

## AN ABSTRACT OF THE THESIS OF

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To quantify the reproductive behavior of male and female gray-tailed voles, *Microtus canicaudus*, under conditions of natural estrus, sexually experienced pairs were observed in a standardized behavior test for 10 hours. The total duration of sexual interactions averaged 223 minutes, which is considerably longer than previously published reports on microtines. The average mount latency, the time from pairing to first mount, also was found to be longer than previously reported. Receptive females rarely showed proceptive (soliciting) or aggressive behaviors; in contrast, unreceptive females were very aggressive. A majority (67%) of male *M. canicaudus* reached sexual satiety (exhaustion) within a series rather than between series, which is a novel finding for this species.

The effect of prior sexual experience on the reproductive behavior of *M. canicaudus* also was examined. Inexperienced male voles exhibited a significantly longer ejaculatory latency on the first behavioral series than experienced males. In contrast to findings with other rodent species, the effect of sexual experience

on qualitative and quantitative measures of male vole sexual behavior was small and transient. The minor differences between experienced and inexperienced males during the first sexual encounter disappeared quickly, with sexual behavior in series after the first being similar for experienced voles. The sexual behavior shown by receptive female voles was similar for experienced and inexperienced females; however the percent females that were receptive was higher in the experimental animals.

To investigate the effect of familiarity on behavioral responses of females, voles in post-partum estrus were paired with familiar or unfamiliar males. Sexual behaviors were indistinguishable for females that were paired with the two types of males, supporting the hypothesis indicating that M. canicaudus do not pair-bond.

To investigate whether luteinizing hormone-releasing hormone (LHRH) influences the sexual behavior of male M. canicaudus, subcutaneous injections of LHRH (500 ng) were given to intact males and to castrated males with different levels of testosterone replacement. Intact voles, as well as castrated voles with Silastic capsules of testosterone propionate, showed significant facilitation of several parameters of masculine sexual behavior 2 hours after LHRH injection, compared to saline controls. Castrated voles without testosterone replacement showed no sexual behavior, even when injected with LHRH.

The observation that in M. canicaudus LHRH can enhance masculine behavior supports the hypothesis that LHRH regulates sexual behavior in this species, as has been suggested for other mammals. The

findings also support the hypothesis that the behavioral response to LHRH is mediated by testosterone. The specific behavioral parameters affected suggest that LHRH changes the arousal component of masculine behavior in voles.

**HORMONAL, EXPERIENTIAL AND SOCIAL INFLUENCES  
ON THE REPRODUCTIVE BEHAVIOR OF THE VOLE,  
Microtus canicaudus**

by  
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## TABLE OF CONTENTS

	<u>Page</u>
CHAPTER I.....	1
GENERAL INTRODUCTION.....	1
Sexual Behavior in Male Mammals.....	2
The Role of Luteinizing Hormone-Releasing Hormone in the Control of Sexual Behavior.....	10
Summary.....	19
References.....	21
CHAPTER II.....	27
EFFECT OF PRIOR SEXUAL EXPERIENCE ON THE REPRODUCTIVE BEHAVIOR OF VOLES, <u>Microtus canicaudus</u> .....	27
Introduction.....	27
Methods and Materials.....	30
Results and Discussion.....	34
Summary.....	45
References.....	51
CHAPTER III.....	54
THE EFFECT OF LUTEINIZING HORMONE-RELEASING HORMONE ON THE MASCULINE SEXUAL BEHAVIOR OF THE VOLE, <u>Microtus canicaudus</u> .....	54
Introduction.....	54
Materials and Methods.....	57
Results.....	62
Discussion.....	65
References.....	85
CHAPTER IV.....	88
GENERAL DISCUSSION.....	88
BIBLIOGRAPHY.....	90
APPENDICES.....	98
Appendix A. Testosterone Radioimmunoassay Protocol.....	98
Appendix B. Chromatography Protocol.....	104
Appendix C. Miscellaneous Data.....	107

## LIST OF FIGURES

<u>Figure</u>		<u>Page</u>
1	Behavioral Latencies in Intact Male Voles after LHRH or Saline Injection.....	69
2	Behavioral Frequencies in Intact Male Voles after LHRH or Saline Injection.....	71
3	Behavioral Latencies in Castrated Male Voles with Small Testosterone Implants after LHRH or Saline Injection.....	73
4	Behavioral Frequencies in Castrated Male Voles with Small Testosterone Implants after LHRH or Saline Injection.....	75
5	Behavioral Latencies in Castrated Male Voles with Large Testosterone Implants after LHRH or Saline Injection.....	77
6	Behavioral Frequencies in Castrated Male Voles with Large Testosterone Implants after LHRH or Saline Injection.....	79

## LIST OF TABLES

<u>Table</u>	<u>Page</u>
1	Summary of Sexual Behavior Parameters in Experienced and Inexperienced Males.....47
2	Measures of Copulatory Behavior in Experienced Male <u>M. canicaudus</u> .....48
3	Measures of Copulatory Behavior in Inexperienced Male <u>M. canicaudus</u> .....49
4	Sexual Behavior of Female <u>M. canicaudus</u> with Different Types of Prior Sexual Experience.....50
5	Effect of LHRH or Control Injection on Plasma Testosterone Levels in Castrated Male Voles or Castrated, Testosterone- Implanted Male Voles.....81
6	Summary of Sexual Behavior After LHRH or Saline Injection in Castrated Male Voles with Different Levels of Testosterone Replacement.....82
7	Behavioral Latencies Before or After Treatment with Saline or LHRH in Castrated, Testosterone-Implanted Male Voles.....83
8	Behavioral Frequencies Before and After Treatment with Saline or LHRH in Castrated, Testosterone-Implanted Male Voles.....84
9	Plasma Testosterone Levels in Male Voles at Time Zero (Experienced and Inexperienced) and at 24, 48, and 72 Hours and 1 Week and 3 Weeks Following Bilateral Castration.....108
10	Sexual Behavior of Female <u>M. canicaudus</u> when Exposed to Familiar or Unfamiliar Males.....109



**HORMONAL, EXPERIENTIAL AND SOCIAL INFLUENCES  
ON THE REPRODUCTIVE BEHAVIOR OF THE VOLE,  
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**GENERAL INTRODUCTION**

**Chapter I.**

Sexual behavior is a complex behavior with many points of possible control and modulation by internal and external factors. The purpose of this introductory chapter is to provide background information on male sexual behavior patterns, the control of behavior by steroids and to review literature on the role of the neuropeptide luteinizing hormone-releasing hormone (LHRH) in modulating reproductive behavior.

Great variation in components of copulatory behavior exists among male mammals. Even within a given species variation in behavioral parameters are observed. Different components of the behavior pattern are independently controlled by neurophysiological factors, such as steroid hormones and neuropeptides. Hormones affect sexual behavior in a variety of ways and at many points in the neural circuitry, from the reception of sensory stimuli to the firing of motor neurons. Testicular steroids are required for the display of sexual behavior in male mammals, however steroids alone are not sufficient for the execution of the normal pattern of behavior. In addition to testosterone, LHRH has been implicated in

the control of certain aspects of sexual behavior in a few species of vertebrates.

## SEXUAL BEHAVIOR IN MALE MAMMALS

### Masculine Mating Patterns

Male mammalian sexual behaviors are generally stereotyped within species but vary widely between species (Dewsbury, 1972). This variation exists in both the temporal patterning of the three basic components of sexual behavior - mounts, intromissions, and ejaculations - and in the presence or absence of four additional characteristics: a mechanical lock or tie, intravaginal thrusting during intromission, multiple intromissions to ejaculation or multiple ejaculations per encounter. Simple classification of copulatory behavior of male mammals based on these variations suggests several questions about the hormonal and neural control of behavioral expression. Do the same hormones control the expression of very diverse behavior patterns? What neurophysiological factors account for the re-arousal of males after ejaculation in some species but not others? For example, baboons, macaques, and voles each have evolved a remarkably similar pattern of sexual behavior with no lock, the presence of intravaginal thrusting, and the presence of multiple intromissions and ejaculations (Dewsbury, 1972). Within the order Rodentia, however, virtually every combination of behavioral characteristics is observed.

Within a given species, sexual behavior is relatively stereotyped. The same basic motor patterns are shown by virtually all mammalian species. In rodents, mounts are recognized as when the male approaches the rear of the female, places his forelegs on the flanks of the stimulus female and gives shallow repetitive pelvic thrusts. Intromission is defined as a mount that involves a deep pelvic thrust and insertion of the penis into the vagina. Ejaculation is identified by a mount with intromission terminated by a deep pelvic thrust that is maintained at its most rostral extent for several seconds while repeated flexures of the hindquarters occur (Dewsbury, 1972). A typical series in rodents consists of a number of mounts and intromissions culminating in ejaculation. Ejaculation is followed by a refractory period that lasts several minutes. The post-ejaculatory refractory interval may then be followed by another series of mounts and intromissions that end with ejaculation. Microtine rodents generally show intravaginal thrusting, no lock, and multiple ejaculations. Multiple intromissions may be required for ejaculation and a broad range of quantitative differences exists (Dewsbury, 1978).

Within a species, quantitative differences in the occurrence of copulatory events are common; i.e., the number of mounts and intromissions to ejaculation or the length of the post-ejaculatory interval. Such seemingly subtle differences can be critically important to successful pregnancy. For example, if 1 to 4 intromissions precede ejaculation in laboratory rats, only 5 percent of the females become pregnant; whereas 83 percent become pregnant

if 13 to 16 intromissions precede the ejaculation (Adler, 1969). Resumption of copulatory activity by the male within 1 to 15 minutes following ejaculation produces an appreciable decrease in uterine sperm counts in rats (Adler and Zoloth, 1970). In induced ovulators (it appears that all voles of the genus Microtus are induced ovulators; Gray, Davis, Zerylnick, and Dewsbury, 1976), copulatory stimuli is of critical importance to stimulate ovulation. Microtine females require multiple intromissions and, usually, multiple ejaculations to initiate ovulation and pregnancy initiation (Dewsbury, 1978). Therefore, quantitative differences in sexual behavior can be just as critical to successful reproduction as qualitative differences in copulatory pattern.

Different components of the copulatory pattern seem to be controlled by different mechanisms; some components vary independently of others. Beach (1956) inferred from descriptive data that a minimum of control mechanisms are involved in regulating sexual behavior of males. The sexual arousal mechanism ("motivation") was believed to control the male's arousal prior to his first intromission, as well as the male's re-arousal prior to the resumption of copulation after each ejaculation. The copulatory (consummatory or "performance") mechanism was postulated to act to maintain copulation so that repeated intromissions occurred, and to sum the excitation from the intromissions until ejaculation occurred (Sachs, 1978). In addition to these two mechanisms proposed by Beach, several lines of evidence also support the idea of an inhibitory mechanism that further modifies male mammal sexual

behavior (Beyer, 1976). It seems likely that these three major factors in the expression of sexual behavior are under different kinds of hormonal and neural control.

### Hormonal Control of Male Sexual Behavior

Hormones can affect behavior in a variety of ways (Leshner, 1978). They can alter the general metabolic state of the animal and thereby change behavior. Also, hormones can change the sensitivity of sensory neurons and receptors, can alter the central nervous system circuits that integrate sensory information, or can modify motor neurons and anatomical structures. Neuropeptides and gonadal steroids affect sexual behavior through one or more of these mechanisms.

Numerous studies indicate that testicular steroids are required for the display of masculine sexual behavior in mammals (reviewed by Leshner, 1978 and Larsson, 1979). Castration invariably leads to a decrease in sexual activity and in plasma testosterone levels. A decrease in sexual behavior usually occurs shortly after castration, but the complete disappearance of all sexual behavior takes longer -- weeks, months, or even years (Larsson, 1979). In rodents that have been castrated, the ejaculatory pattern is the first to disappear (Davidson, 1966). The intromission pattern disappears simultaneously with, or slightly later than, the ejaculatory pattern; mounting is retained for a significantly longer period of time after castration. The same order of disappearance following

castration has been observed in a number of other species (Larsson, 1979). These results indicate that some components of sexual behavior are more dependent on (sensitive to) testicular hormones than others.

The effects of castration on mating behavior can be reversed to a great extent by testosterone treatment. The reacquisition of behavior in castrated males is gradual, with no effects observed within 24 hours after testosterone administration (Larsson, 1979). The sequence of the types of behaviors that are restored by testosterone is the reverse of the sequence of loss following castration (Davidson, 1972). A dose-dependent restoration in sexual behavior is seen in castrated males until behaviors are restored to precastration levels; further increases in testosterone dosage do not increase behavior above precastration levels further (Grunt and Young, 1953; Larsson, 1966, 1979). These findings support the conclusion that a testicular steroid is necessary for the expression of copulatory behavior in male mammals.

Further evidence for this conclusion comes from the positive correlation between seasonal changes in androgen levels and changes in reproductive behavior. Males in species such as red deer (Cervus elaphus), rhesus monkeys (Macaca mulatta), and sheep have low plasma testosterone concentrations during the season when sexual behaviors are least common (Lincoln and Short, 1970; Lincoln, Guinness, and Short, 1972; Michael and Wilson, 1975). Species which show no change in behavior across seasons likewise show no change in androgens.

The steroids estradiol and dihydrotestosterone also play a major role in the expression of sexual behaviors in male mammals. Testosterone, once it has reached the peripheral tissues, can be converted to dihydrotestosterone by the  $5\alpha$ -reductase enzyme system or to estradiol by the aromatase enzyme system. The behavioral importance of peripheral conversion of testosterone to dihydrotestosterone or estradiol is supported by the presence of both enzyme systems in brain tissue (Naftolin et al., 1975; Naftolin et al., 1975).

Several studies demonstrate that estradiol can restore sexual behavior in castrates of several species of mammals (reviewed in Larsson, 1979). Further, administration of drugs that block aromatization prevents testosterone from restoring sexual behavior in castrates (Morali, Larsson, and Beyer, 1977). These observations support the hypothesis that testosterone is aromatized to estradiol to stimulate sexual behavior in males (Christensen and Clemens, 1974; Davis and Barfield, 1979).

Dihydrotestosterone has a weak stimulatory effect on the male sexual behavior of castrated rats, rabbits, hamsters, and mice (Larsson, 1979). In the guinea pig, however, dihydrotestosterone was as potent as testosterone in stimulating sexual activity (Alsum and Goy, 1974). There appears to be much species variation in behavioral responsiveness to dihydrotestosterone. This is in contrast to the potency of this hormone, in all species, in stimulating growth of tissues of the genital tract (Larsson, 1979). A combined administration of estradiol and

dihydrotestosterone is highly effective in restoring sexual behavior to castrates of a majority of mammal species (Larsson, Sodersten, and Beyer, 1973a,b; Larsson, 1979). A current hypothesis is that estradiol stimulates libido in the brain and dihydrotestosterone maintains penile development and other peripheral structures (Parrott, 1975).

