A Study of the Relationship between Olfaction and Puberty in the Laboratory Rat: Rattus norvegicus

Robert F. Locke
Loyola University Chicago

Recommended Citation
https://ecommons.luc.edu/luc_theses/2549
A STUDY OF THE RELATIONSHIP BETWEEN OLFACTION AND PUBERTY IN THE LABORATORY RAT: RATTUS NORVEGICUS

By

Robert F. Locke Jr.

A Thesis Submitted to the faculty of the Graduate School of Loyola University in Partial Fulfillment of the Requirements for the Degree of Master of Science

May, 1971
BIOGRAPHY

Robert F. Locke was born in Wilmington, North Carolina, on August 10, 1945.

He attended Chaminade College Preparatory School in St. Louis, Missouri, and graduated in June of 1963. He entered St. Procopius College, Lisle, Illinois, where he majored in Sociology and graduated with a Bachelor of Arts degree in August of 1967.

The writer began his graduate study in June of 1969 in the Department of Anatomy at Loyola University Stritch School of Medicine, Maywood, Illinois.
ACKNOWLEDGEMENTS

I wish to express my sincere appreciation to my advisor and Chairman of the examination committee, Dr. Joseph Thomas Velardo, Professor and Chairman of the Department of Anatomy, for his support, professional guidance, and constructive criticism during this investigation.

I am also indebted to Dr. Barbara A. Kasprow, Assistant Professor of Anatomy, and to Dr. Sigfrid Zitzlsperger, Professor of Anatomy for donating their time and assistance as members of the examination committee.
TABLE OF CONTENTS

INTRODUCTION ........................................................................... 1
REVIEW OF THE LITERATURE .................................................. 3
MATERIALS AND METHODS ...................................................... 10
  I. ANIMALS ........................................................................... 10
  II. HOUSING ........................................................................ 10
  III. TREATMENT CATEGORIES ............................................... 10
EXPERIMENTAL RESULTS ....................................................... 13
  A. GROUP I ........................................................................... 13
  B. GROUP II .......................................................................... 13
  C. GROUP III ......................................................................... 15
  D. GROUP IV ......................................................................... 17
  E. GROUP V .......................................................................... 20
  F. GROUP VI .......................................................................... 21
DISCUSSION .............................................................................. 24
SUMMARY AND CONCLUSIONS ............................................... 33
LITERATURE CITED ................................................................ 36
TABLES .................................................................................... 45
  TABLE 1. SUMMARY TABLE ................................................. 45
FIGURES ................................................................................ 14
  FIGURE 1 ISOLATED CONTROL ANIMALS ......................... 14
  FIGURE 2 EXPOSURE TO MATURE MALE RAT URINE ........ 16
  FIGURE 3 DIRECT EXPOSURE TO MATURE MALE RATS ....... 18
TABLE OF CONTENTS (con't)

| FIGURE 4  | DIRECT EXPOSURE TO ORCHIDECTOMIZED, MATURE RATS | 19 |
| FIGURE 5  | DIRECT EXPOSURE TO ORCHIDECTOMIZED, MATURE RATS GIVEN TESTOSTERONE | 22 |
| FIGURE 6  | DIRECT EXPOSURE TO MATURE MALE MICE | 23 |

| PHOTOMICROGRAPHS | 42 |
| PLATE I  | VAGINAL SMEAR, DIESTRUS | 42 |
| PLATE II | VAGINAL SMEAR, PROESTRUS | 42 |
| PLATE III | VAGINAL SMEAR, ESTRUS | 43 |
| PLATE IV | VAGINAL SMEAR, METESTRUS | 43 |
| PLATE V  | VAGINAL SMEAR, ESTRUS WITH ABUNDANT SPERMATOZOA | 44 |
ABSTRACT

A series of experiments was undertaken for the purpose of determining whether or not direct or indirect exposure of young, female rats to mature, intact male rats or mature, bilaterally orchidectomized rats could advance pubertal onset in the females. It is known that both direct and indirect contact (i.e., contact with the soiled bedding material of male mice) with mature male mice advances pubertal onset in the exposed female mice.

Grouped, twenty-one day old female albino rats were exposed from weaning to one of the following stimuli: 1) direct, constant exposure to one mature, male rat of the same strain; 2) direct, constant exposure to the urine of one mature, male rat of the same strain; 3) direct, constant exposure to one mature, orchidectomized rat of the same strain; 4) direct, constant exposure to one mature, orchidectomized rat of the same strain but injected daily with 1.5 mg. testosterone propionate in sesame oil; 5) direct, constant exposure to two mature, male mice. Control females were isolated from both contact with male rats and male rat odor. Vaginal canalization and first vaginal estrus were used as indices of pubertal onset. Exposure to the various odor stimuli lasted until the first fully cornified vaginal smear could be recorded.

The results indicate that the basic reactions of young, female albino rats in this study and under these environmental conditions parallel those of young, female mice even though the reactions are by no means as dramatic. Exposure of young, female rats to both mature, male rats and their urine resulted in an advanced first estrus. Vaginal canalization was not
advanced. First estrus in the direct exposure group and the urine exposure group occurred at 35.9 days and 35.5 days respectively in contrast to a mean of 37.9 days in the isolated, control group.

Direct exposure of young, female rats to orchidectomized rats with and without testosterone injection did not advance canalization but rather, delayed the establishment of the vaginal orifice. First estrus in the aforementioned exposure groups was not significantly affected by the gonadectomized male rats. Canalization occurred at mean ages of 35.7 days and 38.2 days respectively in contrast with a mean age of 34.2 days in the isolated, control females.

