THE EMBRYONIC VIABILITY OF THE CONSTANT ESTRUS MOUSE

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ABSTRACT OF THESIS

THE EMBRYONIC VIABILITY OF THE CONSTANT ESTRUS MOUSE

This investigation was undertaken in an attempt to compare the litter size and sexual receptivity of the constant estrus mouse with the normal estrus mouse. Vaginal smears were taken from mice in constant light to determine when the mice were in estrus. When this condition persisted longer than 3 days the mice were considered in constant estrus. Female mice were also left in a normal light regime of 14 hr light and 10 hr dark. Mice in each category were super-ovulated with Pregnant Mare Serum (PMS) and Human Chorionic Gonadotrophin (HCG), while others were sacrificed and ova counted. Embryonic viability was determined from the average litter size and from the number of ova ovulated from each pair of ovaries.

No appreciable difference occurred in litter size in the constant estrus mice when compared with the same treatment in the normal estrus mice.

The incidence of breeding or sexual receptivity of the constant estrus mouse was approximately 66% as compared with the 25% in the normal cycling female mouse.

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Date

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INTRODUCTION

The mouse displays periodic cyclic changes in the reproductive tract with the onset of maturity. This phenomenon, which occurs many times a year, is termed the estrous cycle. Animals showing this phenomenon are referred to as polyestrous animals. Numerous follicles, at various stages of development, and corpora lutea from past cycles occur within the ovaries.

The mouse normally displays an estrous cycle six days in length (8,36,3). The stages of the estrous cycle in the rodent were first studied by Stockard and Papanicolaou (36) who observed cellular contents of the vagina. By observing the vaginal smears the stages of estrous could be depicted and the onset of heat determined.

Allen (3), first studied the estrous cycle in the mouse and described several phases within the normal estrous cycle. At the same time, Long and Evans (25) published a very thorough study of the rat.

The stage of the estrous cycle which is the onset of heat in the mouse can be accurately determined by the "copulatory response" (43,3). This stage termed estrus is an anabolic stage, and coincides with the greatest development of ovarian follicles (21). The duration of this stage is three days at the maximum (31,32,33,34,35).

The next stage in the sequence is metestrus, a catabolic stage characterized by degenerative changes in the genital tract (3,32,34,35) and a newly formed corpora lutea within the ovaries (29). The duration of this stage is one to five days (8,34,35).

Diestrus characterized by a period of quiescence or slow growth for two to four days (8,34,35) follows metestrus.

The final stage in this sequence is proestrus, an anabolic stage, with active growth in the genital tract (3,6,8, 34). The duration of proestrus is one to one-and-one-half days (34,35,8).

By subjecting the mouse to external forces, the whole physiological, psychological, and endocrine events of reproduction can be altered. If mice are exposed to constant light, the normal estrous cycle disappears within a short time after exposure and mice show what is called "continuous estrus" (29). No corpora lutea are present in the ovaries, but the follicles are extremely large. Ovulation does not occur due to a deficiency in LH (2), the uterine horns enlarged and the vagina shows constant cornification (29).

The purpose of this study is to test the embryonic viability of these ova that are prolonged in a mature state in the follicle. This may be done by testing sexual receptivity and the resulting litter size after a controlled period of time in constant light.

LITERATURE REVIEW

The study of the estrous cycle, began with the work of Stockard and Papanicolaou (36) in 1917. This study dealt with the histological and physiological changes in the reproductive cycle of the guinea pig. Kingery's (23) work in 1917 with oogenesis in the white mouse remains fairly accurate to date. Allen (3) pursuing these earlier accomplishments published his findings on the estrous cycle of the mouse in 1922. This was the first work on the mouse and to this date is considered the standard reference for the estrous cycle. Allen (3) points out that mice of different strains have distinct modes of cycle length. Long and Evans (25) following the same line, did a thorough study of the estrous cycle of the rat and its associated phenomena.

Brambell and Parkes (8,31,32) making a thorough study of the time element associated with the estrous cycle noted that ovulation takes place at the beginning of estrus. Allen (3), however, considered that ovulation in the mouse occurs at the end of estrus. Another important observation shows that the estrous stimulus is exerted about forty-eight hours before the onset of estrus. This implies that removal of the ovaries toward the end of diestrus does not interfere with the development of the next estrus.