How do steroid hormones influence the brain to affect sexual behavior? The brain of the male rat contains nuclei with receptors for androgens, estrogens or both (McEwen et al., 1982). These steroid concentrating sites correspond to the sites that have been shown, primarily through electrical lesion and stimulation studies, to be critical to the display of sexual behavior (Larsson, 1979). Steroid-concentrating neurons occur throughout the entire neural pathway that is involved in the reproductive behavior of frogs and birds (Kelley, 1978). Auditory and vocal nuclei, as well as motor neurons, all contain steroid receptors. Steroids operate via direct effects on neural membranes or the induction of gene products in neural tissue (McEwen et al., 1982).

Although steroids are clearly necessary for the display of male sexual behavior, there is no direct relationship between steroid levels and behavior levels. In intact male rats and guinea pigs, no correlation has been found between quantitative or qualitative aspects of sexual behavior and circulating testosterone levels (Harding and Feder, 1976; Damassa et al., 1977). It appears as though once a "threshold" level of testosterone is present, differences in sexual behavior are independent of plasma



testosterone titers. In castrated rats, the amount of testosterone required to maintain all parts of normal sexual behavior is less than 10% of the normal circulating level (Damassa et al., 1977). This "overshooting" of the amount of androgen required for maximal behavior could account for the lack of correlation between testosterone and behavior levels. Grunt and Young (1953) observed guinea pigs belonging to two different strains, one strain exhibiting a high level of sexual activity and the other a low level. Animals with the high level of sexual activity retained behavior longer after castration than the less active strain. Following the administration of testosterone, both strains returned to pre-castration levels of behavior. These findings raise the possibility, that other factors besides testosterone, may account for the differences in behavior among individual animals.

In a number of species other than the rat, testosterone replacement is unable to restore the normal mating behavior to castrated males. The differences in behavior range from quite subtle (i.e., longer post-ejaculatory intervals in the castrated, testosterone-treated hamster; Whalen and DeBold, 1974) to as distinct as in the rhesus monkey (M. mulatta) where ejaculation frequency remained at 50% less than in intact animals despite identical circulating testosterone levels as intacts (Michael and Wilson, 1975).

The changes in sexual activity seen in seasonally breeding animals are not solely the result of changing testosterone levels with gonadal regression and recrudescence. In red deer

(C. elaphus), testosterone is only capable of inducing sexual activity during the breeding season although social aggression, another androgen-dependent behavior, can be activated with testosterone injections at any time of the year (Lincoln, Guinness and Short, 1972). In this species, sexual behavior can be elicited at any time of the year by the presence of an estrous female.

## THE ROLE OF LUTEINIZING HORMONE-RELEASING HORMONE IN THE CONTROL OF SEXUAL BEHAVIOR

### Introduction

The brain controls reproductive physiology as well as reproductive behavior. A neuropeptide synthesized and secreted in the hypothalamus - luteinizing hormone-releasing hormone (LHRH) - coordinates many aspects of reproductive function. LHRH is a decapeptide produced by neurons and released primarily from the median eminence where it enters a portal system and is transported to the anterior pituitary. In the anterior pituitary LHRH causes the release of luteinizing hormone (LH) and follicle stimulating hormone (FSH). LH and FSH then act on the gonads in the male and female to stimulate steroidogenesis and gametogenesis. High concentrations of gonadal steroids feed back on the pituitary and hypothalamus to influence the release of LHRH. The physiological effects of LHRH have been extensively investigated, but the behavioral effects of LHRH have been studied less extensively.

Moss, Riskind and Dudley (1979) give three lines of evidence to support the hypothesis that LHRH is directly involved in regulating sexual behavior: First, there is the anatomical overlap between the neural tissue producing LHRH and regulating mating behavior. Significant amounts of LHRH are present in the medial preoptic area (MPOA) and arcuate-ventromedial area (Moss et al., 1975). LHRH has also been demonstrated to be present in the mesencephalon, and para-olfactory complex and other extra-hypothalamic areas (Mauk et al., 1980). The MPOA in the base of the hypothalamus, is critical to the display of sexual behavior. Lesions in this area abolish sexual behavior and electrical stimulation activates the behavior (Ryan and Frankel, 1978). The preoptic-anterior hypothalamic area shows a greater uptake of labelled testosterone and estrogen than other brain areas. Small implants of testosterone into the MPOA of castrated male rats can restore sexual behavior when implants in other parts of the brain cannot. Exposure of a male rat to sexual stimuli (estrus females) results in an increase in plasma levels of LH and testosterone (Kamel et al., 1977). This increase still occurs in animals with MPOA lesions although sexual behavior itself is abolished (Kamel and Frankel, 1978; Ryan and Frankel, 1978). This supports the idea that endocrine and behavioral responses to LHRH occur in different parts of the brain.

The second supporting observation is the presence of neurons responsive to LHRH (they change their firing rate when small quantities of peptide are applied) throughout the hypothalamus (Moss et al., 1979). Many such neurons project from the MPOA and arcuate

nucleus to areas other than the median eminence (Moss et al., 1979). Such neurons could mediate extrapituitary functions such as mating.

Lastly, there is the temporal relationship between the LHRH-triggered pre-ovulatory LH surge in female rats and the onset of sexual receptivity. In the 4-day cyclic rat, behavioral heat follows 3 to 5 hours after the gonadotropin surge and persists for 8 to 10 hours. The time course of potentiation of mating behavior with exogenous LHRH is very similar (Moss and McCann, 1975; Moss et al., 1979). LHRH was first examined for effects on sexual behavior in female rats (Moss and McCann, 1973; Pfaff, 1973). Work was then extended to other female rodents and male rats as well as birds, reptiles, and amphibians (see, for example, Moss et al., 1975; Cheng, 1977; Alderete et al., 1980; Mauk et al., 1980; Kelley, 1982; Moore et al., 1982).

### Studies in Male Mammals

Little information is published on the effect of LHRH on sexual behavior in male mammals. Male laboratory rats show a demonstrable effect of LHRH and related analogs on copulatory behavior, and male primates also show an effect under certain circumstances (reviewed by Mauk et al., 1980).

In intact male rats, subcutaneous injections of LHRH (500 ng) can alter reproductive behavior, specifically latency to first intromission and to first ejaculation (Moss et al., 1975). The

injection of LHRH into the lateral ventricle of intact male rats is also able to significantly increase the number of mounts (Dorsa and Smith, 1980). Whether LHRH is critical to the display of copulatory behavior in intact males has not been proven conclusively, however, treatment of male rats with an LHRH antagonist inhibits the display of copulatory behavior (Dorsa et al., 1981). Intact, analog-treated male rats show longer intromission and mount latencies in early tests and eventually ceased to show sexual behavior at all. (The analog did decrease testis weight and plasma LH and testosterone levels.) After castration, the gradual decline in behavior usually observed could be markedly accelerated by LHRH analog treatment. These results by Dorsa et al. (1981) indicate that LHRH can maintain sexual behavior after castration.

Evidence for a physiological role for LHRH in regulating sexual behavior of male rats is contradictory. When male rats are castrated and treated with testosterone (at doses too low to fully restore sexual behavior), subcutaneous LHRH injection causes a significant decrease in time to achieve ejaculation (Moss et al., 1975). Although LHRH facilitates behavior, it does not restore it to the same levels as those observed in intact males or castrates receiving high testosterone replacement levels. Optimal levels of testosterone replacement result in a maximum display of behavior and LHRH is incapable of making any further increases (Dorsa, Smith, and Davidson, 1981). Based on these results, Dorsa et al. (1981) conclude that LHRH has little, if any, function in the control of sexual behavior in a low testosterone environment and act as a

'buffer' against fluctuations in endogenous testosterone levels. LHRH does not induce masculine copulatory behavior in the absence of steroids (Moss et al., 1975).

Based on observation of specific behavioral parameters affected, Dorsa and Smith (1980) proposed that LHRH acts on the arousal mechanism of male sexual behavior. They used a penile anesthetization paradigm to remove ejaculatory "reward" and examine the level of arousal more directly. Using this procedure, they report that males given LHRH intracerebroventricularly display more mounting than controls injected with saline.

Regarding studies of LHRH effects on sexual behavior of human males, results have been contradictory or inconclusive (reviewed in Mauk et al. 1980). Lengthy treatment with LHRH analogs reportedly increases sexual potency in men with certain sexual dysfunctions (Mortimer, McNeilly, Fisher, Murray, and Besser, 1974; Benkert, Jordon, Dahlen, Schneider, and Grammel, 1975; Schwarzstein, Aparicio, Turner, Calamera, Mancini, and Schally, 1975; Moss, Riskind, and Dudley, 1979). Other studies, however, report that LHRH administration has no effect on normal men or men with psychogenic sexual dysfunction (Davies, Mountjoy, Gomez-Pen, Watson, Hanker, Besser, and Hall, 1976; Ehrensing and Kastin, 1976). Moss, Riskind, and Dudley (1979) have reported a personal communication from Doring, McGinnis, Kraemer, and Hamberg stating that an LHRH analog has no effect on the sexual behavior of adult male chimpanzees.

### Studies in Female Mammals

The first reports of induction of mating behavior in female rats by LHRH were made independently by Pfaff (1973) and Moss and McCann (1973). They quantified the effects of LHRH on lordosis, a reflexive curvature of the back displayed by sexually receptive females when mounted by a male. Moss and McCann (1973) treated ovariectomized rats with dosages of estrogen too low to induce lordosis. Subsequent subcutaneous injections of LHRH to these ovariectomized, estrogen-primed rats caused 86% of the females to display lordosis. Pfaff (1973) used ovariectomized, estrogen-primed rats that had been hypophysectomized and found the same facilitation of behavior. LHRH has also been shown to facilitate behavior in ovariectomized, estrogen-primed mice (Luttge and Sheets, 1977) and (slightly) in guinea pigs, but LHRH has not been observed to have an effect in hamsters (Carter and Davis, 1977). The nature of the LHRH activation of sexual behavior has been most thoroughly detailed in female rats. Moss et al. (1975) describe the time course of the response and the effect of different doses of LHRH on lordosis of female rats.

Considerable evidence supports the hypothesis that LHRH has a direct effect on the central nervous system in female rats. Adrenalectomized female rats (ovariectomized and estrogen-primed) still display lordosis when treated with LHRH (Moss et al., 1975). Therefore the effect on sexual behavior is independent of adrenal steroid hormones, like progesterone. Furthermore, results in

hypophysectomized animals (and the LH, FSH, and TRH controls of Moss and McCann, 1973) demonstrate that the facilitation of behavior is not caused by changes in LH or FSH levels brought about by LHRH injections (Pfaff, 1973). Intracerebroventricular injections of LHRH into female rats also induces sexual behavior (Tennent et al., 1982).

Other studies in female rats localize the site of action of LHRH. Microinfusions of LHRH into the medial preoptic area (MPOA) and arcuate nucleus facilitate behavior while infusions into the lateral hypothalamus and cerebral cortex have no effect (Moss and Foreman, 1976; Rodriguez-Sierra and Komisaruk, 1982). Inhibition is observed after LH or TRH infusion into the MPOA or arcuate only (Moss et al., 1979). Intrahypothalamic infusion studies have not been done in males.

The mechanism of action of LHRH on sexual behavior appears to be via only part of peptide and to be distinct from the endocrine effects of LHRH (Dudley et al., 1983). A differentiation between the effects of LHRH on the pituitary and on the central nervous system has been demonstrated using synthetic LHRH analogs. These analogs can be stimulatory, inhibitory or inactive in releasing LH and FSH from the anterior pituitary. The pituitary effect of a certain analog can not be used to predict the effect on behavior in female rats (Kastin et al., 1980; Dudley et al., 1981). Analogs from all three groups are capable of stimulating behavior; some have no effect on behavior. Intraventricular administration of various LHRH fragments indicates that LHRH may contain functionally



different elements and that the active sequence for facilitation of mating behavior is contained in the last half of the decapeptide (Dudley, Vale, Rivier, and Moss, 1983). The presence of a lag time (approximately 2 hours) between LHRH injection and the appearance of behavioral effects suggests that LHRH is likely not acting through a simple monosynaptic mechanism (Moss et al., 1975).

LHRH antibody studies support the hypothesis that LHRH plays a physiological role in the initiation of sexual receptivity. The infusion of an antibody to LHRH into the lateral ventricle or midbrain central gray of ovariectomized, estrogen and progesterone-treated rats suppresses lordosis (Kozlowski and Hostetter, 1978; Sakuma and Pfaff, 1980; Dudley, Vale, Rivier, and Moss, 1981). However, LHRH has no effect on lordosis in intact female rats (Moss et al., 1975). It does not alter levels of behavior during mating in normal proestrus animals and cannot advance the onset or prolong the period of sexual receptivity. In estrogen-primed, LHRH-injected females, lordosis behavior is not significantly different from intacts or estrogen and progesterone treated animals (Moss et al., 1975). This contrasts with the castrated male rat where LHRH facilitates behavior but has not been shown to restore it to the same level as intacts.

Studies of the behavioral effects of LHRH in female rats do note that the behavior displayed is qualitatively different from normal. In particular, females do not display as much hopping and darting behavior (proceptive behaviors) and do display some hindkicking and squealing which are characteristic of 'non-

receptive' diestrous females (Moss et al., 1975). LHRH appears to facilitate receptive behavior but not proceptive behavior (Tennent, Smith and Dorsa, 1982). Although the proceptive and receptive aspects of female sexual behavior may not be analogous to the arousal and copulatory components of male sexual behavior, it is interesting to speculate that LHRH may control discrete parts of the mating pattern in both sexes.

### Studies in Non-Mammalian Vertebrates

Few mammalian species are reported to be sensitive to the induction or augmentation of reproductive behavior by LHRH. The fact that LHRH can also facilitate the sexual behavior of a variety of non-mammalian vertebrates supports the contention that this phenomenon may be fairly common.

In ovariectomized ring doves, high and low levels of estrogen replacement result in an abolishment of courtship behavior (Cheng, 1977). Treatment of both groups with exogenous LHRH restores behavior. A similar study in male ring doves found no effect of LHRH on courtship behavior - likely due to optimal replacement doses of testosterone after castration (McDonald, 1979). This experiment attempted to correlate changing photoperiod (ring doves being seasonal breeders) with changes in LHRH. Dorsa et al. (1981) suggest a role for LHRH in maintaining sexual behavior in the face of a low testosterone environment. In seasonally breeding animals

such a situation would occur at the beginning and end of the breeding season during gonadal recrudescence and regression.

In the amphibian, Taricha granulosa (rough-skinned newt), intracerebroventricular injections of LHRH into intact (but sexually inactive) males increases the incidence of sexual behavior (Moore et al., 1982). An LHRH antagonist suppresses behavior in intact newts. Interestingly, the LHRH stimulation of behavior could only be elicited during the early part of the breeding season. A female amphibian, Xenopus laevis, also shows an increase in sexual receptivity after LHRH injection into both intact females and ovariectomized, estradiol and progesterone-treated females (Kelley, 1982).

