

THE ROLE OF REPEATED COPULATIONS IN REDUCING  
THE EFFECTS OF COMPETITIVE INSEMINATIONS  
AMONG MALE LABORATORY RATS

By

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While male laboratory rats (Rattus norvegicus) typically display several ejaculatory series when mating with a female rat, the possible adaptive significance or benefits of such a behavioral pattern have heretofore been unclear. The present research investigated the possibility that such multiple ejaculatory series serve to prevent sperm displacement or sperm dilution by other males. Interpretations of previous research on sperm displacement in rodents were rendered tenuous by inadequate observation of behavioral phenomena or by fertility differences between strains. In Experiment I, six combinations of four strains were examined in tests of successive matings of two different strains. Two strains of nearly equal fertility were chosen for subsequent experimentation. Moreover, frequent sperm displacement was observed for these males when only one

ejaculatory series was allowed for each male. In Experiment II, the first male sired most of the offspring (79 per cent for one strain and 60 per cent for the other), when the first male was allowed to mate for five ejaculatory series prior to the copulation of a male of the other strain for five ejaculatory series with the same female. However, the first male sired few offspring (12 per cent for one strain and 17 per cent for the other) when the first male was only allowed one ejaculatory series. Experiment III examined whether successive inseminations, vaginal stimulation, or time alone was responsible for this effect. Neither time alone nor vaginal stimulation without additional sperm transfer offered any protection from sperm displacement. The proposed mechanism for the present effect is sperm dilution. These data suggest that the behavioral pattern of multiple ejaculatory series has evolved through the process of natural selection since males mating for several ejaculatory series can have a selective advantage over males mating for one series in species such as rats which have polygamous mating systems.

## INTRODUCTION

Despite the well known stereotypy of the complex, species-characteristic copulatory patterns of male animals, very few studies have investigated the adaptive significance of these patterns. For example, each male mammal of a species displays only one of a possible sixteen different patterns based on a classification system devised by Dewsbury (1972). In this classification system, each species can be readily categorized on the basis of the following four criteria: (1) Is there a lock (i.e., mechanical tie between the penis and vagina)? (2) Does intravaginal thrusting occur? (3) Are multiple intromissions prerequisite for ejaculation? and (4) Do multiple ejaculations occur? While a large number of mammalian species have been classified using this system (Dewsbury, 1972, 1975), the adaptive functions of the specific pattern of copulation displayed by a particular species have rarely been examined. The present thesis will consider the possible adaptive significance of the diverse and often complicated patterns of copulation present among muroid rodents and test a hypothesis regarding one



additional adaptive function of copulatory behavior in male rats.

Males of most muroid rodent species display copulatory behavior after the first ejaculation (Dewsbury, 1975). There are few data indicating that such behavior is necessary for successful reproduction for females of the same species. The theoretical proposals and experimental data which could account for such disparity seem to be inadequate.

The new hypothesis to be tested is that males which have several ejaculations with a particular female decrease the probability that another male will sire offspring by the same female.

#### Behavioral Stimuli Influencing Successful Reproduction in Muroid Rodents

Specific quantitative aspects of the male mating pattern in some muroid rodents have been shown to be important in (1) initiating pregnancy responses; (2) increasing the number of ova released by mature follicles; and (3) reducing the receptivity of females.

In many rodent species initiation of successful pregnancy may require more than the passing of sperm from male to female. For example, the critical copulatory stimuli which initiate the physiological states necessary for

pregnancy vary among these species. While an ejaculatory reflex by a male is sufficient for the initiation of pregnancy in female housemice (Land and McGill, 1967), female laboratory rats require the multiple intromissions (discrete vaginal insertions) which are normally shown by male rats prior to an ejaculation (Adler, 1969; Chester and Zucker, 1970; Wilson, Adler and LeBoeuf, 1965). Copulatory stimulation which occurs after the first ejaculation increases the probability of pregnancy in hamsters (Lanier, Estep and Dewsbury, 1975), cactus mice (Dewsbury and Estep, 1975), montane voles (Davis, Gray, Zerylnick and Dewsbury, 1974), and northern grasshopper mice (Lanier and Dewsbury, 1977).

In female rats, which ovulate spontaneously, at least two factors influence the number of ova released. Rogers (1971) has noted that the number of ova shed can be increased if females are mated at particular times in the day-night cycle. Second, Davis (1974) has demonstrated that more ova are released after several ejaculatory series than after only a single ejaculatory series is allowed when a male mates with an aged, multiparous female.

Copulation and associated stimuli have been shown to be effective in reducing the receptivity of females of many rodent species. Hamsters show a decline in receptivity after

vaginal stimulation (Carter, 1972, 1973). A single male hamster often appears to reduce the receptivity of the female to a level such that a second male attains very few, if any, complete ejaculatory series (Estep, Lanier and Dewsbury, Note 1; Oglesby, Lanier and Dewsbury, Note 2). Similarly, female laboratory rats show a reduction in receptivity that is inversely related to the number of previous intromissions (Hardy and DeBold, 1972).