Allen (3), Brambell (8), and Parkes (31,32) worked out the following time schedule for the phases of the average estrous cycle: proestrus (18 hr), estrus (42 hr), metestrus (12 hr), and diestrus (3 days). These values represent (to the nearest half-day) those found for 1000 cycles in unmated normal mice.

Exposure of female mice to constant light causes them to enter periods of prolonged estrus, as is indicated by large numbers of keratinized scales in the vaginal smears (9,16,17,39,40,10). This response normally depends in large measure upon the eyes as light receptors (20,18, 37,24). If rats are blinded and small glass fibers are implanted into regions of the hypothalamus, the opposite ends of the fibers projecting above the skull surface, exposure to light induces constant estrus (24). The work with light and its relation to estrous rhythms first began in the late thirties with the largest amount of work occurring during the early sixies. Fiske (18). in 1941 showed that light falling directly upon hypothalamic neurons can evoke the release of pituitary gonadotrophins which produce functional changes in the gonads. Lisk (4), in 1964 said that light appears to act upon the same areas of the hypothalamus which have been shown to respond to implanted sex steriods and influence the estrous cycle and behavior of the rat. Following the earlier work, Wurtman,

Axelrod, and Phillips (41) discovered that the pineal synthesizes melatonin in the absence of light. Chu, Wurtman, and Axelrod (10) in 1964, hypothesized that melatonin, a specific product of the pineal gland, exerts an inhibitory effect upon gonad functions. According to Fiske, Bryant, and Putman (17), the increased ovarian weight and constnat estrus produced by continuous illumination might be a consequence of the reduced output of melatonin by the pineal under these conditions. Biochemical studies in 1960-63 showed that melatonin, found only in the pineal gland, is synthesized from sertonin through the catalytic action of hydroxyindole-O-methyl transferase (HIOMT). Although sertonin is found in many tissues, HIOMT is present only in the pineal gland (17,41). Light perceived by the retina is believed to set up impulses which travel through unknown pathways to the superior cervical ganglia and then the pineal gland. The discharge of impulses in the pineal gland alters the rate of synthesis and release of melatonin. The site of melatonin action is not known; it might act directly on the gonads or indirectly through the central nervous system and anterior hypophysis (16,40). Fiske, Bryant, and Putman (17) showed in 1960 that rats maintained in constant light have small pineals, and these contain much less HIOMT and less melatonin than those kept in darkness. Furthermore, Whitten (37) in 1956, showed

that after destruction of the retina, photoperiods have no effect on the pineal enzymes and the quantities of melatonin synthesized. The participation of the pineal gland in the mediation of light stimuli is a fruitful area of investigation. Although the majority of work has been carried out with the rat, the clarification of certain aspects of the theory can be related to the mouse. It seems justifiable to extend these observations to a wider variety of vertebrates. This reasoning is an important aspect of this study.

The viability of the mouse embryo studied in 1926, by MacDowell and Lord (26) who attempted to correct the assumption that the maleness increases the chances of foetal death. The data came from litters with no fewer individuals than the corresponding corpora lutea of pregnancy; hence litters that would reveal the primary sex ratio directly. The results indicated a 1:1 ratio. MacDowell and Lord (26), found an average of seven mature ova or corpora lutea per ovulation, Allen (3), 9.2. Brambell and Parkes (8), found that the average number of maturing follicles in each pair of ovaries is approximately 8.75. This figure is roughly in agreement with the observed mean size of litter at birth (31). Falconer (15) makes the statement that litter size corresponds almost directly to the number of corpora lutea, thus assuming the ova ovulated

are almost always fertilized. Danforth and DeAberle's (11) study in 1928 of the functional interrelation of the ovaries as indicated by the distribution of foetuses in the mouse uteri showed that for litters of any particular size, the foetuses show a mathematically random distribution between the two horns of the uterus. From this it is assumed that each foetus has equal chance for viability. Falconer (15) exerts that complexity arises mainly from the fact that the character belongs partly to the parental generation and partly to the filial generation; that is to say, the number of young born in a litter depends partly on the fertility of the parents-chiefly the female- and partly on the viability of the embryos that will constitute the litter. Falconer, et al (15), noted that natural estrous mice shed approximately 10.32 eggs per set of ovaries, and superovulators approximately 12.87 eggs. There was a negative correlation in the eggs shed by the two ovaries after natural ovulation but positive after superovulation. From this comprehensive study the normal litter size can be inferred to range from 8 to 10 young.