In the female lizard, Anolis carolinensis, LHRH and TRH are both able to induce sexual receptivity in ovariectomized and estrogen-primed animals (Alderete, Tokarz, and Crews, 1980).

### SUMMARY

Great variation in components of copulatory behavior exists among male mammals. Even within a given species variation in behavioral parameters has been observed. This complexity raises the possibility that there are many points of possible control and modulation of sexual behavior by internal and external factors. A thorough understanding of reproductive behavior therefore, requires answers to questions about those critical control points and the factors that influence them. In particular, what does the normal

sexual behavior of the species look like? Can we discern separate components of that behavior that may be separately controlled? What environmental factors -- such as photoperiod, olfactory cues, prior experience with the opposite sex in general or with certain individuals, genital stimuli -- can change sexual behavior? By what mechanism do these environmental factors affect an individual's behavior? Perhaps the effects are via changes in neurophysiological factors such as steroid hormones and neuropeptides? The purpose of this thesis is to examine some of these points in detail for the species Microtus canicaudus, the gray-tailed vole. The first manuscript (Chapter II) is primarily descriptive: the sexual behaviors of M. canicaudus are quantified and the effects of prior sexual experience on the reproductive behavior of both sexes are determined. The second manuscript (Chapter III) approaches the problem from the opposite perspective. It focuses on the behavior of male M. canicaudus and describes the changes in sexual behavior that follow experimental manipulation of testosterone and luteinizing hormone-releasing hormone.

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EFFECTS OF PRIOR SEXUAL EXPERIENCE ON THE REPRODUCTIVE  
BEHAVIOR OF VOLE, MICROTUS CANICAUDUS

Chapter II.

INTRODUCTION

The patterns of copulatory behavior shown by different vole species (Microtus) are extremely diverse (Dewsbury, 1978). Vole social systems are also varied. Certain species display stable social organizations and monogamous associations, such as M. ochrogaster (Thomas and Birney, 1975; Getz and Carter, 1980). Other species are mostly solitary and less likely to be monogamous, such as M. xanthognathus (Wolff, 1980) and M. pennsylvanicus (Madison, 1980). A thorough understanding of the sexual behavior of microtines and the social factors affecting that behavior are necessary before proximate and ultimate determinants of reproduction and reproductive behavior can be understood.

Experience can alter the reproductive capacity of an animal in important ways (Dewsbury, 1978). Such factors as length of exposure of females to males and subtle differences in copulatory pattern (e.g. ejaculation frequency) result in critical changes in the internal reproductive physiology of both sexes (see, for example, Kranz and Berger, 1975; Milligan, 1975a,b). Copulatory patterns of male M. canicaudus have been described in detail (Dewsbury and Hartung, 1982), however the sexual behavior of the female and some

basic factors affecting behavioral interactions have not been described.

Social systems, and especially mating systems, also influence reproductive behavior. Pair-bonding has not been observed in M. canicaudus, as it has for the closely related species M. montanus when at very low densities (Jannett, 1980). M. ochrogaster has been shown through laboratory and field trapping data to form relatively permanent pair bonds (Getz, Carter, and Gavish, 1981); M. xanthognathus and M. pennsylvanicus are polygynous (Madison, 1980; Wolff, 1980).

In all mammals that have been studied, sexual experience influences masculine behavior (Larsson, 1979). The effects of sexual experience on masculine behavior has not been reported for any microtine rodent.

Natural sexual behavior appears to be different from hormone-induced behavior in a number of microtine species. For example, in M. ochrogaster females, progesterone has been reported to have an inhibitory effect on the display of lordosis (Dluzen and Carter, 1979). Gray and Dewsbury (1973), however, observed no differences in the sexual behavior of that species between natural- and hormone-induced estrus. Another microtine rodent, the brown lemming (Lemmus trimucronatus), shows significant differences in social and sexual behavior between females in natural estrus and ovariectomized animals that were treated with estrogen or estrogen plus progesterone (Huck, Carter, and Banks, 1982). In the only previous study on M. canicaudus sexual behavior, females were brought into

sexual receptivity with injections of estradiol benzoate and progesterone (Dewsbury and Hartung, 1982). The possibility exists that the sexual behaviors of the hormone-treated voles were qualitatively and quantitatively different from the naturally occurring sexual behavior in this species.

This study was designed to identify and quantify the reproductive behaviors of male and female M. canicaudus under conditions of natural estrus and to determine the effects of previous sexual experience on the reproductive behavior of this species. In addition, the behavioral responses of females to familiar and unfamiliar males were compared to investigate the possibility that this species exhibits pair-bonding.

## METHODS AND MATERIALS

Laboratory born M. canicaudus (formerly M. montanus canicaudus; Hall, 1981) were maintained on a phase-shifted photoperiod of 16L:8D hours (lights out at 1500 hr). Prior to the experiments, all voles were housed singly for at least 3 weeks in 30 x 35 x 17 cm clear plastic cages containing hardwood shavings. Rat chow, rabbit chow and water were available ad libitum.

The sexual behavior of voles was observed and quantified. Male sexual behavior motor patterns included mounts (male mounts female from the rear and displays pelvic thrusting), intromissions (mounts with penile insertion), and ejaculations (mounts with intromissions characterized by a series of short vigorous thrusts followed by a longer thrust and a period of relative immobility) (described by Dewsbury, 1973). Behaviors recorded were mount and intromission latency (time from start of test to first mount or intromission), ejaculation latency (time from first mount of a series to ejaculation), mount, intromission, and ejaculation frequency (total number per test) and post-ejaculatory interval (time from an ejaculation to the next mount).

Lordosis, which is characterized in M. canicaudus by an immobile stance with a slightly elevated perineum (Dewsbury, 1973), was recorded for females. Two measures of aggression, threat-leap and fighting behavior (as described by Dluzen and Carter, 1979), were also noted for females.

### Sexual Behavior at First Interaction

Multiparous females, 5-7 months of age, whose most recent litter was born 21-30 days previously, were used. Males were sexually experienced and 4-5 months old. To facilitate behavioral estrus (sexual receptivity) in females, males and females were first housed opposite each other for 3 days in cages (16 x 15 x 58 cm) that were divided with hardware cloth (Richmond and Conaway, 1969; personal observation). On the fourth day, males were transferred to the female's side of the cage. The reproductive behavior of both sexes was recorded for 10 consecutive hours (the entire dark phase under red light plus one hour before and after under white incandescent light). Only those pairs where the female exhibited behavioral estrus, 67% of the females tested, were included in the analysis.

### Effect of Experience

The sexual behavior shown by experienced males described above was compared with that of age-matched, sexually inexperienced males using the same procedures.

The role of experience in female sexual behavior was examined using females that were 100-120 days of age. Sexually inexperienced females were exposed to one of four experimental conditions for 2 weeks: housed alone in a clean cage (naive females), housed alone in a cage with soiled shavings from a male's cage (pheromone-exposed females), housed with a vasectomized male (vasectomized male-exposed

females), and housed with an intact male (experienced females). All females that were housed with an intact male became pregnant, subsequently raised litters, and were housed with pups for 2 weeks after parturition and alone for another week to 2 weeks. Females in all other groups were housed alone for 3-4 weeks after treatment before being moved to the divided cages opposite sexually experienced males and tested as above.

The sexual behavior of females at post-partum estrus was observed to determine if the sexual behavior of female M. canicaudus is influenced by whether the male is familiar or unfamiliar to the female. Stud males were housed with females continuously from mating, throughout gestation, and only removed 6-12 hours before birth of the litter. Within 6 hours following parturition, females were moved to a neutral cage (without pups) and the stud male or an unfamiliar, sexually experienced, non-paired male was introduced. Behavior was observed during the dark phase for 3 hours (using a red light).

### Statistical Analyses

Freidman's non-parametric two-way analysis of variance (ANOVA) was used to check for sequence effects (changes across ejaculatory series) on male behavior, and post-hoc pair-wise comparisons (within parameters showing significant differences on the ANOVA) were done with the Mann-Whitney U Test. The Mann-Whitney U Test was also used to compare sexually experienced and inexperienced animals, and the



response of females to familiar and unfamiliar males. Kendall's Coefficient of Concordance was used to determine if there were significant associations between any parameters (Seigel, 1956). Student's T-test was used to compare behavior on complete versus incomplete series. Percent receptive animals per group were compared with chi-square test and paired comparisons of groups were done with Fisher's Exact Test.

## RESULTS AND DISCUSSION

### DESCRIPTION OF SEXUAL BEHAVIOR AT FIRST INTERACTION

#### General Parameters

Experienced M. canicaudus pairs showed great variation in latency to initiate sexual behavior (range of 4.6 to 404 minutes). Total duration of copulatory interactions was quite long, due primarily to long post-ejaculatory intervals (Table 1). Most measures of copulatory behavior were relatively similar; for example, average mount frequency per series ranged from 13.4 to 15.5 (Table 2) and ejaculation frequency per test ranged from 4 to 9 (Table 1).

The present study, using a 10-hour observation period, found that the total duration of copulatory interactions averaged 223 minutes, which is considerably longer than previously published reports on microtines (see references below). This difference may be due to procedural differences. In particular, the previous studies used a restrictive "satiety criterion", 30 minutes with no copulations as a basis for considering behavioral interactions were complete (Gray and Dewsbury, 1973, 1975; Dewsbury, 1973, 1976; Kenney et al., 1979; DeJonge and Ketel, 1981; Dewsbury and Hartung, 1982). Because the average post-ejaculatory interval was longer than 30 minutes in the present study (Table 1), a 30-minute satiety criterion would have ended observations prematurely.

In addition, this difference in length of copulatory interactions may be due to the fact that the female voles used in previous studies were injected with progesterone (studies cited above). Progesterone injection decreases sexual receptivity in female M. ochrogaster and Lemmus trimucronatus (Dluzen and Carter, 1979; Huck et al., 1982). Thus injections of progesterone may have affected the length of time females were receptive and prematurely ended sexual encounters in prior studies.

The long duration of sexual activity that was observed here in M. canicaudus pairs may serve several purposes. Increasing numbers of series significantly increases probability of ovulation and successful pregnancy, especially in induced ovulators such as microtine rodents (see for instance Davis et al., 1974; Milligan, 1975b; Dewsbury, 1978). Male presence for an extended amount of time post-copulation also increases percent conception significantly in M. ochrogaster and M. montanus, presumably due to the importance of the continued presence of olfactory cues (Kranz and Berger, 1975; Richmond and Stehn, 1976).

The mount latency that was found in the present study is longer than that previously reported for M. canicaudus (Dewsbury and Hartung, 1982). This difference could be due to the use of females that had been treated with hormones in the study by Dewsbury and Hartung (1982). When female voles were treated with hormones to induce estrus in this lab, latencies were typically 200-300 seconds (unpublished observations), which is more similar to Dewsbury and Hartung's observations. Thus, it appears that the normal mount

latency for M. canicaudus is longer than had been reported previously.

### Description of Female Behavior

Observation of receptive behavior indicates that female M. canicaudus show lordosis only when mounted, and lordosis does not extend beyond dismount. Latency to first lordosis, therefore, is the same as mount latency, and lordosis frequency equals mount frequency. It is extremely difficult for a male to mount an unreceptive female; mounts were not observed in this study unless the female exhibited lordosis.

In addition to receptive sexual behavior, females also were observed for the occurrence of any proceptive or aggressive behavior. Female M. canicaudus in natural estrus rarely showed darting or other proceptive (soliciting) behaviors. Aggression was rare in receptive females, but common in unreceptive females. It has been suggested that in M. ochrogaster increases in female aggressive behavior cause cessation of mating (Gray and Dewsbury, 1975; Dluzen and Carter, 1979). M. canicaudus females did not show a change in aggression after several ejaculatory series nor was there a change in lordosis response to mounts. Thus, it appears unlikely that an increase in aggression by females ends the copulatory interaction.

### Description of Male Behavior

The present study confirms the earlier observations by Dewsbury and Hartung (1982) that male M. canicaudus exhibit a behavioral pattern with multiple intromissions, multiple ejaculations, repeated thrusting during intromissions and no copulatory lock. M. canicaudus exhibit multiple intromissions prior to ejaculation, as do M. montanus and M. ochrogaster (Dewsbury, 1973; Gray and Dewsbury, 1973). Other microtines sometimes exhibit ejaculation during the first intromission (Dewsbury and Hartung, 1982).

Measures of male copulatory behavior in the first four series are summarized in Table 2. Post-ejaculatory intervals changed significantly across series; series one was significantly shorter than series two, three, or four. The post-ejaculatory intervals of the last three series did not differ from each other. No significant correlation exists between mount frequency, ejaculation latency or post-ejaculatory interval, but mount frequency and intromission frequency are positively correlated ( $W = 0.963$ ,  $P < 0.05$ ).

The quantity of behavior (mount frequency, intromission frequency, and ejaculation latency) within the first four series is consistent, and the only indication of satiety appears to be changes in length of the post-ejaculatory interval. This description of male M. canicaudus sexual behavior is similar to that described by Dewsbury and Hartung (1982), except for the longer post-ejaculatory intervals recorded here.

M. canicaudus showed little variation in mount frequency and intromission frequency across series (Table 2), until the start of the incomplete satiety series. M. pennsylvanicus shows a progressive decrease in these parameters in later series; a "facilitation" of behavior because ejaculation is attained after less stimulation (Gray and Dewsbury, 1975). Other microtine males, however, show a pattern similar to M. canicaudus (Dewsbury, 1973; Gray and Dewsbury, 1973; Dewsbury and Hartung, 1982). Regarding intromission frequency per series, male M. canicaudus are somewhat intermediate among microtines. For example, M. ochrogaster is similar to M. canicaudus (Gray and Dewsbury, 1973). M. oeconomus and M. xanthognathus show 1-5 intromissions per series, whereas M. montanus have 15-25 intromissions per series (Dewsbury, 1973; Dewsbury and Hartung, 1982). Gray and Dewsbury (1973, 1975) proposed that male copulatory patterns and female vaginal stimulus requirements for initiation of pregnancy are coadapted and species-typical. Their proposal has been supported by data in the laboratory mouse (Diamond, 1970) and M. californicus (Kenney et al., 1979).

When males eventually cease mating, they are said to have become sexually exhausted or reached sexual satiety. Upon reaching sexual exhaustion, 67% of male M. canicaudus show incomplete series. That is, they reach sexual satiety within a series and fail to complete it with an ejaculation, which is different from the more usual pattern of simply failing to start a new series at satiety (Dewsbury, 1973). Of the other 33% that did fail to start a new

series at satiety, 20% of those showed the behavior pattern typical of incomplete series (see below) but did eventually attain ejaculation. Incomplete series comprised 11.36% of all series. On average, sexual behavior ceased within or after the sixth series.