#### Sexual Selection and Competitive Inseminations

The term "sexual selection" was first used by Darwin (1871) to refer to those advantages which individuals have over other members of the same species with regard to reproduction. While "sexual selection" is now regarded as a type of natural selection rather than a separate process (Mayr, 1972), Darwin may be properly credited with originating this term and with making the observation that males compete for females in many species. Trivers (1972) has suggested that this type of competition is a logical outcome for species in which the burdens of reproduction and parental care rest primarily on the female; ". . . where one sex invests considerably more than the other, members of the latter will

compete among themselves to mate with members of the former" (p. 173). All of the adaptive functions of rodent copulatory behavior discussed above probably fall into the general category of sexual selection since these adaptations increase the probability or efficiency of pregnancy responses evoked by particular males. However, competition among males is particularly clear in those situations where two males have copulated with one female and each have left sperm in her reproductive tract.

In an article reviewing the literature on similar competition among insects, Parker (1970) defined "sperm competition" as ". . . the competition within a single female between the sperm from two or more males for the fertilization of ova" (p. 527). Two opposing evolutionary forces were noted. First, selection would favor those males which could displace the sperm of a male which had mated previously with a female. On the other hand, selection would also favor mechanisms whereby the first male could prevent displacement of his sperm.

The present experiments on competitive inseminations in rodents will explore an adaptive function of repeated ejaculations which is probably common to a number of taxa. While Parker's (1970) examples of sperm mixtures and sperm

displacement in insects illustrate that similar processes are present in these animals, the term "sperm competition" has not been presently adopted since this term rhetorically implies that the individual sperm vie equally in a race to the ova. What is competitive about most of these inseminations and behaviors by these males is that one male may displace or "dilute" the semen of another male.

Males of avian species probably are subject to some of the same kinds of internal reproductive competition. In one such study of sperm mixtures among birds, Martin, Reimer, Lodge and Dzuik (1974) found that the ratio of sperm of each of two strains of domestic cocks was directly related to the probability that each would sire the resulting broods. In short, the pursuit of the general problem of competitive inseminations may shed new light on the evolutionary significance of repeated matings.

#### Evidence of Competitive Inseminations in Rodents

The present hypothesis is that the repeated ejaculatory series of male rodents may have the adaptive function of reducing a second male's ability to compete successfully in siring offspring. There are indications that more than

one male frequently mates with the same female in the natural habitat. Competitive inseminations are of obvious biological significance for at least some rodents since more than one male frequently mates with a single female as is documented by the protein analysis of blood samples of litters born to pregnant, field-caught deermice, Peromyscus maniculatus (Birdsall and Nash, 1973), and observations of multiple matings in wild and semi-natural populations of rats, Rattus norvegicus (Barnett, 1958; Calhoun, 1962).

Multiple inseminations frequently occur in laboratory situations in which two males are housed with a single female housemouse (Levine, 1958; Levine and Lasher, 1964; Levine and Krupa, 1966) or rat (King, 1929). These experiments have used coat color as a convenient genetic marker of which male has sired the resulting offspring. For example, when a homozygous albino female is mated with both a homozygous albino male and a homozygous pigmented male, the coat color of the offspring will always reflect the strain of the father since albinism is recessive. Unfortunately, actual copulation in these experiments was not usually observed.

In two laboratory experiments, one ejaculation by male rodents of two different strains with the same female was

observed. In housemice, Levine (1967) noted that albino males mating with albino females sired more of the resulting offspring than pigmented males, particularly when the albino male mated first (95 per cent versus 70 per cent). However, the amount of time between ejaculations by successive males did not appear to be a critical factor (range = 16-181 minutes). By contrast, Adler and Zoloth (1970) found that a pigmented male rat which mated with an albino female within 15 minutes after the single ejaculation of an albino male fathered 66 per cent of the offspring. However, if mating of the pigmented male was delayed for 30 minutes or longer, less than 20 per cent of the offspring were pigmented. When albino males mated second, they sired most of the offspring regardless of the amount of time between males.

Two additional experiments suggest that prolonged copulation by male rodents decreases the probability that another male can compete successfully for paternity of the offspring. When F344 albino males mated first for only one ejaculatory series with an albino female, an ACI pigmented male that mated second usually sired the offspring. If five ejaculatory series were allowed for the albino male, pigmented males were rarely the fathers of the offspring. However, regardless of the number of ejaculatory

series allowed for pigmented males mating first, they were nearly always displaced by albino males (Estep, Lanier and Dewsbury, Note 1). Somewhat similar data have been collected in hamsters (Oglesby, Lanier and Dewsbury, Note 2). Once again there was a fertility difference between strains. Whereas cream-colored male hamsters of low fertility could effectively displace the sperm of agouti males allowed only one ejaculation, displacement rarely occurred if the agouti males were allowed to mate to satiety before the second male was introduced.

As with previous studies the interpretations of the latter two experiments are tenuous due to fertility differences among strains. These data demonstrate that a male of low fertility mating second with a female has a competitive advantage over a male of high fertility which has mated for one ejaculatory series but does not have an advantage over a male of high fertility which has mated for several ejaculatory series. However, since the reciprocal relationship did not hold, these studies do not necessarily indicate a normal mechanism by which competitive inseminations occur. For example, the last male rat to mate could conceivably sire most of the offspring when males of nearly equal fertility mate sequentially. Nevertheless, since a



male of low fertility can displace the sperm of a more fertile male under some circumstances, one can safely assume that another male of high fertility would be effective at sperm displacement under similar circumstances.