It is important to consider the viability of the foetuses during the gestation period. Most of this work was done in 1963, by McLaren and Michie (27). They asserted that the heavier young were associated with a shorter gestation period (12,27). Biggers, et al, (5), state that litter size effect operates systemically rather than locally; it is

unaffected by the distribution of implanted foetuses between uterine horns, and is not an expression of intra-uterine crowding.

Wiesner and Mirskaia (38) were the first to consider mating behavior on an endocrine basis. Young (42,43) states that mating results as a "copulatory response" during the onset of heat.

Most of the material presented at this time deals with the animal under normal conditions. It can now be surmised what effect will pursue a prolonged continuous factor. The essence of this study is the introduction of a continuous light source. Everett (13) in 1939 launched an investigation of the "continuous estrus" phenomenon in the albino rat under a continuous light source. He noted persistent ovarian follicles and accompanying vaginal cornification, but breeding performance was irregular because of delayed and difficult parturition. As a result, litter size was reduced and the few young born were oversized. It appears that in rats in constant estrus, regardless of how this condition is induced, the breakdown of cyclic reproductive activity occurs because insufficient ovulation-inducing hormone is stored in the pituitary gland (4,16,19).

It may be noted that the identical condition of constant estrus can be produced in at least two other ways., One is to place electrolytic lesions in the anterior region of

the hypothalamus; the other is to administer to prepubertal female rats a single dose of steriod hormone (7,29).

Adler and Bell (2) explored sexual receptivity in constant estrus rats, however their data is not statistically significant for they used only five rats. They concluded that the constant estrus females revealed a disjunction between lordosis and receptivity. Hardy (22) did a more thorough study by comparing animals in a cycling light regime with those in constant light. Normal cycling rats were sexually receptive only one-fourth the time. In the constant estrus rats the sexual receptivity increases with the duration of continuous light.

Since very little if any research has been carried out on the "constant estrus" mouse, the present was designed to explore this area in an attempt to determine means of increasing embryonic viability and the sexual receptivity of laboratory mice.

MATERIALS AND METHODS

Constant Estrus Induction

The constant estrus phenomenon was induced in mice by placing them under a continuous light source. The mice were placed in a plastic laboratory cage with a wire top and an influorescent light was placed approximately one foot over the cage for approximately 30 to 48 days, depending on the particular experiment. Thirty days was set as a standard laboratory length for the light period in the majority of these experiments.

Normal estrus which has a duration of 1 to 3 days (31, 32,33,34,35), is defined as the onset of "heat" in animals. Constant estrus can be defined as "heat" lasting longer than 3 days (33).

Vaginal Smear Technique

The phases of the estrous cycle can best be determined by the vaginal smear. Vaginal smears are best obtained by means of an ordinary pipette, the tip of which has been flamed to a smooth, reduced aperture. A few drops of 0.9% sodium chloride solution is drawn into the pipette. The fluid is transferred to a slide and mounted under a coverslip with a trace of methylene blue to add contrast and

bring out the nuclei. Examination for cell type is carried out under low and then high power magnification, with reduced lighting. This entire technique was adapted from Rugh (34) in his description of the estrous cycle.

Nucleated or cornified epithelial cells and leukocytes can be detected in the vaginal smears. The nucleated epithelial cells are generally oval with obvious nuclei. The cornified epithelial cells are very thin and folded.

As estrus approaches, some mucous may also be present (30), and the cellular type is exclusively cornified epithelial cells. The phases through which the vaginal cells pass represent parallel changes in the entire reproductive tract and are hence diagnostic.