Several differences separate series that end in ejaculation from those that do not. Incomplete series contained significantly more mounts ( $28 \pm 3.5$  mounts versus  $14.6 \pm 0.77$  mounts;  $P < 0.001$ ) and intromissions ( $26.4 \pm 3.3$  intromissions versus  $13.6 \pm 0.79$  intromissions;  $P < 0.001$ ) and lasted significantly longer ( $747.9 \pm 113$  seconds versus  $295 \pm 23$  seconds;  $P < 0.001$ ). The post-ejaculatory intervals preceding an incomplete series were significantly longer than those preceding complete ejaculatory series ( $3179 \pm 677$  seconds versus  $1781 \pm 235$  seconds;  $P < 0.025$ ). Mean latency to the incomplete series was  $13771 \pm 1741$  seconds.

Incomplete series have not been previously reported for M. canicaudus. Two other male microtine rodent species, M. montanus and M. californicus, show incomplete series as well (Dewsbury, 1973; Kenney et al., 1979). Reaching sexual satiety within a series rather than between is relatively rare in other rodents and has been previously assumed to occur in few microtine species and in the golden hamster (Dewsbury, 1973; Bunnell, Boland, and Dewsbury, 1977). The occurrence of incomplete series in the present study, compared to the failure to observe incomplete series in other studies, is perhaps attributable to the long period of testing.

The behavior shown within incomplete series was typically divided into "bouts" of 5-15 mounts and intromissions followed by a

"rest period" of 5-10 minutes and resumption of mounting. Rest periods of this length were never observed in complete series. This pattern was also shown, and finally culminated in ejaculation, on the final or next to final series in 53% of the males. Normal, complete series did not show rest periods of this length. Such a pattern was not described for M. montanus by Dewsbury (1973); although he does note that the mean interval between intromissions increased significantly during the final incomplete series.

#### INFLUENCE OF SEXUAL EXPERIENCE ON MALE SEXUAL BEHAVIOR

In this study of Microtus canicaudus there were no differences between experienced and inexperienced males on latency to first mount and intromission, total time to complete a series (average across all series), post-ejaculatory interval (all series combined), number of series per encounter, ejaculation frequency, and total duration of the copulatory interaction (Table 1). The only difference in copulatory behavior in experienced and inexperienced M. canicaudus was seen in ejaculation latency on the first series (Table 2; Table 3). Inexperienced males show a significantly longer latency than sexually experienced males.

Both the inexperienced and the experienced male voles show a shorter post-ejaculatory interval after the first series than after later series (Table 3). Inexperienced animals also show a significantly decreased mount frequency on series 2 compared to series 1 and later series (Table 3). Experienced males do not show



this. There are not, however, any significant differences in mount frequency between experienced and inexperienced males.

In rats and hamsters, experienced males have shorter mount and intromission latencies and require less total time to ejaculation than inexperienced males (Dewsbury, 1969; Lisk, Zeiss, and Ciaccio, 1972). This was not the case in voles, which supports the idea that experience may be more or less important in the expression of copulatory behavior in different species. The large differences in behavior between experienced and inexperienced male rats parallels the differences in plasma testosterone, penile morphology, and accessory sex organs that has been observed (Taylor, Regan, and Hallen, 1983; Taylor, Weiss, and Komitowski, 1983). Experienced and inexperienced male M. canicaudus show no significant difference in plasma testosterone (see Appendix B). Therefore, experience may be less important in the development of sexual behavior and other reproductive parameters in M. canicaudus than in other mammals. Alternatively, it is possible that no major differences in behavior were observed in this study due to the large amount of individual variation.

Inexperienced male voles show longer ejaculation latencies than experienced males. This is also the case in male rats, although in rats the difference persisted across at least five series (Dewsbury, 1969). In male voles this effect of inexperience on this parameter disappears quickly and later series are comparable to behavior shown by experienced males.

## ROLE OF EXPERIENCE IN FEMALE VOLE SEXUAL BEHAVIOR

Females that had previously mated, either with vasectomized or intact males, were significantly more likely to show sexual receptivity after 4 days housing opposite a male (64% and 67%, respectively), compared to unmated females (naive, 18%, or pheromone-exposed, 10%) (Table 4). These experienced females may have already been in estrus at the start. Post-partum estrus can persist for an unknown length of time if mating does not occur. It is also unknown if a "post-partum" estrus occurs in females after pseudopregnancy. Alternatively, experienced females may be more likely to respond with estrus when paired opposite a male, than inexperienced female voles. Sterile matings are thought to be important for the development of puberty and initiation of full breeding condition in another microtine, Clethrionomys glareolus (Westlin and Gustafsson, 1983). These matings are sterile due to an inadequate endocrine response to copulation.

Naive females and females exposed only to soiled male litter showed low levels of receptivity (18% and 10%, Table 4). Generalized disruptions (such as cage cleaning, handling, or regrouping) can cause 8-17% of naive M. ochrogaster females to show behavioral estrus (Richmond and Conaway, 1969). This type of trauma could have accounted for the small percent of receptive females seen here in M. canicaudus. Exposure to male urine via soiled litter was not effective in inducing estrus in this study. This differs

from M. ochrogaster where 30% of females housed in soiled cages are receptive by the end of one week (Richmond and Conaway, 1969).

Lordosis latency and lordosis frequency shown by receptive animals in vasectomized male-exposed females and experienced females did not differ (Table 4). Latencies and frequencies of receptive animals in naive and pheromone-exposed groups were within the range shown by animals in the other two groups. Therefore the sexual behavior shown by receptive females did not differ based on their prior experience, however, the percent of receptive females increased sharply in animals that had mated previously.

#### EFFECTS OF FAMILIAR AND UNFAMILIAR MALES ON BEHAVIORAL RESPONSES OF FEMALES

When females were exposed to a familiar male or an unfamiliar male, no significant differences were seen in the lordosis latency, lordosis frequency, number of copulatory series or total time from start to completion of behavior nor in any measure of male behavior, between the two groups (see Table 10, Appendix C).

Social patterns of microtine rodents are quite diverse. When a procedure similar to the one in the present study was used in M. ochrogaster, females were significantly more likely to mate, and less likely to engage in aggressive behaviors with their mate than an unfamiliar male (Getz et al., 1981). Trapping data also confirms that M. ochrogaster is monogamous (Thomas and Birney, 1979; Getz et al., 1981). However, M. pennsylvanicus and M. xanthognathus, are

less social and probably do not form monogamous mating associations (Getz, 1978; Wolff, 1980). If M. canicaudus pair-bonded, one would expect more rapid mating of females with familiar than unfamiliar males. This was not the case in this study, which supports the hypothesis that M. canicaudus does not pair-bond. M. montanus, the microtine species perhaps most closely related to M. canicaudus, is facultatively monogamous at very low field population densities and polygynous at high densities (Jannett, 1980). It is possible that the animals in this study construed the restricted cage as a high density situation, and therefore did not show monogamy as they might at low field densities.

## SUMMARY

The total duration of sexual interactions averaged 223 minutes, which is considerably longer than previously published reports on microtines. The average mount latency also was found to be longer than previously reported. A majority (67%) of male M. canicaudus reach sexual satiety (exhaustion) within a series rather than between series, which is a novel finding for this species. These new observations on the sexual behavior of male M. canicaudus are likely due to the use of a long observation period and stimulus females in natural estrus, and points out the importance of these factors when describing the reproductive behavior of a given species.

This study also represents the first description of the sexual behavior of female M. canicaudus. I found that, female M. canicaudus show lordosis only when mounted and lordosis does not extend beyond dismount. Receptive females rarely showed proceptive (soliciting) or aggressive behaviors, in contrast, unreceptive females were very aggressive.

In contrast to findings with other rodent species, the effect of sexual experience on qualitative and quantitative measures of male vole sexual behavior was small and transient. The minor differences between experienced and inexperienced males that were observed during the first sexual encounter disappeared quickly, with sexual behavior in series after the first being similar for experienced sexual behavior in voles. The sexual behavior shown by receptive

female voles was similar for experienced and inexperienced females; however the percent females that were receptive was higher in the experimental animals that had mated previously. Sexual behaviors were indistinguishable for females that were paired with familiar or unfamiliar males indicating that M. canicaudus does not pair-bond.

TABLE 1. Summary of Sexual Behavior Parameters in Experienced and Inexperienced Males.<sup>a</sup>

	MOUNT LATENCY (min)	INTRO LATENCY (min)	TOTAL TIME (min)	POST-EJAC <sup>b</sup> INTERVAL (min)	NO. <sup>b</sup> SERIES	EJAC. <sup>b</sup> FREQUENCY	TOTAL DURATION (min)
EXPERIENCED MALES (N = 15)							
mean	120.0	120.1	4.9	35.4	5.87	5.20	222.7
standard error	29.6	29.6	0.4	4.2	0.35	0.31	26.6
INEXPERIENCED MALES (N = 10)							
mean	101.3	101.5	5.1	29.3	5.10	4.50	153.5
standard error	22.3	22.3	0.4	4.7	0.60	0.52	23.8

<sup>a</sup> No significant differences on any parameter; Mann-Whitney U Test,  $p > 0.05$ .

<sup>b</sup> Post-Ejac Interval = Post-Ejaculatory Interval; No. Series = Number of Series per observation period; Ejac. Frequencies - Ejaculation Frequency.

TABLE 2. Measures of Copulatory Behavior in Experienced Male M. canicaudus.

EJACULATORY SERIES		EJACULATION <sup>b</sup> LATENCY (minutes)	MOUNT <sup>b</sup> FREQUENCY	INTROMISSION <sup>b</sup> FREQUENCY	POST-EJAC <sup>a</sup> INTERVAL (minutes)	N
1	mean	4.28	13.4	12.1	15.13	15
	standard error	0.42	1.2	1.1	2.10	
2	mean	5.45	13.6	12.6	26.98	15
	standard error	1.20	1.1	1.2	4.97	
3	mean	4.10	13.7	12.8	37.72	15
	standard error	0.47	1.4	1.5	9.77	
4	mean	4.97	15.5	15.0	31.13	15
	standard error	0.70	1.8	1.9	5.65	

<sup>a</sup> Series 1 is significantly shorter than series 2, 3, and 4 ( $\chi^2 = 8.04$ ;  $p < 0.05$ ).

<sup>b</sup> Not significantly different by Friedman's Two-Way Analysis of Variance.



TABLE 3. Measures of Copulatory Behavior in Inexperienced Male M. canicaudus.

EJACULATORY SERIES		EJACULATION LATENCY (minutes)	MOUNT FREQUENCY	INTROMISSION FREQUENCY	POST-EJAC INTERVAL (minutes)	N
1	mean	6.68 <sup>a</sup>	14.5 <sup>a</sup>	12.7	11.07 <sup>a</sup>	10
	standard mean	0.87	1.1	1.1	1.83	
2	mean	4.42	10.5	10.3	28.02	10
	standard mean	0.53	1.2	1.2	8.85	
3	mean	5.10	13.2	13.0	32.25	9
	standard mean	0.73	1.6	1.7	10.08	
4	mean	5.45	15.3	14.9	31.55	9
	standard mean	1.22	3.0	2.7	11.05	

<sup>a</sup> Series 1 is significantly different from Series 2 (Mann-Whitney U Test; P <0.05).

TABLE 4. Sexual Behavior of Female M. canicaudus with Different Types of Prior Sexual Experience.

GROUP	N	PERCENT <sup>1</sup> RECEPTIVE	LORDOSIS LATENCY ( $\bar{x} \pm \text{SEM}$ ) (min)	LORDOSIS FREQ ( $\bar{x} \pm \text{SEM}$ )
Naive Females	11	18%	35.63 $\pm$ 14.38	34 $\pm$ 4
Pheromone-Exposed Females	10	10%	44.50 $\pm$ 0	68 $\pm$ 0
Vasectomized Male-Exposed Females	11	64%	35.03 $\pm$ 20.47	85 $\pm$ 18
Experienced Females	15	67%	32.97 $\pm$ 7.13	89 $\pm$ 10

<sup>1</sup> Significant difference ( $\chi^2 = 12.58$ ;  $p < .01$ ); pair-wise comparison with Fishers Exact Test shows that naive does not differ from pheromone-exposed and vasectomized male-exposed does not differ from experienced but naive differs from vasectomized male-exposed and experienced and pheromone-exposed differs from vasectomized-male exposed and experienced females.

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THE EFFECT OF LUTEINIZING HORMONE-RELEASING HORMONE  
ON THE MASCULINE SEXUAL BEHAVIOR OF THE VOLE,  
MICROTUS CANICAUDUS

Chapter III.

INTRODUCTION

Luteinizing hormone-releasing hormone (LHRH) was first shown to facilitate the display of reproductive behavior in female rats (Rattus norvegicus) (Moss and McCann, 1973; Pfaff, 1973). Subsequently, LHRH has been found to stimulate sexual behavior in a variety of species of birds, reptiles, and amphibians (Cheng, 1977; Alderete, Tokarz and Crews, 1980; Kelley, 1982; Moore, Miller, Kubiak and Folkers, 1982). Despite the fact that LHRH can influence sexual behavior in species from four vertebrate classes, very few species of mammals have been shown to be behaviorally sensitive to LHRH.

Reports of behavioral responses to LHRH are restricted to rats, mice, and humans (Mauk, Olson, Kastin and Olson, 1980). The problem has been studied most extensively in female rats and mice, although male rats have been shown to be behaviorally sensitive to LHRH (Moss et al., 1975; Luttge and Sheets, 1977). Studies with guinea pigs (Cavia porcellus) and hamsters (Mesocricetus auratus) indicate that LHRH does not significantly alter sexual behavior in these species (Carter and Davis, 1977; Moss and Dudley, 1980). Likewise, the

sexual behavior of adult male chimpanzees did not respond to LHRH analogs (Doering, McGinnis, Kraemer, and Hamberg personal communication, cited by Moss, Riskind, and Dudley, 1979).

Studies of the effects of LHRH on sexual behavior of human males have been contradictory or inconclusive (reviewed in Mauk et al., 1980). The administration of LHRH analogs to men with certain sexual dysfunctions reportedly increased sexual potency (Mortimer, McNeilly, Fisher, Murray, and Besser, 1974; Benkert, Jordon, Dahlen, Schneider, and Grammel, 1975; Schwarzstein, Aparicio, Turner, Calamera, Mancini, and Schally, 1975; Moss, Riskind, and Dudley, 1979). Other studies, however, found that LHRH had no effect on men who were either normal or had sexual dysfunctions (Ehrensing and Kastin, 1976; Davies, Mountjoy, Gomez-Pen, Watson, Hanker, Besser, and Hall, 1976).

There is evidence that LHRH can act independently of sex steroids, because behavioral effects of LHRH have been observed in castrated and ovariectomized rats (Moss and McCann, 1973; Pfaff, 1973; Moss et al., 1975; Dorsa, Smith, and Davidson, 1981). Furthermore, the behavioral effects of LHRH are independent of pituitary and adrenal hormones, because LHRH has been found to influence the sexual behavior of hypophysectomized and adrenalectomized female rats (Pfaff, 1973; Moss and McCann, 1975).

