These studies examined the role of perinatal androgen in the development of neuroendocrine and behavioral characteristics of gray-tailed voles, *Microtus canicaudus*. When mature, females exhibited alternating vaginal smear patterns or persistent vaginal cornification; estrogen and progesterone concentrations were not correlated with vaginal smear patterns. Corpora lutea were not observed in females showing a variety of vaginal smear patterns, nor were corpora lutea observed after administration of estrogen alone, or in combination with progesterone. These findings indicate that voles do not release cyclic ovulatory surges of luteinizing hormone. Ovariectomized female voles implanted with testosterone displayed masculine copulatory behavior patterns virtually indistinguishable from those of normal
males. These behavioral and endocrine characteristics of female voles are similar to those of other rodent females treated with testosterone during development. The hypothesis that female voles are androgenized by exposure to endogenous testosterone was supported by the finding that female voles have high concentrations of plasma testosterone during development. However, perinatal treatment with antiandrogens or antiaromatases did not affect vaginal smear patterns, ovulatory responses to estrogen and progesterone administration, or the behavioral response to testosterone after ovariectomy.

Castrated male M. canicaudus did not display female-typical behaviors when treated with estrogen or estrogen plus progesterone in adulthood; perinatal androgen manipulations did not affect the potential for female receptive behaviors in males. Specifically, neither castration within 3 hours of birth nor perinatal treatment with antiandrogens or antiaromatases enhanced behavioral responses to estrogen in male voles. In addition, prolonged neonatal testosterone treatment did not decrease the potential for feminine behaviors in female voles. These findings suggest that the development of female receptive behaviors in voles requires more than the absence of perinatal androgen exposure. Female voles did not exhibit feminine behavioral responses to estrogen if ovariectomized prepubertally, suggesting that the
development of female sexual behaviors may require prepubertal exposure to ovarian secretions.
The Role of Perinatal Androgens in the Development of Neuroendocrine and Behavioral Characteristics of the Gray-Tailed vole, Microtus canicaudus.

by

Sandra Petersen Spielvogel

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Redacted for Privacy
Associate Professor of Zoology in charge of Major

Redacted for Privacy

Chairman, Department of Zoology

Redacted for Privacy

Dean of Graduate School

Date thesis is presented May 18, 1984

Typed by researcher for Sandra Petersen Spielvogel
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The Role of Perinatal Androgens in the Development of Neuroendocrine and Behavioral Characteristics of the Gray-Tailed Vole, Microtus canicaudus

CHAPTER I: GENERAL INTRODUCTION

The studies in this dissertation have identified specific behavioral and neuroendocrine characteristics of male and female gray-tailed voles, Microtus canicaudus and have investigated the possibility that the development of these characteristics are influenced by perinatal exposure to testosterone. These studies were designed to investigate, directly and indirectly, the validity of the organizational hypothesis of sexual differentiation in this species of vole. Therefore, studies concerning both the organizational hypothesis, as well as background information about vole reproduction, will be reviewed before specific experiments are described.

Organizational Hypothesis of Sexual Differentiation of the Brain

Sexual Dimorphism of Behaviors and Gonadotropin Release Patterns

Male and female mammals exhibit sexually dimorphic behavior patterns. One explanation for these behavioral differences is that there are sexual differences in the predominant gonadal steroids produced in adulthood. This explanation is incomplete, however, because gonadectomized
males and females that are treated with the same replacement hormones continue to exhibit sexual dimorphism in sexual behavioral responses. For example, castrated adult males exhibit mounting, intromission and ejaculatory behaviors after testosterone treatment (Larsson, 1979); in contrast, ovariectomized females treated with testosterone exhibit incomplete, infrequent or no masculine copulatory behavior patterns (Gerall and Ward, 1966; Ward, 1969; Tiefer, 1970; Swanson and Crossley, 1971; Carter, Clemens and Hoekema, 1972; Sodersten, 1972). Ovariectomized females treated with estrogen and progesterone exhibit species-specific proceptive and receptive behaviors (Beach, 1976). Castrated males may also exhibit female sexual behaviors in response to estrogen and progesterone treatment; however, these behaviors are usually not as frequent or as intense as those of females (Beach, 1945; Feder and Whalen, 1965; Grady, Phoenix and Young, 1965; Whalen and Edwards, 1967).

In addition to behavioral differences, many species also exhibit sexual differences in gonadotropin release in response to gonadal steroid hormone administration. Ovariectomized female rats, but not castrated males, release cyclic surges of luteinizing hormone (LH) when treated with estrogen and progesterone (Gogan, Beattie, Hery, Laplante and Kordon, 1980). As a result of cyclic
release of LH, intact females exhibit spontaneous ovulation and regular changes in vaginal smear patterns (Feder, 1981b). Ovarian and vaginal tissue transplanted into males shows no evidence of cyclic gonadotropin release; ovaries are polyfollicular with no corpora lutea and vaginal smears are persistently cornified (Yazaki, 1960; Harris, 1964; Gorski and Wagner, 1965).

According to the widely-accepted organizational hypothesis of sexual differentiation of the brain, the neural areas controlling sexual behaviors and gonadotropin release patterns are inherently feminine; masculinity is permanently imposed by perinatal exposure to androgen. Therefore, males exhibit masculine sexual behaviors and tonic gonadotropin release as adults, because of early exposure to their own testicular androgens. Females, according to the hypothesis, are not exposed to high levels of androgen and, therefore, retain the capacity to exhibit female behavior and cyclic gonadotropin release as adults (for recent reviews see Baum, 1979; McEwen, 1979; Gorski, 1979); Goy and McEwen, 1980; MacCluskey and Naftolin, 1980; Feder, 1981a).

**Development of the Organizational Hypothesis**

Phoenix, Goy, Gerall and Young (1959) found that prenatal testosterone exposure permanently alters
behavioral characteristics of female guinea pigs. When female guinea pigs are treated prenatally with testosterone, the ability to display female-typical behaviors (specifically, lordosis) in response to estrogen and progesterone is suppressed (defeminization) and the ability to display male-typical copulatory behaviors in response to testosterone is enhanced (masculinization). These authors proposed that, during the prenatal period, testosterone, or a metabolite of testosterone, exerts an organizing effect on the neural tissues which control sexual behavior in adult guinea pigs.

The organizational hypothesis subsequently was tested in rats by Harris and Levine (1962), who determined that neonatally-androgenized females do not exhibit normal female mating behaviors when treated with estrogen and progesterone in adulthood. However, neonatally-androgenized female rats do show male copulatory behaviors in response to testosterone. These investigators interpreted their results to mean that gonadal secretions during the perinatal period influence sexual differentiation in the central nervous system. Barraclough and Gorski (1962) demonstrated that administration of a single dose of 1.25 mg of testosterone to 5-day-old female rats suppressed feminine behavioral responses to estrogen and progesterone treatment.
Barraclough and Gorski proposed that suppression of female sexual behaviors was a result of hypothalamic changes.

In addition to studies that involved treating neonatal females with testosterone, the organizational hypothesis was supported by early studies in which male rats were deprived of androgens. In contrast to intact male rats or males castrated after 10 days of life, males castrated soon after birth exhibit high levels of female receptive behaviors when stimulated with estrogen and progesterone in adulthood (Grady, Phoenix and Young, 1965). In addition, males treated with antiandrogens during prenatal development display enhanced female-typical behavioral responses to estrogen and progesterone in adulthood (Ward, 1972).

Deficits in masculine copulatory behavior are also observed in neonatally-castrated male rats. Males castrated at birth are not as likely to achieve intromission and ejaculation as males castrated in adulthood (Beach and Holz, 1946; Grady, et al., 1965; Whalen and Edwards, 1966, 1967). It has been debated whether these deficits result from central or peripheral effects of androgen deprivation during development (Whalen, 1968; Hart, 1968; 1972).

Early work also suggested that exposure to testicular secretion during development permanently masculinizes
gonadotropin release patterns. Pfeiffer (1936) transplanted testes and ovaries into ovariectomized female rats at birth, and found that ovarian transplants do not contain corpora lutea in adulthood as they do in ovariectomized females without testicular grafts. In addition, Pfeiffer demonstrated that corpora lutea are formed in ovarian transplants of neonatally castrated males, but not in neonatally castrated males with testicular grafts. These results were interpreted to mean that the pituitary of the rat is bipotential at birth and is differentiated as male or female, depending on the gonad present.

Barraclough and Leathem (1954) and Barraclough (1958; 1961) demonstrated that a single injection of testosterone administered to female mice and rats just after birth resulted in a permanent anovulatory sterility; ovaries were polyfollicular with no corpora lutea. Barraclough (1961) suggested that it is not the pituitary, but rather, regions of the hypothalamus controlling gonadotropin secretion that are undifferentiated at birth. Subsequently, Barraclough and Gorski (1961) presented evidence supporting this hypothesis by demonstrating that electrical stimulation of the hypothalamus produces a discharge of gonadotropins from the pituitary sufficient to induce ovulation. Furthermore, they demonstrated that,
in normal females, ovulation is induced by electrically stimulating the suprachiasmatic-preoptic area. In androgen-sterilized females, however, ovulation was induced by stimulating the arcuate-ventromedial nuclei region of the median eminence, not by stimulating the suprachiasmatic-preoptic area. These results were interpreted to mean that there are two hypothalamic regions which control gonadotropin secretion; the anterior preoptic area controls cyclic discharges of luteinizing hormone (LH), and the arcuate-ventromedial nuclei control the tonic discharge of LH. Barraclough and Gorski (1961) suggested that prepubertal androgen treatment alters the function of the preoptic region so that cyclicity of gonadotropin release is abolished, but tonic secretion remains.

The hypothesis that perinatal androgen exerts its effects at the level of the hypothalamus is supported by studies in which testosterone was implanted into specific hypothalamic areas of 5-day-old female rats. Anovulatory sterility with persistent vaginal cornification was seen in these females in adulthood (Nadler, 1968). Similar results were obtained by Wagner, Erwin and Critchlow (1966) using implants of testosterone propionate in doses which did not induce sterility when administered systemically.
All of the above studies support the hypothesis that sexual differentiation of the brain is a result of sexual differences in exposure to, or sensitivity to, testicular androgens during specific periods of development.

**Periods of Maximal Sensitivity to the Developmental Effects of Androgens**

By injecting females with testosterone or castrating males at various times during development, periods of maximal sensitivity to masculinizing and defeminizing effects of testosterone have been determined (Barraclough, 1961; Goy, Bridson, and Young, 1964; Harris, 1964; Swanson and van der Werff ten Bosch, 1964; Gerall, Hendricks, Johnson and Bounds, 1967; Noble, 1973; Coniglio and Clemens, 1976; Huffman and Hendricks, 1981; Corbier, Roffi and Rhoda, 1983). During these periods, males have higher testosterone levels than females (see Table 1.1).

In addition, it has been determined that different areas of the brain control different aspects of sexual differentiation, and that these areas develop at different times during the perinatal period (Nadler, 1968; 1972; 1973; Christensen and Gorski, 1978). Therefore, instead of a single period of maximal sensitivity to androgen, there are a number of "critical periods" during the period of brain development. For this reason, defeminization and
masculinization of behavior and masculinization of gonadotropin release are not necessarily inseparable events. This concept provides one explanation for the finding that female rats, injected with a low dose of androgen during development, show masculinization of gonadotropin release patterns but not defeminization of behavior (Barraclough and Gorski, 1962). It may also explain the finding that female hamsters are behaviorally masculinized by administering neonatal testosterone in doses that do not defeminize behavior (DeBold and Whalen, 1975).

**Species and Individual Differences in Potentials for Heterotypical Sexual Behavior**

Phoenix, Goy and Resko (1968) hypothesized that the presence of androgen does not produce an all-or-none effect on the behavior of an animal; rather, the extent of androgenization depends on the quantity and duration of androgen exposure during critical stages of development. This hypothesis may explain inter- and intraspecies differences in the abilities to display heterotypical sexual behavior.

As examples, male hamsters and ferrets are capable of displaying female-typical sexual behavior after castration and estrogen and progesterone administration in adulthood;
however, they also show male-typical behavior in response to testosterone (Tiefer, 1970; Swanson and Crossley, 1971; Carter, Clemens and Hoekema, 1972; Baum, 1979). It is possible that androgen levels are relatively low in male hamsters and ferrets during the period of maximal sensitivity to defeminizing effects of testosterone, but high during the period of masculinization. This hypothesis is supported by the finding that experimental elevations of plasma testosterone levels in neonatal male hamsters results in behavioral defeminization (Swanson and Crossley, 1971; Carter, et al., 1972). It is also supported by the finding that, in hamsters and ferrets, perinatal androgen levels of males and females are not markedly different throughout perinatal development (Erskine and Baum, 1982; Pang and Tang, 1984).

In rats, perinatal exposure to testosterone affects the ability of progesterone to facilitate a behavioral response to estrogen. Baum (1979) suggests that the potential for female sexual behavior in males will occur only in those species which rely on progesterone for the facilitation of behavior. This hypothesis is consistent with findings that, in species which do not require progesterone (ferrets and primates), males are not defeminized by exposure to endogenous testosterone, nor are females defeminized by administration of testosterone.
during development (Baum, 1976; 1979; Goy, 1978; Baum, Gallagher, Martin and Damassa, 1982). This hypothesis has not been investigated in other species in which females do not require progesterone for the display of sexual receptivity.

In addition to differences among species in the potential for heterotypical sexual behavior, intraspecies differences have been observed. Female rats and mice developing in utero near males display more male behaviors in adulthood and have longer anogenital distances than females developing near other females (Clemens and Coniglio, 1971; Gandelman, vom Saal and Reinisch, 1977; Meisel and Ward, 1981; vom Saal and Bronson, 1978; Clemens, Gladue and Coniglio, 1978). It is possible that these differences result from dissimilar levels of androgen exposure among individuals. The hypothesis that individual behavioral differences are related to differences in perinatal androgen exposure is supported by the finding that female mice developing between males have higher testosterone levels than those developing between females (vom Saal and Bronson, 1980). Furthermore, prenatal treatment with antiandrogen abolishes individual differences in the potential for masculine behavior in female rats (Clemens, Gladue and Coniglio, 1978). Thus, females can be "normally"
masculinized by exposure to endogenous androgens emanating from male littermates.

Reproduction of Voles Compared with that of Other Muroid Rodent Species

Most muroid rodents (i.e., rats, mice and hamsters) exhibit regular ovarian follicular cycles which culminate in spontaneous ovulation (Feder, 1981b). In these females, rising estrogen and progesterone concentrations trigger the release of an ovulatory surge of LH. After ovulation, a subsequent wave of follicular maturation occurs and the cycle is repeated. Associated with these follicular cycles are regular cycles in vaginal smear patterns and behavior. In cycling females, sexual behavior is seen only during periods when estrogen and progesterone are elevated.

The reproductive biology of female voles differs in several ways from that of the muroid rodents described above. Voles do not exhibit spontaneous ovulation, regular cycles in vaginal smear patterns, or regularly recurring periods of sexual receptivity; instead, they ovulate in response to copulation and display vaginal estrus and sexual behavior for prolonged periods of time (Bodenheimer and Sulman, 1946; Greenwald, 1956; Breed, 1967, 1972; Mullen, 1968; Richmond and Conaway,
In addition, ovaries of voles are polyfollicular with no corporal lutea (Peters and Clarke, 1973). These characteristics may be secondary to the inability of voles to respond to rising estrogen levels with ovulatory surges of LH (Milligan 1978, 1981) and, according to Milligan (1978), may reflect the "absence of the sexually differentiated gonadotropin response characteristic of spontaneous ovulators". This hypothesis is supported by the finding that electrical stimulation of the anterior, but not the posterior hypothalamus of the vole, M. agrestis, results in ovulation (Breed and Charlton, 1971). In spontaneous ovulators, electrical stimulation of either region triggers ovulation (Barraclough, 1966).

As discussed above, these characteristics of normal female voles are seen in other species if females are injected with androgens during development. It is possible that female voles are exposed to endogenous androgens that masculinize gonadotropin release patterns. The hypothesis that androgens masculinize gonadotropin release patterns has not been tested in any female that normally lacks cyclic gonadotropin release.

Female voles are also similar to androgenized female
rats in their behavioral response to progesterone. Progesterone does not facilitate the display of female receptive behaviors in female voles (Dluzen and Carter, 1979; S. P. Spielvogel, unpublished). Neonatal testosterone treatment in female rats abolishes the facilitatory effect normally exerted by progesterone on the display of female receptive behaviors (Clemens, Shryne and Gorski, 1970; Gerall and Kenney, 1970). Thus, normal female voles have behavioral characteristics associated with androgen-induced defeminization. No information is available to indicate whether female voles also display characteristics associated with behavioral masculinization (i.e., mounting, intromission and ejaculatory behavior in response to testosterone administration).
Table 1.1 Plasma Testosterone Levels (ng ml$^{-1}$) During Development.

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<tr>
<td>Guinea pig</td>
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Table 1.1 (Cont.) Plasma Testosterone Levels During Development.

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Table 1.1 (Cont.) Plasma Testosterone Levels During Development.

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Table 1.1 (Cont.) Plasma Testosterone Levels During Development.