Sexual Receptivity Determination

Sexual receptivity may be defined as the acceptance of the male by the female during the phase of the estrous cycle known as estrus. Fertilization generally occurs when the female is placed with the male during this phase. Falconer (15) states that if the females are with the males at the time of ovulation, fertilization takes place in about a one to one ratio. Using this inference, a mouse that is sexually receptive will be in estrus and therefore capable of mating with the male. Percentage sexual receptivity may be determined from the number of females

mated when placed in a cage with males (Tables 2,3).

If a female mouse is in constant estrus, ovulation does not take place unless cervical stimulation or exogenous LH is supplied (29). By referring to Table 2, section H, notice that exogenous HCG was injected only to insure an accurate egg count. In every other case of constant estrus, ovulation occurred due to cervical stimulation.

Litter Size Determination

Litter size is referred to as the average number of young born to a set number of females. Only those females that gave birth are considered in this calculation (Tables 2,3).

Litter size can also be determined by the number of ova ovulated from the ovary, and counted when flushed from the oviduct if it is assumed that all ova ovulated will be fertilized.

Superovulation

During the last few years, superovulation has been ideal for providing demonstration or teaching material of ova. In practice, a female is injected intraperitoneally with 5 international units (I.U.) of follicle stimulating pregnant mare serum (PMS) and 44 to 50 hr later with 5 I.U. of human chorionic gonadotrophin hormone (HCG) (34).

Falconer, et al (14), showed that superovulation was used as a basis for increasing litter size. Superovulation was thus used in this research to increase litter size.

Ovum Recovery

The mice from which ova counts were made, were sacrificed by an overdose of Nembutal anesthesia. A midventral incision was made to expose the reproductive tract. Oviducts were removed by cutting the bursa at the ovarian ends and the uterotubal junction at the uterine ends of the oviducts. The oviducts were placed in a watch glass containing saline solution. By inserting a blunted 30 gauge needle into the fimbrial end of the oviduct, flushing of the oviducts was accomplished. The number of ova then counted by placing the watch glass under a stereoscope.

RESULTS

The experimental data obtained in this study are summarized in Tables 1,2,3, and 4 to show the relation between the normal estrous cycle and the phenomenon known as "constant estrus". These two categories are compared on the basis of sexual receptivity and litter size.

This study is designed as nine problems with sexual receptivity and litter size being compared in each.

Problem A. Female mice were subjected to constant light over an extended amount of time (48 days). At the end of this time they were placed with males for 24 hr to determine the litter size and sexual receptivity.

Fifteen mice started the experiment, but before the close of the light treatment, 2 of the mice died. Of the 13 mice left, 6 gave birth to a total of 54 young. The sexual receptivity is approximately 46.153% and the average litter size is 9 young.

Problem B. Female mice were subjected to constant light over a period of approximately 30 days. (Time period was based on the assumption that this would generally be the longest period that mice would be kept like this in the laboratory.) Vaginal smears were taken daily, approx-mately 90% of the mice displayed a state of constant estrus at the end of 14 days. They were placed with males

for 24 hr and 12 of the 20 mice gave birth to 117 young. The sexual receptivity is approximately 60.0% and the average litter size is 9.75 young.

Problem C. Female mice subjected to constant light over an indefinite period of time. Mice were placed in categories of 4 to 8 days depending on the time duration of each phase of estrus. This duration was determined by vaginal smears. These daily vaginal smears presented a problem of cervical stimulation causes nervous impulses to act upon the hypothalamus, which releases neurohumoral substances that are carried via the pituitary portal system to the anterior pituitary. Here they are instrumental in releasing luteotrophic hormone, which is essential to the activation of corpora lutea (1). Pseudopregnancy lasts about half the time of normal pregnancy. The mouse then returns to the normal estrous cycle. Vaginal smears were taken daily on 20 mice, all but 10 appeared pseudopregnant after a period of three weeks, and these were placed with males.

None of the 10 mice from the 4 to 8 day sequence mated, but this was not attributed to a deficiency in the mice. The conclusion was that they were all pseudopregnant, although not appearing so at the time of the last vaginal smear.