Direct exposure of young, female rats to mature, male mice affected neither vaginal canalization nor first vaginal estrus to any significant degree.
INTRODUCTION

Scientists have, for many generations, affirmed that odors play an important role in eliciting specific behavior patterns in many species of animals. Indeed, olfaction in a number of different species of animals serves as a primary means of nourishment, protection and reproduction. The survival of an animal in the wild, therefore, may well depend upon its ability to identify specific odors and respond accordingly.

Until the middle 1950's, very little work had been undertaken to demonstrate the specificity of olfactory responses and relationships in different animal species. The term pheromone was first introduced in 1959 by Karlson and Butenandt to describe specific chemical substances secreted by animals which were capable of altering the behavior of other animals usually of the same species. These chemical substances could be tasted, absorbed through the skin, or perceived by means of olfaction. The olfactory perception of pheromonal substances and their odors appeared to be the most common receptor mechanism.

Perhaps the best illustration of the effects of pheromones and the specificity of pheromonal responses in mammals can be drawn from a number of recent reports of changes in reproductive behavior elicited by odors in rodents.

Laboratory animal breeders and scientists alike have long observed that species specific odors allow other members of the same species to discriminate between sexes, between estrous females and those which are not sexually
receptive, and between juveniles and adults.

The exact mechanism involved in odor receptivity and subsequent response is not known exactly even though the work of Scott and Pfaffman (1967) confirms the general idea that the anatomical connection between the olfactory bulbs and the hypothalamus in the rat is functionally important. Kling, in 1964 reported that olfactory bulb ablation in female mice resulted in a loss of estrous cyclicity.

Thus, it appears obvious that the olfactory sense is intricately involved in the regulation of reproductive behavior in laboratory rodents and much work remains to be carried out in order that mammalian reproduction be better understood. Consequently, a number of experiments were devised so as to explore the role(s) of animal odors in rats, especially since the situation seems so well established in the mouse.
REVIEW OF THE LITERATURE

Just as humans depend to a great extent upon the senses of sight and hearing to exist, interact, and therefore, function in a complex society, many animals rely upon the olfactory sense for their survival. Communication among macrosmatic animals is often accomplished by means of specific secretions and their odors. Bossert and Wilson (1963) attempted with some success to analyze the effects of diffusion of three distinct odors elicited by ants and gypsy moths. These odors were quite distinct in terms of response elicited since one resulted in the communication of fear, the second, in the communication of "recruitment trail" and the third, in sexual attraction and excitation as observed in the gypsy moth. Each odor was the result of a specific, chemical secretion by one or more of the insects.

In contrast, it is believed that although the olfactory potential of some birds is either underestimated or poorly understood (Bang 1960, 1965), the sense of sight rather than that of olfaction appears to be of primary importance to survival in the wild. Most birds, therefore, are regarded as microsmatic rather than macrosmatic.

Perhaps the best illustration of olfactory regulation of behavior is found in the rodents. Indeed, it has been known for some years that most rodents and many larger animals tend to map out a territory and protect this area from intrusion by other animals (Lane-Petter, 1967; Parkes and Bruce, 1961; and Bruce, 1967).
Lane-Petter (1967) reported that a specific secretion of the preputial glands of male mice served to identify or label the territory "claimed" by the animals. Parkes and Bruce (1961) reported that the odoriferous, supra-caudal glands of guinea pigs were much more developed in the male of the species than in the female. Further, they determined the locations of odoriferous glands in different species and noted that odors emanating from the male of many species is capable of producing dramatic effects in the females of the same species.

King (1969) reported that a substance communicating "fear" is present in fecal material of rats at the time of electrical shock. To what extent the suprarenal glands are implicated in this "fear" secretion remains to be examined in detail.

Morrison and Ludvigson (1970) reported a pheromone of "frustration" or "non-reward" to be elicited by rats when food was not found at the termination of an experimental maze. This odor affected a change in direction on the part of the other rats in the same maze.

Odor stimuli can act in at least two ways. First, the immediate reaction on the part of other animals in the category such as fear, excitation, or some similar response. Secondly, and more subtly, however, are those physiological responses which are not immediately observable, such as an acceleration of puberty, pregnancy failure, or synchronization of estrus. Considerable effort, in recent years, has been expended in examining the effects of odor stimuli upon reproduction. The laboratory mouse is an effective model for research and investigation of the role of olfaction in repro-
duction because of its relative sensitivity to a variety of odors.

Lee and Boot (1955, 1956) reported that cyclic estrus in the non-mated, female mouse could be modified by odors of both males and other females. Specifically, when female mice were housed in small groups (4 per cage) away from male mice and their odors, the aforementioned workers observed a uniform suppression of estrus and a significant increase in the number of pseudopregnancies. It was assumed that some pheromone affected the pseudopregnancy by way of olfactory pathways.

Whitten (1957, 1959) elaborated further upon these observations by housing female mice in large groups (30 per cage) in order to determine the effect of community living in the absence of the male upon estrous cyclicity. He reported that suppression of estrus was even more pronounced under these conditions than when smaller groups of females were housed away from males. Estrous cycles became highly irregular and the majority of the mice became anestrous for long periods of time. In 1956, he observed that the introduction of a male mouse effected a synchronization of estrous cycles, and further, that matings of paired females occurred, generally on the third night after the introduction of the male. The synchronization of estrus, he reported, could also be effected by housing a male mouse in the female cage preventing physical contact. The exposure of the female to the excreta of a male mouse for two days was equally effective in initiating and synchronizing estrus in anestrous females. Again, olfactory stimulation was suggested.