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CHAPTER 2: VAGINAL SMEARS, BEHAVIOR AND CONCEPTION IN FEMALE *Microtus canicaudus*

INTRODUCTION

Females of most species of microtine rodents, specifically lemmings and voles, do not exhibit regular ovulatory cycles, but rather are induced to ovulate in response to mating (Bodenheimer and Sulman, 1946; Greenwald, 1956; Breed, 1967 and 1972; Mullen, 1968; Richmond and Conaway, 1969a, b; Clulow and Mallory, 1970; Kirkpatrick and Valentine, 1970; Cross, 1972; Gray, Davis, Zerylnick and Dewsbury, 1974; MacFarlane and Taylor, 1982a, b). Unlike female rodents that ovulate spontaneously, microtine females exhibit considerable individual and species variability in vaginal smear patterns (Hasler, 1975). Using the terminology established for mice (Allen, 1922), voles are reported to have the following vaginal smear patterns: persistent diestrus with no evidence of estrus (Bodenheimer and Sulman, 1946), irregular estrous periodicity with alternating smear types (Hoyte, 1955; Kirkpatrick and Valentine, 1970; Richmond and Conaway, 1969a; Gray *et al.*, 1974; Milligan, 1974; MacFarlane and Taylor, 1982b), regular estrous cycles (Brambell and Hall, 1939; Chitty and Austin, 1957), and persistent vaginal estrus (Breed, 1967; Richmond and Conaway, 1969a; Milligan, 1974). This
variability in vaginal smear patterns has been reported within a single species, as well as among species (Breed, 1967; Milligan, 1974; Hasler, 1975).

One important source of variability in vaginal smear patterns is social environment; specifically, proximity to adult males. Female _M. ochrogaster_ rarely become vaginally perforate in the absence of males; however, if they do become perforate, they typically exhibit diestrous- or proestrous-like vaginal smears when males are not nearby (Hasler and Conaway, 1973). However, if housed near males, but prevented from mating, female _M. ochrogaster_ achieve vaginal opening and estrus within a week and remain in estrus for up to a month (Richmond and Conaway, 1969b). Female _M. agrestis_ also respond to stimuli from males with changes in vaginal smear patterns that culminate in persistent vaginal estrus (Milligan, 1974). Thus, male voles appear to influence vaginal smear patterns of females.

Another source of inter- and intraspecies variation in vaginal smear patterns in isolated female voles may be age. This factor is difficult to evaluate because no systematic studies of vaginal smear patterns have investigated age-related changes in a single species. However, studies of different species indicate that the incidence of vaginal estrus may be higher in older
females. For example, female *Microtus guentheri* housed away from males from weaning show no vaginal estrus for 50 days (Bodenheimer and Sulman, 1946), nor do most isolated female *Microtus ochrogaster* show vaginal estrus at 70-90 days (Hasler and Conaway, 1973). However, persistent vaginal estrus or irregular cycles have been reported in isolated *Microtus agrestis* females (Breed, 1967) and in isolated *Microtus montanus* females (Gray, et al., 1974) after the age of 3 months.

Observations of female *Microtus canicaudus* in this lab suggest that there is a high degree of intraspecies variability in vaginal smear patterns that may be related to age or exposure to males. The present studies were designed to investigate the possibility that variability in vaginal smear patterns is due to age when females are isolated from males. Because age-related changes in vaginal smear patterns were observed, subsequent studies were designed to investigate the physiological significance of these changes. To do this, receptivity and fertility were examined in female voles of several different ages.
MATERIALS AND METHODS

ANIMALS

Gray-tailed voles, *Microtus canicaudus*, used in these studies were from a colony that is maintained in a well-ventilated room with a controlled photoperiod (16L:8D, lights off at 1430 h) at Oregon State University. The animals were housed in fiberglass cages (60x15x15 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.

PROCEDURES

Vaginal Smears

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, leukocytes and cornified cells were counted in three 10X fields and
the mean proportion of each of these cell types per smear was calculated. Female voles have vaginal smear types with cellular associations similar to those observed during the estrous cycles of rats and mice (Allen, 1922; Long and Evans, 1922). These types are: predominantly cornified epithelial cells (this type of smear occurs at estrus in rats and mice), predominantly nucleated epithelial cells (seen on the day of proestrus in rats and mice), predominantly leucocytes (seen on the day of diestrus in rats and mice) and predominantly cornified cells with numerous leucocytes (seen on metestrus in rats and mice). Because these cellular associations do not represent the same ovarian changes and do not reflect the same behavioral changes in voles that are associated with similar vaginal smear types in rats and mice, the use of the terms proestrus, metestrus and diestrus will be avoided in this manuscript. The term "estrus" will be used to describe female voles exhibiting cornified vaginal smears.

Female voles often show a particular vaginal smear type for a prolonged period of time. In these studies, if a particular smear type is observed for at least 10 out of 14 days, the pattern is said to be persistent. If alternating periods of nucleated, cornified and leucocytic smears are seen, the pattern is said to be cycling. It
should be emphasized that the term "cycling" refers only to vaginal smear changes, and is not meant to indicate that regular cyclic ovarian changes occur in voles.

**Behavior Tests**

Behavior tests were conducted one hour after the onset of dark under dim red lights. The test arena consisted of a sheetmetal cylinder (30 cm in diameter and 50 cm high) on an elevated glass floor. A mirror mounted beneath the floor at a $45^\circ$ angle allowed ventral viewing.

Copulatory behavior in *M. canicaudus* has been described in detail by Dewsbury and 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 minutes for exploration. Males had been pretested for the propensity
to display mating behavior, and subsequently, each male was used in only one behavior test. The same male was not used in more than one test, because latency to mount becomes shorter with each successive test regardless of the female used (S. P. Spielvogel, unpublished data).

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

STUDY 1 AGE AND VAGINAL SMEAR PATTERNS

To determine whether vaginal smear patterns change with age, smears were obtained daily (1200 h) for 14 days from isolated females of the following age groups: A) 30-50 days (n=41), B) 90-120 days (n=41) and C) 150-200 days (n=50). During the two weeks of examination, animals were left in the same cages and bedding was not changed, because of the possibility that such a disturbance could result in vaginal smear changes as suggested by Richmond and Conaway (1969a).

STUDY 2 AGE AND PRODUCTION OF OFFSPRING

Results of the first study showed that the incidence
of vaginal estrus increases with age for the first 3-5 months of life if females are isolated from males. The following study was performed to determine whether age-related changes in vaginal smears reflect a maturational process that correlates with increased fertility at 150-200 days, or whether these changes are part of the aging process and correlated with decreased fertility, as is the case for laboratory rats (Everett, 1939).

At the beginning of this study, vaginal smears from females in the three age groups described above were examined (A, n=20; B, n=8; C, n=20). Each female was then placed in the home cage of a reproductively active male. These males had sired offspring within the previous three weeks but had been housed with other males for a 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 and Birney, 1979; Gavish, Carter and 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 1200 h for two weeks. Males were then removed and females were examined for pregnancy by gently palpating their abdomens. Females were not handled again for 10 days, but cages were checked
twice daily to determine the date of parturition. The date of conception was considered to be the day sperm 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 one week, and between one and two weeks after the male was introduced.

STUDY 3 AGE AND BEHAVIORAL RECEPTIVITY

This study was designed to determine whether females showed age-related differences in receptivity. Preliminary studies of intact females determined that females younger than 50 days did not mate and had lower estrogen levels than females that reliably showed receptivity at 150 days of age (S. P. Spielvogel, unpublished). Therefore, ovariectomized females were injected with estradiol benzoate (EB; Sigma Chemical Company, St. Louis, MO.) in doses based on body weight, so that estrogen levels among females of different ages would be similar.

Ovariectomized females representing the three age groups described in Study 1 were used (A, n=28; B, n=11; C, n=23). Bilateral ovariectomies were performed under anesthesia obtained by subcutaneously injecting 125 ug g⁻¹ body weight of ketamine HCl (Vetalar, Parke Davis) and 7 ug g⁻¹ body weight of xylazine (Rompun,
Haver-Lockhart). Ovaries were removed through a single 4-mm mid-dorsal incision. Vaginal smears and body weights were obtained at the time of surgery.

After one week, each female received daily intramuscular injections of 1 ug EB g^{-1} body weight for 3 days. Forty-eight hours after the last injection, females were tested for receptivity as described above. Females ovariectomized between 30 and 50 days of age were retested 1 and 2 months later, using the same EB treatment regimen. The proportion of receptive females and the mean latency to be mounted were compared among the three age groups. Progesterone was not given because, in preliminary studies, it did not affect the incidence or quality of female behavior in females in any age group. Preliminary studies also showed that lower doses of estrogen were not as effective as the dose chosen in inducing estrous behavior in females of various ages (S. P. Spielvogel, unpublished data).

DATA ANALYSIS

These data were analyzed using Fisher's exact tests except for data comparing the latencies to be mounted in Study 3 which were analyzed with Student t tests.
RESULTS

STUDY 1

The data describing age-related vaginal smear patterns are presented in Fig 2.1. Over 95% of females in the 30-50 day age group persistently displayed leucocytic smears. The incidence of persistent leucocytic smears decreased with age and that of persistent cornification increased with age. Approximately two-thirds of females between 90 and 120 days of age showed vaginal cycles. Smear types similar to those reported by Allen (1922) for the mouse were seen in the vole. However, the sequence was not regular and not comparable to that of mice. For example, proestrous-like smears (predominantly nucleated epithelial cells) infrequently preceded and sometimes followed vaginal estrus (cornified cells); metestrous-like smears (cornified epithelial cells and leucocytes) occurred both before and after estrous smears. In cycling female voles, leucocytic predominance generally lasted for 1-3 days and alternated with 1-3 days of cornified smears or 1-2 days of nucleated epithelial cell smears. By 150-200 days, two-thirds of females showed persistent vaginal estrus.

STUDY 2

The incidences of conception after pairing females of
different ages are presented in Figure 2.1. Conception is defined as a mating (indicated by the presence of sperm in the vaginal smear) that resulted in pregnancy. All but 4 of 16 females 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 old became pregnant, conception did not occur until 1-2 weeks after pairing. Only one female in the 30-50 day age group produced young as a result of 2 weeks of pairing with a male. The incidence of conception was significantly greater in females between 150 and 200 days than in either females 30-50 (P=2.91x10^{-8}) or 90-120 days old (P=0.038). The incidence of conception was greater in females 90-120 days old than in those between 30 and 50 days (P=0.015).

The incidence of mating, as indicated by the presence of sperm in vaginal smears, also varied among groups. In females 30-50 days of age, 3 of 20 were inseminated, but only one became pregnant after insemination. In females 90-120 days of age, 2 of the 4 that did not conceive were induced into vaginal estrus and inseminated. Eighteen of 20 females between 150 and 200 days of age were inseminated and all inseminated females conceived.
STUDY 3

The proportion of ovariectomized females showing receptive behavior during a single behavior test are presented in Fig. 2.1. The incidence of mating was significantly greater in females between 150 and 200 days of age than in either of the other groups.

When females ovariectomized between 30 and 50 days of age were retested 1 and 2 months after the first test, the incidence of mating did not change. Only the 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 (108 ± 30 sec) than for those between 90 and 120 days. The mount latency was 275 sec for the single female showing receptive behavior in the 30- to 50-day-old female group.

DISCUSSION

All of the vaginal smear patterns previously described for other species of voles were exhibited by female M. canicaudus isolated from males for 3-5 months after weaning. The variability in vaginal smear patterns seen in this species was related to age. Before 50 days
of age, females showed persistent leucocytosis, but after 90 days estrus occurred periodically. Periods of vaginal estrus increased in frequency and duration as females aged. By 150-200 days, nearly all females displayed persistent vaginal estrus. These age-related changes in vaginal smears were associated with changes in the ability to show receptive behavior and to produce young. Thus, the present findings suggest that female voles, isolated from males, undergo a prolonged period of sexual maturation. The stages of maturation are reflected in vaginal smear patterns.

Between 30 and 50 days of age, most female voles were vaginally perforate and showed diestrous-like smear patterns; estrous smears were rarely observed. These results demonstrate that in female *M. canicaudus*, vaginal perforation and first estrus are not concurrent events as they are in spontaneously-ovulating rodents (Long and Evans, 1922; Blandau and Money, 1943; Donovan and van der Werff ten Bosch, 1965). In another vole, *M. townsendii*, vaginal opening and first estrus may be separated by as many as 36 days when females are separated from males (MacFarlane and Taylor, 1981).

Vaginal opening is often considered to be an event marking the onset of puberty in rodents (Donovan and van der Werff ten Bosch, 1965). However, all female *M.*
canicaudus between 30 and 50 days of age were vaginally perforate, but only one of 20 conceived during two weeks of pairing with adult males. If one defines puberty as that time after which a female can produce young, these findings indicate that vaginal opening is not necessarily coincident with puberty in M. canicaudus.

Between 90 and 120 days, 70% of females had achieved first estrus, and one-half of females paired with males at this age conceived within two weeks after pairing. This conception rate is significantly higher than that of younger females but lower than that of females between 150 and 200 days of age. The most common vaginal smear pattern observed in females 90-120 days was periodic estrus, alternating with diestrous- or proestrous-like smears. This estrous periodicity or "cycling" has been described previously in other Microtus species of unspecified ages (Hoyte, 1955; Chitty and Austin, 1957; Richmond and Conaway, 1969a; Kirkpatrick and Valentine, 1970; Gray, Davis, Zerylnick and Dewsbury, 1974; Milligan, 1974; MacFarlane and Taylor, 1982b). It is unlikely that this estrous periodicity reflects the same ovarian changes that produce cycles in spontaneously-ovulating rodents, because "cycling" voles do not ovulate unless mated (Breed, 1967; 1972; Hasler, 1975).
By 150-200 days, greater than 90% of *M. canicaudus* isolated from males showed periods of estrus and two-thirds exhibited persistent vaginal estrus. All but 2 of 20 females exhibiting prolonged periods of estrus conceived within 48 hours of pairing with males. Persistent vaginal estrus also occurs in female *M. montanus* aged 120 to 160 days that are isolated from males. In addition, fertile matings also occur in persistently estrous female *M. agrestis* as soon as males are introduced (Chitty and Austin, 1957).

The findings described above show that age-related increases in the incidence of vaginal estrus are associated with age-related increases in the ability to reproduce. Although the cause of this infertility in younger females was not specifically investigated, previous studies showed that females younger than 90 days of age are not reliably receptive to mating (S. P. Spielvogel, unpublished). These previous findings were confirmed in the present study. Only 3 out of 20 females between 30 and 50 days of age were mated (as indicated by sperm in vaginal smears) during the 2-week study. In contrast, the incidence of insemination was over 75% in females between 90 and 120 days of 150 and 200 days.

In addition to age-related changes in receptivity, the present study demonstrates that female voles undergo
age-related changes in behavioral responsiveness to estrogen. All but one of 23 females older than 150 days showed receptivity after ovariectomy and estrogen stimulation; however, only one of 28 females younger than 50 days of age and only one-half of females examined between 90 and 120 days were behaviorally active after ovariectomy and estrogen stimulation. Furthermore, the sexual behavior displayed by females in the 150- to 200-day range differed qualitatively as well as quantitatively from that of younger females. All females older than 150 days showed proceptive behavior before the first mount but no females younger than 150 days of age displayed proceptivity until after the first intromission series. The propensity of older females to show soliciting behavior (proceptivity) probably contributed to the decreased mount latency in males paired with females older than 150 days.

The finding that the age at which ovariectomy is performed affects the ability to show female behavior in *M. canicaudus* is different from findings in other mammals. 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 and Cruz, 1970). Sexual receptivity in female rats and hamsters can be induced in adulthood by appropriate
hormone stimulation even if ovariectomies are performed within a week after birth (Lisk and Suydam, 1967; Whalen and Edwards, 1967; Swanson and Crossley, 1971; Gerall, Dunlap and Hendricks, 1972). These findings have been interpreted to mean that ovarian secretions do not play a role in the development of female behavior in rodents (Goy and McEwen, 1980). It may be that the development of female sexual behavior in M. canicaudus differs from that of other rodents and requires exposure to ovarian secretions before puberty.

Age-related differences in the propensity to show receptive behavior and in the behavioral responsiveness to estrogen may explain the age-related differences in conception rate; however, sterile matings in 30- to 50-day-old and 90- to 120-day-old females also contributed to differences. In females 30-50 days of age, four of 20 females entered estrus after a week of pairing and three were inseminated. Only one of these inseminated females became pregnant, indicating that sterile matings had occurred. Sterile matings also were seen in two of the 4 females that did not conceive at 90-120 days of age. No sterile matings were observed in females older than 150 days. Sterile matings just after puberty are characteristic of a number of other microtines (Brambell and Hall, 1939; Hoyte, 1955; Greenwald, 1956; Kirkpatrick
and Valentine, 1970; Mallory and Clulow, 1977; Westlin, 1982; Westlin and Nyholm, 1982; Westlin and Gustafsson, 1983).