Evidence that LHRH can act directly on neural tissue in the brain comes from studies in which infusions of small quantities (50 ng) of LHRH into specific brain areas stimulated lordotic behavior in ovariectomized, estrone-primed female rats (Moss and Foreman,

1976). Furthermore, the infusion of anti-LHRH antiserum or synthetic LHRH antagonists into the lateral ventricle or midbrain central gray has been found to suppress sexual behavior of rats (Kozlowski and Hostetter, 1978; Sakuma and Pfaff, 1980; Dorsa et al., 1981; Dudley et al., 1981).

The facilitation of sexual behavior by LHRH is not completely independent of gonadal steroid hormones. Ovariectomized females do not respond to LHRH until given estrogen replacement (Moss et al., 1975). Similarly, the behavioral response to LHRH in male rats is influenced by testosterone. Castrated male rats are behaviorally responsive to LHRH and its analogs when treated with low, but not high, levels of testosterone (Dorsa and Smith, 1980; Dorsa et al., 1981).

The purpose of this study was to determine whether LHRH can modify the sexual behaviors of male gray-tailed voles (Microtus canicaudus). Intact males were injected with LHRH and their copulatory behaviors were compared with that of saline-injected controls. To investigate the role of the testis and testosterone in the behavioral response to LHRH, castrated voles were implanted with Silastic capsules of testosterone and were injected with LHRH or saline. The effect of injections of LHRH or saline on plasma testosterone was determined to clarify further the relative importance of the neuropeptide and the steroid in the control of male M. canicaudus sexual behavior.



## MATERIALS AND METHODS

M. canicaudus (formerly M. montanus canicaudus; Hall, 1981) were from a laboratory colony that was established in 1973 using locally captured voles. The colony is outbred by periodically adding recently trapped animals. Voles were maintained on a phase-shifted photoperiod of 16L:8D hours (lights out at 1500 hr) and at controlled temperature of 20 to 22°C. Two or three animals were housed in each clear plastic cage (30 x 35 x 17 cm) containing hardwood shavings. Rat chow, rabbit chow and water were available ad libitum.

### Behavioral Testing

The same procedure was used for all tests of sexual behavior. Testing was done between 1700 and 2200 hrs in a 30 cm diameter sheet metal arena with 30 cm sides above which two 40-watt incandescent red light bulbs were suspended. A mirror mounted at a 45° angle beneath the glass floor of the arena allowed ventral viewing of the behaviors. Males were placed in the arena 5 minutes prior to the introduction of the stimulus female. Stimulus females were multiparous females that had been ovariectomized and were injected with 10 µg estradiol benzoate per day for 3 days preceding the day of testing. Females were pretested for sexual receptivity, using non-experimental males, prior to use as stimulus animals. Based on preliminary studies, a 10-minute observation period was determined

to be appropriate for recording sexual encounters in M. canicaudus. Sexual behaviors that were recorded and quantified include mounts (male mounts female from the rear and displays pelvic thrusting), intromissions (mounts with penile insertion) and ejaculations (mounts with intromissions characterized by a series of short vigorous thrusts followed by a longer thrust and a period of relative immobility). The mount, intromission, and ejaculation latencies (time from start of test to first mount, intromission, or ejaculation, respectively) and mount, intromission and ejaculation frequencies (total number per test) were recorded. Total time to complete a series was computed as ejaculation latency minus mount latency. All males were sexually experienced and had been screened for sexual competence by selecting only males that mounted at least once during a 10-minute test period. Males were ranked, based on mount latency on the pretest, and were assigned alternately to treatment and control groups.

### Experiment I: Intact Males

Intact males (7 months of age) received either subcutaneous (sc) injection of synthetic mammalian LHRH (Sigma Chemical Co., 500 ng/0.1 ml saline) or an injection of saline vehicle alone (9 per group). Starting 2 hours after injection, the male was placed in the test arena and sexual behaviors were recorded. A parallel study was conducted to determine the effect of LHRH on plasma testosterone levels. Two hours after injection of saline or LHRH (9 per group),

intact voles were lightly anesthetized with ether. Blood was collected from the supraorbital sinus with a heparinized Pasteur pipette. Blood samples were centrifuged. Plasma was stored at  $-20^{\circ}\text{C}$  until assayed for testosterone using radioimmunoassay.

### Experiment II: Castrated Males

Males (4 to 6 months of age) were bilaterally castrated under xylazine-ketamine anesthesia and then were implanted subcutaneously in the abdominal region with Silastic capsules of testosterone propionate (steroid from Sigma; capsule preparation technique of Moore, 1981; Silastic tubing from Dow Corning; 0.047 x 0.025 inches, o.d. x i.d.). Thirty-seven males received 3 mm capsules (done as two replicate experiments with 20 males in the first and 17 males in the second experiment), a second group ( $n = 19$ ) received 10 mm implants, and a third was implanted with empty capsules (also a replicated experiment with 18 animals per replicate). Small (3 mm) capsules contained  $0.64 \pm 0.02$  mg crystalline hormone; large (10 mm) implants contained  $2.3 \pm 0.05$  mg. Animals within each group were injected twenty days later with LHRH or saline as described above, and sexual behavior was tested 2 hours after the injection. To determine plasma testosterone levels, males were bled immediately after behavior tests. Males in the second replicate of the group that received small capsules were also bled 7 days before behavioral testing. Those samples were assayed for testosterone, males were

ranked based on plasma level, and were assigned alternately to LHRH or saline treatment groups.

### Testosterone Radioimmunoassay

Androgen radioimmunoassay procedures previously described by Moore and Muller (1977) were used (see Appendix A). The antiserum (from Gordon D. Niswender) reacts appreciably with testosterone and dihydrotestosterone (Ismail, Niswender, and Midgley, 1972). Pooled samples from intact and castrated male voles were chromatographed on Celite columns to separate testosterone and dihydrotestosterone fractions (see Appendix B). Because the quantity of androgen measured in the testosterone fraction was not significantly different from that measured in unchromatographed plasma, the amount of dihydrotestosterone in male vole plasma was presumed to be negligible. Therefore plasma samples were not chromatographed, and the hormone levels are reported as testosterone per ml plasma. To correct for losses during the extraction with benzene-hexane, testosterone levels were adjusted according to percent recovery of radiolabeled internal standard (mean extraction efficiency 99%). Intra- and interassay coefficients of variation were 5.4% and 10.4%, respectively. The lower limit of sensitivity of this procedure was 6.25 pg/tube or 0.0625 ng/ml plasma.

### **Statistical Analysis**

Behavioral frequencies and latencies were analyzed using the Wilcoxon Signed Ranks Test for paired comparisons (of pre-test and post-treatment behavior) and the Mann-Whitney U Test for independent comparisons (between groups) (Siegel, 1956). Total frequencies of each behavior were compared using the Chi-square goodness of fit test, and proportions of animals showing a behavior were examined with Fisher's exact test. Student's T test and one-way analysis of variance were used for analysis of radioimmunoassay data.

## RESULTS

### Experiment I: Intact Males

The injection of LHRH facilitated several aspects of sexual behavior in intact male voles. Intromission and ejaculation latencies were significantly shorter for the voles injected with LHRH, compared to saline (Fig. 1). Mounts were significantly more frequent in the LHRH-injected animals compared to the saline-injected animals (Fig. 2). Intromission and ejaculation frequencies in LHRH- and saline-injected voles were not significantly different. The total time from initiation to completion of one ejaculatory series was not significantly different for the LHRH- and saline-injected groups ( $U = 26$ ,  $p > 0.05$ ). The injection of LHRH into intact males resulted in a five-fold increase in plasma testosterone concentration (saline-injected males had  $1.16 \pm 0.16$  ng/ml and LHRH-injected voles had  $5.28 \pm 0.45$  ng/ml;  $t = 3.69$ ,  $p < 0.005$ ).

### Experiment II: Castrated Males

The sexual behavior of castrated, testosterone-implanted males was enhanced by the LHRH injection, compared to saline injection (Table 6). None of the castrated voles that were implanted with empty capsules showed sexual behavior, even when injected with LHRH. In males implanted with small capsules of testosterone, the

number of animals mounting, intromitting, and ejaculating at least once, as well as the total number of mounts and intromissions, was significantly higher in the LHRH-treated males than in the saline-injected voles (Table 6). In castrated males implanted with large testosterone capsules, total number of mounts and total number of intromissions were significantly greater in voles treated with LHRH than in voles treated with saline.

In castrated males with small implants of testosterone, LHRH-injected voles exhibited shorter latencies to first mount and intromission (Fig. 3) and higher frequencies of mounts and intromissions than the saline-injected voles (Fig. 4). In castrates with large testosterone implants, neither latencies nor frequencies were significantly different in the LHRH-treated versus saline-injected voles (Fig. 5, Fig. 6). Due to a large proportion of non-responders in the castrates with small testosterone implants (35%, see Table 6), behavior-positive animals (those exhibiting a given behavior at least once) were compared. No significant differences between the performances of the LHRH- or saline-injected groups was noted.

The sexual behavior of each animal after castration and treatment was compared with that animal's behavior on the precastration screening test. In castrated males with small testosterone implants, sexual performance decreased significantly from precastration levels in saline-injected voles but not in LHRH-injected voles (Tables 7 and 8). In castrated males with large testosterone implants that received saline, measurements of sexual

behavior were not significantly different from precastration levels. In castrates with large implants of testosterone that were injected with LHRH, mount, intromission, and ejaculation latencies were significantly shorter after castration and treatment than before (Tables 7 and 8).

Within a given treatment, there were no significant differences in plasma testosterone levels between LHRH- and saline-injected males (Table 5). The concentration of testosterone in the plasma of saline-injected intact males was significantly lower than castrated males with large testosterone implants and was significantly higher than that of castrates with empty capsules or small testosterone capsules (Table 5). Castrated males with small capsules had a higher concentration of plasma testosterone than castrates with empty capsules. Comparison of plasma testosterone in castrates with small capsules before and after injection (paired t-test) showed no significant difference.



## DISCUSSION

This study demonstrates that an injection of LHRH into gray-tailed voles (M. canicaudus) can enhance masculine sexual behavior. Such an enhancement of masculine behavior has only been previously reported for male rats (Moss et al., 1975; Dorsa and Smith, 1980; Dorsa et al., 1981) and in the human male (see review, Mauk et al., 1980). These findings support the hypothesis that LHRH is involved in the regulation of sexual behavior in a variety of mammals.

The behavioral effects of LHRH in male voles are independent of the testes, because testosterone-implanted castrates responded to LHRH with enhanced sexual behavior. Similarly, Moss et al. (1975) found that castrated male rats that had been treated with testosterone exhibited an increase in sexual behavior following an injection of LHRH.

These studies provide evidence that testosterone can maintain the behavioral response to LHRH in voles. In the absence of testosterone, castrated voles did not exhibit sexual behavior, even after an injection of LHRH. Studies with male rats also found that LHRH did not stimulate sexual behavior in castrates that lacked steroid replacement therapy (Moss et al., 1975). Sex steroids have been shown to maintain the behavioral responses to other neuropeptides such as adrenocorticotrophic hormone and arginine vasotocin (Moore and Zoeller, 1979; Wilson, Thody and Everard, 1979).

Injection of LHRH into intact male voles further increased several measurements of sexual behavior. Intact male rats also respond to LHRH with a facilitation of behavior (Moss et al., 1975). Whether LHRH is critical to the display of copulatory behavior in normal, intact male mammals is unclear; however female rats show a decrement in normal behavior when an LHRH antibody is infused into the brain (Kozlowski and Hostetter, 1978; Sakuma and Pfaff, 1980).

The behavioral effects of LHRH seen in voles are perhaps best considered as changes in the arousal component of masculine sexual behavior. The parameters affected, latencies and frequencies, correspond to the arousal component of masculine sex drive as described by Beach (1956), while another parameter, the total time required to complete a series, is considered part of the completion component. The total time to complete a series was not affected by LHRH. Dorsa and Amith (1980), using a penile anesthetization paradigm to assess arousal more directly, showed that LHRH significantly increases mount frequency in male rats injected intracranially with the peptide.

The hypothesized link between LHRH and the arousal mechanism is further supported by the effects of LHRH on the behavior parameters of the castrated male voles with small testosterone implants. In behavior-positive animals, no differences in the performance of sexual behavior were observed between the saline-treated and the LHRH-treated males. Therefore, the differences between the saline- and LHRH-treated voles can be attributed to the tendency of each

animal to initiate sexual behavior, rather than the performance of the behaviors once initiated.

The potentiation of sexual behavior by LHRH in voles occurs at a variety of plasma androgen levels. This is in contrast to gonadectomized female rats, where LHRH facilitation of lordosis occurs only across a very narrow range in levels of estrogen replacement (Moss et al., 1975). Male voles with high testosterone replacement levels were less responsive to LHRH than either intact males or castrates with low levels of testosterone replacement. As suggested perviously (Dorsa et al., 1981), optimal levels of testosterone replacement may result in a maximum display of behavior; under such a condition, LHRH might be incapable of making further significant increases.

Testosterone replacement to castrated voles resulted in mean plasma levels significantly different from intact males. The concentration of testosterone in the plasma of castrates with large capsules was approximately twice that of intacts, however, sexual behaviors of the two groups were similar. The plasma testosterone levels in castrates with small capsules was too low to restore sexual behavior to precastration levels, although it was sufficient to maintain the behavioral response to LHRH. In castrates with small capsules, comparison of plasma testosterone, before and after the injection of LHRH or saline, revealed no significant differences. These results support the conclusion that the differences in behavior are not caused by differences in plasma

testosterone concentrations and that LHRH did not release a significant amount of adrenal testosterone.

In conclusion, the fact that M. canicaudus responds to LHRH with enhanced masculine behavior supports the contention that this phenomenon may be fairly common in mammals. A physiological role for the peptide is supported by its ability to further increase copulatory behavior in intact voles and in castrated voles with testosterone replacement. Testosterone is necessary for the behavioral response to LHRH to be present; however the effect of LHRH on behavior is independent of the testis. The specific behavioral parameters affected support the hypothesis that LHRH changes the arousal component of masculine behavior in voles.

Figure 1. Behavioral Latencies (mean  $\pm$  SEM) in Intact Male Voles after LHRH or Saline Injection (9 per group). Asterisk (\*) indicates groups significantly different, using Mann-Whitney U Test to compare saline- and LHRH-injected voles.  $U = 16.5$  and  $U = 17$  ( $p < 0.05$ ) on intromission and ejaculation latencies, respectively.  $U = 18.5$  ( $p > 0.05$ ) on mount latency.