Further implicating the role of olfaction in the laboratory mouse was the work of Bruce (1959, 1960). He reported that when newly mated female mice
and others mated with vasectomized males were removed from the cage of the "stud" male and exposed to an "alien" male, both pregnancy and pseudopregnancy failed. Bruce further reported that anosmic female mice did not show this "blocking" reaction. He suggested that the pregnancy block was caused by pheromones acting by way of the olfactory tract. Bruce further reported the pregnancy block only occurred within the first five days after mating, the greater percentage occurring at about three days. It appears, therefore, that the male induced pregnancy block is a block to ova-implantation.

There is striking evidence that androgens or secretions of some androgen dependent gland or glands are the source of the estrus-accelerating pheromone since synchronization of estrus in large groups of isolated female mice can be affected by the introduction of androgenized females (Dominic, 1968). In further support of this hypothesis, Marsden and Bronson (1964), reported that the application of male mouse urine to the external nares of group females for two days resulted in the synchronization of estrus on the following night.

The pregnancy block did not occur when newly mated female mice were exposed to castrated male mice (Bruce, 1965). Androgenized, spayed female mice and urine from these animals, however, appear capable of blocking pregnancy (Dominic, 1965). Thus, there is significant evidence implicating androgens as well as androgenic metabolites as pheromones, the latter affecting the reproductive mechanisms in female mice.

While olfactory pathways have been demonstrated to be the link between stimulus and response, the question of which mechanisms are involved in
eliciting the pregnancy block remains to be determined, as does the mechanism by which the pheromone renders uterine horns incapable of allowing implantation of the fertilized ova. Snyder and Taggart (1967) examined this question in relation to the possible role played by the adrenal glands. They reported that in two strains of mice (domestic and wild), adrenalectomy of the pregnant female prior to exposure to the "alien" male animals resulted in the failure of the pregnancy block. They also reported that pregnancy was sustained in sham-operated pregnant animals similarly exposed.

Bruce and Parkes (1960) and Dominic (1967), demonstrated that graded doses of prolactin, injected daily for five days after mating, protected and sustained pregnancy in spite of the simultaneous exposure to "strange" male mice and "strange" male mouse urine. It was hypothesized by Parkes and Bruce (1961) that the underlying cause of the male-induced pregnancy block is the failure of prolactin secretion by the adenohypophysis.

In 1967, Vandenbergh reported that exposure of immature female mice to adult male mice resulted in accelerated sexual maturation in the females. This exposure, from day twenty-one of life until first vaginal estrus, advanced canalization by three and one-half days over that of isolated controls. First estrus was accelerated by fourteen days. Exposure of immature females to mature females advanced puberty but not as dramatically as exposure to adult males. This same study indicated that pre-weaning exposure to mature male mice was effective but again not as effective as post-weanling exposure.

In a later study, Vandenbergh (1969) designed a series of experiments to determine the extent to which olfaction was implicated in the pubertal accel-
eration observed in previous experiments. He exposed female mice from day twenty-one of life to each of the following stimuli: 1) one adult male mouse placed in direct contact with the females; 2) one adult male mouse, separated from the females by a wire mesh, but in the same cage; 3) one castrated male mouse in direct contact; 4) soiled bedding material from the cages of male mice, or 5) soiled bedding material from the cages of male mice which were in contact with female mice kept in constant heat by estrogen injection. Control females were isolated from contact with male mice and their odor. This set of experimental procedures demonstrated that in every case of exposure to intact, adult, male mice or their soiled bedding material, estrus and canalization were accelerated significantly. Young females exposed to castrated male mice, however, did not open early, but rather, opened and underwent first estrus much later than did the isolated control females. The earliest of the groups to undergo puberty was the group exposed to "activated male odor," that is, the bedding material from the cages of male mice which were exposed to females in constant heat.

Ropartz (1969) performed similar experiments, but exposed female weanling mice to both male and female adult mice from day twenty-one of life. Uterine weights in the experimental females were significantly higher than those of the isolated control females at necropsy on day forty. Vaginal canalization did not occur earlier as reported by Vandenbergh (1967, 1969), and first estrus was not recorded.

Laboratory rats appear to be somewhat less sensitive to pheromones since attempts to demonstrate the pregnancy block phenomenon have not been success-
ful. Similarly, estrous cyclicity in female rats appears not to be influenced by males (Dominic, 1969). Aron and co-workers (1970) determined that estrogenized, female rats, without olfactory bulbs (i.e., they were surgically removed), ovulated after coitus. Five day cyclicity of estrus was reported one month after removal of the olfactory bulbs.

Rosen and Shelesnyak (1937) and Shelesnyak and Rosen (1938) reported that pseudopregnancy could be affected in female laboratory rats by both local anesthesia of the nasal mucosa and application of silver nitrate to the same area. The bilateral removal of the sphenopalatine (pterygopalatine) ganglion produced the same reaction (Shelesnyak et al 1940). In each case, however, the pseudopregnancy was transitory and did not impair reproduction or estrous cyclicity after twenty-four days. These works, however, indicate an immediate naso-genital relationship in female laboratory rats.

In view of the relative lack of information on the pheromonal sensitivities of laboratory rats, it necessarily follows that investigation of olfactory responses should be undertaken. With this need in view, a series of experiments was designed for the purpose of determining whether or not immature female rats respond to male sex odor as do immature female mice. Specifically, these experiments are intended to determine whether or not direct and indirect exposure of 21-day-old female rats to mature male rats can elicit an acceleration of puberty in the females.
MATERIALS AND METHODS

I. Animals

Five groups of female, twenty-one day old Sprague-Dawley derived rats (Charles River Breeding Laboratories) were exposed to a variety of odors and direct stimuli. A sixth group of control animals was isolated from both contact with mature male rats or the odors of mature, male animals. All female rats used in this study were born and raised to weaning age in a room away from both mature male animals and their odors.