The age-related changes in vaginal smear patterns observed in M. canicaudus are also seen in aging female rats. Female rats also become persistently estrous after previously exhibiting cycles; however, it is unlikely for several reasons that these changes in aging female rats are analogous to changes described in female M. canicaudus. First, the vaginal cycles exhibited by female M. canicaudus do not result from the same ovulation-related changes in hormones that produce vaginal cycles in female rats (S. P. Spielvogel, Chapter 3). Secondly, "cycling" voles do not mate or reproduce as readily as persistently estrous females. In contrast, maximum fertility occurs during the period of cyclicity in rats, and the period of persistent estrus marks the cessation of cyclic reproductive activity (Everett, 1939; Huang and Meites, 1975; Lu, Hopper, Vargo and Yen, 1979; Steger, Huang, Chamberlain and Meites, 1980). Finally, the onset of persistent estrus in voles occurs at approximately 3-5 months. This is fairly early in their reproductive lives because voles in the laboratory reproduce for 12 to 16 months (S. P. Spielvogel, unpublished). The onset of persistent estrus in rats
occurs toward the end of their reproductive lives, at 10-20 months of age (Steger et al., 1980). Thus, it is unlikely that the stages observed in female *M. canicaudus* isolated from males represent reproductive senescence.

It is more likely that vaginal smear changes observed in this study in female *M. canicaudus* that were isolated from males represent delayed maturation. Sexual maturation is also delayed in *M. pennsylvanicus* and *M. t. townsendii* housed away from males (Pasley and McKinney, 1973; MacFarlane and Taylor, 1981). Conversely, sexual maturation can be markedly accelerated in female *M. canicaudus* and *M. ochrogaster* housed with unfamiliar adult males from weaning (Hasler and Nalbandov, 1974; Hagen, 1978; Carter, Getz, Gavish, McDermott and Arnold, 1980).

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 can mate and conceive before one month of age (Hatfield, 1935; Hamilton, 1941; Cowen and Arsenault, 1954). Like other species female *M. canicaudus* can also have fertile matings before three weeks of age if housed near adult males from weaning (Hagen, 1978). However, 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 and Conaway, 1969b). These findings suggest that when isolated from males, female *M. canicaudus*, or voles in general, reach maturity much later than other 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 and Lombardi, 1975; Vandenbergh, Finlayson, Dobrogosz, Dills and Kost, 1976).

In summary, results of these studies show that when female *M. canicaudus* are isolated from males from weaning, variability among vaginal smear patterns is related to age. As females mature, vaginal smear patterns progressively change from persistently leucocytic to periodically estrous, and finally, to persistently estrous. Maturational increases in the incidence of vaginal estrus in isolated females are associated with increases in the propensity of intact females to show mating behavior, and with increases in behavioral responsiveness of ovariectomized individuals to estrogen.
In addition, females 150 days of age or older (the age after which most females show persistent vaginal estrus) are more likely to conceive within 48 hours after pairing with adult males than females younger than 150 days of age.
Figure 2.1. Age-Related Changes in Vaginal Smear Patterns, Behavior and Conception Rates in Female *Microtus canicaudus*. Percentage of animals examined (ordinate) are plotted for animals of three age groups (abscissa). Characteristics examined were A) proportion of females showing each of three types of vaginal smear pattern (L=persistently leucocytic; Cy=cycling; C=persistently cornified), B) proportion of females showing behavioral receptivity (as indicated by presence of sperm in vaginal smears) during a 2-week period, C) proportion of females conceiving as indicated by pregnancy after sperm were found in vaginal smears, and D) proportion of females exhibiting sexual receptivity after ovariectomy and estrogen replacement (see text for details).
Figure 2.1
CHAPTER 3: HORMONE LEVELS ASSOCIATED WITH DIFFERENT VAGINAL SMEAR PATTERNS IN Microtus canicaudus FEMALES OF VARIOUS AGES

INTRODUCTION

Plasma hormone levels associated with particular vaginal smear patterns throughout the estrous cycle are well-characterized for several spontaneously-ovulating rodents (Feder, Resko and Goy, 1968; 1969; Leavitt and Blaha, 1970; Lukaszewska and Greenwald, 1970; Challis, Heap and Illingworth, 1971; Baranczuk and Greenwald, 1970; Butcher, Collins and Fugo, 1974; Campbell, Ryan and Schwartz, 1976). Plasma estrogen and progesterone levels in these females change cyclically because of regular follicular cycles associated with ovulation. These regular changes in hormone levels result in predictable cycles in vaginal smear patterns and reproductive behavior in spontaneously ovulating rodents.

Not all rodents ovulate spontaneously; microtine rodents, specifically voles, ovulate only after mating (reflex ovulation) (Bodenheimer and Sulman, 1946; Greenwald, 1956; Breed, 1967, 1972; Richmond and Conaway, 1969a, b; Clulow and Mallory, 1970; Kirkpatrick and Valentine, 1970; Cross, 1972; Gray, Davis, Zerylnick and Dewsbury, 1974; MacFarlane and Taylor, 1982a, b). Despite the absence of spontaneous ovulatory changes, alternating vaginal smear patterns ("cyclicity") have been reported in
several vole species (Brambell and Hall, 1939; Chitty and Austin, 1957; Milligan, 1974; MacFarlane and Taylor, 1982b; Spielvogel, Chapter 1). The cellular associations seen in various vaginal smears of "cycling" voles are similar to those reported for spontaneously-ovulating rodents (Allen, 1922; Long and Evans, 1922); however, vaginal "cycles" observed in voles probably do not result from the same cyclic ovarian changes that elicit vaginal cycles and recurrent sexual behavior in spontaneous ovulators. The hormone levels associated with vaginal smears of "cycling" voles have not been measured nor have hormone levels associated with female receptivity been measured in voles.

As described in the preceding chapter, vaginal "cyclicity" occurs in the gray-tailed vole, M. canicaudus, during a specific period of maturation (between approximately 90 and 120 days). Prior to this period (30 to 50 days), female voles generally show persistently leucocytic vaginal smears and after the period of "cyclicity" (between 150 and 200 days), females generally show persistent vaginal cornification. Thus, vaginal smear patterns in this species change as females mature. Hormone levels associated with these age-related changes in vaginal smear patterns have not been measured in any vole.
The present studies measured estrogen, testosterone and progesterone levels associated with different vaginal smear patterns in \textit{M. canicaudus}. Females were sampled at different ages, and females of the same age were sampled during the stage when vaginal "cyclicity" is observed (90-120 days). In addition, hormonal correlates of female sexual receptivity were examined in \textit{M. canicaudus}.

\textbf{MATERIALS AND METHODS}

\textbf{ANIMALS}

\textit{M. canicaudus} were from a colony maintained at Oregon State University in a well-ventilated room with a controlled photoperiod (16L:8D, lights off at 1430 h). The animals were housed in fiberglass cages (60x15x15 cm) containing hardwood shavings and covered with hardware cloth. Purina Rat Chow, Rabbit Chow and water were available \textit{ad libitum}. All voles were weaned at 18 days of age and housed in sexually-segregated groups of 2 to 3 siblings per cage until used in the studies.

\textbf{PROCEDURES}

\textbf{Sampling}

Blood samples were taken from the retro-orbital sinus using heparinized capillary tubes. Samples were placed immediately on ice, then centrifuged. Plasma was stored
at -20° until assayed for estrogen, progesterone or testosterone.

Vaginal smears were obtained by gently flushing the vagina with warm tap water, using a micropipette. Smears were stained with toluidine blue and examined microscopically. The number of epithelial cells, leucocytes and cornified epithelial cells were counted in 3 microscopic fields. The mean proportion of each of these cell types per smear was calculated.

In all studies, blood samples were taken immediately after an animal was removed from its cage, and vaginal smears were obtained after blood sampling. The bleeding procedure required less than 1 minute to perform.

Behavior Tests
The procedures used for testing behavioral receptivity were described in Chapter 2.

Radioimmunoassays
All antibodies used were supplied by Dr. G. D. Niswender (Ft. Collins, Colo.).

Estrogen
Estrogen was assayed in 100 ul aliquots using procedures previously described (Korenman, Stevens, Carpenter, Robb, Niswender and Sherman, 1974). The small
volume of blood obtained from each female necessitated pooling samples from 2 to 3 females always selected from the same age group and showing similar vaginal smear patterns. Extraction with two 2-ml volumes of diethyl ether resulted in a mean extraction efficiency of 86.0 ± 1.8%. Antibody #244 anti-estradiol-6-BSA serum was used at a dilution of 1:450,000 and approximately 15,000 dpm $^3$H-17B-estradiol (Amersham) were added to each tube in the assay. All solutions were made in 0.1% gelatin (pH 7.4) within a week before use, filtered through 0.45 μm filters (Sartorius Membranfilter) and kept sterile. This procedure resulted in significant reductions in nonspecific binding, compared with assays using nonsterile solutions or solutions prepared more than 1-2 weeks before use.

The range of the standard curve was from 2.5 pg to 250 pg. The 2.5 pg standard was always 2 SD greater than 0, thus the sensitivity of the assay was 2.5 pg per tube or 25 pg ml$^{-1}$ when 100 ul samples were used. Estradiol was consistently undetectable in water blanks. The slope of a dilution curve of 200, 175, 150, 125, 100 and 75 ul of plasma and the slope of the standard curve did not differ, indicating parallelism. Known amounts of estradiol were added to charcoal-stripped plasma, extracted and assayed. The correlation coefficient
between expected vs. observed values was 0.998, and the mean deviation of observed from expected values was 9.8%. The intraassay 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%.

Although the antibody used is highly specific for estradiol, it crossreacts to a limited degree (less than 3%) with estrone (Korenman, et al., 1974). Insufficient plasma was available for chromatography to determine whether significant concentrations of estrone were being measured in addition to estradiol; therefore, values are reported as estrogen.

Progesterone and Testosterone

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

Progesterone was assayed in 25 ul duplicate aliquots of plasma, and testosterone in 50 ul individual aliquots using general procedures previously described (Frankel, Mock, Wright and Kamel, 1975; Koligian and Stormshak,
1977). Charcoal precipitation was used to separate bound from free steroids. All solutions were made in 0.01 M PBS with 0.1% gelatin (pH=7.0).

For progesterone assays, extraction with two 2-ml volumes of diethyl ether resulted in a mean extraction efficiency of 80.0 ± 3.2%. Anti-progesterone-11-BSA serum (#337) was used at a dilution of 1:3750 and approximately 10,000 dpm $^3$H-progesterone (New England Nuclear) was used per tube. The range of the standard curve was 7.8 pg to 1000pg. The 7.8 pg standard was always 2 SD greater than 0, making the sensitivity of the assay 7.8 pg per tube or 312 pg ml$^{-1}$ in 25 ul samples. Progesterone was never detected in water blanks.

The slope of a dilution curve of 200, 100, 50, 25 and 10 ul of plasma did not differ from that of the standard curve. Known amounts of progesterone were added to charcoal-stripped plasma, extracted with ether and assayed. The mean deviation of observed from expected values was 5.6%, and the correlation coefficient between expected and observed values was 0.999. The intraassay CV for 8 replicates of a plasma pool was 4.0% and the interassay CV for 10 assays was 6.8%.

Testosterone was extracted from samples with two 2-ml volumes of benzene-hexane (2:1). Extraction efficiency was 95.1 ± 2.6%. Anti-testosterone-11-BSA (#250) serum
was used at a dilution of 1:90,000 and approximately 12,000 dpm [1,2,6,7,16,17-3H (N)]-testosterone (NET-553, New England Nuclear) were added to each assay tube. Antibody binding was 41.6% of which 1.9% was nonspecific. The intraassay CV was 6.8% and all samples were run in the same assay. The range of the standard curve was 6.25 to 500 pg. The 6.25 pg standard was 2 SD greater than 0, thus the sensitivity of the assay was 6.25 pg per tube or 125 pg per ml in 50 ul samples. No testosterone was detected in water blanks. The slope of a dilution curve of 100, 50, 25, 12.5, 6.25, 3.125 ul plasma and the slope of the standard curve did not differ. Known amounts of testosterone were added to charcoal-stripped plasma and the plasma extracted and assayed. The mean deviation of observed from expected values was 9.8% and the CV between expected and observed values was 0.992.

STUDY 1 PROGESTERONE, AGE AND VAGINAL SMEAR PATTERNS

To determine whether different vaginal smear patterns were related to differences in progesterone levels, and whether progesterone levels changed with age, vaginal smears and blood samples were taken from a total of 95 females aged 30, 45, 60, 75, 90, 120 and 150 days. For this study, vaginal smears were categorized as follows:
L) predominantly leucocytic with occasional epithelial cells (similar to diestrous smears in mice; Allen, 1922), N) nucleated epithelial cells predominantly (similar to prooestrous smears in mice), and C) cornified cells only (similar to estrous smears in mice). Smear patterns and progesterone levels at various ages were then compared.

It is possible that subtle changes in vaginal smear patterns may reflect differences in progesterone levels. For this reason, in the next study, vaginal smear categories were subdivided and progesterone levels were measured in 60 females between 110 and 130 days of age. Vaginal smear patterns were subdivided into six categories as follows: L1) predominantly leucocytes with occasional epithelial cells, L2) leucocytes, epithelial cells and cornified cells present in similar numbers, N) nucleated epithelial cells and mucous, C1) cornified cells and leucocytes, C2) cornified cells, nucleated epithelial cells and leucocytes, and C3) cornified cells only (Fig. 3.1). Blood samples were assayed for progesterone and levels were compared among females in the six categories. In addition, ovaries were examined macroscopically for the presence of corpora lutea.
STUDY 2  ESTROGEN, PROGESTERONE AND TESTOSTERONE IN FEMALES WITH DIFFERENT VAGINAL SMEAR PATTERNS

To determine whether the different vaginal smear patterns of "cycling" females reflect different hormone levels, a group of 85 females 120 days of age were examined. Vaginal smears and blood samples were obtained from all animals and samples were analyzed for estrogen, progesterone and testosterone. Because of the large number of animals required to measure these three hormones, vaginal smears were not subdivided, but rather, all smears were categorized into the three types most commonly seen (L, N, C, described above).

STUDY 3  ESTROGEN LEVELS IN CYCLING AND PERSISTENTLY ESTROUS FEMALES

To determine whether similar vaginal smear patterns are associated with the same estrogen levels in animals of different ages, 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 were "cycling" and had shown only 1 to 2 previous periods of cornification; those at 150 days displayed persistent vaginal cornification.
This study was performed to determine whether hormone levels of unreceptive and receptive females differ. Virgin females between 150 and 180 days of age with vaginal smears showing predominantly cornified cells were used in this study. Reproductive behaviors of females were tested as described in Chapter 2 and females were classified as either receptive or unreceptive. Immediately after the first mount with intromission by the stud male, blood was taken from the receptive female and handled as previously described. The mean time between pairing and bleeding was determined in receptive females, and unreceptive females were bled after a comparable period of exposure to males. Progesterone levels of receptive (n=10) and unreceptive females (n=12) and estrogen levels of receptive (n=5 pools) and unreceptive females (n=5 pools) were compared.

DATA ANALYSIS

These data were analyzed using One-Way or Two-Way Analysis of Variance to determine differences among means. Bonferroni's t-tests were used to determine differences between specific means where appropriate. Student's t-tests were used to determine differences between two means. Regression analysis was used to determine the
relationship between two variables.

RESULTS

STUDY 1

There were no age-associated differences in progesterone or differences in progesterone levels associated with vaginal smear types. Therefore, data from all age groups were combined for each vaginal smear type so that progesterone levels could be compared among types. No differences in plasma progesterone levels were seen among females exhibiting the three major vaginal smear types (Table 3.1) nor among females showing any of the six subcategories of vaginal smear types (Table 3.2). No corpora lutea were found in females displaying any of the vaginal smear types described.

STUDY 2

Both testosterone and estrogen levels were significantly higher in females that have predominately cornified cells than in females that have either leucocytic or nucleated epithelial smear types. As in Study 1, progesterone levels did not differ among females showing any of the three smear patterns observed (Table 3.3).
STUDY 3

Estrogen concentrations were significantly higher in females showing cornified smears than in those showing leucocytic smears at 90 and 150 days (Table 3.4). Significantly higher estrogen levels were found in 150-day-old females than in 90-day-old females, regardless of the vaginal smear pattern. Finally, estrogen concentrations in females displaying vaginal cornification at 90 days did not differ significantly from those of females displaying leucocytic vaginal smears at 150 days.

STUDY 4

The mean progesterone level of unreceptive females (5.8 ± 0.6 ng ml⁻¹) was not significantly different from that of receptive females (6.3 ± 0.9 ng ml⁻¹). However, estrogen was significantly higher (p<0.001) in receptive (290 ± 21 pg ml⁻¹) than in unreceptive (150 ± 15 pg ml⁻¹) females.

DISCUSSION

These results demonstrate that vaginal smear patterns in M. canicaudus are not associated with particular levels of estrogen or progesterone. Females showing the same vaginal smear patterns had different estrogen levels if examined at different ages. Furthermore, when females
of the same age were examined, estrogen levels were similar, regardless of the vaginal smear patterns. The only exception was that vaginal cornification at any age was associated with significantly higher estrogen levels than other smear patterns. In contrast to estrogen levels, progesterone levels were similar in females with different vaginal smears and in females of different ages.

