Age-related changes in plasma oestrogen concentration, behavioural responsiveness to oestrogen, and reproductive success in female gray-tailed voles, *Microtus canicaudus*

Sandra L. Petersen*

Department of Zoology, Oregon State University, Corvallis, Oregon 97331, U.S.A.

Summary. Age-related increases in the incidence of vaginal cornification were associated with increases in the reproductive success of female gray-tailed voles previously isolated from males. The pregnancy rate of females first paired with males at 30–50 days of age was significantly lower than that of females first paired at 90–120 or 150–200 days of age. The improvement was due to increases in propensities to display receptive behaviours and decreases in the incidence of sterile matings. Although plasma oestrogen concentrations increased with age and were higher in receptive than unreceptive females, plasma oestrogen values alone did not account completely for differences in receptive behaviours amongst females of different ages. Females ovariectomized at 30–50 days of age rarely displayed receptive behaviours when treated with large doses of oestradiol benzoate, whereas nearly all females similarly treated were receptive if ovariectomies were performed between 150 and 200 days of age. The receptivity rate of females ovariectomized between 90 and 150 days was intermediate between the rates of the other two groups.

Introduction

Female gray-tailed voles, *M. canicaudus*, show age-related changes in vaginal smear patterns (Petersen, 1986). At 30–50 days of age, females generally exhibit persistently leucocytic vaginal smears; cornified smears are rarely seen. By 90–120 days, most females show vaginal cyclicity with alternating predominance of leucocytes, nucleated epithelial cells or cornified epithelial cells. When examined between 150 and 200 days of age, most females show persistent vaginal cornification. The physiological significance of these changes with reference to reproductive physiology of voles has not been investigated. One explanation for the succession of vaginal smear patterns seen in isolated gray-tailed voles is that they represent reproductive maturation; however, no studies have determined the relationship between vaginal histology and reproductive capacities.

If the age-related changes in vaginal smears of females isolated from males represent maturational changes, one would expect to see increases in reproductive behaviours and fertility. To investigate this hypothesis, the present studies examined behavioural potentials and fertility in female gray-tailed voles of various ages that were isolated from males after weaning. In addition, because oestrogen is known to influence the vaginal epithelium, to increase during maturation of other rodents (Dohler & Wuttke, 1975), and to stimulate expression of receptive behaviours in voles (Dluzen & Carter, 1979; Dewsbury & Hartung, 1982), plasma concentrations of this hormone were measured in females of different ages, and in females of the same age showing different behavioural potentials.

*Present address: Department of Physiology, School of Medicine, University of Maryland, Baltimore, MD 21201, U.S.A.

Materials and Methods

Animals. Gray-tailed voles, *M. canicaudus*, used in these studies were from a colony maintained in a well-ventilated room with a controlled photoperiod (16 h light:8 h dark, lights off at 14:30 h) at Oregon State University. The animals were housed in fibre-glass cages (60 × 15 × 15 cm) which contained hardwood shavings and were covered with hardware cloth. Purina Rat Chow, Rabbit Chow and water were available *ad libitum*.

All voles were weaned at 18 days of age and were housed in sexually-segregated groups of 2–3 siblings per cage until used in the studies. Males and females were housed in the same room but on different racks. All females in a particular study were housed on the same rack.

Behaviour tests. Behaviour tests were conducted 1 h after the onset of dark under dim red lights. The test arena consisted of a sheet-metal cylinder (30 cm in diameter and 50 cm high) on an elevated glass floor. A mirror mounted beneath the floor at a 45° angle allowed ventral viewing.

Copulatory behaviour in gray-tailed voles has been described in detail by Dewsbury & Hartung (1982). In the present studies, a female was considered to be unreceptive if she consistently ran from the male, resisted genital sniffing by avoiding or lunging at the male, and stood on her hind legs and chattered when he approached. A proceptive female typically approached the male, sometimes sniffing, nudging or attempting to mount him. A receptive female allowed genital sniffing, stood still when the male approached from behind, and showed lordosis when mounted.

Before each test, a mature, sexually-experienced male was placed in the arena and allowed 6 min for exploration. Males had been pretested for the propensity to display mating behaviour, and subsequently, each male was used in only one behaviour test because latency to mount becomes shorter with each successive test, regardless of the female used (S. L. Petersen, unpublished data).

At the beginning of each test, the female was introduced, and the behaviours described above were recorded on a 20-channel Esterline-Angus manual recorder. If the male did not investigate the female within 5 min after she was introduced, he was removed and another male substituted. Each test lasted 10 min.