Problem D. Subject female mice to a period of constant

light for a period of 30 days, superovulate with PMS and HCG hormone sequence. Put the females with the males for 24 hr. Determine the incidence of breeding and litter size. The litter size was expected to increase, due to earlier work with superovulation (14). Eight of the 16 females bred with a combined litter size of 88 young. The sexual receptivity is 50.0% and the average litter size is 11 young.

Problem E. Control: Subject females to males that have been under a normal light regime. The purpose was to determine litter size only. The females were left with the males for 10 days, to ensure breeding. Of the 14 females used, 10 conceived and produced a total of 84 young. The average litter size was 8.4 young.

Problem F. Control: Subject female mice to males; that have been under a normal light regime, for a period of 24 hr to determine incident of breeding and litter size. Only 5 of 20 females used were bred, however this is normal considering normal estrus occurs about 25% of the time. The combined litter size was 48. The sexual receptivity is 25.0% and the average litter size is 9.6 young.

Problem G. Control: Female mice that have undergone a normal light regime are superovulated with PMS and HCG hormone sequence. They were placed with males for 24 hr. Incident of breeding and litter size were determined.

This problem was a control to Problem D. Ten females were used, and 6 bred with a total litter size of 69. The sexual receptivity was 60.0% and the average litter size was 11.5 young.

Problem H. Twenty female mice were placed under continuous light for a period of 30 days. At the end of this period, they were injected with exogenous HCG. They were then placed with males for a period of 24 hr. Thirty-six to 48 hr later the mice were sacrificed and the oviducts flushed and an ova count made. The purpose being to determine if any ova being ovulated and not fertilized. The assumption was made that the number of ova present would equal litter size. All females in this group ovulated and produced a total of 179 ova. The sexual receptivity therefore was 100.0% and the average litter size 8.75.

Problem I. Female mice were kept under a normal light regime. Forty females were placed with males and checked for vaginal plugs, and were sacrificed 36 to 48 hr later and the oviducts flushed and ova counted. The ova count was 91, sexual receptivity was 25.0% and the average litter size was 9.1.

Table 1. Generalized scheme for entire study

Number of mice	Time in continuous light	Normal light regime	Hormone treatment	Incident of breeding	Combined litter size
A. 13	48 days*	-	-	6/13	54
B. 20	30 days**	-	-	12/20	117
C. 10	varied***	-	-	0/10	0
D. 16	30 days	-	PMS,HCG	8/16	88
E. 14	-	cycling	-	10/14****	84
F. 20	-	cycling	-	5/20	48
G. 10	-	cycling	PMS,HCG	6/10	69
H. 20	30 days	-	HCG	*****	179 ova
I. 40	-	cycling	-	10/40*****	91 ova

* 48 days being considered the longest time an animal would be in light.
** 30 days were set as a standard for the laboratory.
*** 4 to 8 days duration in constant estrus, with mice in each category.
**** These mice were with males 10 days, the other categories 24 hr.
***** The mice were sacrificed and oviducts flushed for ova count.

Table 2. The constant estrus phenomenon in relation to sexual receptivity and litter size

Number of mice	Number of mice bred	% showing sexual receptivity	combined litter size	young per litter	other comments
A. 13	6	46.153%	54	9	extend light***
B. 20	12	60.0%	117	9.75	-
C. 10**	-	-	-	-	pseudopregnant
D. 16	8	50.0%	88	11	superovulated
H. 20	20	100.0%	179	8.75	HCG****
Totals: 69	46	66.66%	438	9.5217	-

* Sexual receptivity being based on the assumption, that when subjected to males those in estrus would breed.

** These mice were pseudopregnant, due to frequent vaginal smears and were disregarded in the total because fertilization was impossible.

*** Prolonged period of light (48 days).

**** HCG was injected to insure ovulation, here 100.0% sexual receptivity meant that every oviduct flushed had ova. Fertilization was not necessary.