The cellular associations observed in various vaginal smears of female voles between the ages of 90 and 120 days (a period during which vaginal "cyclicity" occurs; S. P. Spielvogel, Chapter 2) were the same as those described for spontaneously-ovulating rodents (Allen, 1922; Long and Evans, 1922). However, vaginal smear pattern changes in "cycling" voles were not associated with the same ovarian hormone changes that occur during the estrous cycles of spontaneous ovulators. For example, nucleated epithelial cell smears in *M. canicaudus* (proestrous smears in rats) were not associated with peak levels of estrogen as they are in rats (Butcher, Collins and Fugo, 1974); in voles, peak estrogen levels were associated with vaginal cornification (estrous smears in rats). Furthermore, female voles, unlike female rats, have similar estrogen and progesterone levels when displaying metestrous-like, proestrous-like and diestrous-like vaginal smears.

The finding that similar vaginal smear patterns in voles and rats are associated with different estrogen and
progesterone levels indicate that vaginal smear patterns in voles reflect ovarian events different from those of rats. Vaginal smear pattern changes in rats reflect regular follicular cycles; as follicles develop, estrogen levels rise and trigger an ovulatory surge release of luteinizing hormone. After ovulation, estrogen levels are low and progesterone is secreted by corpora lutea. In contrast, vaginal changes in voles may result from irregular, nonovulatory follicular changes similar to those described in another reflex ovulator, the rabbit. In rabbits, some follicles are maturing while others are undergoing atresia (Hill and White, 1933). In support of this hypothesis, ovaries of unmated female *Microtus canicaudus* older than 90 days contain similar numbers of developing, mature, and atretic follicles, no matter what types of vaginal smear pattern are exhibited (Spielvogel, unpublished). Similarly, in a closely related species, *Pitymys subterraneus* (another reflex ovulator), the number of large ovarian follicles per female is similar among females regardless of vaginal smear patterns (Jemiolo, 1983).

The hypothesis that vaginal smear changes in "cycling" female *Microtus canicaudus* are nonovulatory is supported by the finding that corpora lutea were not observed in any females between the ages of 90 and 120 days of age. Furthermore, progesterone levels did not
differ among females with different vaginal conditions at any age, including the period during which cyclicity commonly occurs (90-120 days).

Estrogen levels were significantly higher in females at 150 days of age than at 90 days of age. Females were "cycling" at 90 days of age but were displaying prolonged periods of cornification at 150 days of age. Thus, increased estrogen levels in older females may reflect ovarian maturation and may explain the age-related increases in the incidence of vaginal estrus in voles during the first 5 months of life.

Receptive female voles had estrogen concentrations nearly twice as high as unreceptive females, but progesterone levels did not differ. These findings suggest that estrogen, but not progesterone, plays a role in inducing female sexual behavior in the vole. This is supported by the findings that estrogen injections can induce sexual receptivity in ovariectomized female voles, and that progesterone injections do not facilitate the display of female receptivity (Dluzen and Carter, 1979; Spielvogel, unpublished). In other reflex ovulators, progesterone levels remain low and constant during periods of receptivity whereas estrogen levels are elevated (Blatchley and Donovan, 1972; Verhage, Beamer and Brenner, 1976; Shille, Lundstrom and Stabenfeldt, 1979). Like voles, ferrets and cats require estrogen, but not
progesterone, for the display of complete sexual behavior after ovariectomy (Michael and Scott, 1964; Baum, 1979). In contrast, a number of spontaneously-ovulating mammals have elevated levels of both estrogen and progesterone during periods of sexual receptivity (Feder, Goy and Resko, 1967; Feder, Resko and Goy, 1968; Leavitt and Blaha, 1970); these species also require both estrogen and progesterone priming for the display of maximal levels of receptivity after ovariectomy (Dempsey, Hertz and Young, 1932; Boling and Blandau, 1939; Ring, 1944; Frank and Fraps, 1945; Carter, Michael and Morris, 1973; Whalen, 1974; Steel, 1981).

Mean progesterone levels in female M. canicaudus isolated from males ranged between 4.1 and 8.6 ng ml$^{-1}$, regardless of age or vaginal smear pattern. Progesterone levels have been reported in two other female microtines, M. arvalis and M. montanus. Unlike M. canicaudus and M. montanus females, M. arvalis females are reported to ovulate spontaneously (Delost, 1955). The range of progesterone concentrations in M. arvalis is approximately 1 to 8 ng ml$^{-1}$ depending on the stage of the estrous cycle. The highest progesterone levels in M. arvalis appeared at metestrus and the lowest at proestrus (Dobrowolska and Gromadzka, 1978). The absolute values of progesterone in M. arvalis are similar to those of M. canicaudus. Progesterone values reported
for *M. montanus* (9 to 14 ng ml$^{-1}$) (Gray, Davis, Kenney and Dewsbury, 1976) were somewhat higher than those of *M. canicaudus*. Unlike *M. canicaudus*, *M. montanus* females displaying cornified vaginal smears have higher progesterone levels than those with leucocytic smears.

Estrogen levels have not been reported for any other microtine. The estrogen levels of female *M. canicaudus* are generally higher than those reported for other rodents (Challis, Heap and Illingworth, 1971; Butcher, Collins and Fugo, 1974; McCormack and Greenwald, 1974; Shaikh and Shaikh, 1975; Campbell, Ryan and Schwartz, 1976). However, maximum levels of estrogens in the hamster were similar to those of the vole (Baranczuk and Greenwald, 1973). Unpublished work by Dluzen (personal communication) shows that estrogen levels of female *M. ochrogaster* are similar to those of *M. canicaudus* (personal communication).

In summary, the changes in vaginal smear patterns in "cycling" *M. canicaudus* do not result from the same hormonal changes that produce vaginal cycles in spontaneously-ovulating rodents. Estrogen and progesterone levels are fairly constant in "cycling" voles, except during periods of vaginal cornification when estrogen levels increase. Elevations in progesterone levels are not seen in sexually receptive female voles as
they are in spontaneously-ovulating rodents. However, estrogen levels are higher in receptive than in unreceptive female voles, as has been reported in other rodents. These findings indicate that estrogen, but not progesterone, is important in the display of female sexual behavior in voles. Finally, estrogen levels rise as animals mature; age-related increases in estrogen levels may explain age-related increases in the incidence of vaginal cornification and sexual receptivity in voles.
Table 3.1. Plasma Progesterone Levels in Female Microtus canicaudus Exhibiting Different Vaginal Smear Patterns at Different Ages.

<table>
<thead>
<tr>
<th>AGE (DAYS)</th>
<th>L</th>
<th>VAGINAL SMEAR PATTERNS</th>
<th>N</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>4.10 ± 0.89 (5/5)</td>
<td></td>
<td>0/5</td>
<td>0/5</td>
</tr>
<tr>
<td>45</td>
<td>5.91 ± 0.74 (9/9)</td>
<td></td>
<td>0/9</td>
<td>0/9</td>
</tr>
<tr>
<td>75-90</td>
<td>6.53 ± 0.42 (25/53)</td>
<td>6.99 ± 0.51 (15/53)</td>
<td>6.33 ± 0.43 (13/53)</td>
<td></td>
</tr>
<tr>
<td>120</td>
<td>6.48 ± 0.89 (5/21)</td>
<td>8.00 ± 0.77 (7/21)</td>
<td>5.92 ± 0.64 (9/21)</td>
<td></td>
</tr>
<tr>
<td>150</td>
<td>8.65 ± 0.85 (2/7)</td>
<td>7.00 ± 0.40 (2/7)</td>
<td>5.30 ± 1.60 (3/7)</td>
<td></td>
</tr>
<tr>
<td>ALL AGES COMBINED</td>
<td>6.28 ± 0.33 (46/95)</td>
<td>7.29 ± 0.39 (24/95)</td>
<td>6.11 ± 0.39 (25/95)</td>
<td></td>
</tr>
</tbody>
</table>

Progesterone concentrations are expressed as mean ± SEM (ng ml⁻¹). The proportion of females exhibiting particular vaginal smear patterns are given in parentheses. L=predominantly leucocytic smears; N=predominantly nucleated epithelial cells; C=predominantly cornified cells.
Table 3.2. Plasma Progesterone Levels in Female *Microtus canicaudus* Exhibiting Different Subcategories of Vaginal Smear Patterns.

<table>
<thead>
<tr>
<th>VAGINAL SMEAR PATTERN</th>
<th>L1</th>
<th>L2</th>
<th>N</th>
<th>C1</th>
<th>C2</th>
<th>C3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6.79 ± 0.48</td>
<td>7.01 ± 0.99</td>
<td>8.34 ± 0.98</td>
<td>7.83 ± 1.28</td>
<td>6.37 ± 0.49</td>
<td>7.15 ± 0.69</td>
</tr>
<tr>
<td>(n=20)</td>
<td>(n=9)</td>
<td>(n=5)</td>
<td>(n=3)</td>
<td>(n=6)</td>
<td>(n=17)</td>
<td></td>
</tr>
</tbody>
</table>

Plasma progesterone concentrations are expressed as mean ± SEM (ng ml$^{-1}$). See text for description of vaginal smear patterns. (n=number of individuals sampled).
Table 3.3. Plasma Steroid Concentrations in Female Voles Exhibiting Different Vaginal Smear Patterns.

<table>
<thead>
<tr>
<th>HORMONE</th>
<th>VAGINAL SMEAR PATTERN</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>L (n=14)</td>
</tr>
<tr>
<td>ESTROGEN (pg ml$^{-1}$)</td>
<td>56.06 ± 5.58</td>
</tr>
<tr>
<td>TESTOSTERONE (ng ml$^{-1}$)</td>
<td>0.34 ± 0.03</td>
</tr>
<tr>
<td>PROGESTERONE (ng ml$^{-1}$)</td>
<td>6.80 ± 0.66</td>
</tr>
</tbody>
</table>

Gonadal steroid concentrations are expressed as mean ± SEM. For estrogen concentrations, n=number of pools of 2-3 females; for testosterone and progesterone, n=number of individual samples.
Table 3.4 Plasma Estrogen Concentrations in Female Voles Exhibiting Different Vaginal Smear Patterns at Different Ages.

<table>
<thead>
<tr>
<th>AGE (DAYS)</th>
<th>VAGINAL SMEAR PATTERN</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>L</td>
</tr>
<tr>
<td>90</td>
<td>52.83 ± 3.89 (n=12 pools)</td>
</tr>
<tr>
<td>150</td>
<td>139.87 ± 7.20 (n=8 pools)</td>
</tr>
</tbody>
</table>

Estrogen concentrations are expressed as mean ± SEM. Pools consisted of 2 to 3 animals of the same age and vaginal smear pattern.
Figure 3.1. Vaginal Smear Patterns in "Cycling" Female Voles Between 110-130 Days of Age. L1, Predominantly leucocytes with occasional epithelial cells; L2, leucocytes, epithelial cells and cornified cells present in similar numbers; N, nucleated epithelial cells and mucus; C1, cornified cells and leucocytes; C2, cornified cells, nucleated epithelial cells and leucocytes; C3, cornified cells only.
Figure 3.1
CHAPTER 4: EVIDENCE FOR MASCULINIZATION IN FEMALE Microtus canicaudus

INTRODUCTION

According to the organizational hypothesis of sexual differentiation of the mammalian brain (Phoenix, Goy, Gerall and Young, 1959), exposure to androgen during development permanently alters sexual behavior and gonadotropin release patterns in adulthood. It is believed that these tissues are inherently feminine, but that androgen stimulation alters brain function in such a way that permanent masculinity is imposed. Presumably, the active androgen present during early development is testosterone (Resko, Feder and Goy, 1968).

If injected with large doses of testosterone during specific periods of perinatal development, female rats, hamsters, mice and guinea pigs lose the ability to ovulate spontaneously in adulthood. These androgenized females exhibit no regular follicular cycles, possess polyfollicular ovaries with no luteal tissue, and exhibit persistently cornified vaginal smears (Barraclough and Leathem, 1954; Barraclough, 1961; 1966; 1967; Harris, 1964; Brown-Grant and Sherwood, 1971; Swanson and Crossley, 1971). These characteristics are also seen in vaginal and ovarian tissue grafted into males, and appear to be secondary to the inability of either males or androgenized females to release cyclic surges of
gonadotropins in response to elevated serum estrogen and progesterone levels (Gorski and Wagner, 1965; Mennin and Gorski, 1975; Gogan, Beattie, Hery, LaPlante and Kordon, 1980). In addition to exhibiting a masculine, tonic pattern of gonadotropin release, androgenized female rodents have an increased propensity to display masculine mounting, intromission and, in some species, ejaculatory behaviors when stimulated with testosterone in adulthood (Phoenix, et al., 1959; Harris and Levine, 1965; Crossley and Swanson, 1968; Edwards and Burge, 1971; Carter, Clemens and Hoekema, 1972; Sachs, Pollak, Krieger and Barfield, 1973).

Female voles, untreated during development, possess characteristics that are similar to those of androgenized female rodents. These include: an absence of spontaneous ovulation, no regular estrous cycles, polyfollicular ovaries with no luteal tissue, and vaginal smears showing persistent cornification (Bodenheimer and Sulman, 1946; Greenwald, 1956; Breed, 1967, 1972; Richmond and Conaway, 1969a, 1969b; Clulow and Mallory, 1970; Kirkpatrick and Valentine, 1970; Cross, 1972; Gray, Davis, Zerylnick and Dewsbury, 1974; MacFarlane and Taylor, 1982a, 1982b; Spielvogel, Chapters 2 and 3). The finding that LH release is not facilitated by estrogen and progesterone in one vole species, *Microtus arctis* (Milligan, 1978), supports the idea that the neuroendocrine control of
gonadotropin release in voles is similar to that of androgenized female rodents.

The present studies were conducted to determine whether treatment with estrogen and progesterone can facilitate ovulatory surges of LH in adult gray-tailed voles, *Microtus canicaudus*. In addition, these studies investigated the potential for masculine copulatory behavior in female voles treated with testosterone after ovariectomy in adulthood. Initial studies demonstrated that females do not ovulate as a result of estrogen and progesterone priming, and that female voles do exhibit male copulatory behavior in response to testosterone. To determine whether these characteristics result from perinatal androgen exposure, flutamide, a potent antiandrogen lacking androgenic, estrogenic or progestagenic effects in rats (Neri, Florance, Koziol and van Cleave, 1972), was administered perinatally. Because aromatization of testosterone to estrogen may be involved in brain masculinization (Doughty, Booth, McDonald, Parrott, 1975; Naftolin, Ryan, Davies, Reddy, Flores, Petro and Kuhn, 1975; Lieberburg, Wallach and McEwen, 1977; McEwen, Lieberburg, Chaptal and Krey, 1977; Vreeburg, van der Vaart and van der Schoot, 1977; Booth, 1978; Sodersten, 1978; Whalen and Olsen, 1981), other female voles were treated perinatally with an antiaromatase, ATD.
ANIMALS

Microtus canicaudus used were from a colony maintained on a 16L:8D photoperiod (lights off at 1430 h). Purina Rat Chow, Purina Rabbit Chow and water were available ad libitum.

MATERIALS

Estradiol benzoate (EB), testosterone (T), carboxymethylcellulose, polysorbate 80 and benzyl alcohol were obtained from Sigma Chemical Co., St. Louis. Flutamide (4'-nitro-3'trifluoromethylisobutyranilide, batch number 10485-95) was donated by Schering Corp., Bloomfield, N.J. ATD (1,4,6-androstatrien-3,17-dione) was purchased from Steraloids, Inc., Wilton, N.H. Vetalar (ketamine HCl) was obtained from Parke-Davis, Morris Plains, N.J. and Rompun (xylazine) from Haver-Lockhart, Shawnee, Kansas. Silastic tubing and Silastic 382 Medical Grade Elastomer were purchased from Dow Corning Corp., Midland, Mich.

PROCEDURES

Hormone Administration

Both flutamide and ATD crystals were suspended in an aqueous vehicle composed of 0.9% NaCl, 0.5%
carboxymethylcellulose, 0.4% polysorbate 80, and 0.9% benzyl alcohol (Neri, et al., 1972) for oral or parenteral administration. Silastic capsules of ATD or testosterone were made by sealing one end of a 1-cm. piece of Silastic tubing (i.d., 1.47 mm; o.d., 1.96 mm) with Silastic Medical Grade Elastomer, packing the tubing with crystalline steroid and sealing the open end with Elastomer (Moore, 1981).

The doses, times and modes of administration of flutamide were based on results of preliminary studies. I found that a dose of 5 mg flutamide administered daily to pregnant females was just as effective as 7.5 mg and more effective than either 0.5 or 2 mg in preventing genital masculinization in offspring. Implantation of flutamide capsules (three 1-cm capsules) was not as effective as a 5 mg oral dose. Daily doses rather than twice daily doses were given because abortion always resulted when pregnant females were handled more than once daily.

Surgical Procedures

Implantation of ATD capsules into pregnant females and ovariectomies of adult females were performed under anesthesia produced by injecting 7 ug Rompun gm⁻¹ body weight, and 125 ug Vetalar gm⁻¹ body weight. Bilateral ovariectomies were performed through a single dorsal midline incision.
Behavior tests

Tests for the ability to show male behaviors were performed 1 h after the onset of the dark period under dim red lights. Stimulus females were prepared by injecting ovariectomized females with 30 ug estradiol benzoate in 0.1 ml corn oil 24 and 48 h before the test. Only females showing receptivity in brief pretests with active males were included as stimulus animals.