The Adaptive Significance of Male  
Rat Copulatory Behavior

Despite the numerous studies of copulatory behavior of laboratory rats (Rattus norvegicus), the adaptive significance of this copulatory pattern is unclear. When a male laboratory rat is placed with a receptive female, the male typically displays several mounts with shallow pelvic thrusting towards the female's vaginal orifice. On some of these mounts, the male briefly inserts his penis into her vagina. Approximately ten of these mounts with intromission occur prior to an insertion on which he ejaculates. This event concludes the first ejaculatory "series." Sperm and a seminal coagulate collectively called a "plug," are deposited in the female's vagina (Beach and Jordan, 1956). A male rat typically displays seven ejaculatory series prior to obtaining a satiety criterion of 30 minutes without mounting.

The multiple intromissions of male rats have been shown to aid in successful reproduction in several ways.

Adler (1973) reviewed the available literature and concluded that multiple intromissions were required to: (1) to facilitate sperm transport in the female's reproductive tract (Adler, 1969; Chester and Zucker, 1960); (2) to initiate a "neuroendocrine reflex" that results in the necessary hormonal states for a luteal phase of ovarian activity; and (3) to initiate a mammary gland development 3-5 days after mating (Dilley and Adler, 1968).

The adaptive or beneficial functions of multiple ejaculations for female rats are particularly unclear. Whereas Zucker and Wade (1968) suggested that multiple ejaculations may have evolved as a "safety factor," this view seems to be untenable (see Dewsbury, 1971).

Davis (1974) demonstrated that multiple ejaculatory series greatly increase the probability of pregnancy for aged (approximately 11-16 months old) multiparous female rats. In addition, the number of ova released in these rats was directly related to the number of ejaculatory series they received. While these data suggest that multiple ejaculatory series may have an important effect on reproduction for a rather limited period in a female's reproductive life, one may question how frequently a female rat actually perseveres to such an age in more natural habitats.

Nevertheless, these data suggest that under some kinds of "biological stress" multiple ejaculatory series may be important for success in reproduction. In more recent work, Davis and Conner (1977) found that multiple ejaculatory series were more effective than a single series for females mating in post partum estrus. The "stresses" of recently bearing a litter and lactating during pregnancy may be responsible for this apparent reduction of sensitivity of reproductive responsiveness. Similarly, Ball (1934) found that female rats subjected to surgical "stress" to their reproductive tracts were more likely to bear litters if more than one copulatory plug was present in their vaginae.

#### The Hypothesis To Be Tested

The present experiments were designed to test the hypothesis that multiple ejaculatory series can aid in the successful reproduction of a male mating with a healthy, young female rat. In particular, an individual male's probability of siring offspring may be increased by multiple ejaculatory series in those instances where another male mates with the same female.

Natural selection of a pattern of multiple ejaculations would be expected if males showing this pattern had some

selective advantage over males displaying only a single ejaculatory series. Although a female might have maximally successful reproduction after mating with a male for only a single series, the results on an individual male's chances of reproduction could be disastrously affected if another male subsequently mated with the same female and displaced or diluted the first male's sperm. In this regard, the present study differs from most previous work: the relative reproductive success of males rather than females will be examined.

## EXPERIMENT I

The primary purpose of this experiment was to find strains of nearly equal fertility for subsequent experimentation. This selection of strains seemed particularly important since fertility differences among populations or strains have rendered interpretations of most previous experiments on sperm displacement in rodents somewhat tenuous.

Replication of the finding that one male rat can displace the sperm of another male rat under laboratory conditions would be expected. This effect, previously observed by Lanier, Estep, and Dewsbury (Note 3) differs from that of Adler and Zoloth (1970) in that the displacement of the first male's sperm occurs even when an interval of 10-15 minutes or more has occurred between matings.

Six combinations of two males of different strains mating with the same albino female were used (see Table 1). Males of each of three pigmented strains were allowed to mate for one ejaculatory series before an albino male was allowed to mate with the same female for one ejaculatory

Table 1. Test Combinations for Experiment I of Two Males Each Mating for One Ejaculatory Series with the Same Female.

First Male	Second Male	Female
Long-Evans <sup>1</sup>	F344	F344 Albino
F344 <sup>2</sup>	Long-Evans	F344 Albino
Mai <sup>3</sup>	F344	F344 Albino
F344	MAX	F344 Albino
BR <sup>4</sup>	F344	F344 Albino
F344	BR	F344 Albino

1. Long-Evans: hooded, outbred.

2. F344: Fischer albinos, inbred.

3. Mai: Microbiological Associates' hooded, outbred.

4. BR: nonagouti brown, inbred.

series on 30 tests. On an additional 30 tests the strain of male mating first was reversed.

### Methods

#### Subjects

The four strains of males and their origins are listed in Table 1. When males in each of these strains were mated with a homozygous albino female, the resulting offspring would bear the coat color of the sire since albinism is recessive. The suppliers of these strains indicated that it was unlikely that any of the pigmented males carried a recessive gene for albinism. As described below, the coat color of the offspring always resembled the coat color of the sire on these pretests.

Males were 4-6 months old and females were approximately 3 months old at the beginning of the experiment. All subjects were housed individually in Wahmann stainless steel cages in an air-conditioned, windowless room with a 14:10 light-dark cycle with light onset at 1200 hr. Purina lab chow and water were continuously available.

## Procedure

Females were given daily tests for receptivity (Adler, 1968). By applying firm flank stimulation to females and assessing the degree of lordotic responsiveness receptivity of females to males could usually be predicted. Tests were discontinued if the test female was unreceptive.

