitary homogenate were parallel to the standard curve and the recovery of ovine LH added to *M. domestica* plasma was within 10% of the expected value. The intra-assay coefficient of variation was 8.2% ($n = 12$).

Experimental design

Two experiments were performed to determine hormonal changes in females undergoing various reproductive cycles. In Expt 1, the changes in concentration of progesterone in plasma were determined in pregnant and non-pregnant females and in isolated females. In Expt 2, the presence and timing of the pre-ovulatory LH surge was determined. In both experiments, changes in body weight and in the appearance of the vulva were recorded. Initially, changes in the proportions of cell types in vaginal smears were to be used to determine oestrus, but the technique proved unsatisfactory because most smears contained too few cells for reliable diagnosis.

Experiment 1. Three groups of females (50–90 g) were established. Females of group 1 ($n = 9$) were paired with intact males and those of group 2 ($n = 6$) with vasectomized males to examine changes in concentration of progesterone in plasma in pregnant and nonpregnant cycles respectively. The day of pairing with a male was designated day 0.

Beginning on day 0, body weights were recorded daily and for the next 8–12 days changes in the size of the urogenital opening (vulval swelling) were assessed. Males were removed between days 13 and 20. Approximately 8 days after the maximum swelling of the vulva was attained the females were palpated to assess the size of the uteri. The palpation was performed without knowledge of the expected reproductive state of the female. Blood samples were taken three times each week for analysis of progesterone in plasma.

Another group of five females (group 3) was isolated from all contact with males for 35 days to determine whether females underwent oestrous cycles in the absence of males. Vulval swelling was assessed daily, body weight changes were recorded on each weekday, and blood was sampled for progesterone determination three times a week. Animals were palpated as described for group 1.

Experiment 2. The relationship between vulval swelling, body weight changes and the pre-ovulatory LH peak was determined by pairing females ($n = 5$) with intact males and blood samples were taken twice a day (at 08:30 h and 17:00 h) beginning after the vulval rating reached 2–3 and ending after the vulval rating decreased to <4; these samples were analysed for LH. Because the body size of *M. domestica* is small, no blood samples were taken for analysis of progesterone throughout the cycle, but animals were palpated 8 days after maximal vulval swelling and body weights were recorded for 25 days after pairing.

Statistical analyses

Paired *t* tests were used to determine differences among various parameters for each reproductive state. The parameters compared were the intervals from pairing to the first rise in progesterone and to birth, and the concentrations of progesterone at different times during pregnant and nonpregnant cycles. All results are presented as means $\pm$ SEM.

Three criteria were used to assess stimulation by a male: (i) changes in the swelling of the vulva, (ii) changes in body weight and (iii) a subsequent increase in progesterone from <0.5 to >1 ng ml⁻¹.

Results

Changes in vulval size and body weight in the presence of males

Before association with a male the urogenital opening/vulva was pale and small (Fig. 1a, rating 1–2). Within 2–5 days of pairing, there were distinct changes in all stimulated animals in the colour and degree of enlargement of the everted vulva (Fig. 1b, rating 3). The maximum size of the vulva was reached within 4–8 days in most animals (Fig. 1c, rating 5). During the next 2–3 days the size of the vulva decreased to rating 1–2.

Parallel increases and decreases ($11.4 \pm 2\%$; $n = 13$) in body weight accompanied these changes in the vulva (Figs 2b, d, 3b and 5a–e). After the vulval score had fallen to rating 1–2, there was a second increase in body weight ($21.7 \pm 3.3\%$; $n = 13$) which reflected the increase in the size of the uterus. In all females stimulated by the presence of a male, the uteri could be readily palpated 8 days after maximal vulval size (Figs 2b, d and 3b). Unstimulated or isolated females showed no significant changes in body weight or size of the vulva, and the uteri remained small (Fig. 4).

Experiment 1. Progesterone profiles in stimulated and unstimulated females

Group 1. Eight of nine females paired with an intact male showed evidence of stimulated breeding activity, and five of these gave birth. The remaining female was not stimulated by the presence of a male and remained unstimulated when monitored for a further 35 days in the absence of a male. Progesterone concentrations in plasma showed a marked increase within two days of the maximal vulval score. The changes in the progesterone profile were very similar for all stimulated females (Figs 2a, c and 3a). The major difference between individuals both within and between groups was the day of the first increase above 1 ng ml⁻¹. In group 1 this occurred on day 7 (range 5–13 days) (Fig. 2a) and in group 2 on day 9 (range 7–12 days) (Fig. 2c). Thereafter maximum concentrations and the pattern were similar. After the initial peak in progesterone ($3\text{--}4$ ng ml⁻¹), a second peak of lower magnitude (2 ng ml⁻¹) occurred 9–10 days later. Basal concentrations (<0.5 ng ml⁻¹) were reached 14–15 days after the first increase above 1 ng ml⁻¹. In females that produced a litter, progesterone concentrations in plasma were always less than 1 ng ml⁻¹ on the day of or day after parturition (Figs 2a and 3a). In group 1, birth occurred in individual females 19, 20, 22, 23 and

Fig. 2. Progesterone concentrations (■) in plasma (mean $\pm$ SEM) for female *Monodelphis domestica* (a) during a pregnancy after pairing with an intact male ($n = 5$) and (c) during a nonpregnant cycle after pairing with a vasectomized male ($n = 5$). Values have been aligned on the mean day of the first increase in progesterone > 1 ng ml⁻¹. Solid arrow indicates mean day of birth of litters. Corresponding changes in concentration of progesterone in plasma, during (b) pregnancy and (d) the nonpregnant cycle, respectively.