Sampling. Vaginal smears were obtained by gently flushing the vagina with warm tap water expelled from a micropipette. Smears were stained with toluidine blue and examined microscopically. Nucleated epithelial cells, leucocytes and cornified cells were counted in three fields at × 10 magnification and the mean proportion of each cell type per smear was calculated.

Radioimmunoassays for oestrogen and progesterone. Plasma samples were assayed for oestrogen as previously described (Korenman *et al.*, 1974; Petersen, 1986). The sensitivity of the assay was 2.5 pg per tube or 25 pg/ml when 100 µl samples were used. Oestradiol was consistently undetectable in water blanks. The intra-assay coefficient of variation (CV) for 8 replicates was 6.1% and the interassay CV among 6 assays was 3.6%. Nonspecific binding averaged 3.5%.

Samples of plasma pools from animals of various ages and showing different vaginal smear patterns were assayed for progesterone after celite chromatography (Weisz & Ward, 1980), or were assayed without chromatography. No differences were seen between values obtained using these two methods, so all samples were assayed without chromatography.

Progesterone was assayed in 25 µl duplicate aliquants of plasma using general procedures previously described (Koligian & Stormshak, 1977; Petersen, 1986). The sensitivity of the assay was 7.8 pg/tube or 312 pg/ml in 25 µl samples. Progesterone was never detected in water blanks. The intra-assay CV for 8 replicates of a plasma pool was 4.0% and the interassay CV for 10 assays was 6.8%.

Study 1: age and conception rate. At the beginning of this study, vaginal smears from females of three age groups were examined: (A) 30–50 days (N = 20), (B) 90–120 days (N = 8), (C) 150–200 days (N = 20). After the initial examination, each female was placed in the home cage of a

reproductively-active male. These males had sired offspring within the previous 3 weeks, but had been housed with other males for 1 week before pairing with females in this study. This procedure was followed because at least one other species of vole, *M. ochrogaster*, shows pair bonding (Thomas & Birney, 1979; Gavish, Carter & Getz, 1981) which could interfere with mating if a male is taken from the cage of one female and placed with another.

Vaginal smears were obtained daily at 12:00 h for 2 weeks. Males were then removed and females were examined for pregnancy by gentle abdominal palpation. Females were not handled again for 10 days, but cages were checked every 12 h to determine the date of parturition. The date of conception was considered to be the day spermatozoa were found in the vaginal smear. The different age groups were compared in the proportions of females conceiving within 48 h, between 48 h and 1 week, and between 1 and 2 weeks after the male was introduced.

Study 2: plasma oestrogen concentrations in cyclic and persistently oestrous females of different ages. To determine whether oestrogen concentrations change with age, plasma was obtained from females showing either cornified or leucocytic vaginal smears at 90 days of age (N = 50; 20 pools) and at 150 days of age (N = 50; 23 pools). Females showing vaginal cornification at 90 days of age were 'cyclic' and had shown only 1–2 previous periods of cornification; those at 150 days displayed persistent (at least 7 days) vaginal cornification. All samples were obtained between 09:00 and 10:00 h.

Study 3: relationship between receptivity and ovarian hormone concentrations in plasma. Virgin females between 150 and 180 days of age with predominantly cornified cells in the vaginal smear were used in this study. Reproductive behaviours of females were tested as described above and females were classified as receptive or unreceptive. Immediately after the first mount with intromission by the stud male, blood was taken from the receptive female. The mean time between pairing and bleeding was ~3 min; therefore, unreceptive females were bled after 3 min of exposure to reproductively-active males. Progesterone concentrations of receptive (N = 10) and unreceptive females (N = 12) and oestrogen concentrations of receptive (5 pools; 2 animals/pool) and unreceptive females (5 pools; 2 animals/pool) were compared.

Study 4: age and behavioural receptivity in ovariectomized, oestrogen-treated females. The results of Studies 1 and 2 showed that females younger than 150 days of age did not reliably mate. In addition, females between 90 and 120 days of age had lower oestrogen concentrations than did females showing receptivity at 150 days of age. To determine whether differences in behavioural potentials were based on differences in circulating hormone values, ovariectomized females were injected with oestradiol benzoate (Sigma Chemical Company, St Louis, MO) in doses based on body weight, so that oestrogen concentrations amongst females of different ages would be similar. The doses used were based on the results of preliminary behavioural studies and on doses previously reported as effective in eliciting behaviour in female voles (Dewsbury & Hartung, 1982).