Table 3. The normal light regime in relation to sexual receptivity and litter size

Number of mice	Number of mice bred	% showing sexual receptivity	combined litter size	young per litter	other comments
E. 14	10	71.428%	84	8.4	*
F. 20	5	25.0%	48	9.6	**
G. 10	6	60.0%	69	11.5	superovulated
I. 40	10	25.0%	91	9.1	***
Totals: 84	31	30.0%****	292	9.419	-

* The females were left with males 10 days in order to determine litter size, can not be used in sexual receptivity due to extended time.

** All the results obtained in this section were expected beforehand.

*** The mice were sacrificed and the oviducts flushed and ova counted.

**** Total for sexual receptivity can only be considered in three categories, due to a varied time limit.

Table 4. Comparison of the totals of the normal light regime and the constant estrus phenomenon

	constant estrus	normal light regime
% sexual receptivity	66.66%	30.0%
young per litter	9.5217	9.4193

Difference in sexual receptivity:

constant estrus	66.66%
normal regime	-30.00%
	36.66%

Difference in young per litter:

constant estrus	9.5217
normal regime	-9.4193
	0.1024

Conclusion: Constant estrus increases sexual receptivity and there is no appreciable difference in young per litter.

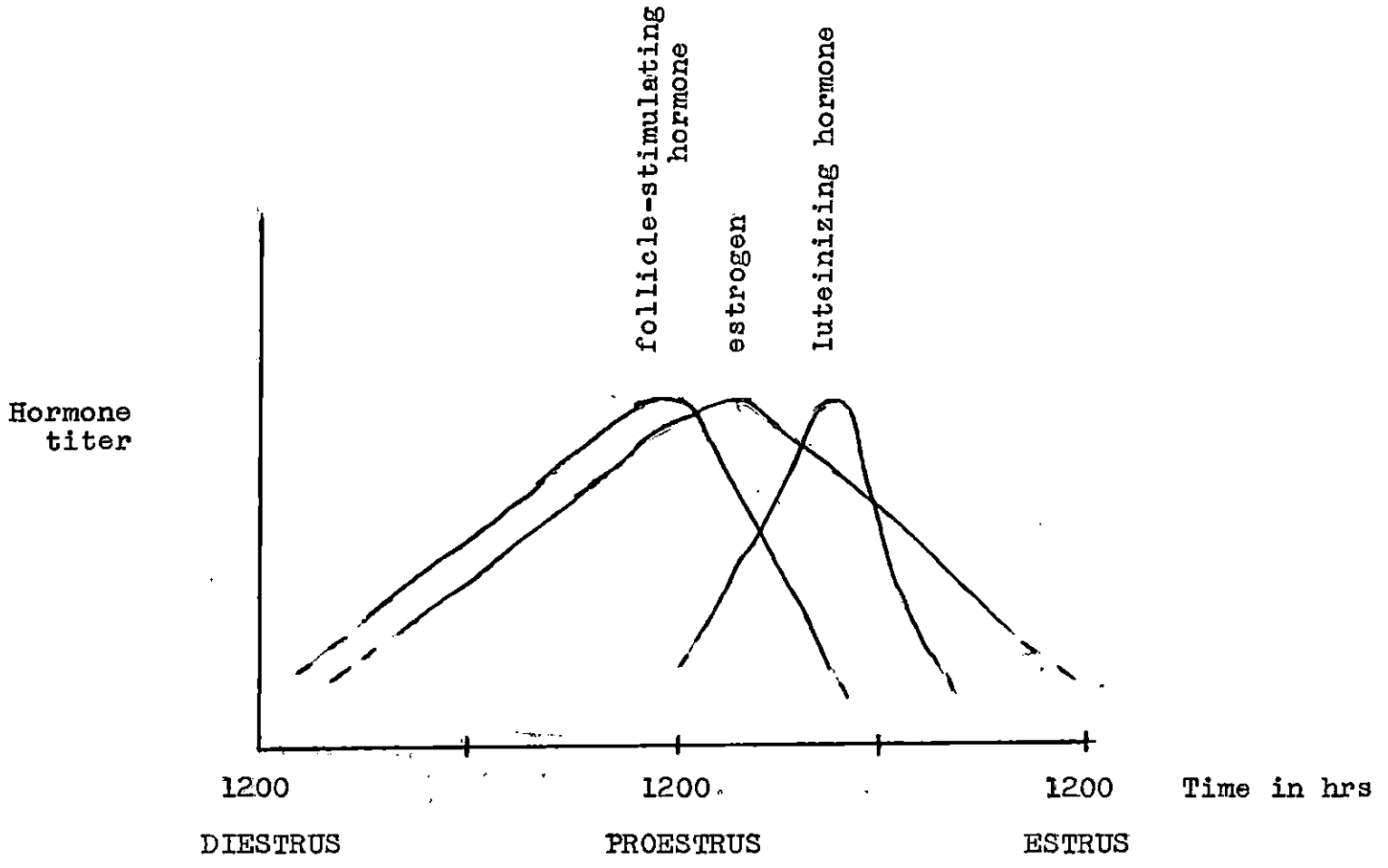

Fig. 1. Hormone titers during the diestrus to estrus sequence in the normal estrous cycle