In pretests, batteries of virile males and fertile females were established. In each of four strains, males were selected on the basis of three criteria:

1. Each of these males was required to initiate copulation within 10 minutes after the introduction of a receptive female on one of four tests. While this procedure may have eliminated a few sluggish but "normal" copulators, the males that were selected had reasonably short intromission latencies. Predictable intromission latencies were important for this and subsequent experiments since the second male of a test was required to mate with the test female within a short period of time after the first male was removed from the female.

2. After the initiation of copulation, each male was required to mate for at least three complete ejaculatory series. Most males displayed five or six ejaculatory



series. Requiring males to mate for at least three ejaculatory series helps to eliminate ambiguity in interpretation caused by including males which mated to satiety but displayed only one or two ejaculatory series.

3. Males were also excluded if after two tests with copulation with a receptive female, pregnancy did not result for at least one female. The resultant litters verified that albinism was recessive to all of the pigmented coat colors and that none of the pigmented sires carried the recessive gene for albinism.

Tests were conducted in circular plexi-glass arenas of 18 x 2.54 cm diameter with a floor cover of San-i-cel, a commercial corncob bedding. Tests were initiated 4-6 hours after light offset by the introduction into an arena, approximately 10 minutes after the introduction of the test female to the arena.

The behavioral events (mounts, intromissions and ejaculations, see Beach and Jordan, 1956) were recorded by the experimenter on an Esterline-Angus event recorder. Males were allowed to copulate freely with the female until the occurrence of the first ejaculation. If the male failed to copulate with the female within 5 minutes after the female

was introduced, or if the female failed to show a lordotic posture in response to vigorous mounting by the male, the test was disregarded.

After a male achieved one ejaculation with a female, that male was removed. Ten minutes later, a male of a different strain was introduced and allowed to copulate until the occurrence of the first ejaculation. If copulation failed to occur within five minutes after the introduction of the second male, the test was terminated and disregarded.

In short, every female received one ejaculatory series from a male of one strain and 10-15 minutes later one ejaculatory series with a male of another strain was initiated.

### Results and Discussion

As can be seen in Table 2, fertility varies among these strains. Nearly all albino pups resulted regardless of strain order after tests of successive matings of Mahooded males and F344 albino males. However, more pigmented than albino pups were born when Long-Evans males mated after these albino males. In successive matings of non-agouti brown males and F344 albino males, the proportion of albino pups was intermediate between those obtained using the other two pigmented strains.

Table 2. The Total Number of Pups of Each Coat Color and the Per Cent Albino Pups Which Resulted from Two Successive Matings of Two Males from Different Strains.

	Strain of Male					
	Long-Evans and F344		Brown and F344		Mai and F344	
	Order		Order		Order	
	Long-Evans First	F344 First	Brown First	F344 First	Mai First	F344 First
Number of Albino Pups	55	35	53	56	72	34
Number of Pig- mented Pups	<u>29</u>	<u>37</u>	<u>16</u>	<u>23</u>	<u>0</u>	<u>5</u>
Total	84	72	69	79	72	39
Number of Litters	9	9	9	9	8	6
Per Cent Albino	65.5	48.6	76.8	70.8	100.0	87.2

Application of chi-square tests for each combination of strains revealed no significant difference in fertility between the Long-Evans and F344 strains ( $\chi^2 = 3.19$ ,  $df = 1$ ,  $p > .05$ ). However, there were significant differences between the Brown and F344 strains ( $\chi^2 = 33.53$ ,  $df = 1$ ,  $p < .001$ ) and between the Mai and F344 strains ( $\chi^2 = 91.67$ ,  $df = 1$ ,  $p < .001$ ).

A descriptive analysis of the litter composition of these tests shows a similar trend (See Table 3). Successive matings of Mai-hooded males and albino males resulted in thirteen all albino litters and one mixed litter. After successive matings of Long-Evans and F344 males, about an equal number of albino, mixed and all pigmented litters resulted.

Individual data for this experiment are presented in Appendix I (Table A-1).

On the basis of this experiment, the Long-Evans strain was chosen for subsequent experiments since males of this strain appeared to be closer in fertility to the F344 albino males than any other pigmented strain.

The results also indicate that sperm displacement frequently occurs when a second male is allowed only one

Table 3. Experiment I, the Frequency of Each of Three Types of Litters after Successive Matings of Males of Two Different Strains.

Type of Litter	Strains					
	Long-Evans and F344		Brown and F344		Mai-Hooded and F344	
	Order		Order		Order	
	Long-Evans First	F344 First	Brown First	F344 First	Mai First	F344 First
All Albino	3	3	6	3	8	5
Mixed	4	4	1	5	0	1
Pigmented	<u>2</u>	<u>2</u>	<u>2</u>	<u>1</u>	<u>0</u>	<u>0</u>
Total	9	9	9	9	8	6

ejaculatory series, 10-15 minutes after the end of a single ejaculatory series by the first male.

## EXPERIMENT II

This experiment critically tested the hypothesis that some behavioral or physiological aspect of the several ejaculatory series typically displayed by a male rat is more effective than a single ejaculatory series in preventing sperm displacement. The design of this experiment is represented in Table 4. The first male was allowed to complete one or five ejaculatory series. The second male was always allowed to complete five ejaculatory series. On half of the tests in each condition, an albino male mated first, while on the other half of the tests, a pigmented male mated first.