Ovariectomized females representing the 3 age groups described in Study 1 were used (A, N = 28; B, N = 11; C, N = 23). Bilateral ovariectomies were performed under anaesthesia obtained by subcutaneously injecting 125 µg ketamine HCl/g body weight (Vetalar: Parke Davis, Morris Plains, NJ) and 7 µg xylazine/g body weight (Rompun: Haver-Lockhart, Shawnee, KS). Vaginal smears and body weights were obtained at the time of surgery.

After 1 week, each female received daily intramuscular injections of 1 µg oestradiol benzoate/g body weight for 3 days. At 48 h after the last injection, females were tested for receptivity as described above. Females ovariectomized between 30 and 50 days of age were retested when they were 90–120 days, using the same oestradiol benzoate treatment regimen. The proportion of receptive females and the mean latency to be mounted were compared amongst the 3 age groups. Progesterone was not given because, in preliminary studies, it did not affect the incidence or quality of feminine behaviours in any age group.

Data analysis. The incidences of behaviour or insemination were analysed using Fisher's exact tests or χ^2 tests (when $N > 50$). Hormone concentrations were analysed using one- or two-way analysis of variance and Bonferroni's *t* tests were used to determine differences between specific means when appropriate. Student's *t* tests were used to determine differences between two means.

Results

Study 1

All but 2 of 20 females at 150 days of age or older conceived within 48 h of pairing with a male. In addition, only females 150 days old or older conceived within 1 week of pairing. Although one-half of the females between 90 and 120 days of age became pregnant, conception did not occur until the 2nd week after pairing. Only one female in the 30–50-day age group produced young after pairing with a male for 2 weeks. The incidence of conception was significantly greater in females of 150–200 days of age (18/20) than in those 30–50 days old (1/20; $P < 0.001$) or 90–120 days old (4/8; $P < 0.05$). The incidence of conception was greater in females 90–120 days old than in those between 30 and 50 days of age ($P = 0.015$).

The incidence of mating, as indicated by the presence of spermatozoa in vaginal smears, also varied amongst groups. In females 30–50 days of age, 3 of 20 were inseminated, but only one became pregnant. In females 90–120 days of age, 2 of the 4 that did not conceive exhibited vaginal oestrus and were inseminated. Of 20 females aged 150–200 days, 18 were inseminated and all conceived. The incidence of mating was therefore significantly lower in females between 30 and 50 days of age than in 90–120-day-old females ($P = 0.023$) or 150–200-day-old females ($P < 0.001$).

Study 2

Oestrogen concentrations were significantly higher in females with cornified smears than in those with leucocytic smears at 90 and 150 days (Table 1). Significantly higher oestrogen concentrations were found in 150-day-old females than in 90-day-old females, regardless of the vaginal smear pattern. Finally, oestrogen concentrations in females displaying vaginal cornification at 90 days did not differ significantly from those of females displaying leucocytic vaginal smears at 150 days.

Table 1. Plasma oestrogen concentrations (pg/ml) in female gray-tailed voles exhibiting leucocytic (L) or cornified (C) vaginal smear patterns at 90 or 150 days of age

Age (days)	Vaginal smear pattern	
	L	C
90	52.83 $\pm$ 3.89 (12 pools)	165.17 $\pm$ 10.27 ^a (6 pools)
120	139.87 $\pm$ 7.20 ^a (8 pools)	247.13 $\pm$ 16.49 ^{a,b,c} (8 pools)

Values are mean $\pm$ s.e.m. for 2–3 animals/pool.

^a $P < 0.01$ compared to L at 90 days.

^b $P < 0.01$ compared to C at 90 days.

^c $P < 0.01$ compared to L at 120 days.

Study 3

The mean progesterone concentration of unreceptive females (5.8 $\pm$ 0.6 ng/ml) was not significantly different from that of receptive females (6.3 $\pm$ 0.9 ng/ml). However, oestrogen concentration was significantly higher ($P < 0.001$) in receptive (290 $\pm$ 21 pg/ml) than in unreceptive (150 $\pm$ 15 pg/ml) females.

Study 4

The proportion of oestrogen-treated ovariectomized females showing receptive behaviours during a single test was significantly greater in Group C (17/23) than in Group A (1/28; $P < 0.001$, χ^2 test) or in Group B (4/11; $P < 0.05$). In addition, the proportion of receptive females in Group B (4/11) was significantly greater than that of Group A (1/28; $P = 0.02$). When females ovariectomized between 30 and 50 days of age were retested ~ 2 months after the first test, the incidence of receptivity did not change; only the single female that had mated during the first test mated in subsequent tests.