II. Housing

Both experimental and control female rats were housed in clear plastic cages (Maryland Plastics, Inc.) on San-i-cel bedding (Paxton Laboratories). The cages were changed and sterilized as needed. All animals were fed ad libitum with a standard rat diet (Purina Rat Chow). Room temperature was maintained at 72 degrees F. ± 2 degrees and light/dark cycles were maintained at 12 hours on and 12 hours off. Each experimental group of female rats was housed separately in a room containing no other animals and equipped with a separate air exchange system.

III. Treatment Categories

After weaning at twenty-one days of age (Farris and Griffith, 1949), female rats were placed into one of five treatment categories. Individual treatment groups had the following characteristics:

A. Controls. Fifty immature, female rats were housed, three per cage (16
cages containing 3 animals each and one cage containing 2 animals), in a room which was separated from any direct or indirect contact with mature, male rats.

B. Twenty-five immature female rats were housed, three per cage (7 cages containing 3 animals each and one cage containing 4 animals), in a separate room and treatment consisted of introducing three gauze sponges soaked with the urine of a mature, male rat of the same strain. The sponges were placed in the cages at 8:00 A.M. and 8:00 P.M. each day. Special care was taken to make sure that the females were exposed to the urine of the same male rat each day. Treatment lasted from day twenty-one of life until first vaginal estrus could be recorded. Standard rat metabolic cages (Acme Research Products) were used for daily urine collection.

C. Twenty-four immature, female rats were similarly housed, with the exception that from day twenty-one of life they were directly exposed to one mature, male rat of the same strain. The exposure was constant and lasted until first vaginal estrus could be recorded.

D. Twenty-four immature, female rats were likewise housed, with the exception that from day twenty-one of life they were exposed directly to one mature, bilaterally orchidectomized rat. Sufficient time was allowed after castration for atrophy of the androgen supported glands (Zarrow et al., 1964). Again, exposure was constant and lasted until first vaginal estrus could be recorded.

E. Twenty-five immature female rats were housed, three per cage (7 cages containing 3 animals each and one cage containing 4 animals), with the exception that from day twenty-one of life they were exposed directly to one mature, orchidectomized rat of the same strain which was injected daily with 1.5 mg.
testosterone propionate in sesame oil (Holmes Serum Co.).

F. Twenty-four immature female rats were housed, four per cage in direct, constant contact with two mature, male mice (Swiss-Webster) of proven virility and known reproductive history. The duration of exposure was identical to that of the other experimental groups.

All of the animal groups were examined daily for the establishment of the vaginal orifice (canalization). From the day of vaginal canalization, a daily vaginal levage was taken (Long and Evans, 1922) until the first fully cornified smear was observed. Plates I through V illustrate the stages of the estrous cycle observed and identified. All vaginal smears were read at the time they were taken.
EXPERIMENTAL RESULTS

A. Control Females (Group I)

Reports concerning the mean ages of vaginal canalization and first estrus in albino rats appear somewhat varied. Long and Evans (1922) reported that canalization occurred at a mean age of 72 days. First estrus, they reported, occurred at a mean age of 77 days with a range of 34 to 109 days. Wistar rats (albino) underwent puberty within a range of 35 to 50 days as reported by Farris and Griffith (1942). Gorski and Adaniya (1971) reported that young, female Sprague-Dawley rats underwent canalization and first estrus simultaneously at a mean age of 39 days. It appears, therefore, that the age of pubertal onset in the laboratory rat may vary in accordance with a number of environmental factors including source, and strain. Similarly, the age of vaginal canalization in Long-Evans rats has been shown to vary according to the physical location (geographic) and environment of the nursing mother (Ellet, 1970).

In the present study isolated, control female rats underwent vaginal canalization at a mean age of 34.2 days but only in a few animals was canalization and first estrus observed to occur simultaneously. First estrus occurred at a mean age of 37.9 days (figure 1). This is almost one full estrous cycle after the establishment of the vaginal canal. In addition, it was observed that most of the control female rats showed a diestrous smear on the day of vaginal canalization.

B. Group II (Exposure to mature, male rat urine)
FIGURE 1

Percentages at different ages of immature, female rats showing vaginal canalization and first estrus when caged in isolation from male rats.
The exposure of weanling female rats to the urine of one mature male rat from day twenty-one of life failed to accelerate the age of vaginal canalization to any significant degree (table 1). Canalization occurred at a mean age of 33.6 days as compared to a mean of 34.2 days recorded in the isolated, control females (figure 2).

While this exposure failed to advance canalization in young, female rats there was a significant acceleration of first estrus. Female rats in this experimental group showed first estrus at a mean age of 35.5 days in contrast to a mean age of 37.9 days observed in the isolated, control group. The student T test for statistical validity revealed that first vaginal estrus was accelerated significantly at the 0.02 level of predictability.

C. Group III (Direct exposure to mature, male rats)

The direct exposure of weanling female rats to one mature, male rat from day twenty-one of life failed to advance the age of vaginal canalization in immature female animals. Canalization occurred at a mean age of 34.5 days. This mean did not differ significantly from that reported in the control group.

First estrus, however, in this experimental group occurred at a mean age 35.9 days in contrast to a mean age of 37.9 days in the isolated, female control animals. The level of predictability revealed by the student T test was 0.05. It was further observed that first estrus and canalization often occurred simultaneously since spermatozoa were often observed in the vaginal smear on the day of vaginal canalization (figure 3). The appearance of sperm in the vaginal smears was observed more often than was the presence of the vaginal plug, and in most cases, the first mating resulted in successful
Figure 2

Percentages at different ages of immature, female rats showing vaginal canalization and first estrus when exposed to the urine of intact, mature male rats.