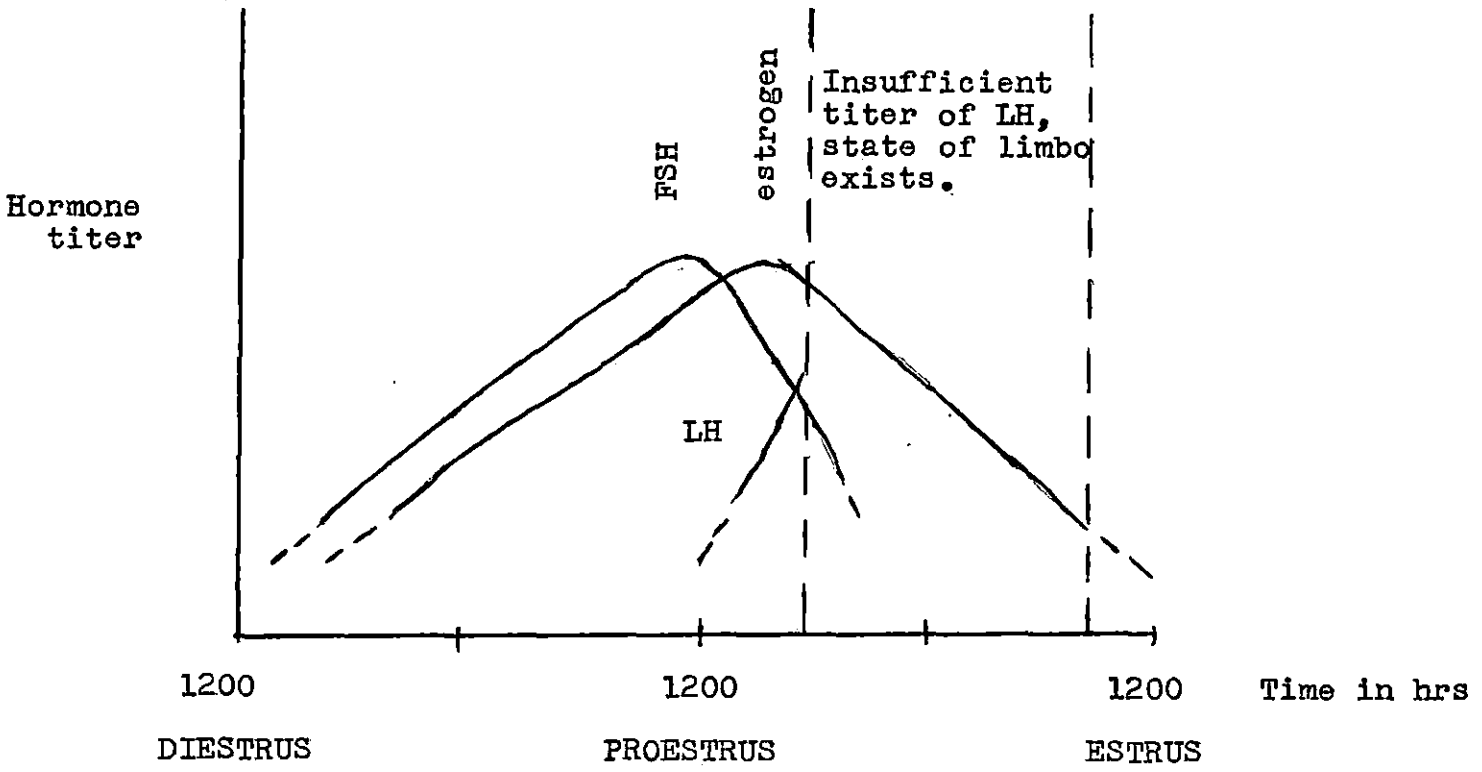

Fig. 2. Hormone titers during the diestrus to estrus sequence in the constant estrus mouse

DISCUSSION

The purpose of this study was to compare the embryonic viability of the constant estrus mouse with that of the normal estrus mouse. It was determined that this could best be accomplished by calculating the average litter size in each category. During the course of this study there appeared a definite increase in sexual receptivity. This was determined on the basis of the percentage of females that mated when subjected to males.

Nalbandov (29) has described the hormone sequence during the normal estrous cycle. An attempt was made to illustrate this sequance in Fig. 1. According to Nalbandov (29), FSH starts rising 1500 hr of diestrus and acts to inhibit FSH. The contraversial hormone in this study; LH, peaks during early estrus thus causing ovulation. During the constant estrus phenomenon the follicles are extremely large, the uteri enlarged and the vagina show constant cornification (29). This leads to the inference in Fig. 2 that LH is all that is inhibited. This being the case, all the ova that had recieved the inital FSH response would remain in a state of limbo during the phase of continuous estrus. It seems probable to assume that this prolonged state may present

a problem of viability to these particular ova. However, from the results of this investigation it is indicated that constant light has no effect on litter size. Next, the problem arises that perhaps a large number of ova had matured due to this stimulus and only a percentage were capable of fertilization and development. This problem was resolved by sacrificing the females and counting the ova that were ovulated and comparing with the control. This was the purpose of Problem H and I, Tables 1,2, and 3. It was shown that there was no appreciable differnce in the number ovulated. For a differnce to be recognized, the assumption must be made that FSH is secreted at a continuous high titer. But it must be pointed out here that the mice in Problem H had been in constant light for various length of time and showed no relative difference from day to day. Thus it may be inferred that Fig. 1 and 2 are correct unless another method is devised.