The test arena consisted of a sheetmetal cylinder (30 cm in diameter and 50 cm high) sitting on an elevated glass floor. A mirror mounted at a 45° angle beneath the floor allowed ventral viewing. The animal to be tested for male behaviors was placed in the arena for 6 minutes prior to introducing the stimulus female. The pair was observed for 10 minutes or until one ejaculatory series was completed. An ejaculatory series was defined as a mating sequence that ended with ejaculation (males) or pseudoejaculation (females) and that was followed by a pause exceeding 90 seconds. Behaviors were coded and recorded on a 20-channel Esterline-Angus manual event recorder. The following sexual behaviors were quantified: 1) mount latency (ML), the time between the introduction of the stimulus female and the first mount 2) intromission latency (IL), the time between introduction of the stimulus female and the first intromission 3) ejaculation
latency (EL), the time between the introduction of the stimulus female and ejaculation, and 4) intromission frequency (IF), the number of intromission bouts in an ejaculatory series.

**Vaginal Smears**

Vaginal smears were obtained using warm water and a fire-polished micropipette. A drop of water was placed at the vaginal opening and then gently aspirated. Smears were stained with toluidine blue, examined and classified as persistent diestrus, persistent estrus or "cycling" as described in Chapter 2.

**Blood Sampling and Testosterone Radioimmunoassay**

Blood was obtained in heparinized capillary tubes from the suborbital capillary plexus, placed on ice and centrifuged. Plasma was stored at -20°C until assayed for testosterone.

Testosterone was assayed in a RIA that was validated as described in Chapter 3. Samples (50 ul) were extracted with benzene-hexane (1:2) and values obtained from the assay were corrected for a mean extraction efficiency of 96.4%. Extracted water blanks had no measurable testosterone. All samples were analyzed in the same assay. The intraassay CV was 4.9% and the sensitivity of the assay was 7.5 pg tube\(^{-1}\) or 0.15 ng ml\(^{-1}\).
DATA ANALYSIS

Data comparing the incidences of male behaviors or ovulation were analyzed using Fisher's exact probability tests. Data comparing components of male behavior were analyzed using One-Way Analysis of Variance.

STUDY 1

The present study was designed to quantify masculine sexual behaviors in females. This study also investigated whether masculinization was dependent upon perinatal testosterone activity by administering drugs which block the action of androgens in other species.

Methods

A. Flutamide and Masculine Behavior in Female Voles

Nonpregnant females were paired with adult males and vaginal smears obtained at 0730, 1530 and 2130 h daily. Males were removed after sperm were found in the smear; this time was considered to be the time of conception (day 0 post-coitum, p.c.). On day 11 p.c., females were assigned randomly to one of the seven treatment groups described in Table 4.1. For prenatal treatments, flutamide (5 mg in 0.2ml vehicle) or vehicle (0.2 ml) was administered daily to pregnant females by gavage through a straight feeding needle from day 11 p.c. through day 20 p.c. (the day before parturition). Postnatally, flutamide
or vehicle was administered by subcutaneous injection; injection sites were sealed with collodion. Beginning at 30 minutes after birth (Day 0) and continuing through Day 4, pups were injected daily with 0.125 mg flutamide (approximately 0.06 mg gm\(^{-1}\) body weight) or 0.05 ml vehicle. From Day 5 through Day 9 (the day of eye-opening), pups were injected daily with 0.25 mg flutamide (approximately 0.06 mg gm\(^{-1}\) body weight) or 0.1 ml vehicle.

The anogenital distances of males and females treated prenatally, or prenatally plus neonatally, were measured at 15 days of age using vernier calipers. At 18 days of age, all pups were weaned and subsequently housed with 2 to 3 littermates of the same sex, until 45 days of age when females were transferred to individual cages. (The subsequent treatment of experimental males is described in Chapter 5.) Vaginal smears were obtained daily for 15 days, beginning at 90 days of age. At 105 days, all females were ovariectomized and implanted subcutaneously in the mid-dorsum with a 1-cm capsule of testosterone (containing approximately 2 mg). Untreated males were left intact so that masculine behaviors of treated females could be compared with those of normal males.

B. ATD and Masculine Behavior in Female Voles

To determine whether aromatization of testosterone to estrogen is important for the development of the potential
for masculine behavior in female voles, subjects were treated perinatally with ATD.

On Day 12 p.c., three 1-cm capsules of ATD or three empty capsules were implanted subcutaneously in pregnant females. Within an hour after birth and for the next 9 days, offspring of ATD-implanted females were injected with 1.25 mg ATD suspended in 0.05 ml vehicle. Pups from control females were injected with 0.05 ml vehicle. All pups (treated and controls) were crossfostered to untreated mothers soon after birth, because previous studies demonstrated that mothers implanted with ATD are not consistently able to raise pups to weaning (S. P. Spielvogel, unpublished).

At 18 days of age, pups were weaned and subsequently housed in sexually-segregated groups of 3 to 4 siblings. At 45 days of age, body weights and anogenital distances were measured in treated and control females. At 100 days of age each female was bilaterally ovariectomized and implanted with a 1-cm capsule of testosterone. Body weights and penile sheath lengths were recorded 4 weeks after testosterone was implanted. Behavior tests were performed on all animals 2 days after these measurements were made, and weekly for the next two weeks. Seven controls and 11 treated animals survived the entire experiment.
Results, Study 1

Untreated males had significantly greater \((p<0.0005)\) mean anogenital distances than untreated females and vehicle-treated females (Fig. 4.1). Anogenital distances of flutamide-treated males were not different from those of vehicle-treated females. In addition, females treated only prenatally with flutamide had a significantly shorter mean anogenital distance \((p<0.05)\) than vehicle-treated controls.

The genitalia of ATD-treated females appeared to be masculinized (Fig. 4.2); the clitoris was large and protruded from a clitoral sheath. In addition, there was no vaginal introitus and a structure similar to a scrotal sac was apparent. No genital abnormalities were seen in flutamide-treated females before ovariectomy and testosterone implantation.

Flutamide treatment did not affect vaginal smear patterns. The patterns commonly observed were alternating days of nucleated epithelial, cornified or leucocytic smears \((38.1\%)\), persistent vaginal cornification \((42.9\%)\) or persistent leucocytic smears \((19.0\%)\). Vaginal smear patterns were defined as "persistent" when they occurred during at least 10 of the 15 days of examination.

In ovariectomized females treated with testosterone, the incidence of male copulatory behavior was similar among treatment groups (flutamide and ATD) and did not
differ from vehicle-treated or untreated controls (Fig. 4.3). (The incidence of behavior among control groups did not differ so data were combined for presentation.) In addition, the incidence of behavior shown by ovariectomized females treated with testosterone did not differ from that of intact males. There were also no differences among the experimental groups in ML, IL, EL, or IF (Table 4.2).

The ejaculatory behavior of intact males and ovariectomized testosterone-implanted females differed in a subtle way. At ejaculation, males thrust more frequently and then showed a final deeper thrust followed by genital grooming; the next mount was delayed for at least 90 seconds following ejaculation. Testosterone-implanted females ended a series of intromissions with an increased frequency of thrusting, followed by genital grooming and a pause exceeding 90 seconds. Thus, the only difference between intact males and ovariectomized testosterone-treated females was that the deep thrust shown by males at ejaculation was not apparent in females.

Testosterone levels were not significantly different in implanted females that showed male behavior (1.62 ± 0.53 ng ml⁻¹) compared to those not showing such behavior (2.44 ± 0.52 ng ml⁻¹). In addition, mean testosterone levels were similar in intact males (1.75 ±
0.22 ng ml\(^{-1}\)) and testosterone-implanted ovariectomized females from all groups (2.06 \pm 0.28 ng ml\(^{-1}\)).

**STUDY 2**

**Methods**

**A. Effect of Estrogen and Progesterone on Ovulation**

The ability to ovulate in response to rising estrogen and progesterone levels is abolished by perinatal androgenization (See Introduction). To determine whether female *M. canicaudus* exhibit positive feedback, several series of EB or EB plus P treatments were given to animals at 60, 90 or 150 days of age. All hormones were given subcutaneously; EB was dissolved in corn oil, and P suspended in physiological saline. Animals from each of these age groups received treatments A, B, and C, as described in Table 4.3. Ovaries were examined macroscopically for corpora lutea.

**B. Perinatal Flutamide and ATD Effects on Ovulatory Potential**

To determine whether interference with perinatal androgen activity results in the development of positive feedback in voles, females were treated with either flutamide or ATD during development, and tested for estrogen- and progesterone-facilitated ovulation in
adulthood.

Flutamide or vehicle was administered pre- and postnatally using the same methods and dosages as described in Study 1. Treatments were begun on day 11 p.c. and continued until the day of eye-opening (Day 9 or 10 after birth). ATD was also administered as described in Study 1; pregnant females were implanted on day 12 p.c. with either three 1-cm capsules of ATD or three empty capsules. Offspring were injected with 1.25 mg ATD suspension or 0.05 ml vehicle beginning on the day of birth and continuing until eye-opening.

At 90 days of age, one group of ATD-treated females (n=7), the flutamide-treated females (n=6) and corresponding controls for each (n=7 per group) were injected with 30 ug EB. A second injection of EB and an injection of P (500 ug) was administered 3 days after the first injection. These females were ovariectomized 4 days later. A second group of ATD-treated females (n=6) and controls (n=7) received 150 ug EB at 90 days of age and were ovariectomized 4 days later. Ovaries of all females were examined macroscopically for corpora lutea.

Results, Study 2

Corpora lutea were never seen in response to EB or to EB in combination with progesterone treatment in female voles untreated during development (0/52). In addition,
no control females or ATD-treated females possessed corpora lutea, indicating that ovulations had not occurred after EB or EB plus P treatments. Ovulation occurred in 2 of six females treated perinatally with flutamide. However, the proportion of females ovulating did not differ significantly between flutamide-treated females and controls (P=0.192).

DISCUSSION

Female *M. canicaudus* that were ovariectomized and treated with testosterone in adulthood displayed masculine behaviors. The temporal patterns and frequencies with which these behaviors were displayed by testosterone-treated females did not differ from those of intact males. Intact female voles exhibited persistent vaginal cornification in adulthood and, when treated with estrogen and progesterone, failed to exhibit signs of ovulation. Thus, female *M. canicaudus* exhibit characteristics that are typical of experimentally androgenized female rodents (Baum, 1979; Goy and McEwen, 1980).

The finding that female voles treated with testosterone in adulthood show masculine behaviors virtually identical to those of males distinguishes voles from other species. Female hamsters rarely mount or show
ejaculatory behavior when treated with testosterone in adulthood (Tiefer, 1970; Swanson and Crossley, 1971; Carter, Clemens and Hoekema, 1972; Paup, Coniglio and Clemens, 1972). Female guinea pigs mount, but not as frequently as males (Phoenix, Goy, Gerall and Young 1959; Goy, Phoenix and Meidinger, 1967; Goldfoot and van der Werff ten Bosch, 1975). Female rats display male mounting and intromission behavior, but the patterns and frequency of display are not the same as male counterparts (Gerall and Ward, 1966; Whalen and Robertson, 1968; Ward, 1969; Sodersten, 1972).

The ability of female voles to express nearly complete masculine sexual behavior in response to hormone treatment in adulthood differs from that of female rats, hamsters and guinea pigs. However, the propensity to show masculine behaviors can be enhanced in these other rodents if they are treated with androgen during the perinatal period. Like masculine copulatory behaviors in ovariectomized female voles treated with testosterone in adulthood, behaviors of perinatally-androgenized female rats differ from those of males only in the ejaculatory component (Sachs, Pollak, Krieger and Barfield, 1973; Thomas, Howard and Barfield, 1978; Thomas, McIntosh and Barfield, 1980; Thomas, Barfield and Etgen, 1982). The male behaviors of perinatally-androgenized female hamsters and guinea pigs are not as complete as those of
androgenized female rats or normal female voles; however, the frequency of mounting and intromission behaviors in guinea pigs and hamsters is increased by androgenization (Phoenix, Goy, Gerall and Young, 1959; Carter, Clemens and Hoekema, 1972).

A second difference between voles and other rodents concerns the control of ovulation and gonadotropin release patterns. In the present study, female voles showed no evidence of positive feedback when estrogen was administered alone or in combination with progesterone; corpora lutea formation was not induced by these treatments. Estrogen and progesterone are also ineffective in facilitating the release of ovulatory surges of LH in _M. agrestis_ (Milligan, 1978, 1981). The apparent absence of positive feedback in _M. agrestis_ and _M. canicaudus_ is consistent with the findings that these voles do not have regular vaginal cycles and ovulate reflexively after copulation, instead of spontaneously (Breed, 1967, 1972; Hasler, 1973; S. P. Spielvogel, unpublished). In addition, ovaries of adult female voles are polyfollicular with no corpora lutea (Breed and Charlton, 1971; Breed, 1972; Peters and Clarke, 1973; S. P. Spielvogel, unpublished data). These findings suggest that female voles have a tonic pattern of gonadotropin release as a result of the inability of gonadal hormones to exert a postive feedback effect on LH release. In
contrast, rising estrogen levels from developing ovarian follicles induce cyclic surges of LH in spontaneously-ovulating rodents (Feder, 1981) and regular vaginal cycles result from these cyclic changes in hormone levels (Challis, Heap and Illingworth, 1971; Baranczuk and Greenwald, 1973; Butcher, Collins and Fugo, 1974; Kalra and Kalra, 1974). If females of these spontaneously-ovulating species are treated with androgen during development, they resemble normal female voles. Specifically androgenized female rats, guinea pigs and hamsters do not respond to increased estrogen levels with an ovulatory surge of LH; as a result, ovaries are polyfollicular with no corpora lutea and vaginal smears are persistently cornified (Pfeiffer, 1936; Bradbury, 1941; Harris, 1964; Swanson and van der Werff ten Bosch, 1964; Barraclough, 1966; Swanson, 1966; Gorski, 1968; Brown-Grant and Sherwood, 1971; Gogan, Beattie, Hery, La Plante and Kordon, 1980).

Similarities between androgenized rodents and normal female voles in potential for the display of masculine sexual behavior and pattern of gonadotropin release may result from exposure of female voles to masculinizing levels of endogenous androgens during development. Consistent with this hypothesis is the finding that female M. canicaudus have perinatal androgen concentrations very similar to those of adult males. In addition, female
voles exhibit a sudden increase in testosterone just after birth that is indistinguishable from the postnatal surge seen in male counterparts (S. P. Spielvogel, Chapter 6).

To determine if the masculine sexual behavior and gonadotropin release exhibited by female voles is a consequence of perinatal exposure to endogenous testosterone, I administered the antiandrogen, flutamide, to developing females. Prenatal flutamide treatment decreases the masculinizing effects of endogenous testosterone in female rats. For example, female rats located near males in utero exhibit longer anogenital distances at birth and have an increased potential for masculine copulatory behaviors when treated with testosterone as adults; prenatal flutamide treatment blocks this masculinization (Clemens, Gladue and Coniglio, 1978). However, perinatal flutamide treatment of female voles did not significantly reduce their ability to express masculine sexual behaviors in response to testosterone administration as adults. In addition, vaginal smear patterns were not affected and gonadal hormone treatment did not significantly facilitate ovulation in flutamide-treated voles. The finding that two females treated perinatally with flutamide did ovulate after estrogen and progesterone treatment was not statistically significant; however, this finding is intriguing because no corpora lutea were found in females of other groups.
It may be that flutamide reached the brains of developing fetuses in concentrations insufficient for completely blocking androgen effects in voles. The doses of flutamide used in voles were approximately 5-10 times higher (based on body weights) than those used to block masculinization of female rats (Clemens, Gladue an Coniglio, 1978), but flutamide was given by injection in rats and orally in voles. Because oral flutamide treatment abolished sexual differences in anogenital distance in voles, it is unlikely that mode of administration accounts for the inability of this drug to modify neuroendocrine functions.

The failure of perinatal flutamide to reduce the masculine behavioral potentials or to alter gonadotropin release mechanisms of female voles may indicate that masculine levels of testosterone circulating in the plasma of developing females do not play a role in the development of these characteristics. However, there are several possible technical explanations for the ineffectiveness of flutamide in blocking masculinization. Flutamide abolished sexual differences in anogenital distances, indicating that it reached the fetuses. However, flutamide may not have exerted a central effect. Such dissociation of central and peripheral effects has been described in at least two other systems. Flutamide suppresses prostate and seminal vesicle weight without
affecting libido in adult rats (Neri, et al., 1972) and antagonizes methyltestosterone-stimulated maintenance of seminal vesicle and prostate weights in castrated mice without affecting methyltestosterone-stimulated aggression (Heilman, Brugmans, Greenslade and DaVanzo, 1976).

A second possibility is that flutamide was not available in sufficient amounts at the time when masculinization occurred. Although flutamide was injected within an hour of birth, it may not have been absorbed in time to block the effects of the androgen peak seen at 1 hour after birth. However, it seems unlikely that masculinization of behavior and gonadotropin release could result from such a brief exposure to androgens. In rats, masculinization requires androgen exposure for 48 to 72 hours (Hayashi and Gorski, 1974). It is also unlikely that masculinization occurred before flutamide treatment was begun on day 11 p.c., or after eye-opening when treatment was discontinued, for two reasons. First, the rate of development of voles appears to be similar to that of rats, and testicular production of androgens in rats does not occur until after 14 to 15 days p.c. (Warren, Haltmeyer and Eik-Ness, 1973). In addition, in no mammals studied can behavioral potentials be significantly modified by hormone exposure after eye-opening (Goy and McEwen, 1980).