Cumulative % of Population (25 animals)
gestation and parturition. Attempted mounting activity on the part of the mature, male animals was not observed prior to canalization, indicating that the males were able to discriminate between mature and immature female animals. Instances of cannibalism or destruction of the young were not observed in this group.

D. Group IV (Direct exposure to bilaterally orchidectomized rats)

The direct exposure of immature, female rats from day twenty-one of life to mature, orchidectomized rats did not advance the time of vaginal canalization in female rats (figure 4, table 1). Rather, this exposure resulted in a significant delay of vaginal opening. Canalization occurred at a mean age of 35.7 days in contrast to a mean of 34.2 days of age observed in isolated, control females. This difference is significant at the 0.02 level of predictability. It was further noticed that the females of this group showed a much higher incidence of hyperkinesis than any of the other animal groups. The difference in the age of vaginal canalization in this experimental group is reminiscent of that observed by Vandenbergh in 1969.

First estrus in this experimental group was not accelerated as reported in the two preceeding groups. The mean age of first estrus was 39.0 days and did not significantly disagree with that of the isolated, control females.

Orchidectomized rats in this treatment category were allowed a minimum of three to four weeks after castration before they were placed in direct contact with the immature females. Mounting activity on the part of the orchidectomized rats was not observed before or after vaginal canalization of the females was recorded. Similarly, there were no semen plugs found in the vaginas of the young females or in the bedding material of the cages.
FIGURE 3

Percentages at different ages of immature, female rats showing vaginal canalization and first estrus when caged in constant, direct contact with mature, intact male rats.

Cumulative % of Population (24 animals)
Figure 4

Percentages at different ages of immature, female rats showing vaginal canalization and first estrus when caged in direct, constant contact with previously orchidectomized, mature rats.

Cumulative % of Population (24 animals)

- First Estrus
- Canalization

Days of age
E. Group V (Direct exposure, orchidectomized rat with testosterone treatment)

Both androgens and the secretions of androgen-supported glands have been implicated as possible pheromonal sources affecting reproduction in mice (Dominic, 1965). Vandenberg's work (1969) further supports this hypothesis since every group of female mice exposed directly or indirectly to intact, mature male mice underwent an accelerated puberty.

For the purpose of determining whether large doses of testosterone propionate in orchidectomized rats could accelerate puberty in exposed female rats, one group of 25 animals was so exposed in accordance with same experimental regimen. Vaginal canalization was not accelerated in the exposed females in spite of the constant, direct contact with injected male animals. Further, the mean age of canalization was again significantly delayed as reported in the preceding treatment group (table 1). The experimental females showed vaginal canalization at a mean age of 38.2 days, in contrast to a mean of 34.2 days of age observed in the isolated, control females. The level of predictability revealed by the student T test was 0.001. First estrus in this group occurred at a mean age of 39.33 days. This mean is not significantly later than that of the isolated control group but is in extremely close agreement with the mean first estrus of the females exposed to orchidectomized rats without testosterone propionate treatment (figures 4 and 5).

Mounting behavior was not observed prior to the establishment of the vaginal orifice in spite of the fact that subcutaneous testosterone injection was continued for at least twenty days. Six of the twenty-one day old females were killed by the injected male rats shortly after exposure. The females
and the injected males were replaced immediately and no further problems were encountered. In ten of the twenty-five female rats in this experimental group, vaginal plugs were found on or soon after the day of vaginal canalization. Dienstrous smears were recorded thereafter. Plugs in each case were found in the vaginal canal and not in the bedding material. The day on which plugs were observed was recorded as the day of first estrus.

F. Group VI

Neither canalization nor first estrus was accelerated by direct, constant exposure to mature, male mice. In fact, both indices of pubertal onset closely paralleled those of the isolated, control female rats (figure 6, table 1). Canalization occurred at a mean age of 35.0 days and first estrus occurred at a mean age of 37.3 days.

The housing of young, female rats with adult, male mice did not present any problems since the two rodent species appeared quite compatible during the experimental period.
FIGURE 5

Percentages at different ages of immature, female rats showing vaginal canalization and first estrus when caged in constant, direct contact with previously orchidectomized, mature rats given testosterone daily.
FIGURE 6

Percentages at different ages of immature, female rats showing vaginal canalization and first estrus when caged in constant, direct contact with mature, intact male mice.
DISCUSSION

A. The Environment

Morton, Denenberg, and Zarrow (1963) reported that first estrus in female rats could be advanced by ten days by the mere handling of the animals for three minutes per day from day one to day twenty-four of life. The question of the possible effects of animal handling in a study of this nature should be considered. Until day 29 or 30 of life, female rats in the present study were handled only on the days that the cages needed to be changed. This occurred about once every week or ten days. The duration of handling was only a matter of seconds and, in most cases, the bedding material and the female pups were scooped from the cage and physical contact with the technician was thus avoided. This type of handling prevents possible killing of the young on the part of the mother which often occurs due to the "strange" odor of the clean cage and fresh bedding material. Handling after day 30 of life occurred each day between 4:00 P.M. and 5:00 P.M. and only lasted long enough for visual examination for canalization and smearing. Animals of all treatment groups were similarly treated in this context.

Rats of the Sprague-Dawley strain which were procured from another source or breeding laboratory and raised to puberty in our laboratory are reported to undergo canalization much later than the animals in this study (Adaniya and Gorski, 1971). Environmental factors will, of necessity vary among different breeders in different parts of the country as will the different light cycles in the different breeding laboratories. Sprague-Dawley rats, for example,
raised at Sprague-Dawley Inc. in Madison, Wisconsin currently are exposed to constant lighting. Animals in this experiment are exposed to cycled lighting from birth. Caging systems also vary and clear, plastic cages allow more light than do wire cages. Twelve female rats were housed in suspended, steel cages with wire fronts at 21 days of age and isolated from male rats or their odors. Canalization in each individual case occurred before day 36 of life. The range of canalization was identical to that of the isolated, control female rats reported herein.