The mean latency between the time the male was introduced and the time that the female was mounted was significantly less ($P < 0.001$) for females between 150 and 200 days of age (108 ± 30 sec) than for those between 90 and 120 days (210 ± 36 sec). The latency to be mounted was 275 sec for the single female showing receptive behaviour in females between 30 and 50 days of age.

Discussion

Previous studies demonstrated that female gray-tailed voles isolated from males after weaning experience age-related increases in the incidence of vaginal cornification (Petersen, 1986). The results of the present studies demonstrate that isolated females also experience age-related increases in plasma oestrogen concentrations, behavioural receptivity, and the incidence of fertile matings. Furthermore, these studies demonstrate a relationship between the incidences of vaginal cornification and conception. Only 5% of females aged 30–50 days show vaginal cornification (Petersen, 1985) and 5% conceived in the present study. By 90–120 days of age, $\sim 70\%$ show some periods of vaginal oestrus (Petersen, 1985) and $\sim 50\%$ of females in the same age group conceived in the present studies. Finally, $\sim 90\%$ of females between 150 and 200 days of age show at least periodic vaginal oestrus (Petersen, 1985) and the same percentage of females in the present study conceived during 2 weeks of pairing with males. Therefore, both the incidence of vaginal oestrus and the rate of reproductive success increase with age. These results are consistent with the hypothesis that the changes in vaginal smear patterns previously reported in gray-tailed voles represent maturational changes (Petersen, 1986).

The differences in reproductive success amongst age groups in the present study appeared to result from at least two factors—the proportion of females exhibiting reproductive behaviours and the proportion experiencing fertile matings increased with age. When intact females were paired with males, only 3 of 20 females between 30 and 50 days of age were mated during the 2-week study. In contrast, the incidence of insemination was over 75% in females between 90 and 120 days of age and 90% in females over 150 days of age. The presence of spermatozoa in the vaginal smear was taken as evidence that females were behaviourally receptive because unreceptive females never allow mating (S. L. Petersen, unpublished). These results suggest that differences in receptivity account, in part, for the differences seen in reproductive success among females of the three age groups examined. The age-related increase in the propensity of intact voles to show receptive behaviour may result from age-related increases in oestrogen concentrations. This hypothesis is supported by the finding that females had higher oestrogen values at 150 days than at 90 days, regardless of whether animals with leucocytic or cornified vaginal smears were examined. Furthermore, behaviourally receptive females have significantly higher oestrogen concentrations than unreceptive females of the same age. In contrast, progesterone values neither change with age (Petersen, 1986) nor were they different between receptive and unreceptive females in the present studies. These findings are consistent with work of Dluzen & Carter (1979) showing that oestrogen alone is required for the stimulation of receptive behaviour and that administration of progesterone does not facilitate expression of receptive behaviour in another vole, *M. ochrogaster*. It seems likely from the results of the present studies that females in the 150–200-day age group had higher plasma oestrogen concentrations and, for this reason, exhibited a higher incidence of receptive behaviours than did younger animals.

The finding that ovariectomized females show different rates of behavioural responsiveness to oestrogen, depending on the age at the time of ovariectomy, suggests that plasma oestrogen values alone do not explain age-related differences in behavioural potentials. No females ovariectomized before 50 days of age showed receptive behaviour after treatment with high doses of oestrogen, even when testing was repeated when animals were between 90 and 120 days of age. In contrast, even though females of different ages were given the same dose of oestrogen based on body weight, nearly half of females ovariectomized between 90 and 120 days and over three-quarters of females > 150 days of age were behaviourally receptive. In addition, the quality of behaviour was highest in females between 150 and 200 days of age; all females >150 days of age showed proceptive behaviours before the first mount, but no females in either of the other age groups displaced proceptivity until after the first intromission series. This propensity of older females to show soliciting behaviour (proceptivity) probably contributed to the decrease in time to first mount in females older than 150 days compared with those younger than 150 days. In other mammals, the age at which ovariectomy is performed does not affect the ability to show feminine behaviour. For example, female rabbits show similar levels of receptivity regardless of whether they are ovariectomized at 30, 60 or 90 days of age (Beyer, Rivaud & Cruz, 1970). In addition, sexual receptivity in female rats and hamsters can be induced in adulthood by appropriate hormone stimulation even if ovariectomies are performed within 1 week after birth (Lisk & Suydam, 1967; Whalen & Edwards, 1967; Swanson & Crossley, 1971; Gerall, Dunlap & Hendricks, 1972). The results of the present studies indicate that the development of the ability to display feminine sexual behaviour in gray-tailed voles may require exposure to ovarian secretions before puberty.