A final possibility for the lack of a flutamide
effect on behavior and gonadotropin release pattern in the vole is that testosterone is converted to estrogen before it acts. A number of studies support this aromatization hypothesis (Doughty, Booth, McDonald and Parrott, 1975; Naftolin, Ryan, Davies, Reddy, Flores, Petro and Kuhn, 1975; Lieberburg, Wallach and McEwen, 1977; McEwen, Lieberburg, Chaptal and Krey, 1977; Vreeburg, van der Vaart and van der Schoot, 1977; Sodersten, 1978; Whalen and Olsen, 1981). The findings that the antiaromatase, ATD did not alter behavior or neuroendocrine regulation of ovulation in the vole are not consistent with the aromatization hypothesis. However, the failure of ATD to interfere with masculinization is not unprecedented. A number of studies using rats have shown that ATD does not reduce mounting or intromission behavior (Vreeburg, van der Vaart and van der Schoot, 1977; Davis, Chaptal and McEwen, 1979; Thomas, McIntosh and Barfield, 1980; Whalen and Olsen, 1981) nor does it always prevent masculinization of gonadotropin release patterns (McEwen et al., 1977; Vreeburg et al., 1977). These results are surprising because perinatal administration of estrogen or aromatizable androgens can readily masculinize behavior (Levine and Mullins, 1964; Docke and Dorner, 1975; Christensen and Gorski, 1978) and defeminize the pattern of gonadotropin release of rats.

One explanation for the inability of ATD to affect
behavior or gonadotropin release patterns in voles in the present study is that it exerted an effect other than blocking aromatization. At the doses given, ATD appeared to actually masculinize the genitalia of females. Evidence of an androgenic effect of ATD has been reported by other investigators; ATD induces an increase in seminal vesicle and prostate weights in immature male rats (Brodie, Wu, Marsh and Brodie, 1979), and maintains precastration levels of copulatory behavior in castrated male rats (Landau, 1980). In addition, it has been determined that animals may metabolize ATD to a polar derivative that has androgenic activity, and can displace testosterone from an antibody to testosterone (W. Ellinwood, personal communication; Donaldson and Forest, 1980).

In summary, female voles have characteristics that support the hypothesis that they are androgenized during normal development. Specifically, female voles are exposed to high perinatal testosterone concentrations; they exhibit nearly complete male copulatory behavior after testosterone treatment in adulthood; they do not appear to ovulate after estrogen and progesterone administration; they show persistent vaginal cornification in adulthood; and they possess polyfollicular ovaries with no luteal tissue. The hypothesis that masculine behavioral potentials and gonadotropin release patterns
are a result of exposure to perinatal testosterone is not supported by the findings that these characteristics were not affected by perinatal treatment with flutamide and ATD. The relationship between the high perinatal levels of testosterone normally seen in female voles and the presence of masculine traits in female voles will require further investigation.
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Table 4.2. Components of Masculine Copulatory Behavior In Male and Female *M. canicaudus*

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<td>Pre+Neo</td>
<td>$\bar{x}$ 127.6</td>
<td>137.0</td>
<td>305.0</td>
<td>25.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Flu</td>
<td>S.E. 30.1</td>
<td>27.8</td>
<td>32.2</td>
<td>0.6</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>N 8</td>
<td>8</td>
<td>3</td>
<td>3</td>
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<tr>
<td></td>
<td>F</td>
<td>Pre+Neo</td>
<td>$\bar{x}$ 133.4</td>
<td>133.4</td>
<td>360.9</td>
<td>21.5</td>
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<tr>
<td></td>
<td></td>
<td>ATD</td>
<td>S.E. 27.1</td>
<td>27.1</td>
<td>39.3</td>
<td>4.6</td>
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<td>N 8</td>
<td>8</td>
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</tr>
</tbody>
</table>

Components of masculine copulatory behavior are ML, mounting latency (seconds); IL, intromission latency (seconds); EL, ejaculation latency (males) (seconds); PL, pseudoejaculation latency (females) IF, intromission frequency (number). Treatments include flutamide (Flu) and ATD administration prenatally (Pre) and neonatally (Neo). Females were ovariectomized and implanted with testosterone 4 weeks before behavior tests were performed. Males were intact.
Table 4.3. Experimental Treatments for Females in Study 2A

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day</th>
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<tbody>
<tr>
<td>A</td>
<td>EB 30 ug</td>
<td>EB 30 ug</td>
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<tr>
<td></td>
<td>45 days, n=5</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>90 days, n=5</td>
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<td></td>
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<tr>
<td></td>
<td>150 days, n=5</td>
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<tr>
<td>B</td>
<td>EB 60 ug</td>
<td>EB 60 ug</td>
<td>EB 60 ug</td>
<td>P 500 ug</td>
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<tr>
<td></td>
<td>45 days, n=6</td>
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<tr>
<td></td>
<td>90 days, n=7</td>
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<tr>
<td></td>
<td>150 days, n=6</td>
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<tr>
<td>C</td>
<td>EB 150 ug</td>
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<td>45 days, n=6</td>
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<td></td>
<td>90 days, n=7</td>
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<tr>
<td></td>
<td>150 days, n=5</td>
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</tbody>
</table>

Hormones, estradiol benzoate (EB) and progesterone (P), were administered to female voles of different ages. Females were ovariectomized (ovx) on either day 5 or day 7.
Figure 4.1. Anogenital Distances of Weanling Voles Which Had Received Different Perinatal Treatments.

Anogenital distances were measured from the base of the penile or clitoral sheath to the anus in male and female voles at 15 days of age. VM, vehicle-treated males; VF, vehicle-treated females; F_pM', males treated prenatally with flutamide; F_pF', females treated prenatally with flutamide; F_pnM', males treated prenatally and neonatally with flutamide; F_pnF', females treated prenatally and neonatally with flutamide. Treatments are described in detail in the text (Study 1, Methods A).
Figure 4.1
Figure 4.2. Genitalia of Male and Female Voles Subjected to Different Treatments during the Perinatal Period. A, vehicle-treated, ovariectomized female four weeks after receiving testosterone implant; B, intact male; C and D, ATD-treated, ovariectomized females four weeks after receiving testosterone implants. Treatments are described in detail in the text.
Figure 4.3. Incidence of Masculine Copulatory Behaviors in Intact Males and Ovariectomized, Females Implanted with Testosterone Capsules in Adulthood after Receiving Different Perinatal Treatments. IM, intact males; UF, females receiving no perinatal treatment; V, females treated perinatally with vehicle; \( F_p \), females treated prenatailly with flutamide; \( F_n \), females treated neonatally with flutamide; \( F_{pn} \), females treated prenatally and neonatally with flutamide; ATD, females treated prenatally and neonatally with ATD.
Figure 4.3
CHAPTER 5: ABSENCE OF FEMALE-TYPICAL BEHAVIORS IN MALE VOLES

INTRODUCTION

Feminine sexual behaviors have been categorized into attractivity, proceptivity and receptivity (Beach, 1976); these behaviors can be elicited in adult female mammals by injecting estrogen or estrogen in combination with progesterone (Morali and Beyer, 1979). The ability to express feminine sexual behaviors in response to hormones is not limited to genetic females. Males of some species show female receptive behaviors after treatment with estrogen and progesterone in adulthood; however, the potential for these behaviors in males is species specific. In adult, castrated male rats and guinea pigs, for example, feminine sexual behaviors are rarely observed in response to estrogen and progesterone treatment (Beach, 1945; Feder and Whalen, 1965; Grady, Phoenix and Young, 1965; Gerall, Hendricks, Johnson and Bounds, 1967; Goldfoot, Resko and Goy, 1971). In contrast, male ferrets and hamsters exhibit high levels of feminine sexual behaviors when castrated and treated with estrogen (Baum, 1979; Tiefer, 1970; Swanson and Crossley, 1971).

Goy and McEwen (1980) considered the possibility that species differences among males, in the ability to show feminine sexual behaviors, result from differences in the level of, or sensitivity to, androgen exposure during
perinatal development. This possibility is based on the organizational hypothesis of brain differentiation, which states that the ability to display female behaviors is inherent in both sexes but suppressed by exposure to androgen during development (Phoenix, Goy, Gerall and Young, 1959). In support of this hypothesis, if male rats are deprived of neonatal testosterone by castration soon after birth, as adults they show typical female behaviors when injected with estrogen and progesterone, (Thomas and Gerall, 1966; Corbier, Roffi and Rhoda, 1983). Furthermore, male hamsters show high levels of female behaviors when injected with estrogen and progesterone after castration in adulthood (Tiefer, 1970), but if testosterone levels are elevated experimentally in neonatal hamsters, the potential for female receptive behaviors is suppressed (Eaton, 1970; Swanson and Crossley, 1971; Carter, Clemens and Hoekema, 1972).

The organizational hypothesis is also supported by demonstrations that the potential for female sexual behaviors can be suppressed in some female mammals by perinatal testosterone administration (Baum, 1979). However, just as there are species differences in the ability of males to exhibit female behavior, the degree to which female mammals can be defeminized by perinatal testosterone administration differs among species. Female rats, hamsters, and guinea pigs treated with testosterone
during perinatal development have decreased responsiveness to hormonal stimulation of female receptive behavior (Phoenix, Goy, Gerall and Young, 1959; Barraclough and Gorski, 1962; Harris and Levine, 1965; Swanson and Crossley, 1971; Carter, Clemens and Hoekema, 1972). In contrast, female ferrets, rabbits, rhesus monkeys and marmosets do not appear to be sensitive to the defeminizing effects of perinatal testosterone (Campbell, 1965; Anderson, 1970; Baum, 1976; 1979; Goy and Resko, 1972; Goy, 1978; Abbott and Hearn, 1979). Baum (1979) proposed that defeminization will occur in only those species that rely on progesterone for the facilitation of behavior. This hypothesis is consistent with the findings that in ferrets and rhesus monkeys, species that do not require progesterone, males are not defeminized by exposure to endogenous testosterone nor are females defeminized by injecting testosterone during development (Baum, 1976; 1979;).

Because voles do not require progesterone for the display of female receptivity (Dluzen and Carter, 1979; S. P. Spielvogel, unpublished), one would predict from Baum's hypothesis that male voles will exhibit female behavior when castrated and treated with estrogen in adulthood. Another prediction is that female voles will not be defeminized by neonatal testosterone exposure. The present study tested these predictions in the gray-tailed
vole, *M. canicaudus*, by exposing females to testosterone during the neonatal period, and by testing for feminine behaviors in adult male voles that were castrated and treated with estrogen. Because it was determined that males did not show female receptive behaviors, subsequent studies investigated the role of perinatal exposure to testosterone (or its estrogenic metabolites) in the suppression of female behaviors. This was done by castrating males within 3 hours after birth, by administering an antiandrogen (flutamide) to males before and/or after birth, and by administering an aromatase inhibitor (ATD) to males before and after birth. Flutamide was used because it reportedly blocks androgen receptors and lacks estrogenic, androgenic and progestagenic effects (Neri, Florance, Koziol, and Van Cleave, 1972; Liao, Howell and Chang, 1974). The aromatase inhibitor, ATD, was used to determine whether aromatization of testosterone plays a role in defeminization of male voles as it does in male rats (McEwen, Lieberburg, Chaptal and Krey, 1977; Vreeburg, van der Vaart and van der Schoot, 1977; Clemens and Gladue, 1982).
MATERIALS AND METHODS

ANIMALS

Gray-tailed voles (M. canicaudus) used in these studies were from a colony established from locally-collected animals (Hagen, 1978). The colony was maintained on a controlled photoperiod (16L:8D, lights off at 1430 h). Purina Rat Chow, Purina Rabbit Chow and water were available ad libitum.

MATERIALS

Estradiol benzoate (EB), testosterone (T), carboxymethylcellulose, polysorbate 80 and benzyl alcohol were obtained from Sigma Chemical Co., St. Louis. Flutamide (4'-nitro-3'trifluoromethylisobutyranilide, batch number 10485-95) was donated by Schering Corp., Bloomfield, N.J. ATD (1,4,6-androstatrien-3,17-dione) was purchased from Steraloids, Inc., Wilton, N.H. Vetalar (ketamine HCl) was obtained from Parke-Davis, Morris Plains, N.J. and Rompun (xylazine) from Haver-Lockhart, Shawnee, Kansas. Silastic tubing and Silastic 382 Medical Grade Elastomer were purchased from Dow Corning Corp., Midland, Mich.
PROCEDURES

Hormone Administration

Both flutamide and ATD were suspended in an aqueous vehicle composed of 0.9% NaCl, 0.5% carboxymethylcellulose, 0.4% polysorbate 80, and 0.9% benzyl alcohol (Neri, et al., 1972) for oral or parenteral administration. Silastic capsules containing ATD or testosterone were made by sealing one end of a 1-cm piece of Silastic tubing (i.d., 1.47 mm; o.d., 1.96) with Silastic Medical Grade Elastomer, packing the tubing with crystalline steroid and sealing the open end with Elastomer (Moore, 1981). Estradiol benzoate was administered subcutaneously in corn oil vehicle and progesterone in 0.9% saline.

Behavior Tests

Tests were performed 1 h after the onset of the dark period under dim red lights. The test arena consisted of a sheetmetal cylinder (30 cm in diameter and 50 cm high) sitting on an elevated glass floor. A mirror mounted at a 45° angle beneath the floor allowed ventral viewing. A sexually active male was placed in the arena and allowed 6 minutes for exploration. The animal to be tested for female behavior was then introduced and the pair was observed for 10 minutes. Female behaviors were recorded on a 20-channel Esterline-Angus manual event recorder.
These behaviors have been categorized during preliminary observations of mating in intact female and male voles (S. P. Spielvogel, unpublished). When the male approaches, a receptive female remains stationary and allows the male to sniff her face. She then turns away from the male. When the male begins to investigate her genitalia, the female stands still briefly and then darts forward. This alternation between standing still and darting forward continues until the male mounts. Receptive females usually show lordosis on every mount. In addition to behaviors described above, proceptive behaviors displayed by a female consists of approaching the male and sniffing or nudging him. If the male does not respond, females will sometimes attempt to mount the males.

**STUDY 1**

The following studies were performed to determine whether castrated male voles exhibit female receptive behaviors when treated with estradiol benzoate (EB) or with EB plus progesterone (P). A variety of doses and administration regimes were used as described below. Two different age classes were tested because female voles show age-related differences in the ability to show female behavior (S. P. Spielvogel, Chapter 2) and it was hypothesized that the same may be true of males.

**Methods**
Castrations were performed under anesthesia produced by injecting 5-7 ug Rompun per gm body weight and 125 ug Vetalar per gm body weight. Testes were removed through a transverse incision midway between the base of the penis and the anus.

A. Males (n=10) were castrated at 30 to 35 days of age and then tested weekly for the ability to show male behavior. Male behavior disappeared in all animals by 5 weeks after castration. The following week males began receiving hormone injections and were tested for female behavior according to the following schedule (Day 1 is the day of the first EB injection):

Week 1: Days 1, 2, and 3  60ug EB injected subcutaneously daily at 1000 h.
        Day 4  500 ug P injected subcutaneously 4 hours before behavior test
        10-minute behavior test

Week 2: Days 8, 9, and 10  60 ug EB
        Day 11 Behavior test

Week 3: Days 15, 16, and 18  60 ug EB
        Day 18 Behavior test

Week 4: Days 22, 23, and 24  60 ug EB
        Day 25 Behavior test

Week 5: Day 29, 30, and 31  60 ug EB
        Day 32 Behavior test
Week 6: Day 36, 37, and 38 90 ug EB
Day 39 Behavior test

B. Adult males were castrated at 90 days of age (n=10) and adult females (n=8) were ovariectomized at the same age to be used as controls. Hormone injections were begun 6 weeks later. The schedule was as follows:

Week 1: Days 1, 2, and 3 60 ug EB given daily by subcutaneous injections at 1000 h.
Day 4 Behavior test
Week 2: Days 8, 9, and 10 60 ug EB
Day 11 Behavior test
Week 3: Days 15, 16, and 17 60 ug EB
Day 18 Behavior test
Week 4: Days 22, 23, and 24 60 ug EB
Day 25 500 ug P given 4 hours before the behavior test
Day 25 Behavior test

C. Males were castrated at 90 to 110 days of age (n=10) and female siblings (n=8) were ovariectomized at the same time. Instead of waiting for 6 weeks (as in the above study), EB injections were begun the day after surgery and continued according to the following schedule:

Days 1, 2, 3, and 4 15 ug EB
Day 5 Behavior test
Days 5, 6, 7, and 8 15 ug EB
Day 9 Behavior test
Days 9, 10, 11, and 12  60 ug EB

Day 12 Behavior test

Results, Study 1

Neither castration at 30 to 35 days nor at 90 days elicited the display of female sex behavior by males; none of the 30 males tested responded to EB or EB and P priming by showing female receptive behaviors. Neither the dosage of EB nor the time of initiation of EB treatment affected the propensity to show behavior. The same dosages and testing regimens used in males reliably induced sexual behavior in female controls. Females treated with EB beginning 6 weeks after ovariectomy showed a similar incidence of receptive behavior (5 of 8) as those treated with EB beginning the day after surgery (6 of 8).