The bedding material used throughout the experiments was composed of dried, crushed corn cob, and therefore, contained no natural estrogenic material. Purina rat chow was used as a staple diet from weaning age and was used as a standard diet for the nursing mothers as well. Naturally-occurring estrogens are controlled in this laboratory diet.

B. Treatment Categories

It is known that the olfactory bulbs, which are well developed in the different rodent species, receive axons from the olfactory nerve cells of the nasal mucosa. Secondary and tertiary fibers from the olfactory bulbs terminate within the cerebral cortex as well as the hypothalamus as reported in the laboratory rat (Craigie, 1925). Kling (1964) demonstrated that the reproductive function is related to neural connections in the rhinencephalon, since rhinencephalic lesions resulted in delayed puberty in young, female rats. Olfactory bulb ablation in female mice resulted in a loss of estrous cyclicity as reported by Whitten (1956). The exact mechanism by which odor is interpreted and response is elicited neurally is not known. The neural connection between the olfactory bulbs and the hypothalamus gives adequate indication that
the hypothalamic-hypophyseal-ovarian interrelationships may be affected by olfactory stimuli. The works of Whitten (1956), Bruce and Parkes (1960), Lee and Boot (1955, 1956), and Vandenergh (1967, 1969), give considerable support to this hypothesis.

Releasing factors secreted by the hypothalamus which affect secretion of various tropic hormones by the pituitary have been affirmed in recent years. Watanabe and McCann (1968) prepared crude extracts from the stalk median eminence of the rat hypothalamus which produced significant increases of follicle stimulating hormone (FSH) release by the adenohypophysis. FSH releasing factor, they suggested, is localized in the stalk median eminence of the rat hypothalamus. Similarly, Evans and Nikitovich-Winer (1969) demonstrated that median eminence extracts, infused intra-muscularly, were capable of reactivating pituitary grafts (autotransplants) in the left renal capsule. Both ovarian and vaginal hypoplasia were observed in the control rats which were infused with oxytocin and vasopressin. Corbin and Danels (1969) suggested that puberty in the female rat involves a liberation of FSH-releasing factor (RF) by the stalk median eminence and the subsequent release of FSH by the adenohypophysis. Estrogen secreted by the ovarian follicle, probably is a significant factor in awakening the FSH-RF mechanism at pubertal onset. Receptor molecules for estrogen were found in the regions of the preoptic and arcuate nuclei of the hypothalamus by McGuire and Lisk in 1969. McCann and Ramirez (1964) reported that hypothalamic control of Luteinizing Hormone (LH) secretion is localized in the basal and tuberal hypothalamus.

Whether the acceleration of puberty in female mice reported by Vandenergh (1969) involves a stimulation of FSH-RF and LH-RF secretion by the hypothalamus
and subsequent FSH and LH release by the adenohypophysis due to olfactory stimulation alone cannot be concluded as definitive; yet, gonadotropin release on the part of the pituitary is necessary for both pubertal onset and reproductive function (Velardo, 1958).

In a similar sense, neural involvement of the hypothalamus and the olfactory nerve probably provide an explanation for the male-induced pregnancy block reported by Bruce (1960) in female mice. McCann and Ramirez (1964) reported secretions of the hypothalamus which promote the release of FSH and LH by the pituitary but inhibit the secretion of prolactin (LTH). Grosvenor, Mena, Dhariwal and McCann (1967) reported that a specific LTH inhibiting factor was localized in the stalk median eminence of the female rat hypothalamus. The failure of prolactin secretion by the pituitary was suggested as a possible explanation of the male-induced pregnancy block as was reported by Bruce and Parkes (1960) and Dominic (1967). The possible liberation of prolactin inhibiting factor may provide a convenient explanation for these observations since exogenous LTH injection for the first five days after mating prevented the pregnancy block elicited after exposure for the newly-mated female to an "alien" male (Dominic, 1967).

The actual source of the estrus-accelerating pheromone is not known even though the ablation of the testes in the male resulted in failure of both pubertal acceleration and the male-induced pregnancy block. It becomes evident that testicular androgen production is probably involved either directly or indirectly. It has been well established that androgen supported glands (i.e. the seminal vesicles and prostate) regress following orchidectomy (Zarrow, 1964), yet whether or not their secretions in the intact, male rodent
contribute to the pheromone or the pheromonal potential is not known. Zarrow and colleagues (1969) gave some indication of the potential of testosterone by showing that direct injection of testosterone propionate into immature, female rats resulted in a precocious vaginal opening as well as an advanced first estrus. Again, the hypothalamic-hypophyseal-ovarian axis seems to have been involved and pubertal preciosity could be blocked by lesion of the anterior, ventrolateral hippocampus.

In the present study, the acceleration of vaginal canalization did not occur. More specifically, in the immature female rats which were used in this investigation, exposure to intact, adult, male animals and the urine of these animals did not induce an early vaginal opening; indeed, canalization appeared unrelated to sex odor of intact males.

First estrus, however, was significantly accelerated in young, female rats exposed to adult, male rats or the urine of adult males. In contrast to the isolated, control females in which a mean 3.7 day delay of first estrus after canalization was observed, the experimental groups II and III normally underwent first estrus the day after canalization was observed. In a similar sense, the frequency of simultaneous canalization and first estrus in the exposure groups was much greater than that of isolated, control females. These observations are somewhat reminiscent of the estrous suppression and synchronization phenomena (Lee and Boot, 1955 and Whitten, 1956).