### Methods

#### Subjects

The 11 Long-Evans males, 12 F344 males and 54 F344 females used in this experiment had served in Experiment I. Housing conditions and light cycle were as in that experiment. Each of the females was used once and only once in this experiment. An additional 16 females either failed

Table 4. Strain and Number of Ejaculatory Series Allowed for Each Male in Experiment II.

Test Condition	First Male		Second Male	
	Strain	Number of Series	Strain	Number of Series
1	Pigmented	1	Albino	5
2	Albino	1	Pigmented	5
3	Pigmented	5	Albino	5
4	Albino	5	Pigmented	5



to respond to flank stimulation or vigorous mounting by a male.

The combination of males, one of each of two strains was essentially random. No male was used more than twice in any test condition. Males were allowed two weeks for recovery between tests.

### Procedure

Tests were conducted in the same arenas as in Experiment I. In each test the first male was allowed to mate until either the completion of the first or the fifth ejaculatory series, depending on the test condition. Immediately after the completion of the appropriate ejaculatory series, the test male was removed and replaced by a male of the other strain. Females were left in the test chamber undisturbed until the end of the test. If the second male failed to achieve intromission within 5 minutes after being placed with the test female, that male was removed and replaced by another male of the same strain. Tests were terminated whenever a lag of more than 10 minutes occurred between the last ejaculation of the first male and the first intromission of a male of the other strain.

The last male on these tests was allowed to mate until five ejaculatory series were completed or until a satiety criterion of 30 minutes without an intromission was reached. However, only those tests in which satiety was reached after three or more complete ejaculatory series were included in some of the statistical analyses below.

### Results and Discussion

Litter composition, number of litters and mean percentage of pups sired by the first male are shown in Table 5. A Kruskal-Wallis nonparametric analysis of variance (Siegel, 1956) on the mean percentage of pups in each litter sired by the first male demonstrated there were significant differences between at least two of the groups ( $H_c = 23.68$ ,  $df = 3$ ,  $p < .001$ ). Mann-Whitney U-tests (Siegel, 1956) further revealed that Long-Evans males sired significantly more pups ( $U = 11.0$ ,  $n_1 = 12$ ,  $n_2 = 11$ ,  $p < .001$ ) if five ejaculatory series rather than one ejaculatory series were allowed prior to the introduction of the F344 male. Similarly, F344 males sired significantly more pups ( $U = 11.5$ ,  $n_1 = 10$ ,  $n_2 = 10$ ,  $p < .01$ ) if five ejaculatory series rather than one ejaculatory series were allowed prior to the introduction of the Long-Evans male. Thus, regardless

Table 5. Litter Composition, Number of Litters, and Mean Percentage of Pups Sired by the First Male in Experiment II.

Measure	F344		Long Evans	
	1E	5E	1E	5E
Mean Percentage of Pups Sired by 1st Male	12	79 (74)	17	60 (56)
Number Sired by 1st Male	7/53	74/98 (56/82)	12/83	39/66 (35/66)
Number of Litters	11	12 (10)	10	10 (9)

Numbers in parenthesis refer to results when the data from three tests on which neither of two males of the other strain mated after the first male.

of the strain of male mating first, the first male had a high probability (79 or 60%) of siring offspring if 5 ejaculatory series were allowed but a low probability of siring offspring (12 or 17%) if only one ejaculatory series was allowed.

Individual data for this experiment are presented in Appendix II (Table A-2).

While these data give clear evidence that multiple ejaculatory series can greatly reduce the probability of sperm displacement or dilution, the mechanism is not clear. In order to more critically test the hypothesis that the behavior or physiological changes occurring in the second through fifth series of the second male were approximately balanced across groups, three tests, in which the first male mated for 5 ejaculatory series but in which both a second and third male of the other strain failed to mate with the test female, were excluded and the data were reanalyzed. The apparent reduction in female receptivity which appears to accompany prolonged copulation may be an interesting phenomenon. However, these three cases muddle the question "do multiple ejaculatory series reduce the probability of sperm competition with another male when a second male has also mated and deposited viable sperm in the female's reproductive tract."

The results were essentially the same when the three tests are excluded where only one male mated with the female. Application of a Kruskal-Wallis analysis of variance revealed that there were significant differences between at least two of the groups ( $H_C = 20.71$ ,  $df = 3$ ,  $p < .001$ ). Mann-Whitney U-tests demonstrated that one ejaculatory series was not as effective as five ejaculatory series in maximizing the probability that Long-Evans males mating first would sire the offspring ( $U = 11$ ,  $n_1 = 10$ ,  $n_2 = 11$ ,  $p < .01$ ) or that F344 males mating first would sire the offspring ( $U = 13.5$ ,  $n_1 = 9$ ,  $n_2 = 10$ ,  $p < .01$ ).

The analysis with the three tests excluded demonstrates that multiple ejaculatory series reduce the probability that the second male's sperm will successfully fertilize the female even when these sperm have been deposited in the female's reproductive tract. While the first male's copulatory stimulation may occasionally be sufficient to terminate receptivity, consideration of these few instances is not necessary to account for the phenomenon that multiple ejaculatory series reduce the probability of sperm competition.

### EXPERIMENT III

While Experiment II clearly demonstrated that "something" which occurs during the time interval from the end of the first to the end of the fifth ejaculatory series of a male rat's mating sequence reduces the probability that subsequent males will sire offspring with the same female, the critical elements of the first male's mating behavior that mediate this effect are unclear.