The behaviors shown by gonadectomized animals treated with EB were similar to those shown by intact animals as described above. Stud males approached and pursued castrated, EB-treated males as they did EB-treated females. In addition, castrated EB-treated males and unreceptive ovariectomized females injected with EB responded to stud males in the same manner. Unreceptive animals allowed face sniffing but never turned away from the male. If the stud male approached from behind, the unreceptive animal turned abruptly toward the stud male and emitted a high-pitched vocalization. The shrieking
was followed by chattering and lunging at the stud male if he continued pursuit. No castrated, EB-treated males approached, sniffed or attempted to mount stud males.

STUDY 2

The following study was performed to determine whether gonadal hormones during the neonatal period are involved in defeminization of sexual behavior.

Methods

Within 3 hours of birth, males were castrated (n=20) or sham-castrated (n=16), and females were implanted with 5mm T capsules (n=16) or cholesterol capsules (n=15). Castration and capsule implantations in neonatal voles were performed under cryoanesthesia with the aid of a dissecting microscope. For castrations, a 3 mm midline transverse incision was made just below the umbilicus and the testes were removed. Sham castrations consisted of making an incision and exposing the testes. Intact, neonatal females were implanted subcutaneously with 5mm Silastic capsules of testosterone or cholesterol. Wounds were closed with nylon suture and sealed with collodion.

There was high post-operative mortality, reducing the castrate group to 6 males and the other groups to 5 animals each. Beginning at 120 days of age, all animals received daily injections of 60 ug EB for three days. Behavior tests were performed 24 hours after the last EB
injection. After the behavior tests, each female was left with a stud male for 30 days or until she had produced a litter.

**Results, Study 2**

None of the six males castrated within three hours after birth exhibited female receptivity in response to EB when tested in adulthood. These males exhibited behaviors similar to those of males castrated as adults (Study 1).

Female voles treated with testosterone from the day of birth until 1 month exhibited normal female behavior as adults. Four of 5 cholesterol-implanted females and 5 of 5 testosterone-implanted females showed receptive behavior in at least one of 3 tests. The receptive behaviors shown by these females did not differ from those previously observed in normal females. All darted forward and then stopped to allow genital sniffing and all showed lordosis every time they were mounted by stud males. In addition, 4 of 5 females in the cholesterol-implanted group and 4 of 5 females treated neonatally with testosterone conceived during the month of housing with males. All that conceived were able to raise young to weaning.

**STUDY 3**

This study was performed to determine whether the administration of drugs that interfere with androgen
action prenatally and/or postnatally, would increase the propensity of males to show female behaviors.

Methods

Nonpregnant female voles were paired with adult males and vaginal smears obtained at 0730, 1530 and 2130 h daily from the day after pairing. Males were removed after sperm were found in the vaginal smear; this time was considered to be the time of conception (day 0 post-coitum, p.c.). On day 11 p.c., pregnant females were assigned randomly to one of the six treatment groups described in Table 5.1. For prenatal treatments, flutamide (5 mg in 0.2 ml vehicle) or vehicle (0.2 ml) was administered daily to pregnant females by gavage through a straight feeding needle from day 11 p.c. through day 20 p.c. (the day before parturition). For postnatal treatments, flutamide or vehicle was administered by subcutaneous injections; injection sites were sealed with collodion. From 30 minutes after birth (Day 0) through Day 4, pups were injected with 0.125 mg flutamide (approximately 6.25 ug per gm body weight) in 0.05 ml vehicle and from Day 5 through Day 9, pups received 0.25 mg (approximately 6.25 ug per gm body weight) in 0.1 ml vehicle; controls received vehicle in these volumes.

Anogenital distances of males and females treated prenatally or prenatally and neonatally were measured at 15 days of age. Pups were weaned at 18 days of age and
subsequently housed with 2 to 3 littermates of the same sex. Males were castrated at 110 days of age and injected with 30 ug EB on 3 consecutive days. These males were tested for female behavior 24 and 48 hours after the last injection. Estrogen priming and testing continued for 3 consecutive weeks. On the day of the last test, 500 ug P was given four hours before testing.

Results, Study 3

Anogenital distances in males treated with prenatal or prenatal plus neonatal flutamide were significantly shorter than those of vehicle-treated males. These data are shown in Figure 4.1. The anogenital distances of treated males were similar to those of vehicle-treated females. Females treated prenatally with flutamide had significantly shorter anogenital distances than vehicle-treated females. No treated males (n=19) or controls (n=20) displayed female receptive behaviors. The behaviors of flutamide- and vehicle-treated males were similar to behaviors of adult- or neonatally-castrated males described in the previous studies.

STUDY 4

This study was designed to determine whether aromatization is involved in defeminization of behavior in
males.

Methods

On Day 12 p.c., pregnant females were implanted with either three 1-cm capsules of ATD or with three empty capsules. Within 3 hours after birth and for the next 9 days, offspring of ATD-implanted females were injected with 1.25 mg ATD suspended in 0.05 ml of aqueous vehicle (n=11); pups from females implanted with empty capsules were given injections of 0.05 ml vehicle only (n=9). Previous studies had demonstrated that mothers implanted with ATD are not consistently able to raise pups to weaning (S. P. Spielvogel, unpublished); therefore, all pups (treated and control) were crossfostered to untreated mothers soon after birth.

At 18 days of age, pups were weaned and subsequently housed in sexually-segregated groups of 3 siblings each. All males were castrated at 110 days of age. One week later castrated males were injected with 30 ug EB for 3 consecutive days and tested 24 and 48 hours after the last injection. This regimen was continued for 3 weeks.

Results, Study 4

Perinatal ATD treatment did not result in the display of female receptive behaviors by males. The behaviors of ATD-treated males, following castration and injection with estrogen, were similar to those described for castrated,
estrogen-injected males in all previous studies; i.e., no female proceptive or receptive behaviors were observed. ATD treatment masculinized the genitalia of females but did not affect that of males (See Fig. 4.2).

DISCUSSION

Prenatal and neonatal treatment with flutamide or ATD did not result in the display of female receptivity in response to estrogen in male *M. canicaudus*. These results do not support the organizational hypothesis of sexual differentiation in voles. However, it is possible that the drugs used to block prenatal testosterone activity (flutamide and ATD) were not exerting central activity, or were not exerting the same pharmacological activity that they do in other species. These results also demonstrate that female voles exposed to prolonged neonatal testosterone treatment display normal receptivity in adulthood, and that male voles castrated within three hours after birth do not exhibit female behaviors when treated with estrogen in adulthood.

When male voles were castrated at approximately one month of age or at 3 months of age, estrogen or estrogen in combination with progesterone was ineffective in eliciting female receptive behaviors. In contrast, similar treatments reliably stimulated receptivity in
3-month old female siblings. The absence of female sexual behaviors in estrogen-primed, castrated males is not a result of testing with disinterested stud males. In the present studies, stud males showed similar affiliative behaviors when they were paired with estrogen-primed castrated males or ovariectomized females; all experimental subjects were approached, investigated and followed.

The failure of male voles to show female receptive behaviors when castrated as neonates or adults distinguishes them from other mammals studied. Male hamsters and ferrets that have been castrated and treated with estrogen as adults exhibit high levels of female behaviors (Hoekema, Carter and Clemens, 1970; Tiefer, 1970; Swanson and Crossley, 1971; Baum and Vreeburg, 1973; Baum, 1979). Male rats and guinea pigs do not show high levels of female behavior in response to estrogen and progesterone if castrated in adulthood (Gerall, Hendricks, Johnson and Bounds, 1967; Goldfoot, Resko and Goy, 1971) but they do if castrated within 6 hours of birth (Grady, Phoenix and Young, 1965; Thomas and Gerall, 1966; Whalen and Edwards, 1967; Dunlap, Gerall and Hendricks, 1972; Corbier, Roffi and Rhoda, 1983).

One hypothesis to explain species differences in the ability of males to show female behavior is that males of various species have differences in sensitivity to, or
exposure to early testosterone (Goy and McEwen, 1980). Consistent with this hypothesis is the finding that male rats and voles (which show little female receptive behavior after adult castration) have perinatal androgen concentrations similar to, or exceeding levels, of androgen in sexually active adult males (Dohler and Wuttke, 1975; S. P. Spielvogel, Chapter 6). In contrast, developing male hamsters and ferrets (which show high levels of female behavior) have testosterone levels nearly 10-fold lower than those reported for sexually-active adult males (Macrides, Bartke, Fernandez and D'Angelo, 1974; Neal, Murphy, Moger and Oliphant, 1977; Erskine and Baum, 1982; Pang and Tang, 1984). The hypothesis that male hamsters can exhibit female behavior because they are exposed to perinatal testosterone concentrations insufficient for defeminization is further supported by the findings that injections of testosterone levels into developing male hamsters decreases their abilities to show female behavior (Carter, Clemens and Hoekema, 1972; Johnson, 1975; Coniglio and Clemens, 1976).

Another explanation for species differences in the propensity of males to show female receptive behavior has been proposed by Baum (1979). Baum hypothesized that early testosterone exposure would not affect female behavior in species that do not require progesterone for the display of such behavior. The finding, in the present
study, that male voles did not show female sexual behavior after a variety of estrogen treatments is inconsistent with the hypothesis. However, the finding that female voles showed normal receptivity and were able to conceive despite postnatal treatment with testosterone is consistent with Baum's hypothesis. Female voles, ferrets and rabbits all exhibit full receptive behavior in response to estrogen alone; progesterone is not required (Sawyer and Everett, 1959; Palka and Sawyer, 1966; McDonald, Vidal and Beyer, 1970; Baum, 1979; Dluzen and Carter, 1979; S. P. Spielvogel, unpublished). As predicted by Baum's hypothesis, receptive behaviors in female voles are unaffected by treatment with testosterone after birth. Similarly perinatal testosterone exposure does not defeminize female ferrets and rabbits (Campbell, 1965; Anderson, 1970; Baum, 1976).

The findings that neonatal castration of males and that neonatal testosterone treatment of females did not affect female-typical behavior in adulthood may indicate that a period of maximal sensitivity to androgen occurs before birth, rather than after. Consistent with this hypothesis is the finding that male voles have higher levels of testosterone than females prenatally, but similar levels are seen in males and females just after birth (S. P. Spielvogel, Chapter 6). However, the idea that prenatal testosterone exposure blocks the development
of the potential for female behavior in the male vole was not supported by studies using flutamide.

Because flutamide blocks androgen receptors (Neri, Florance, Koziol and Van Cleave, 1972; Liao, Howell and Chang, 1974), it was predicted that perinatal treatment with this substance would feminize the male vole. However, neither prenatal nor neonatal flutamide treatment increased the potential for female receptivity in male voles. This finding is different from results of studies in which developing rats were treated with antiandrogens. For example, the potential for female sexual behavior can be induced in male rats by prenatal treatment with the antiandrogen, cyproterone acetate, or with flutamide (Nadler, 1969; Ward, 1972; Ward and Renz, 1972; Gladue and Clemens, 1982).

The doses of flutamide used to treat male voles prenatally were 5 to 10 times as high (based on approximate body weights) as those used prenatally to feminize male rats (Gladue and Clemens, 1982). However, in rats, flutamide was given by injection to pregnant females after dissolving it in propylene glycol. This vehicle proved to be toxic in voles, so flutamide was administered orally following a procedure described by Neri et al. (1972). The finding that flutamide treatment in voles completely abolished sexual differences in anogenital distances supports the idea that the doses
used in this study were sufficient to reach fetuses. It may be that the timing of flutamide treatments (Day 11 p.c. to eye-opening) did not coincide with the timing of the neural differentiation involved in suppressing female behavior. However, rats and voles appear to have similar rates of development and rat testes do not begin producing testosterone until 14.5 days p.c. (Noumura, Weisz and Lloyd, 1966; Warren, Haltmeyer and Eik-Ness, 1973; Picon, 1976). Therefore, it seems unlikely that vole testes secrete enough testosterone before 11 days p.c. to affect development of female behavior.

It may also be that flutamide was unable to exert a central effect in voles; the finding that genital differentiation was affected does not necessarily indicate that flutamide was able to reach or affect central structures. A dissociation between central and peripheral effects of flutamide has been previously reported (Neri, et al., 1972; Heilman, Brugmans, Greenslade and DaVanzo, 1976), and was discussed in Chapter 4.

The ineffectiveness of flutamide treatment in increasing the propensity of male voles to show female behavior may mean that early testosterone exposure is not responsible for the lack of such behavioral potential. However, another explanation for the lack of a flutamide effect is that testosterone may be aromatized to estrogen in the brain before it exerts its effects, as appears to
be the case in male rats (Doughty, Booth, McDonald, Parrott, 1975; Naftolin, Ryan, Davies, Reddy, Flores, Petro and Kuhn, 1975; Lieberburg, Wallach and McEwen, 1977; McEwen, Lieberburg, Chaptal and Krey, 1977; Vreeburg, van der Vaart and van der Schoot, 1977; Clemens and Gladue, 1978; Sodersten, 1978; Whalen and Olsen, 1981). If this is the mechanism whereby testosterone acts, blocking androgen receptors with flutamide would not be effective in feminizing male voles; however, inhibiting aromatization should have such an effect. ATD is a potent anti-aromatase that has been used to increase the potential for the display of lordosis by male rats. Male rats treated with ATD either before or after birth were behaviorally feminized (McEwen, et al., 1977; Vreeburg, et al., 1977; Gladue and Clemens, 1982; Whalen and Olsen, 1981). However, ATD treatment during the perinatal period did not affect male voles in a similar manner. Differences in results obtained with voles in this study and rats in the studies described above may be due to differences in dosage or route of administration. However, it is also possible that ATD was ineffective in feminizing male voles because it exerted an effect other than inhibiting aromatization.

In voles, ATD appeared to act as an androgen or to stimulate androgen production. Other reports also suggest an androgenic activity for ATD. ATD induces an increase
in seminal vesicle and prostate weights in immature male rats (Brodie, Wu, Marsh and Brodie, 1979) and maintains precastration levels of copulatory behavior in castrated male rats (Landau, 1980). Furthermore, serum from ATD-treated animals contains a polar derivative of ATD that has androgenic activity and displaces testosterone from a testosterone antibody in a radioimmunoassay (W. Ellinwood, personal communication; Donaldson and Forest, 1980). Thus, the absence of an increase in the potential for female behavior in ATD-treated animals may be due to androgenizing effects of the drug.

In view of the findings that perinatal androgen manipulations did not affect the development of female behavior in voles, one other hypothesis must be considered. It is possible that the development of female behavior in this species requires stimulation by prepubertal ovarian hormones, not just the absence of androgen action during development. This idea is supported by the finding that female voles ovariectomized as late as 30 days after birth fail to display receptive behaviors in response to estrogen when tested soon after ovariectomy or two months later. If ovariectomies are delayed until 150 days of age, nearly all female voles exhibit receptive behaviors (S. P. Spielvogel, Chapter 2). The finding that the timing of ovariectomy effects the ability to show female behavior is quite different from
findings in rats or rabbits. Unlike voles, female rats treated with estrogen and progesterone show high levels of receptivity even after neonatal ovariectomy (Lisk and Suydam, 1967; Gerall, Dunlap and Hendricks, 1972). Female rabbits show similar levels of sexual behavior when ovariectomies are performed 30, 60 or 90 days after birth (Beyer, Rivaud and Cruz, 1970). The presence of ovaries during development does, however, increase receptivity somewhat in neonatally androgenized female rats and in neonatally castrated male rats (Farrell, Gerall and Alexander, 1977; Dunlap, Gerall and Carlton, 1978). In addition, neonatally ovariectomized female rats are less sensitive to estrogen stimulation of behavior than are females ovariectomized after puberty (Sodersten, 1976).

In summary, male voles do not show female behavior in response to estrogen, even if castrated within three hours after birth. Furthermore, perinatal treatment with antiandrogen or antiaromatase does not increase the incidence of female receptive behaviors in male voles treated with estrogen. These data do not support the organizational hypothesis of sexual differentiation nor do they support the hypothesis that defeminization will be observed only in animals which require progesterone for facilitation of behavior (Baum, 1979). It is possible that the development of female receptive behaviors in voles requires exposure to prepubertal ovarian secretions.
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<td>Prenatal and Neonatal Flutamide</td>
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CHAPTER 6: PLASMA TESTOSTERONE CONCENTRATIONS DURING PERINATAL DEVELOPMENT OF Microtus canicaudus

INTRODUCTION

The organizational hypothesis states that the areas of the mammalian brain controlling sexual behaviors and gonadotropin release patterns are intrinsically feminine, but that exposure to androgens during development can permanently impose masculinity (Phoenix, Goy, Gerall and Young, 1959). This hypothesis was originally based on evidence obtained by experimentally elevating plasma testosterone levels of developing female guinea pigs and observing effects on behavior and ovulatory potential in adulthood. Similar experiments have since been performed using other laboratory rodents and some generalizations have been made. In general, if androgen levels are experimentally elevated in plasma of developing female mammals, the ability to respond to gonadal hormones in adulthood with feminine sexual behavior and surges of gonadotropin release is suppressed (defeminization) (Phoenix et al., 1959; Barraclough and Gorski, 1962; Harris, 1964; Swanson and van der Werff ten Bosch, 1964; Barraclough, 1966; Clemens, Hiroi and Gorski, 1969; Davidson and Levine, 1969; Clemens, Shryne and Gorski, 1970; Gerall and Kenney, 1970; Gorski, 1971; Flerko, 1975; Mennin and Gorski, 1975; Sodersten, 1976; Gogan, Beattie, Hery, Laplante and Kordon, 1980). Furthermore, the
ability of females to show male-typical behaviors is enhanced by perinatal androgenization (masculinization) (Phoenix et al., 1959; Harris and Levine, 1965; Crossley and Swanson, 1968; Edwards and Burge, 1971; Carter, Clemens and Hoekema, 1972; Paup, Coniglio and Clemens, 1972; Sachs, Pollak, Krieger and Barfield, 1973).