It appears, therefore, that in every group of immature female rats exposed either to adult male rats directly or to the urine of mature male rats, first vaginal estrus was advanced. Vaginal canalization was not advanced in either group. Corbin and Daniels (1969) suggested that estrogen is probably the most
significant factor in activating the FSH-RF mechanism at pubertal onset, but olfactory stimulation of the hypothalamus is somehow involved in advancing first estrus in mice, and, in this study, in rats.

It has been consistently reported by Whitten (1957), Bruce (1965) and Vandenberg (1969) that the pheromonal substance causing estrous synchronization, precocious puberty and pregnancy failure is absent in male mice after orchidectomy. The indication would be that because ablation of the testes results in degeneration of androgen-supported glands and more importantly, loss of testicular androgens, the suspected pheromonal constituents are eliminated. Such androgen deprivation generally requires about two weeks after surgery (Zarrow, 1964). Female rats did not show the acceleration of first estrus when exposed to orchidectomized rats. Rather, first estrus occurred at about the same time as that of isolated, female control animals. This is consistent with the observations reported for laboratory mice (Vandenbergh, 1969) and further, demonstrates that adrenal androgens alone cannot accelerate first estrus or vaginal canalization.

Less clear, however, is the mechanism involved in delaying the onset of vaginal opening. Canalization, in Group IV, occurred later than that of the isolated, control females. The delay was statistically significant and raises a question of the possibility of a new pheromone in male rats and mice after orchidectomy due to a change in the endocrine environment. The time interval between vaginal opening and first estrus closely resembled that of the isolated, control animals since both groups showed a period of over 3.5 days between canalization and first estrus.
It is known that pituitary gonadotropic hormone levels rise rather dramatically after removal of male and female gonads in laboratory animals and in humans. Treatment with testicular and ovarian hormones such as estradiol and testosterone will affect a decrease in pituitary gonadotropins (Velardo, 1958).

The full effect of high blood and urinary gonadotropic hormone titers in the castrated male rat is not known fully but it is doubtful that these tropic hormones would act as puberty-delaying pheromones.

Exogenous testosterone propionate injection in orchidectomized rats would, therefore, reduce the peripheral and urinary titers of gonadotropins and also restore the androgen supported glands which deteriorate in the absence of this hormone. Similarly, testosterone would be restored in the daily urinary output. Female rats exposed to the castrated male rats injected daily with testosterone propionate showed no acceleration in the age of pubertal onset. In spite of the large doses (1.5 mg. daily) of testosterone in the male animals, vaginal canalization in the exposed females was significantly delayed. In fact, the delay in canalization was even greater than the delay reported when females were exposed to castrated males without replacement therapy. First estrus was not significantly later than either that of the isolated, control females or that of the females exposed to the castrated males without testosterone. The approximate 3.5 day time-interval between canalization and first estrus which was observed in the previous two groups was not observed in the testosterone-treated group. The occurrence of vaginal plugging was frequently observed on the day of canalization in this group indicating that testosterone is probably effective in advancing first estrus in relation to canalization.
It has been reported by Barnafi and Croxatto (1966) that after orchi­
dectomy, the weights of both the pituitary and the adrenal glands of male rats
show a significant elevation. The elevation of adrenal weights is an indi­
cation of some adrenal hypertrophy. In a similar sense, hypophyseal adreno­
corticotropic hormone (ACTH) increases (Kitay, 1963). The total significance
of this change is not yet known.

Hyperadrenalism and ACTH have both been shown to decrease reproductive
ability in a number of different animal species. Large amounts of ACTH in­
jected for ten days caused follicular atresia and decreased uterine weights in
mature mice. ACTH also inhibited maturation of the male and female deer mouse
and white-footed mouse (Christian et al., 1965). It is conceivable that hyper­
trophy of the adrenal medulla may also be involved. The effect of epinephrine
upon reproduction is less well understood even though injection of this hor­
mone into female rats is reported to cause diestrus to last from give to 29
days (Perry, 1941). The pregnancy block, or implantation block, can also be
elicited by epinephrine injection in some strains of mice (De Fries, 1965).

The previous cogitations are speculative at best since the full effect
of castration upon the total endocrine environments of male rats is not known.
The adrenal, however, would provide a good point of origin for further study.
It would be of interest to know whether this delay in vaginal canalization is
augmented or diminished as a function of time after orchidectomy.

The relative failure of direct exposure of young, female rats to mature
male mice to elicit either an advance in vaginal canalization or first estrus
is of some interest. That female rats did not respond to odor stimuli eli­
cited by the mature mice gives further support to the observation that phero-
mones and odor stimuli are "species specific," at least in the case of exposure of male mice to female rats. Whether male rat odor is capable of advancing puberty in young female mice remains to be examined.
SUMMARY AND CONCLUSIONS

(A) The direct exposure of young, female rats to adult, intact male rats does not significantly advance vaginal canalization in the females but does significantly advance first vaginal estrus over that of isolated, control female animals. First vaginal estrus occurred at a mean of 35.9 days of age in contrast to a mean of 37.9 days of age in the isolated, control females. The predictability level was 0.05.

(B) The direct exposure of young, female rats to the urine of adult, intact male rats does not significantly advance vaginal canalization but does significantly advance first vaginal estrus over that of the isolated, control female animals. First vaginal estrus occurred at a mean of 35.5 days of age in contrast to a mean of 37.9 days of age in the isolated, control females. The predictability level was 0.02.

(C) The direct exposure of young, female rats to adult, orchidectomized rats neither advances vaginal canalization nor first estrus over that of isolated, control female animals. Rather, exposure to adult orchidectomized rats significantly delays the establishment of vaginal orifice. Canalization in exposed females occurred at a mean of 35.7 days of age in contrast to a mean of 34.2 days of age in isolated, control female animals. The level of predictability was 0.02.