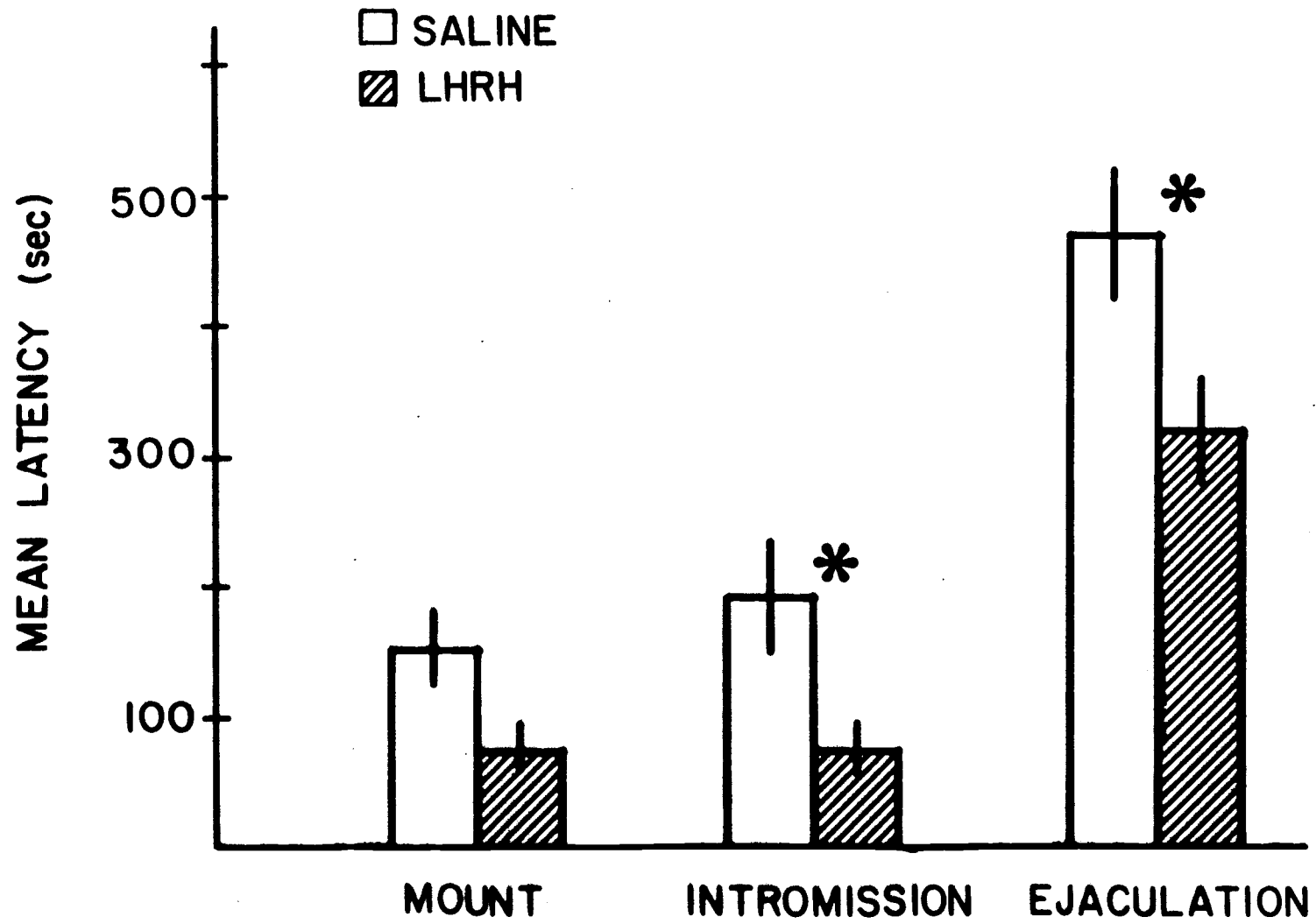


Figure 1.

Figure 2. Behavioral Frequencies (mean  $\pm$  SEM) in Intact Male Voles after LHRH or Saline Injection (9 per group). Asterisk (\*) indicates groups significantly different, using Mann-Whitney U Test to compare saline- and LHRH-injected voles.  $U = 16$  ( $p < 0.05$ ) on mount frequency.  $U = 20$  and  $U = 31$  ( $p > 0.05$ ) on intromission and ejaculation frequencies, respectively.

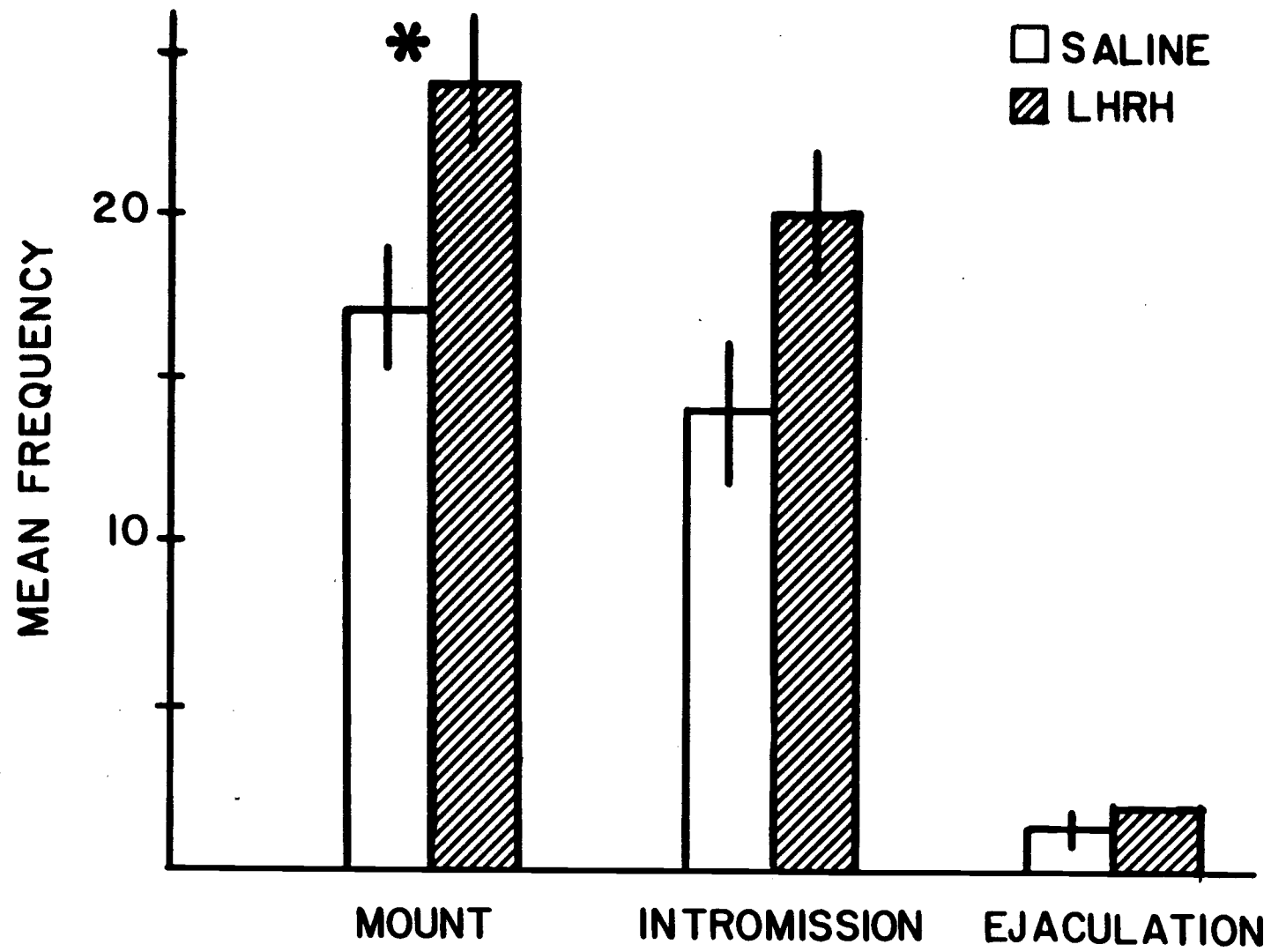


Figure 2.



Figure 3. Behavioral Latencies (mean  $\pm$  SEM) in Castrated Male Voles with Small Testosterone Implants after LHRH (n = 19) or Saline (n = 18) Injection. Asterisk (\*) indicates groups significantly different, using Mann-Whitney U Test to compare saline- and LHRH-injected voles. U = 66 ( $p < 0.02$ ) on mount and intromission latencies. U = 113 ( $p > 0.05$ ) on ejaculation latency.

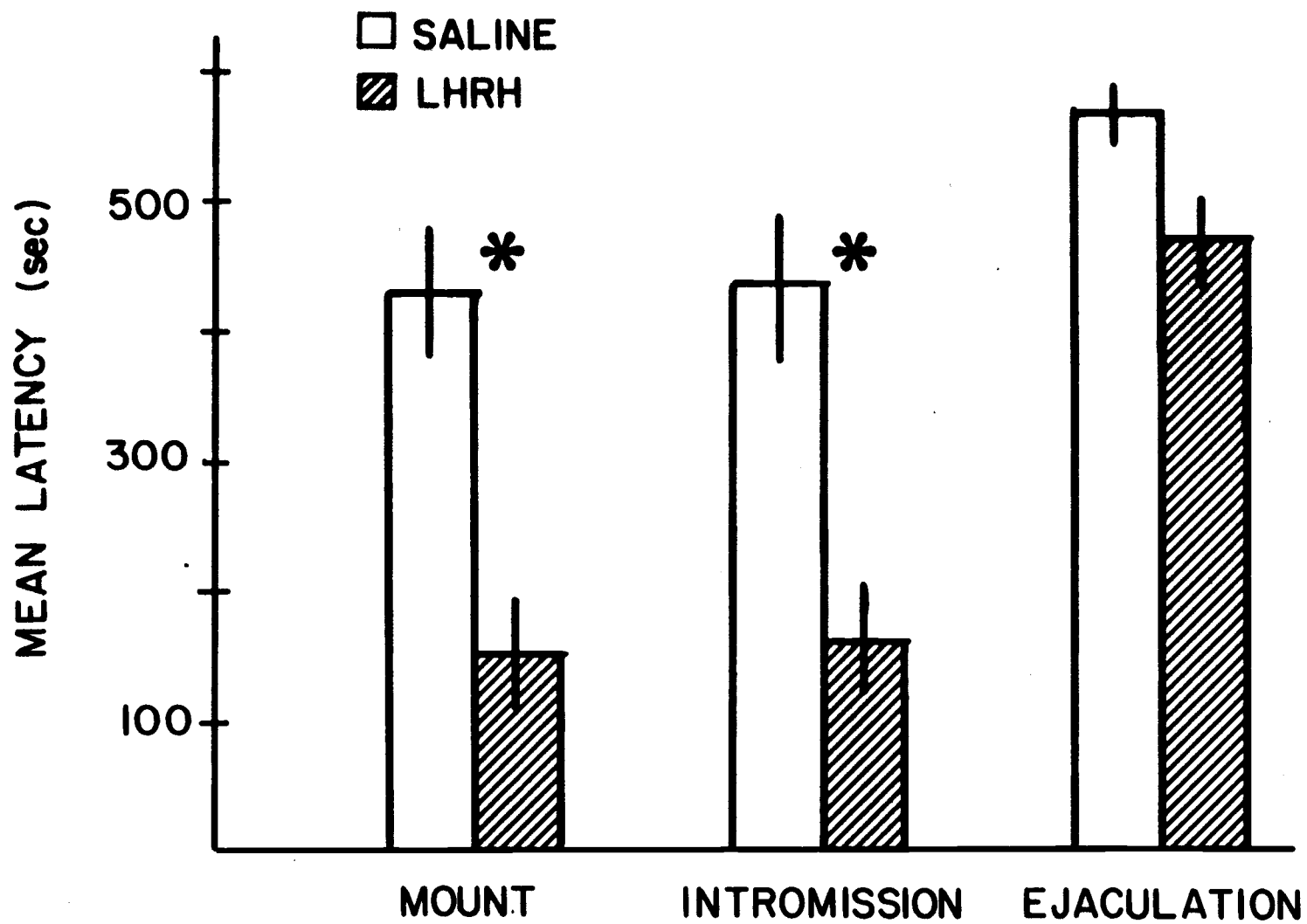


Figure 3.

Figure 4. Behavioral Frequencies (mean  $\pm$  SEM) in Castrated Male Voles with Small Testosterone Implants after LHRH (n = 19) or Saline (n = 18) Injection. Asterisk (\*) indicates groups significantly different, using Mann-Whitney U Test to compare saline- and LHRH-injected voles. U = 98 (p < 0.05), U = 84 (p < 0.02), and U = 99 (p < 0.05) for mount, intromission, and ejaculation frequencies, respectively.

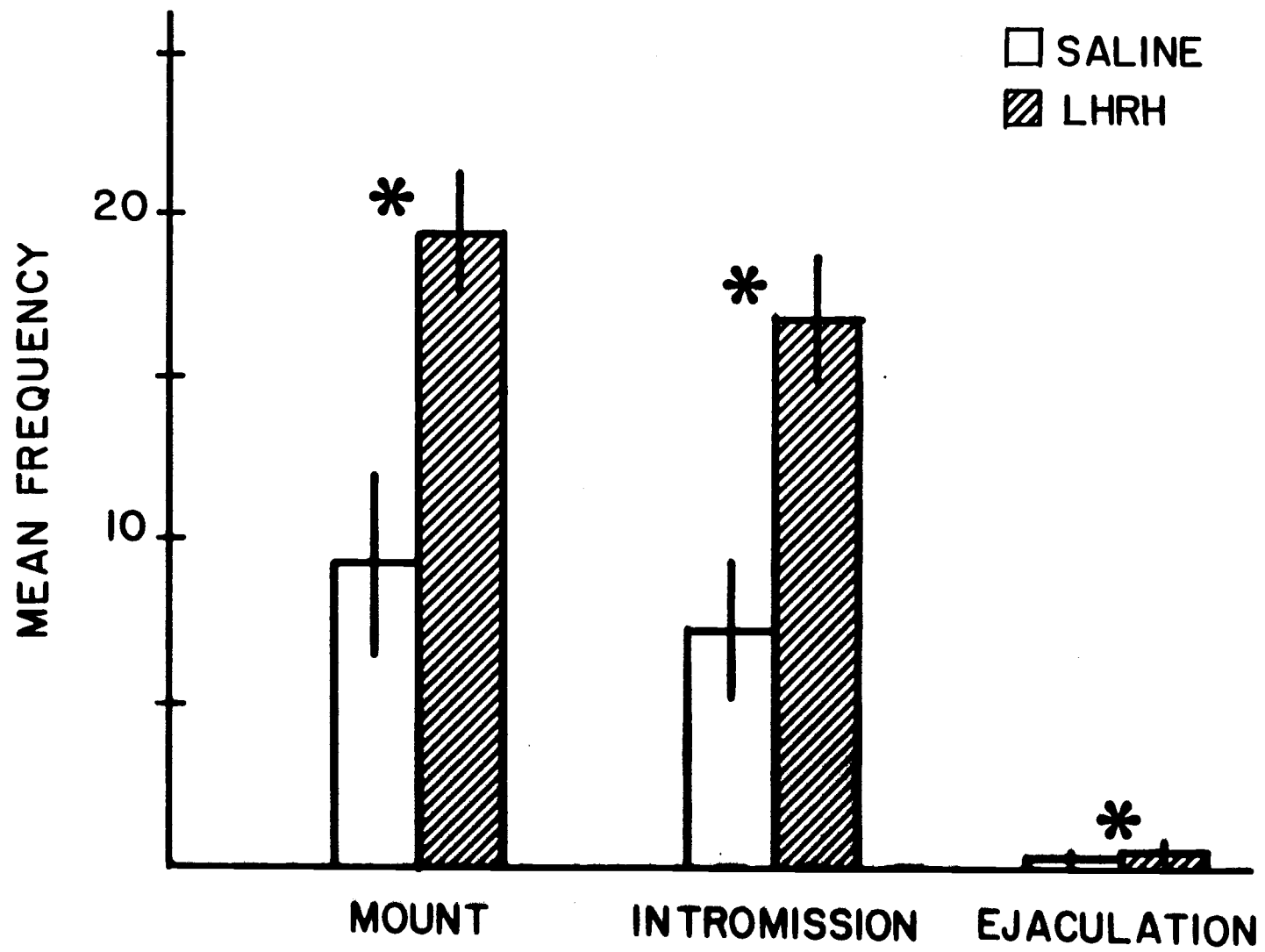


Figure 4.