The organizational hypothesis is also supported by the finding that when androgen levels are experimentally decreased in developing males by castration, feminine characteristics are exhibited in adulthood. Neonatally castrated males can release cyclic surges of gonadotropins and show female-typical behavior in response to estrogen and progesterone in adulthood (Yazaki, 1960; Harris, 1964; Grady, Phoenix and Young, 1965; Gerall, Hendricks, Johnson and Bounds, 1967; Neill, 1972; Brown-Grant, 1973). These neonatally-castrated males also exhibit a reduction in the ability to express normal masculine behavior when treated with testosterone in adulthood (Beach and Holz, 1946; Whalen and Edwards, 1966, 1967; Grady et al., 1965; Gerall et al., 1967).

The experiments described above tested the androgenization hypothesis by artificially elevating testosterone levels in females or lowering testosterone levels in males during development. In the course of these experiments, it was determined that there are specific developmental periods during which brain tissues
are maximally sensitive to the defeminizing and masculinizing effects of testosterone. In addition, it was determined that different areas of the brain are involved in the processes of masculinization and defeminization, and that these areas are maximally sensitive to hormone effects at different times during development (Christensen and Gorski, 1978).

Experiments defining periods of maximal sensitivity to androgens make it possible to test the androgenization hypothesis by comparing endogenous androgen levels in males and females during these periods. As predicted, perinatal testosterone levels are significantly higher in males than females in species in which males, but not females, are defeminized and masculinized (Turkelson, Dunlap, MacPhee and Gerall, 1977; Buhl, Pasztor and Resko, 1979; Butte, Moore and Kakihana, 1979; Pang, Caggiula, Gay, Goodman and Pang, 1979; Slob, Ooms and Vreeburg, 1980; Corbier, Rhoda, Kerdelhue and Roffi, 1981; Groeneveld, Post and Ooms, 1983; Pang and Tang, 1984). In contrast, in species which show high levels of heterotypical sexual behavior, sexual differences in testosterone levels are not dramatic during development. For example, male hamsters show high levels of female-typical behavior after castration and hormone stimulation in adulthood and, as predicted, testosterone levels in males and females just after birth are not
widely divergent (Vomachka and Greenwald, 1979; Pang and Tang, 1984). If, however, these sexual differences are magnified by injecting neonatal male hamsters with testosterone, males no longer show high levels of heterotypical behavior in adulthood (Eaton, 1970; Swanson and Crossley, 1971; Carter, Clemens and Hoekema, 1972.

Gray-tailed voles, *Microtus canicaudus*, provide an example of a species in which both males and females have masculine characteristics. Females show high levels of male mating behavior and appear to have a tonic pattern of gonadotropin release (Chapter 4). The present study tests the prediction that male and female voles have similar levels of testosterone during development.

**MATERIALS AND METHODS**

**ANIMALS**

*Microtus canicaudus* were from a colony that is maintained at Oregon State University under a controlled photoperiod of 16L:8D (lights off at 1430 h) and temperature of 20 to 22° C. Each fiberglass cage (60x15x15cm) was covered with hardware cloth and contained hardwood shavings and cotton batting for nesting material. Purina Rat Chow, Purina Rabbit Chow and water were available ad libitum.
PLASMA COLLECTION

Nonpregnant females were paired with males and vaginal smears were checked every 12 h for the presence of sperm. The male was removed when sperm were found in the vaginal smear; this day was considered to be the date of conception. Pregnant females were isolated until fetuses were surgically removed or spontaneously delivered, 21 days after insemination.

Pregnant females were sacrificed by cervical dislocation and fetuses were quickly removed through a midline abdominal incision. Trunk blood was collected in heparinized capillary tubes from male and female fetuses at 17, 18, 19 or 20 days post-conception (p.c.). The tubes were placed on ice, centrifuged and plasma stored at 

\[-20^\circ C\] until testosterone concentrations were measured by RIA. The sex of each fetus was determined by examination of anogenital distance and gonadal structure (Schlegel, Farias, Russo, Moore and Gardner, 1967). Examples of gonads from 19-day male and female fetuses are shown in Figure 6.1.

Blood was collected in the same manner from neonatal male and female voles at 1 or 6 h after birth (Day 0) or on Day 1, 2 or 3. Most births occurred between 1100 h and 1500 h; blood was collected between 1300 h and 1400 h, except from animals sampled at 1 or 6 hours of birth.

The times chosen for sampling were based on samplings
in similar studies that described perinatal testosterone profiles of rats (Pang, Caggiula, Gay, Goodman and Pang, 1979; Slob, Ooms and Vreeburg, 1980; Corbier, Rhoda, Kerdelhue and Roffi, 1981). Gestation lengths and the time of eye-opening differ between rats and voles by only one to two days; therefore, rates of testicular and brain development are probably comparable.

Testosterone levels were also measured in reproductively active adult male and female voles (60 days of age) for comparison with those of perinatal males and females.

RADIOIMMUNOASSAY FOR TESTOSTERONE

Testosterone was assayed in 50 ul aliquots of plasma (pooled from 2-4 individuals) using procedures previously described (Frankel, Mock, Wright and Kamel, 1975). Anti-testosterone-11-BSA serum (provided by Dr. G. D. Niswender) was used at a dilution of 1:90,000, and approximately 12,000 cpm \( [1,2,6,7,16,17-3H(N)] \)-testosterone (NET-553, New England Nuclear) were added to each assay tube. All solutions were made in 0.01 M PBS with 0.1% gelatin (pH=7.0). Mean antibody binding was 52.1%, of which 2.7% was nonspecific. The intraassay coefficient of variation was 6.8%, and the interassay coefficient of variation was 6.9% for five assays. Plasma sample values were corrected for an
extraction efficiency of 95.0 ± 2.1% (mean ± SEM). Testosterone was never found in extracted water blanks.

The assay was validated using pooled plasma from male neonates and pools from female neonates. Initially, samples were run with or without Celite chromatography (Weisz and Ward, 1980). No differences in testosterone values were seen between samples run with or without chromatography in plasma from either males or females. Therefore, all subsequent analyses were done without chromatography.

Dilution curves for samples from neonatal males and females were parallel to each other and to the standard curve. Known amounts of testosterone were added to charcoal-stripped plasma. The spiked plasma was then extracted and assayed for testosterone. The mean deviation of observed from expected values was 10.1% and the correlation coefficient between expected and observed values was 0.995.

DATA ANALYSIS

A Two-Way Analysis of Variance for unequal cell sizes was used to analyze data.

RESULTS

Plasma androgen levels are shown in Figure 6.2. There was a significant effect of time ($F_{18,171}=4.82$;
p<0.001) and sex (F_{11,177}=11.92; p<0.001). There was also a significant time x sex interaction (F_{18,171}=6.65; p<0.001).

The significant interaction between time and sex reflects the finding that mean testosterone concentrations were lower in females than in males at all times except on the day before birth and at 1 and 6 h after birth when levels were similar between sexes. For both males and females, plasma androgen levels were higher before birth than after, except for a peak in both sexes at 1 h after birth. Testosterone levels in females before birth ranged between 0.75 ± 0.09 and 0.93 ± 0.10 ng ml\(^{-1}\); those of males ranged between 1.09 ± 0.12 and 1.47 ± 0.07 ng ml\(^{-1}\). Postnatal levels of testosterone on Days 1-3 ranged between 0.25 ± 0.03 and 0.59 ± 0.16 ng ml\(^{-1}\) in females; those of males ranged between 0.52 ± 0.05 and 1.04 ± 0.03 ng ml\(^{-1}\).

Mean testosterone levels of adult males were 0.98 ± 0.20 ng ml\(^{-1}\) and those of females were 0.41 ± 0.03 ng ml\(^{-1}\).

**DISCUSSION**

These results demonstrate that there are periods, during perinatal development, when male *M. canicaudus* have significantly higher plasma testosterone levels than females. However, there are also periods during which
testosterone levels are similar in both sexes. Plasma testosterone levels were significantly lower in females than in males on days 17, 18 and 19 p.c. But, on the day before birth (day 20 p.c.), and at 1 and 6 hours after birth, females had testosterone concentrations similar to those of males. Plasma testosterone levels were highest in both sexes at 1 hour after birth. These findings may explain previously observed sexual differences in adult behavioral potentials of voles. Furthermore, because these patterns differ from those described in other rodents, the present findings may explain behavioral and neuroendocrine differences that exist between female voles and females of other rodent species.

Males of a number of other species have significantly higher levels of circulating testosterone than female counterparts during specific periods of maximal sensitivity to the virilizing effects of testosterone (Turkelson, Dunlap, MacPhee and Gerall, 1977; Buhl, Pasztor and Resko, 1979; Butte, Moore and Kakihana, 1979; Pang, Caggiula, Gay, Goodman and Pang, 1979; Slob, Ooms and Vreeburg, 1980; Corbier, Rhoda, Kerdelhue and Roffi, 1981; Erskine and Baum, 1982; Vreeburg, Groeneveld, Post and Ooms, 1983; Pang and Tang, 1984). The present findings that male voles have significantly higher levels of testosterone during the prenatal period may indicate that defeminization of male voles occurs before birth.
The observations that male voles castrated within 3 hours after birth do not exhibit female behaviors after estrogen treatment in adulthood, and that neonatal testosterone treatment does not defeminize females supports this hypothesis (S. P. Spielvogel, Chapter 5).

The present finding, that male and female voles have similar levels of testosterone during period around birth, may explain the observed masculine characteristics of female voles. Voles of both sexes show male-typical behavior in response to testosterone administration after gonadectomy in adulthood (S. P. Spielvogel, Chapter 4). In addition, adult female voles do not appear to ovulate after estrogen and progesterone injections (Milligan, 1978; S. P. Spielvogel, Chapter 5), nor do they ovulate spontaneously (Breed, 1967; 1972; Hasler, 1975). Instead, voles show persistent vaginal cornification and polyfollicular ovaries (Hasler, 1975; S. P. Spielvogel, Chapter 3), characteristics seen in other female rodents that have been masculinized by testosterone treatment during development (Barraclough, 1961; Harris, 1964; Swanson and Crossley, 1971).

If exposure to endogenous testosterone produces the masculine characteristics seen in female voles, masculinization probably occurs just before and/or after birth, when testosterone levels of females are similar to those of male littermates. However, it is also possible
that the masculinizing process is accomplished over a longer period by prenatal exposure to relatively high levels of testosterone in addition to exposure to the early postnatal surge of testosterone. This interpretation is supported by the observation that prenatal testosterone concentrations of developing males and females are in the range of adult males.

The finding that female and male voles have similar testosterone levels just after birth distinguishes them from other female rodents studied (Slob, Ooms and Vreeburg, 1980; Vreeburg, Groeneveld, Post and Ooms, 1983; Pang and Tang, 1984). Furthermore, no other developing female rodents have been reported to have androgen levels in the range of reproductively-active adult males. These species differences in plasma testosterone levels, during the perinatal period, may account for species differences in the ability of females to exhibit masculine sexual behaviors in adulthood. Female voles treated with testosterone after ovariectomy in adulthood exhibit mounting, intromission and pseudoejaculatory behaviors that are virtually indistinguishable from behaviors displayed by normal males (S. P. Spielvogel, Chapter 4). The level of male behavior observed in female voles is not observed in females of other species. For example, female rats that have been treated with testosterone mount and sometimes show intromission behavior; however, the
proportion of females showing the behaviors and the frequency with which these behaviors are displayed are lower than those observed in tests of normal male rats (Whalen and Edwards, 1967; Nadler, 1969; Sodersten, 1972; Thomas, McIntosh and Barfield, 1980). Female hamsters and guinea pigs rarely mount or show intromission behaviors in adulthood (Phoenix, Goy, Gerall and Young, 1959; Swanson and Crossley, 1971; Goldfoot and van der Werff ten Bosch, 1975). Thus, female voles display masculine behaviors to a greater degree than other female rodents studied.

The idea that exposure to endogenous perinatal testosterone has a masculinizing effect on female voles is supported by data from other species. Female rats and mice developing near males in utero have a greater capacity to show male behavior in adulthood than those developing near females (Clemens and Coniglio, 1971; Gandelman, vom Saal and Reinisch, 1977; Clemens, Gladue and Coniglio, 1978). These differences in behavioral potentials can be eliminated in female rats by treating them prenatally with antiandrogens (Ward and Renz, 1972; Clemens, Gladue and Coniglio, 1978).

The source of testosterone in fetal and neonatal voles was not investigated in this study. However, the fact that female voles had higher testosterone concentrations before than after birth (except at 1 hour after birth) indicates that the source may be male
littermates or the mothers. In rats, testes are believed to produce most of the circulating testosterone in males (Warren, Haitmeyer and Eik-Nes, 1973; Picon and Ktorza, 1976; Feldman and Bloch, 1978), and at least some testosterone in female rats and mice comes from male littermates (Clemens, Gladue and Coniglio, 1978; vom Saal and Bronson, 1980). In addition, the placentae contribute testosterone to developing female rats (Vreeburg, Groeneveld, Post and Ooms, 1983).

In summary, testosterone concentrations are higher in male gray-tailed voles than in females before birth, indicating that this period may be the time during which behavioral defeminization occurs in males. Female voles are exposed to relatively high levels of endogenous androgens during development. This exposure may be the basis of masculine characteristics seen in adult female voles.
Figure 6.1. Gonadal Structures of Female and Male Microtus canicaudus at 19 Days Gestation. A, gonadal structures of females (O, ovary); B, gonadal structures of males (T, testis).
Figure 6.1
Figure 6.2. Serum Testosterone Levels in Male and Female Voles during Perinatal Development. Values are presented as mean ± SEM.
These studies of gray-tailed voles, *Microtus canicaudus*, investigated the role of perinatal testosterone exposure in the development of potentials for heterotypical behaviors in males and females, and in the development of the gonadotropin release pattern of females. It was determined that ovariectomized adult females exhibit male-typical behavior when implanted with capsules that maintained testosterone levels in the physiological range for sexually-active males. In addition, it was determined that female *M. canicaudus* do not have regular vaginal cycles and that ovulation was neither spontaneous nor was it stimulated by elevating estradiol and progesterone levels with exogenous hormones. These behavioral and neuroendocrine characteristics described for normal female voles are similar to those of other female rodents that have been treated perinatally with testosterone. It is possible that female voles are masculinized and defeminized by exposure to endogenous androgens during development. This hypothesis is supported by the finding that female voles have masculine levels of testosterone just before and after birth. However, the findings that perinatal treatment with antiandrogens or antiaromatases do not block the potential for masculine copulatory behavior in females are
inconsistent with the hypothesis that masculinization is caused by perinatal testosterone exposure.

Male *M. canicaudus* did not show any feminine sexual behaviors when castrated in adulthood and treated with estradiol benzoate in doses that reliably stimulated sexual behavior in adult, ovariectomized females. In addition, neonatal castration of males did not facilitate the display of female behavioral responses to estrogen nor did neonatal testosterone treatment of females suppress the display of female sexual behaviors. If interpreted in terms of the organizational hypothesis, these results may mean that the potential for female sexual behaviors (presumably inherent in both sexes) is blocked in males by exposure to testosterone before birth. This hypothesis is supported by the finding that males have significantly higher levels of testosterone than females before birth. However, the organizational hypothesis of sexual differentiation is not supported by the findings that neither perinatal treatment with antiandrogen nor with antiaromatase facilitates the display of feminine sexual behavior by males. In addition, the finding that feminine sexual behavior cannot be elicited in female voles if ovaries are removed before two months of age does not support the organizational hypothesis.

The data regarding the development of masculine behavior in female voles can be reconciled with the
organizational hypothesis if technical difficulties such as dosage, route of administration or side effects explain the ineffectiveness of antiandrogen and antiaromatase in preventing masculinization. If the results of these drug studies are ignored, one could speculate that the high levels of androgens observed in females during development are responsible for the masculine characteristics seen in adulthood.

It is more difficult, however, to reconcile the data on the development of female sexual behavior with the organizational hypothesis, even if drug studies are ignored. The organizational hypothesis states that the neural substrate responsible for the display of female sexual behavior exists in both males and females; no hormone stimulation is required for its development. However, the results of these studies suggest that feminine sexual behavior in female voles cannot be reliably elicited with ovarian hormones unless ovariectomy is delayed until two to five months after birth. Thus, in female voles, development of feminine sexual behavior may be different from that of other mammals studied, because it may require ovarian hormone stimulation during the prepubertal period. If this speculation is correct, it is understandable that neonatally-castrated male voles do not show feminine sexual behavior in response to ovarian hormone administration as adults.
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