27 days after pairing (22.2 ± 1.4 days, mean $\pm$ SEM), about 15 days after the first evidence of a decrease in the swelling of the vulva (Figs 2a, b and 3).

One female in group 1 was retained with the same male although it was evident from palpation that she was not pregnant. She underwent a second cycle of stimulation as indicated by parallel changes in body weight and vulval size and increases in progesterone concentrations as in the first cycle. However she did not produce a litter. Another stimulated, but nonpregnant, female showed no subsequent changes in plasma progesterone after the intact male was removed from the cage.

Group 2. Five of the six females paired with a vasectomized male (group 2) showed evidence of stimulation. The remaining female showed an initial increase and decrease in both vulval size and body weight but no subsequent changes in body weight and no increases in plasma progesterone.

The profiles of progesterone concentration in plasma of activated females were similar to those of group 1 animals: the initial peak of progesterone was of similar magnitude and duration, and the

Fig. 3. Results for an individual female *Monodelphis domestica* during pregnancy. (a) Progesterone concentrations (ng ml⁻¹) in plasma (■), and (b) changes in body weight (g) (○) and vulval rating (●). Solid arrow indicates day of birth of litter.

Fig. 4. Results for five female *Monodelphis domestica* isolated from males for 35 days. Progesterone concentrations (ng ml⁻¹) in plasma (mean $\pm$ SEM) (■), percentage change in body weight (mean $\pm$ SEM) (○) and changes in vulval rating (mean $\pm$ SEM) (●).

second peak of lesser magnitude was also present (Fig. 2c). Progesterone concentrations decreased to <0.5 ng ml⁻¹ about 15 days after the vulval rating began to decline. The increases in body weight (Fig. 2d) in the first few days after pairing and subsequently were greater than for group 1

Fig. 5. Relationship between concentrations of luteinizing hormone (LH) in plasma (■), changes in body weight (○) and vulval ratings (●) in individual female *Monodelphis domestica* after pairing with intact males. Arrow indicates day of birth of litter.

females paired with intact males (Fig. 2b). The group 2 females were younger at the time of pairing and had not reached their mature body weight. Thus the changes in body weight associated with the cycle stimulated by the presence of a male occurred presumably along with a component of growth in these females.

When females of groups 1 and 2 were palpated eight days after the swelling of the vulva had begun to decrease, pregnant and nonpregnant females could not be distinguished on the basis of uterine size. Five days later, the uteri of nonpregnant females were distinctly smaller than those of females that subsequently gave birth.

Group 3. None of the females isolated from males showed any evidence of stimulation during 35 days of observation. No changes in body weight, appearance of the vulva or increases in plasma progesterone were observed (Fig. 4). The absence of changes in the two females (one each from groups 1 and 2), which were kept in the presence of a male were identical to those of group 3.

Experiment 2. Detection of the pre-ovulatory LH peak

An LH peak was observed in three of five animals monitored twice daily after pairing with a male. The peak, ranging in magnitude from 8.4 to $>50 \text{ ng ml}^{-1}$, occurred in two consecutive samples in two of three animals; LH concentrations remained low to undetectable at all other sampling times. All females showed evidence of stimulation in terms of body weight changes and in the swelling of the vulva (Fig. 5). Where the LH peak was detected it was coincident with the maximal swelling of the vulva and was followed by a second increase in body weight (Fig. 5a, b, d). In the absence of a detectable LH peak subsequent changes in body weight were not observed (Fig. 5c, e). Only one female gave birth and this occurred 17 days after the occurrence of the LH peak (Fig. 5d).

Discussion

In previous studies of the reproductive physiology of *Monodelphis domestica*, activation of reproduction in females has been shown to follow the introduction of a female to a male (Fadem *et al.*, 1982; VandeBerg, 1983; Fadem, 1985) or to his odours (Fadem, 1987). In this study, we provide the first hormonal evidence confirming that female *M. domestica* are induced ovulators. The introduction of either an intact or vasectomized male was equally effective in stimulating breeding activity in females. Activation was indicated by dramatic changes in the appearance of the vulva and corresponding changes in body weight, followed by a second rise in body weight and increases in plasma progesterone concentrations. By contrast, none of the females isolated from males for 35 days showed any of the above diagnostic changes.