In order to test whether successive inseminations, vaginal stimulation, time alone or other factors were primarily responsible for the effect found in Experiment II, Experiment III was initiated.

#### Methods

##### Subjects

All of the 14 F344 males, 7 vasectomized F344 males and 10 normal Long-Evans males had served in Experiments I and II and were vigorous copulators. The 29 F344 females were virgins approximately 4 months of age. Housing and

general testing conditions were as in Experiments I and II.

Two ventral incisions were made on each male to be vasectomized while under Penthrane anesthetic. The vas deferens was exposed, tied off with surgical thread in two places near the epididymis and a cut between the ties was made with surgical scissors.

#### Procedure

Each test of this experiment followed the same general format. A single albino female was allowed to mate with an albino male until the completion of one ejaculatory series. Next an intermediate treatment was given to each female. Finally a hooded Long-Evans male was allowed to mate for five ejaculatory series with this same female.

The three intermediate treatment conditions were as follows:

1. A normal albino male was allowed to mate with the test female for four complete ejaculatory series. This male was introduced one minute after the completion of the first ejaculation of the first male, at which time the first male was removed. If the second male failed to initiate copulation within 10 minutes, the test was terminated.

2. This treatment condition was exactly as above except that the albino male used for the intermediate condition was vasectomized. Thus, these males were capable of relatively normal copulatory behavior, but deposited copulatory plugs which did not contain sperm. Each of these vasectomized males had been pretested at least two weeks prior to the test in order to ascertain that these males had relatively normal copulatory behavior, and deposited copulatory plugs but did not deposit sperm.

3. In this intermediate treatment condition, a restrained albino male was introduced one minute after the first male's ejaculation. These males were restrained in wire cages and remained in the test arena for a period of 45 minutes prior to the introduction of the Long-Evans male. This group was to control for the possibility that time alone, auditory stimulation or olfactory cues from males of the albino strain might influence the Long-Evans male's probability of siring offspring.



### Results and Discussion

Table 6 shows the number of litters, the mean percentage of pups sired by the first male and the total number of pups of each type of coat color for each test condition. An overall chi-square test on the total number of pups of each coat color for each condition revealed that at least two groups were statistically different ( $\chi^2 = 21.28$ ,  $df = 2$ ,  $p < .001$ ). Subsequent pairwise chi-square comparisons revealed that the restrained male and vasectomized male groups did not differ ( $\chi^2 = 0.926$ ,  $df = 1$ ,  $p > .05$ ) but that both the restrained male and vasectomized male groups differed statistically from the normal male group ( $\chi^2 = 12.65$ ,  $df = 1$ ,  $p < .001$ ;  $\chi^2 = 15.82$ ,  $df = 1$ ,  $p < .001$ , respectively). While the trend of these data appears to show that the mean percentage of pups sired by the albino males was highest after a normal male was in the intermediate condition, intermediate when a vasectomized male was used and least when a restrained male was used, Kruskal-Wallis nonparametric analysis of variance revealed no significant differences between these groups ( $H_c = 1.95$ ,  $df = 2$ ,  $p > .05$ ).

Individual data for this experiment are presented in Appendix III (Table A-3).

Table 6. Summary of the Results of Experiment III in Which an Albino Male Mated for One Ejaculatory Series, an Intermediate Treatment Was Given and Then a Hooded Male Was Allowed to Copulate for Five Ejaculatory Series.

Measure	Intermediate Treatment		
	Restrained Male	Vasectomized Male	Normal Male
Mean Percentage of Pups Sired by the First Male	19.7	31.4	44.1
<u>Pups' Coat Color</u>			
Pigmented	56	64	44
Albino	13	13	38
Number of Litters	9	10	10

In short, it appears that vaginal stimulation alone is not sufficient to account for the present effect. Vasectomized males were not as effective as normal males in preventing sperm displacement. However, these data could be easily misinterpreted since the vasectomized males were doing more than adding additional copulatory stimulation. An analysis of the intromission latencies revealed that all vasectomized males mated within 300 seconds after their introduction to the female. As discussed below, these males effectively removed the copulatory plug of the first male and replaced the plug with one containing no sperm. Copulatory behavior and plug dislodgement occurring less than 4-6 minutes after an ejaculation has been shown to greatly reduce the number of sperm reaching the female's uterine horns (Matthews and Adler, 1977). Thus, the present data indicating that copulatory behavior and plugs of vasectomized males have no effect greater than time alone may be misleading, since the group of females in the 45 minute restrained male group did not have the plugs removed. In the intermediate group with normal males, the plug was removed by the second albino male's copulation but these intermediate males deposited additional plugs and sperm during the course of his four ejaculatory series.

While there may be conditions under which copulatory plugs may serve as a physical barrier to later sperm deposition and behavioral stimuli may inhibit later sperm transport, the current data suggest the total number of sperm from successive ejaculations is the primary factor in preventing later sperm competition among laboratory rats.

## GENERAL DISCUSSION

The three present experiments demonstrate and replicate a clear effect: while a male rat mating for only one ejaculatory series with a female rat frequently can have his sperm displaced by those of another male rat, such displacement is unlikely when the first male has completed five ejaculatory series. These data suggest that the probable adaptive significance of multiple ejaculations in rats and closely related species is to reduce the probability of sperm displacement.