Figure 5. Behavioral Latencies (mean  $\pm$  SEM) in Castrated Male Voles with Large Testosterone Implants after LHRH (n = 9) or Saline (n = 10) Injection. No significant differences, using Mann-Whitney U Test to compare saline- and LHRH-injected voles. U = 29.5 (p > 0.05) for mount and intromission latencies. U = 44.5 (p > 0.05) for ejaculation latency.

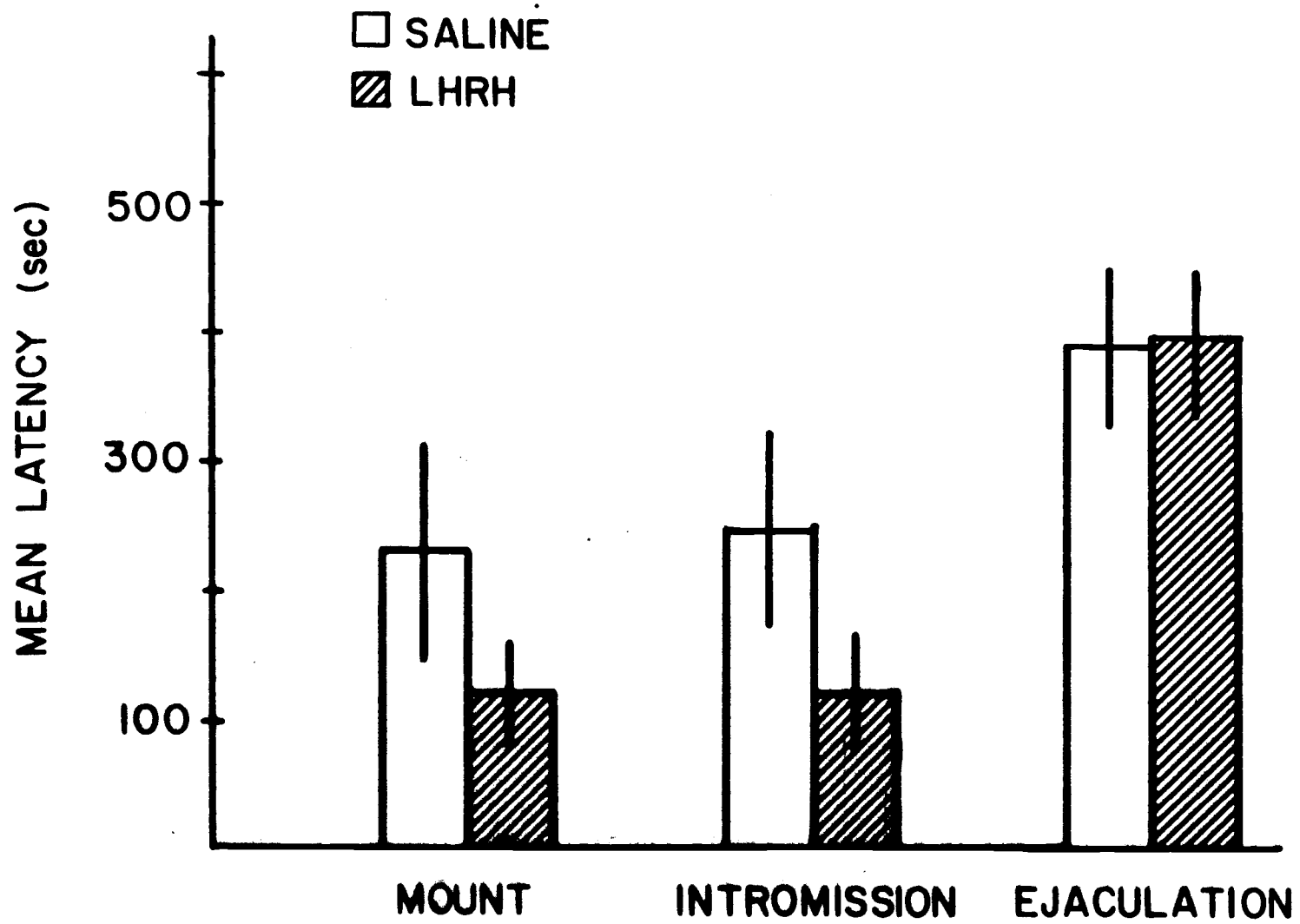


Figure 5.

Figure 6. Behavioral Frequencies (mean  $\pm$  SEM) in Castrated Male Voles with Large Testosterone Implants after LHRH (n = 9) or Saline (n = 10) Injection. No significant differences, using Mann-Whitney U-Test to compare saline- and LHRH-injected voles. U = 27.5, U = 27, and U = 40.5 ( $p > 0.05$ ) for mount, intromission, and ejaculation frequencies, respectively.

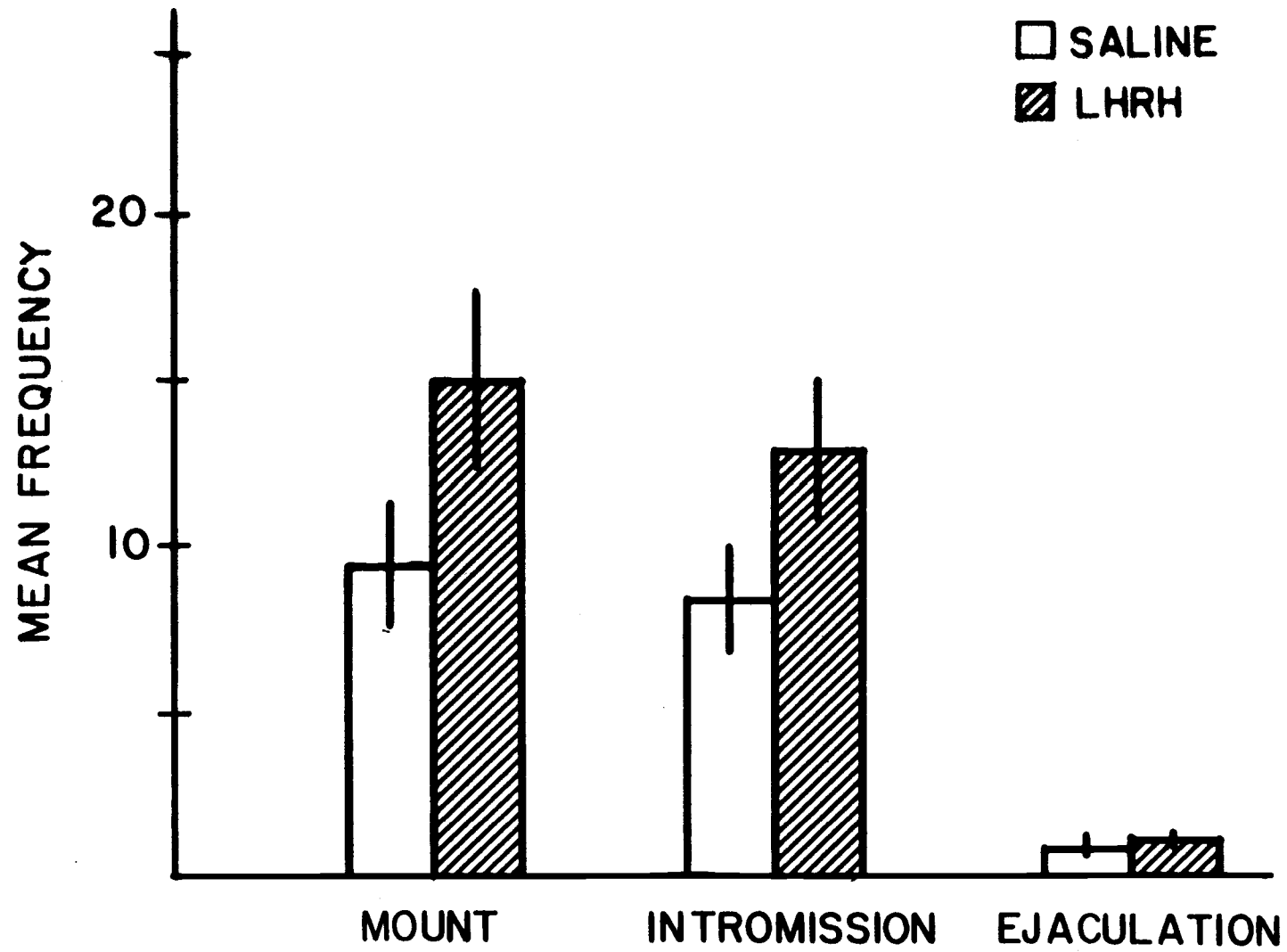


Figure 6.



TABLE 5. Effect of LHRH or Control Injection on Plasma Testosterone Levels in Castrated Male Voles or Castrated, Testosterone-Implanted Male Voles.<sup>a</sup>

Treatments	PLASMA TESTOSTERONE (ng/ml; mean $\pm$ SE)		n
	Saline	LHRH (500 ng)	
Castrates + Empty Capsules	0.17 $\pm$ 0.01	0.17 $\pm$ 0.02 <sup>b</sup>	9
Castrates + Small Testosterone Capsules			
7 days before injection	0.51 $\pm$ 0.07	0.60 $\pm$ 0.08	8
After injection	0.36 $\pm$ 0.05	0.52 $\pm$ 0.15 <sup>b</sup>	8
Castrates + Large Testosterone Capsules	2.20 $\pm$ 0.33	2.26 $\pm$ 0.27 <sup>b</sup>	9

<sup>a</sup> Two-way ANOVA;  $F_{\text{row}} = 55.9$ ,  $p < 0.01$ ;  $F_{\text{column}} = 0.38$ ,  $F_{\text{interaction}} = 0.07$ ,  $p > 0.05$ .

<sup>b</sup> Saline and LHRH treatment data were combined and castrate groups with difference capsules were compared with a T-test. Empty capsule group versus small capsule group after injection,  $t = 3.50$ ,  $p < 0.005$ . Small capsule group after injection versus large capsule group,  $t = 7.43$ ,  $p < 0.001$ . Empty capsule group versus large capsule group,  $t = 9.97$ ,  $p < 0.001$ . Castrates plus small capsule group, before versus after,  $t = 1.15$ ,  $p > 0.05$ .

TABLE 6. Summary of Sexual Behavior After LHRH or Saline Injection in Castrated Male Voles with Different Levels of Testosterone Replacement.

Treatments		No. Diff. n Males Mount.	No. Diff. Males Intro.	No. Diff. Males Ejac.	Total Mounts	Total Intromissions
Cast + Small Capsules <sup>a</sup>						
Saline	18(10/8)	7(4/3) <sup>b</sup>	7(4/3) <sup>b</sup>	3(2/1) <sup>c</sup>	172(97/75) <sup>d</sup>	134 (61/73) <sup>d</sup>
LHRH	19(10/9)	17(9/8)	17(9/8)	9(5/4)	373(195/178)	328(152/176)
Cast + Large Capsules						
Saline	10	8	8	7	96 <sup>e</sup>	85 <sup>e</sup>
LHRH	9	9	9	8	134	119
Cast + Empty Capsules						
Saline	18	0	0	0	0	0
LHRH	18	0	0	0	0	0

<sup>a</sup> Values in parentheses are results of separate replicates, i.e., (first replicate/second replicate)

<sup>b</sup> Fisher's exact test comparing saline and LHRH treatments,  $p = 0.002$ .

<sup>c</sup> Fisher's exact test comparing saline and LHRH treatments,  $p = 0.041$ .

<sup>d</sup> Chi-square test comparing saline and LHRH treatments,  $\chi^2 = 74.13$  for total mounts and  $\chi^2 = 81.46$  for total intromissions,  $p < 0.005$

<sup>e</sup> Chi-square test comparing saline and LHRH treatments,  $\chi^2 = 6.28$  for total mounts and  $\chi^2 = 5.67$  for total intromissions,  $p < 0.025$ .

TABLE 7. Behavioral Latencies Before or After Treatment with Saline or LHRH in Castrated, Testosterone-Implanted Male Voles (mean  $\pm$  SEM).

Treatments	n	ML <sup>a</sup> Before	ML After	IL <sup>a</sup> Before	IL After	EL <sup>a</sup> Before	EL After
Cast + Small Capsules							
Saline	18	217 $\pm$ 33	429 $\pm$ 54 <sup>b</sup>	226 $\pm$ 34	434 $\pm$ 53 <sup>b</sup>	472 $\pm$ 30	569 $\pm$ 21 <sup>b</sup>
LHRH	19	223 $\pm$ 38	151 $\pm$ 39	232 $\pm$ 40	159 $\pm$ 39	428 $\pm$ 33	467 $\pm$ 35
Cast + Large Capsules							
Saline	10	232 $\pm$ 59	231 $\pm$ 71	249 $\pm$ 59	241 $\pm$ 70	424 $\pm$ 52	383 $\pm$ 55
LHRH	9	226 $\pm$ 55	117 $\pm$ 39 <sup>c</sup>	255 $\pm$ 54	119 $\pm$ 39 <sup>c</sup>	504 $\pm$ 42	392 $\pm$ 44 <sup>c</sup>

<sup>a</sup> ML = mount latency, IL = intromission latency, EL = ejaculation latency (expressed in seconds).

<sup>b</sup> Comparison of before and after yields  $T = 14$  (ML),  $T = 12$  (IL), and  $T = 15$  (EL);  $p < 0.002$ .

<sup>c</sup> Comparison of before and after yields  $T = 5$  (ML),  $T = 1$  (IL),  $T = 0$  (EL);  $p < 0.05$ .