It may also be pointed out that vaginal smears were taken with extreme care, and neglected whenever possible. Acker (1) proved conclusively that taking vaginal smears induces pseudopregnancy. The lack of sexual receptivity of the females in Problem C was probably the result of pseudopregnancy.

Mice were injected with exogenous HCG to induce ovulation, and superovulation to increase litter size.

Superovulation plus constant estrus increases litter size as well as sexual receptivity.

Constant estrus mice (B Table 2) have a marked increase in sexual receptivity over the controls (F Table 2), but litter size is relatively the same in both. Comparing Problem D and G, superovulation shows very little difference in the two categories. Problems H and I show a marked difference in sexual receptivity, but not in litter size.

In Table 2, Problem C and Problem E in Table 3, were not included in the totals for only those females subjected to males for 24 hr were considered. Superovulation was not disregarded for it was used in both categories. From the results summarized in Table 4, it may be concluded that constant estrus increases sexual receptivity as much as 36.66% with relatively no change in litter size and does not alter the viability of the embryo. In addition, the constant estrus phenomenon has certain qualities that can be utilized in the laboratory for increasing the incidence of breeding.

SUMMARY

The object of the study was to compare the embryonic viability of the constant estrus mouse with the normally cycling estrus mouse. Constant estrus was defined as the duration of "heat" lasting longer than three days. This was detected by the use of vaginal smears.

Testing of embryonic viability was done in two phases: (1) counting the ova ovulated from the ovary, (2) counting the number of young per litter.

Sexual receptivity was considered as a method of improving laboratory breeding procedures.

The results were that constant estrus had no effect on embryonic viability. However, sexual receptivity was increased sufficiently to warrant adoption in the laboratory.

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