Changes in the appearance and size of the vulva, in conjunction with parallel changes in body weight, provided an excellent indication of stimulation by a male. However, the appearance of these changes does not provide unequivocal evidence that ovulation will follow. For example in group 1, one female showed signs of stimulation 4–7 days after pairing, but no subsequent increase in body weight or changes in concentration of progesterone in plasma occurred. Similarly in Expt 2, in two females there were changes in vulval size and body weight, but no LH peak was detected and no subsequent increase in body weight was observed. We conclude that ovulation did not occur in any of these females. Body weight changes in the pro-oestrous period are also seen in other marsupials, including *Dasyuroides byrnei* (Fletcher, 1989) and another South American marsupial, *Marmosa robinsoni* (Godfrey, 1975). In the latter species, during pro-oestrus the vaginal complex forms a sixth of the body weight of the animal, and the cloaca also becomes swollen and red. Female *M. domestica* in pro-oestrus have significantly higher concentrations of oestradiol in plasma than females that are not in oestrus (Fadem, 1989). Thus, the change in the size of the vulva in *M. domestica* and in other marsupials probably occurs in response to oestrogens produced by the growing follicles. Certainly there was a rapid decline in the vulval size coincident with or soon after the LH peak was detected, at which time, on conventional premise, the follicle would stop producing oestrogens and ovulation would occur (Harder *et al.*, 1985).

The second increase in body weight in *M. domestica*, during the period when progesterone concentrations were high, was probably due to the increase in the size of the uteri which were easily

detected by palpation at this stage. Until close to term the size of the uteri were similar in pregnant and nonpregnant females. Body weight changes during pregnancy and the oestrous cycle have also been reported for the kowari, *D. byrnei* (Fletcher, 1989).

In this study, as described in earlier studies (Fadem *et al.*, 1982; Fadem, 1985, 1987), not all females became activated when associated with a male, and only about half of the pairings with intact males resulted in the birth of a litter. Fadem & Rayve (1985) also report that isolated females undergo either short or long oestrous cycles (14 or 32 days), as determined from changes in cell types in vaginal smears. However, in the present study different criteria were used to assess reproductive activity and isolated females showed no evidence of ovulatory cycles nor were there increases in progesterone during the period of isolation. Because of the lack of progesterone measurements in the study of Fadem & Rayve (1985), and our inability to obtain consistent vaginal smears, the results of the two studies cannot be reconciled. Clearly some females associated with males show vulval enlargement, but no subsequent progesterone increases which would indicate normal ovulation. Thus, although vaginal cornification may occur it is not accompanied by behavioural oestrus or ovulation, because pheromonal input was presumably inappropriate. Despite this interpretation, it is not clear why oestrous cycles of two lengths were observed in isolated females by Fadem & Rayve (1985), unless, as in *Marmosa robinsoni* (Godfrey, 1975), the short cycles are anovular and the longer cycles are normal oestrous cycles.

The profile of concentration of progesterone in plasma was characterized by two peaks. The first peak occurred within 7–9 days after pairing and the second one, slightly lower in magnitude, 9–10 days later, about 16–19 days after initial pairing. This pattern is different from that reported for other species of this reproductive grouping (group 1, see Tyndale-Biscoe & Renfree, 1987), the opossum (*Didelphis virginiana*), possum (*Trichosurus vulpecula*), kowari (*Dasyuroides byrnei*), eastern quoll (*Dasyurus viverrinus*) (see Hinds, 1990 for review) and woolly opossum (*Caluromys philander*) (Perret & Ben M'Barek, 1991), in which the profile is clearly unimodal. The significance of the different pattern of progesterone concentration in *M. domestica* is unknown. Nevertheless, in *M. domestica*, as in the other group 1 species, pregnancy occupies the luteal phase only, and the profiles are similar in pregnant and nonpregnant females indicating that the lifespan of the corpora lutea appears to be unaffected by pregnancy. However, the results of the present study do suggest that the gestation period of *M. domestica* may be longer than 14–15 days (Fadem *et al.* 1982). The period of high progesterone concentration was never less than 15 days in any stimulated female, and birth always occurred after plasma progesterone concentrations had declined to less than 1 ng ml^{-1} . Furthermore, in Expt 2, the only animal that gave birth did so 17 days after the LH peak was detected.

Induced ovulation is relatively common among eutherian species (Whitten, 1966; Milligan, 1980). However, the phenomenon has been described for only two marsupial species, the South American grey opossum, *M. domestica* (Fadem *et al.*, 1982; VandeBergh, 1983) and the Australian potoroid, *B. penicillata*, of the Superfamily Macropodoidea (Smith, 1992; Hinds & Smith, 1992). In the latter species induced ovulation had not been suspected because females were presumed to enter *post-partum* oestrus and ovulate spontaneously, as described for other species of kangaroo, wallaby and rat-kangaroo with a group 3 reproductive pattern (see Tyndale-Biscoe & Renfree, 1987). Until recent studies compared concentrations of progesterone in plasma in *B. penicillata* females associated with, or isolated from, males in the *post-partum* period, it was not realised that the presence of males was critical for successful *post-partum* ovulation (Hinds & Smith, 1992; Smith, 1992). Closer study of more marsupials may reveal other species in which spontaneous oestrus and ovulation does not occur in the absence of males.

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