This behavioral pattern, multiple ejaculatory series, has the necessary concomitant physiological adaptations. Rats have relatively large testes and complex accessory sex glands (Arata, 1964). The large testes, seminal vesicles and coagulating glands of male rats are necessary for delivering successive ejaculates of appropriate size. These popularly studied structures of rats probably owe their large size to this same adaptive function of multiple ejaculatory series.

There may be other adaptive functions or benefits of multiple ejaculatory series. In aging, multiparous rats

and lactating females mating in post partum estrus, multiple ejaculatory series increase the probability of pregnancy for such females when compared to similar females receiving only one ejaculatory series (Davis, 1974; Davis and Conner, 1977). However, sperm displacement may well also be an important factor for males mating with lactating and multiparous females.

Wilson (1975) has outlined the possible types of competition among males including sperm displacement and sperm plugs. These modes range from infanticide to ". . . one extraordinary species, the certo-pognoid fly Johannseniella nitida, the body of the male itself serves as the plug . . ." (Wilson, p. 321). Since a single copulatory plug of a male rat does not provide reliable protection from later inseminations, male rats have evolved a behavior pattern of multiple ejaculations. One can easily envision a reproductive battlefield of male rats and their progenitors. As males evolved more effective physiological mechanisms for preventing sperm displacement (such as the deposition of copulatory plugs), males within the same population evolved more effective means of plug dislodgement. While other adaptive functions have been suggested for the multiple intromission pattern (as was discussed above), these pre-ejaculatory intromissions may serve to dislodge the plugs

of previous matings. If so, this may account for the reduction of intromission frequency in the second through fifth ejaculatory series of male rats; selection would not favor male rats which totally dislodged their own plugs and displaced their sperm from previous matings. The ineffectiveness of vasectomized males in preventing subsequent inseminations as observed in Experiment III of the present thesis, suggests that successive copulatory plugs do not serve as complete physical or chemical barriers but confer selective advantages on males mating first for several series.

While Adler and Zoloth (1970), and Matthews and Adler (1977) have shown that the first 4-6 minutes after an ejaculation is the critical period in preventing disruption of "sperm transport" to the uterus which occurs when the copulatory plug is removed from a female rat, the present data on the ineffectiveness of restrained males in preventing competitive inseminations suggest that the period of vulnerability of a female to subsequent inseminations is longer than 45 minutes. Despite Adler and Zoloth's (1970) data indicating that roughly a quarter of a million sperm remain in a female rat's uterus when a plug is not displaced until 45 minutes later, the present data indicate

that a second male can still dilute the first male's sperm and sire most of the offspring.

Adler and Zoloth rarely found sperm displacement when an interval of 45 minutes had elapsed between matings. The strains of rats used by Adler and Zoloth differed from those presently employed. Nevertheless, there is another critical factor which could account for the present finding of sperm displacement when an interval of 45 minutes elapses between matings. In the present study, the second male was allowed to mate for five ejaculatory series, while only one ejaculatory series was allowed in Adler and Zoloth's study. Thus, the second male had a much greater opportunity of diluting the first male's sperm in the present study. While this speculation can only be substantiated by future research, comparisons across these two studies suggest that the current effect is probably the result of sperm dilution in utero rather than some other mechanism.

Since the major difference between the matings of intermediate normal males and intermediate vasectomized males in Experiment III was that the former deposited additional sperm, these data also suggest that sperm dilution is the mechanism by which the last test male can sire offspring.



Three other plausible mechanisms which could mediate the present effect have little support. The accumulated material from copulatory plugs does not make an effective barrier since vasectomized males do not prevent later inseminations.

It is also unlikely that total or partial plug displacement alone can account for this phenomenon. First, sperm displacement frequently occurred in Experiment III when 45 minutes occurred between mating of successive males. Moreover, males mating for several series in Experiments II and III dislodged their own plugs from previous ejaculations in their own successive ejaculations in their own successive matings.

Finally, there does not appear to be any evidence that active sperm transport by the reproductive system of the female was inhibited by behavioral, mechanical or temporal factors. Females generally bore litters of normal size sired by the last male despite the previous matings of vasectomized males or the imposition of a 45 minute time interval between successive matings.

In short, the present data suggest an extremely important adaptive function of multiple ejaculatory series for male rats. This behavioral pattern appears to reduce the

probability of sperm dilution by other males which subsequently mate with the same female. Since analogous mechanisms are known to exist in insects and birds, it appears likely that multiple ejaculatory series may have similar adaptive functions in a number of mammalian species where more than one male mates with a given female.

APPENDICES

APPENDIX I

Table 1-A. Individual Data from Experiment I; Each of Two Males Mated for One Ejaculatory Series with the Same Female.