Analysis of the results of the present studies indicates that an increase in the propensity to respond to oestrogen with receptive behaviour was the main factor contributing to the age-related increase in reproductive success. However, the increase in the incidence of fertile matings seen in older females also appears to be a factor. In females 30–50 days of age, 75% of matings that occurred were sterile (only 1 of 4 conceived). In contrast, only 33% of matings experienced by females between 90 and 120 days of age were sterile and no sterile matings occurred in females older than 150 days. Age-related differences in fertility have also been observed in female bank voles (Westlin & Gustafsson, 1984): females of this species mated at 30–50 days of age show a very low fertility rate compared with females mated after the age of 100 days of age, and this low rate appears to be associated with a greater incidence of sterile matings. In addition, sterile matings just after puberty are characteristic of a number of other microtines (Brambell & Hall, 1939; Hoyte, 1955; Greenwald, 1956; Kirkpatrick & Valentine, 1970; Mallory & Clulow, 1977; Westlin, 1982a; Westlin & Nyholm, 1982; Westlin & Gustafsson, 1983). Fertility of young female bank voles can be increased by treatment with prolactin or progesterone after mating (Westlin, 1982b). In addition, mating-induced prolactin secretion appears to be part of a luteotrophic complex in the field vole, *M. agrestis* (Milligan & MacKinnon, 1976; Charlton, Milligan & Versi, 1978). Based on these observations, it was suggested that sterile matings may be a result of an immaturity of the mechanism activating the release of prolactin (Westlin & Gustafsson, 1984). It is possible that a similar situation exists in young gray-tailed voles. No work has examined the effect of varying oestrogen concentrations on the post-coital release of prolactin in the vole; however, it is known that oestrogen is required for the pro-oestrous surge of prolactin in rats (Neill, Freeman & Tilson, 1971; Freeman, Reichert & Neill, 1972). Perhaps the high oestrogen values seen in females >150 days of age in this study account for the absence of sterile matings in this group.

Another explanation for the differences in conception rates amongst groups may be that younger animals received fewer copulatory stimuli than did older animals. It is known that ovulation in female voles requires a certain amount of copulatory stimulation and that the intensity of stimulation required for ovulation varies amongst species (Sawrey & Dewsbury, 1985). If younger females in the present study did not receive the same number of intromissions as older females, it might be expected that the effectiveness of the mating stimulus would be decreased and ovulation would not occur. However, receptive female gray-tailed voles are treated similarly by experienced males

during mating tests, regardless of age or body weight (S. L. Petersen, unpublished). Therefore, it seems unlikely that differential mating stimulation could account for differences in conception rate amongst groups in these studies.

Female voles have long been considered to be among the earliest-maturing mammals known (Greenwald, 1956). This conclusion is based on studies showing that female voles, presumably exposed to males from weaning, can mate and conceive before 1 month of age (Hatfield, 1935; Hamilton, 1941; Cowan & Arsenault, 1954). Like other species, female gray-tailed voles can also have fertile matings before 1 month of age if housed near adult males from weaning (Hagen, 1978). However, the results of the present study indicate that the time required for maturation may be 2 months or more if females are isolated from males at weaning. Likewise, female *M. ochrogaster* reared with parent or sibling males rarely breed before 60 days of age (Richmond & Conaway, 1969). These findings suggest that isolated female gray-tailed voles, or female voles in general which are reared in the laboratory, reach maturity much later than do some other laboratory rodents isolated from males. For example, puberty in female rats and mice is attained between 30 and 40 days in the absence of males, but can be accelerated by approximately 9 days in rats and 20 days in mice by exposure to unfamiliar adult males (Vandenbergh, 1967; Vandenbergh, Whitsett & Lombardi, 1975; Vandenbergh, Finlayson, Dobrogosz, Dills & Kost, 1976). Female voles may therefore be more dependent on males than are most other female rodents for the induction of maturation, just as they are dependent upon males for the induction of ovulation (Breed, 1967) and maintenance of early pregnancy (Berger & Negus, 1982). In the field, it seems likely that females would receive early exposure to males and would, therefore, undergo early maturation as has been previously reported (Hatfield, 1935; Hamilton, 1941). However, the results of the present studies suggest that when maturation of female voles is investigated in a laboratory setting, the conditions of housing and exposure to males must be considered in evaluating responses of females.

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