(D) The direct exposure of young, female rats to adult, orchidectomized rats injected with 1.5 mg. of testosterone propionate, in sesame oil, failed to advance either vaginal canalization or first estrus over that of the isolated, control female animals. Rather, a delay in establishment of
the vaginal orifice was observed. Canalization in exposed females oc-
curred at a mean of 38.2 days in contrast to a mean of 34.2 days of age
in the isolated, control female animals. The level of predictability
was 0.001.

(E) The direct exposure of young, female rats to mature, intact male mice did
not significantly advance either vaginal canalization of first vaginal
estrus over that of isolated, control female animals.

This investigation gives some indication that young, female rats of the
Sprague-Dawley strain are considerably less sensitive to male sex odors than
are mice. The indices of pubertal onset established by Vandenberg (1967, 1969)
for female mice were identical in this study yet the first of these,
vaginal canalization, does not appear to be affected by the presence of mature
males or the urine of mature male animals. The second of these indices (first
estrus) is significantly accelerated by both direct exposure to male rats and
direct exposure to the urine of male rats.

Whether or not a new pheromone is involved in delaying the vaginal canal-
ization of female rats exposed to castrated male rats is only speculation, yet
the delay is significant.

The hypothesis that androgen-supported glands and androgens in the male
animal may be sources of sex odor is supported, at least in part, by this work
since the absence of testicular androgens in orchidectomized rats resulted in
a failure to accelerate first estrus. However, testosterone treatment alone
was not an effective substitute for the presence of gonads. Further, the
time interval between canalization and first estrus in all groups in which
intact males or testosterone-administered, orchidectomized rats were used as odor stimuli was relatively short. This would indicate that testicular androgens alone or as a pheromonal constituent are probably effective in initiating first estrus in exposed female rats. When testosterone was absent, the time interval in this context was at least 3.5 days. This represents nearly one full estrous cycle.

It would appear, therefore, that testicular androgens, specifically testosterone, is probably a necessary constituent of the pheromonal substance which activates the hypothalamic-pituitary-ovarian mechanism in female rats through olfactory pathways.
LITERATURE CITED


Bruce, H. M. 1960 A block to pregnancy in mice caused by the proximity of strange males. J. Reprod. Fertility, 1: 96-103.


Bruce, H. M. 1965 The effects of castration on the reproductive pheromones of male mice. J. Reprod. Fertility, 10: 141-143.


Lee, S. Van der and Boot, L. M. 1955 Spontaneous pseudopregnancy in mice. 

Lee, S. Van der and Boot, L. M. 1956 Spontaneous pseudopregnancy in mice II. 


PLATE I Typical diestrous vaginal smear stained with Giemsa.

PLATE II Typical proestrous vaginal smear stained with Giemsa.
PLATE III Typical estrous vaginal smear stained with Giemsa.

PLATE IV Typical metestrous vaginal smear stained with Giemsa.
PLATE V  Typical estrous vaginal smear with abundant spermatozoa. Giemsa stain.
INFLUENCE OF MALE URINE AND TESTOSTERON: ON VAGINAL CANALIZATION AND ESTROUS CYCLES

SUMMARY TABLE

<table>
<thead>
<tr>
<th>TREATMENT</th>
<th>n</th>
<th>CANALIZATION (DAYS)</th>
<th>P</th>
<th>S.D. ± S.E.</th>
<th>FIRST ESTRUS (DAYS)</th>
<th>P</th>
<th>S.D.</th>
<th>S.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Controls</td>
<td>(50)</td>
<td>34.20</td>
<td>-</td>
<td>1.92</td>
<td>37.94</td>
<td>-</td>
<td>3.44</td>
<td>.49</td>
</tr>
<tr>
<td>II. Urine Exposure</td>
<td>(25)</td>
<td>33.68</td>
<td>.40</td>
<td>2.42</td>
<td>35.56</td>
<td>.02</td>
<td>4.02</td>
<td>.80</td>
</tr>
<tr>
<td>III. Direct Exposure</td>
<td>(24)</td>
<td>34.51</td>
<td>.70</td>
<td>3.56</td>
<td>35.95</td>
<td>.05</td>
<td>3.59</td>
<td>.73</td>
</tr>
<tr>
<td>IV. Direct Exposure (Orchidectomized Rat)</td>
<td>(24)</td>
<td>35.71</td>
<td>.02</td>
<td>3.25</td>
<td>39.00</td>
<td>.40</td>
<td>(NS) 7.13</td>
<td>1.49</td>
</tr>
<tr>
<td>V. Direct Exposure (Orchidectomized Rat with Testosterone)</td>
<td>(25)</td>
<td>38.28</td>
<td>.001</td>
<td>3.68</td>
<td>39.33</td>
<td>.20</td>
<td>(NS) 3.79</td>
<td>.77</td>
</tr>
<tr>
<td>VI. Direct Exposure (Mature Male Mice)</td>
<td>(24)</td>
<td>35.00</td>
<td>.20</td>
<td>2.04</td>
<td>37.37</td>
<td>.60</td>
<td>(NS) 3.33</td>
<td>.68</td>
</tr>
</tbody>
</table>

n = number
P = Probability Values
S.D. = Standard Deviation
S.E. = Standard Error of the Mean
APPROVAL SHEET

The thesis submitted by Robert F. Locke Jr. has been read and approved by three members of the faculty of the Graduate School.

The final copies have been examined by the chairman of the thesis committee and the signature which appears below verifies the fact that any necessary changes have been incorporated, and that the thesis is now given final approval with reference to content, form and accuracy.

The thesis is therefore accepted in partial fulfillment of the requirements for the degree of Master of Science.

May 24, 1971
Date

[Signature of Advisor]