Condition	Strains			Offspring	
	MAX	F344	Female	Pigmented	Albino
	First Male	Second Male			
Number	Number	Number			
Pigmented male first	8	64	46	0	5
	2	54	16	0	10
	3	42	27	0	10
	4	37	66	0	10
	6	46	40	0	10
	1	46	55	0	10
	9	52	49	0	5
	10	47	21	0	2
	7	61	24	0	10

Condition	Strains			Offspring	
	F344	MAX	Female	Pigmented	Albino
	First Male	Second Male			
Number	Number	Number			
Albino male first	39	4	32	0	6
	44	3	15	0	4
	45	6	23	0	9
	38	8	28	0	4
	47	10	4	5	1
	48	9	5	0	10

Table 1-A (Extended)

Condition	Strains			Offspring	
	Long-Evans	F344	Female	Pigmented	Albino
	First	Second			
	Male	Male	Number	Number	Number
Pigmented	13	48	36	0	11
male first	14	52	22	1	9
	15	61	11	0	7
	16	64	47	0	10
	17	56	2	8	0
	18	57	6	6	0
	20	47	41	3	7
	21	65	1	3	8
	22	39	59	8	3

Condition	Strains			Offspring	
	F344	Long-Evans	Female	Pigmented	Albino
	First	Second			
	Male	Male	Number	Number	Number
Albino male	56	13	48	4	7
first	54	15	64	8	3
	55	16	45	0	6
	46	17	50	3	7
	47	18	53	0	4
	61	19	13	5	2
	64	20	29	0	6
	38	21	56	9	0
	66	22	3	8	0

Table 1-A (Extended)

Condition	Strain			Offspring	
	Brown	F344	Female	Pigmented	Albino
	First	Second			
	Male	Male			
Number	Number	Number			
Pigmented	26	47	114	0	11
male first	28	39	67	0	8
	29	48	57	1	11
	30	42	42	4	0
	31	43	20	11	0
	32	44	18	0	7
	33	45	33	0	9
	34	46	119	0	6
	22	61	34	1	1

Condition	Strains			Offspring	
	F344	Brown	Female	Pigmented	Albino
	First	Second			
	Male	Male			
Number	Number	Number			
Albino	42	26	26	1	11
male first	38	28	30	7	0
	39	29	31	0	1
	52	30	37	0	9
	43	31	60	5	6
	44	32	10	2	9
	45	33	8	7	2
	54	34	52	1	9
	37	27	7	0	6
	42	9	38	0	9

APPENDIX II

Table 2-A. Individual Data for Experiment II, listing Subject Numbers and Number of Offspring of Each Coat Color for Each Condition.

Condition	First	Second	Female Number	Offspring	
	Male Number	Male Number		Pigmented	Albino
Pigmented male mating first for five ejaculatory series	17	52	4	1	8
	18	54	30	0	1
	19	58	8	1	4
	20	56	6	0	10
	21	64	66	0	2
	24	61	40	2	7
	21	61	21	0	6
	24	64	46	3	1
	13	44	44	0	2
	14	48	54	0	3
	15	47	58	0	2
Pigmented male mating first for one ejacu- latory series	13	14	26	7	1
	15	46	3	0	8
	13	48	13	7	0
	14	44	18	5	7
	16	46	52	7	0
	17	54	14	5	0
	18	52	22	3	0
	20	56	38	9	0
	13	46	10	9	1
	14	47	11	7	3
	15	48	16	10	2
	16	44	27	5	2

Table 2-A (Extended)

Condition	First	Second	Female Number	Offspring	
	Male Number	Male Number		Pigmented	Albino
Albino male	59	21	19	11	0
mating first	61	22	31	10	0
for one	64	24	36	10	0
ejaculatory	52	18	33	3	1
series	56	19	34	7	0
	58	20	49	1	5
	47	13	15	11	0
	46	14	24	4	6
	44	15	59	8	0
	45	16	65	6	0
Albino male	45	13	42	5	0
mating first	46	14	32	1	6
for five	44	15	56	6	2
ejaculatory	59	21	28	5	4
series	61	24	48	1	2
	58	18	27	3	5
	56	18	50	2	7
	52	19	51	1	5
	54	20	62	0	4
	58	17	41	3	4



APPENDIX III

Table 3-A. Contains Individual Subject Numbers and Number of Pups of Each Coat Color for Each Condition in Experiment III.

Condition	First	Second	Third	Female	Offspring	
	Male	Male	Male		Pigmented	Albino
	Number	Number	Number	Number		
Restrained male	64		19	80	9	0
	59		21	92	7	2
	61		24	81	8	4
	46		14	87	4	3
	66		15	78	1	0
	55		14	99	2	1
	56		16	94	4	2
	65		19	118	6	0
	66		15	97	6	1
Vasecto- mized male	59	38	18	97	0	5
	54	44	15	102	13	0
	58	36	24	72	13	0
	55	49	14	95	0	3
	59	38	13	86	12	3
	58	44	14	116	10	0
	52	41	13	111	7	0
	48	38	15	77	6	1
	47	49	14	101	0	4
	52	36	19	99	3	0

Table 3-A (Extended)

Condition	First	Second	Third	Female	Offspring	
	Male	Male	Male		Pigmented	Albino
	Number	Number	Number	Number		
Normal	59	52	18	98	7	3
male	45	61	2	90	8	0
	52	46	21	91	5	5
	45	66	19	93	1	9
	56	59	13	101	3	0
	45	46	19	77	1	6
	59	66	18	107	9	1
	56	46	20	88	4	3
	54	61	21	82	6	3
	55	52	16	96	0	8

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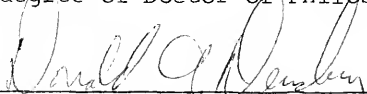
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## BIOGRAPHICAL SKETCH


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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

  
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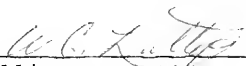
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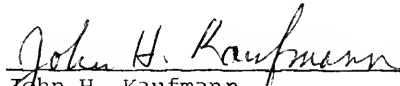
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June 1978

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