TABLE 8. Behavioral Frequencies Before and After Treatment with Saline or LHRH in Castrated, Testosterone-Implanted Male Voles (mean  $\pm$  SEM).

Treatments	n	MF <sup>a</sup> Before	MF After	IF <sup>a</sup> Before	IF After	EF <sup>a</sup> Before	EF After
Cast + Small Capsules							
Saline	18	17.1 $\pm$ 2.0	9.6 $\pm$ 3.1	14.0 $\pm$ 1.5	7.5 $\pm$ 2.5	0.8 $\pm$ 0.1	0.2 $\pm$ 0.1
LHRH	19	14.5 $\pm$ 1.5	19.6 $\pm$ 2.2	13.6 $\pm$ 1.6	17.3 $\pm$ 2.0	0.7 $\pm$ 0.1	0.5 $\pm$ 0.1
Cast + Large Capsules							
Saline	18	11.4 $\pm$ 2.9	10.6 $\pm$ 1.9	9.6 $\pm$ 2.2	9.5 $\pm$ 1.8	0.6 $\pm$ 0.2	1.8 $\pm$ 0.2
LHRH	19	11.7 $\pm$ 1.9	15.9 $\pm$ 3.0	9.3 $\pm$ 1.6	14.2 $\pm$ 2.6	0.4 $\pm$ 0.2	1.9 $\pm$ 0.1

<sup>a</sup> MF = mount frequency, IF = intromission frequency, EF = ejaculation frequency.

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## GENERAL DISCUSSION

### Chapter IV.

The present findings on normal sexual behavior for Microtus canicaudus differ from previously published data on this species. These differences are likely due to the use of a long observation period and stimulus females in natural estrus. This points out the importance of the use of more naturalistic procedures if normal behavior is to be most accurately described. The studies also show effects of prior sexual experience on reproductive behavior. Such a finding raises new questions on the mechanism of such an effect and the importance of it in the field. It also indicates that such factors need to be controlled in experimental studies of sexual behavior. Preliminary evidence presented here supports the hypothesis that M. canicaudus does not pair-bond. Further studies, especially field studies, are necessary to fully resolve this question however.

The neuropeptide LHRH had a demonstrable effect on male M. canicaudus sexual behavior. This work now needs to be extended in two important directions. The first question is clarification of the physiological importance of LHRH to the sexual behavior of the normal male. The second critical question is on the mechanism by which LHRH affects copulatory behavior.

Complete understanding of M. canicaudus sexual behavior would require integration of the present studies with future work on other environmental factors -- such as olfactory stimuli and photoperiod cues -- and their effect on the brain, and hence behavior. It would



also require understanding the role other brain peptides play in the control of sexual behavior.

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## **APPENDICES**

## APPENDIX A

## TESTOSTERONE RADIOIMMUNOASSAY PROTOCOL

## A. Preparation

1. Number 15 x 85 mm glass culture tubes for each plasma sample to be assayed.
2. Pipet appropriate aliquots of serum into each sample tube.

## B. Incubation

1. Add 1000 cpm of 1,2,6,7,16,17<sup>3</sup>H-testosterone (tracer) in 0.025 ml Ethanol (25  $\mu$ l) to each sample tube for "recovery." Include pool samples.
2. Add 0.025 ml (25  $\mu$ l) of tracer to two scintillation vials as well, for total tracer values.
3. Vortex all tubes for 10 seconds.
4. Equilibrate at room temperature for 30 minutes.

## C. Extraction and Recovery

1. Add 2 ml of benzene-hexane (1:2) (under hood) to each tube (using 1000  $\mu$ l Eppendorfs).
2. Vortex carefully for 30 seconds.
3. Cover tightly and freeze at -20°C for a minimum of one hour to allow for freezing of the aqueous phase. Be sure to place rack on floor of freezer for proper freezing.

4. Number 12 x 75 mm disposable glass culture tubes to correspond with the numbers on the 15 x 85 mm sample tubes.
5. Remove sample tubes from the freezer (2 to 4 at a time) and decant the solvent phase of the frozen extract into the corresponding 12 x 75 mm culture tubes.
6. Dry the solvent phase in the 12 x 75 mm sample culture tubes under air at 40°C.
7. Rinse the sides of the dried sample tubes with 200  $\mu$ l of benzene-hexane (1:2). Remove 50  $\mu$ l from each tube with a 50  $\mu$ l Eppendorf and place in a scintillation vial for recovery values. (Caps must be numbered accordingly).
8. Air dry the solvent in the recovery scintillation vials and total tracer scintillation vials. After drying, add four (4) ml of toluene-based scintillation fluid to each vial.
9. Air dry the remaining solvent in the 12 x 75 mm sample culture tubes at 40°C.

#### D. Standards

1. Label 12 x 75 mm disposable glass culture tubes for two sets of standards, two NSB (non-specific binding) tubes, two total count tubes, and two blanks.
2. Pipet 100  $\mu$ l of each standard (0, 6.25, 12.5, 25, 50, 100, 250, 500, 1000, 2000 pg testosterone/100  $\mu$ l Ethanol) into appropriately labeled culture tubes.
3. Dry under air at 40°C.

### E. Competitive Binding

1. Add 200  $\mu$ l of PBSG to the two NSB and two total count tubes.
2. Add 200  $\mu$ l of diluted antiserum (1:50,000 in PBSG) to two blank tubes, two sets of standards, and all sample tubes.
3. Vortex all tubes for 5 seconds.
4. Incubate at room temperature for 20 minutes.
5. Add 100  $\mu$ l of competitor  $^3\text{H}$ -testosterone (approx. 3,500 cpm/100  $\mu$ l in PBSG) to all tubes in the assay.
6. Vortex all tubes 5 seconds.
7. Cover tightly and incubate 1.5 hours at room temperature or 16-22 hours at 5°C.
8. Place rack of tubes in an ice bath for 10 minutes.
9. Using a 1000  $\mu$ l Eppendorf:
  - a. add 1.0 ml PBS buffer to both total count tubes
  - b. add 1.0 ml dextran-charcoal to all other tubes in the assay.
10. Vortex all tubes 5 seconds.
11. Incubate rack of tubes in ice bath for 10 minutes.
12. Centrifuge all tubes for 10 minutes at 4°C at 3,000 rpm (2,500 x g).

13. Decant 0.4 ml of the supernatant from each tube into a scintillation vial using a Repipet. Also add 3.6 ml of toluene: Triton X scintillation fluid to each vial using the Repipet.
14. Vortex vials well before counting.
15. Operate scintillation counter in the preset tritium mode. Count for four or ten minutes and 10,000 counts.

## TESTOSTERONE RADIOIMMUNOASSAY REAGENTS

## 1. Antiserum (11-BSA S250 Antitestosterone)

Undiluted antiserum is stored at  $-20^{\circ}\text{C}$ .

Diluted antiserum 1:50,000/200  $\mu\text{l}$  is made by adding 1 ml of stock solution (at 1:1000 Ab dilution) to 48 ml of PBS-G.

Store at  $-20^{\circ}\text{C}$ .

## 2. Competitor

Dry under air at  $40^{\circ}\text{C}$ , 2.5 ml  $^3\text{H}$ -testosterone stock ([1,2,6,7,16,17]); 0.25 mCi/250 ml EtOH). Add 23.5 ml PBS-G to yield approximately 3,500 cpm/100  $\mu\text{l}$ .

Store at  $5^{\circ}\text{C}$ .

## 3. Recovery Tracer

Dry under air at  $40^{\circ}\text{C}$ , 0.1 ml  $^3\text{H}$ -testosterone stock ([1,2,6,7,16,17]); 0.25 mCi/250 ml EtOH). Add 4 ml redistilled EtOH to yield approximately 1,000 cpm/25  $\mu\text{l}$ .

Store at  $5^{\circ}\text{C}$ .

## 4. Standards

Dissolve steroid in 100% redistilled EtOH.

Store at  $-20^{\circ}\text{C}$ .

## 5. Phosphate Buffered Saline (PBS)

Dissolve 3.45 gm  $\text{NaH}_2\text{PO}_4$ , 7.1 gm  $\text{Na}_2\text{HPO}_4$ , 71.5 gm NaCl and 8.75 gm Sodium azide in 8.75 l distilled water, adjust to pH 7.0 with NaOH.

## 6. PBS-Gelatin (0.1%)

Dissolve Knox gelatin in PBS at  $37-40^{\circ}\text{C}$  and then bring up to final volume with PBS on ice.

Store at  $5^{\circ}\text{C}$ .

#### 7. Dextran-coated Charcoal

Add 0.25 gm Dextran T-70/l and 2.50 gm Neutralized Norit/l of PBS to volume with cold PBS and mix well for approximately two hours before use if used the same day. The dextran charcoal should be kept on ice while in use.

#### 8. Scintillation Fluor

Dissolve 21.0 gm PPO (7.0 gm/l) in 2 l toluene. Mix with 1 liter Triton X-100.



**APPENDIX B**  
**CHROMATOGRAPHY PROTOCOL**

**A. Packing Columns**

6 gms Celite/10 column

1. Heat Celite to 1,000 degrees for at least 24 hours
2. Using 5 ml serological pipet (Kimble; VWR), pack water trap: Add 2 mls distilled water to 6 gms. Celite. Place glass bead in column and pack 1/2 ml as water trap.
3. Packing remainder of column:

Add 3 mls of propylene glycol: Ethylene glycol 1:1 to 6 grams of Celite and grind in mortar. Pack 1.5 mls on top of water trap already packed.

**B. Preparing columns**

Do not distill iso-octane (2, 2, 4-trimethyl pentane)

Distill ethyl acetate day before using (lasts 4 weeks)

Keep solvents out of light.

1. Run 4 mls iso-octane through columns
2. Repeat with another 4 mls.

**C. Plasma Extraction - Use numbered 15 x 85 mm glass tubes**

1. Measure plasma samples (all same size or bring all to volume of 300  $\mu$ l with distilled water if samples very small.)
2. To all samples add tracers:  
25  $\mu$ l of hot T (1,000 cpm 25  $\mu$ l) and 20  $\mu$ l of hot DHT (1,000 cpm/10  $\mu$ l).
3. Pipette duplicate samples of 20  $\mu$ l each hot hormone into two vials. (Two vials, each containing 20  $\mu$ l \*T and 20  $\mu$ l \*DHT)
4. Make water blanks of 0.5 ml distilled water. Allow samples and blanks to incubate at least 1/2 hour (or overnight).
5. Add 2 mls redistilled benzene-hexane (1:2) to each tube.
6. Vortex for 30 sec.

7. Cover tightly and freeze at  $-20^{\circ}\text{C}$  for a minimum of 1 hour to freeze aqueous phase.
8. Number 12 x 75 mm disposable culture tubes to correspond with numbers on the 15 x 85 mm sample tubes.
9. Remove sample tubes from freezer and decant solvent phase of frozen extract into corresponding 12 x 75 mm culture tubes.
10. Air dry at  $40^{\circ}\text{C}$  in these 12 x 75 mm culture tubes.
11. Rinse the sides of the dried sample tubes with 50  $\mu\text{l}$  of redistilled benzene-hexane (1:2) and dry again under air at  $40^{\circ}\text{C}$ .
12. To each extract, add 1 ml 2% ethyl acetate in iso-octane.
13. Whirl mix for 10-20 sec to dissolve extract and add to top of column using individual Pasteur pipets. Repeat steps 12 and 13 using 0.5 ml of 2% ethyl acetate.
14. For blanks at both ends of columns, add 1 ml of distilled water through same procedure.

D. Running the columns.

1. Run the 1.5 ml of plasma extract into the column (must not be more than 1.5 ml of sample extract or sample will start to come off). Run in at 1 drop/7 seconds (about 10 min.). This rate is very important.
2. Add 4.0 mls of pure iso-octane, apply gas pressure and run through. This gives progesterone fraction.
3. Add 4.5 mls of 10% ethyl acetate in iso-octane and run through. This gives DHT fraction.
4. Add 4.0 mls of 20% ethyl acetate and run through. This gives testosterone fraction.
5. Add 4 mls of 20% ethyl acetate and run through. This gives corticosterone fraction.
6. Take tubes with extracts and evaporate to dryness. Can stop at this point and seal tubes in refrigerator for a few days. (Can leave at room temperature overnight).
7. Add 2 mls ethyl acetate in iso-octane to dried extracts and whirl mix well. For T use 20% ethyl acetate; DHT, 10%; corticosterone, 50%.

8. Assay each plasma sample in duplicate. Add 400  $\mu$ l of extract to two tubes. 200  $\mu$ l remains for recovery.
9. For recovery: Add 5 mls scintillation fluid to the 200  $\mu$ l extract (in scintillation vial) and count. (No. of counts x 5 is total counts recovered. Counts recovered divided by counts added x 100% is recovery - should be 60-80%.)
10. Evaporate 400  $\mu$ l samples to dryness and proceed with assay.

**APPENDIX C**  
**MISCELLANEOUS DATA**

TABLE 9. Plasma Testosterone Levels in Male Voles at Time Zero (Experienced and Inexperienced) and at 24, 48, and 72 Hours and 1 Week and 3 Weeks Following Bilateral Castration.

Treatment	n	Mean Plasma Testosterone <sup>a</sup> (ng/ml) ( $\pm$ SEM)
Time Zero (Intact		
Inexperienced	10	2.67 $\pm$ 0.48
Experienced	10	1.94 $\pm$ 0.40 <sup>b</sup>
24 Hour Castrates	9	0.42 $\pm$ 0.07 <sup>b</sup>
48 Hour Castrates	8	0.13 $\pm$ 0.01 <sup>b,c</sup>
72 Hour Castrates	12	0.17 $\pm$ 0.01 <sup>c</sup>
1 Week Castrates	10	0.19 $\pm$ 0.01
3 Week Castrates	18	0.17 $\pm$ 0.01

<sup>a</sup> One-Way ANOVA ,  $F = 17.93$ ;  $p < 0.01$ .

<sup>b</sup> T-test,  $p < 0.005$ .

<sup>c</sup> T-test,  $p < 0.025$ .

TABLE 10. Sexual Behavior of Female M. canicaudus when Exposed to Familiar (n=9) or Unfamiliar (n=9) Males.<sup>a</sup>

Behavioral Parameter	Females + Familiar Males ( $\bar{x} \pm \text{SEM}$ )	Females + Unfamiliar Males ( $\bar{x} \pm \text{SEM}$ )
Lordosis Latency (sec)	226 $\pm$ 71	439 $\pm$ 245
Lordosis Frequency	31 $\pm$ 4	30 $\pm$ 11
Number of Copulatory Series	3.6 $\pm$ 0.2	3.3 $\pm$ 0.3
Total Time of Sexual Interaction (sec)	2807 $\pm$ 439	1939 $\pm$ 479

<sup>a</sup> No significant differences when compared with Mann-Whitney U Test.