SUPPRESSION OF OESTRUS IN GROUPED MICE: THE EFFECTS OF VARIOUS DENSITIES AND THE POSSIBLE NATURE OF THE STIMULUS

A. K. CHAMPLIN*

Department of Biology, The University of Rochester, Rochester, N.Y., U.S.A.

(Received 23rd November 1970)

Summary. The effects of various degrees of grouping on the oestrous cycle of C57BL/6 and BALB/Ci mice were examined. The results support the findings of others which have shown that suppression of oestrous occurs as a result of female grouping and indicate that oestrus can be suppressed by housing females even in pairs. Strain differences were found in the oestrous response to grouping and in the density at which maximum suppression occurred. In both strains, the suppression took the form of a relative lengthening of the dioestrous phase of the cycle. A second study revealed that the effects of grouping on the oestrous cycle could be imitated by housing single females in cages recently soiled by groups of females, and suggested that a pheromone may be the stimulus for suppression.

INTRODUCTION

Stimuli from the social environment have been shown to influence both the frequency and composition of the oestrous cycle in the mouse (Whitten, 1966, 1969; Bronson, 1968; Whitten & Bronson, 1970). While the presence of a male appears to accelerate the oestrous cycle, an all-female environment suppresses the cycle. This suppression was first reported by Andervont (1944) and has since been examined by a number of investigators who have used several strains of inbred mice as well as randomly bred mice, and a diversity of caging conditions and group sizes. It is not surprising, therefore, that the reported effects of female grouping on the oestrous cycle have varied from an extension of the cycle length (Lamond, 1958, 1959) to the induction of a spontaneous pseudopregnant condition (van der Lee & Boot, 1955, 1956; Dewar, 1959; Mody, 1963) to the occurrence of anoestrus during the entire experimental period (Whitten, 1959). Because of these inconsistencies, one of the objectives of the present study was to examine, in a systematic manner, the effects of several degrees of female grouping on the length and composition of the oestrous cycle in two genetically unrelated strains of inbred mice.

The nature of the stimulus affecting the suppression is unknown. Other effects

* Present address: Department of Biology, Colby College, Waterville, Maine, U.S.A.

of the social environment on reproduction in mice have been attributed to the involvement of pheromones (see reviews cited above). There is, however, no evidence for a pheromonal basis for the suppression of oestrous which occurs in grouped females, although it has been suggested that such substances may be involved (Whitten, 1966; Bronson, 1967). This suggestion is partially supported by the finding of Whitten (1959) that the suppression stimulus is probably not mediated through visual, auditory or tactile sensations. However, Dewar (1959) has proposed that the 'extra-coital' pseudopregnancy which he observed among grouped females may have been induced by the male-like chasing and mounting behaviours which are occasionally observed among grouped females. Therefore, the second section of the present study was designed as an examination into the possible existence of a suppression pheromone and as a test of the suggestion that suppression of oestrous is induced by male-like mounting behaviour.

MATERIALS AND METHODS

Subjects and facilities

Virgin C57BL/6 and BALB/Ci female mice were used in both parts of the investigation. The C57BL/6 females were derived from brother $\times$ sister matings of the descendants of C57BL/6J mice obtained from the Jackson Laboratory. The animals of both strains were bred and housed in $7 \times 11 \times 5$ in. clear plastic cages with straight wire tops. Litters were weaned at 25 to 30 days of age and the females were housed in groups of four until used for experimentation.

The room housing the breeding colony adjoined the experimental room which never contained any males. The two rooms were ventilated in a manner which generally permitted a one-way flow of air from the experimental room to the breeding room. Both rooms were illuminated on a 12 hr light/12 hr dark schedule, with the lights off between 01.00 and 13.00 hours.

The effects of various numbers of females

The 120 female subjects from each strain ranged in age from 68 to 104 days at the start of the experiment, at which time they were placed in equal numbers into five housing conditions: one, two, four, six or eight females per cage. They were put into previously unused, clean cages and transferred to the experimental room, where they remained undisturbed for 15 days except for weekly changes of cage bedding. Littermates were distributed, where possible, among the five housing conditions.

Following the 15 days allowed for adaptation, a vaginal smear was taken daily between 13.00 and 18.00 hours from each female for 28 days by the lavage method (Snell, 1941). From the types and relative concentrations of cells in the smears, three parameters were determined for each female: the number of complete oestrous cycles during the 28-day period, the length of each cycle and the relative lengths of the cycle components. The latter was determined by counting the total number of days that each animal spent in the combined pro-oestrous-oestrous-metoeestrous phase and in dioestrus, and comparing the five housing conditions on each of these measures. This is actually a measure of the likelihood that the experimenter would find an animal in each of these

phases when a vaginal smear was taken, and reflects the proportion of the total cycle represented by each of the two components.

An examination into the nature of the stimulus

Twelve females of each strain were used. The C57BL animals were between 147 and 156 days of age and the BALB females between 93 and 96 days of age at the start of the experiment.

In separate experiments, the females from each strain were divided into two groups of equal size. The experimental females in each strain were placed individually into cages in which adult females of the same strain had been housed for 4 days. These were clear plastic cages with Ab-Sorb-Dri bedding (Ab-Sorb-Dri, Inc., Garfield, N.J.) which had never housed males, and which had been soiled by four females in the experiment with the C57BL mice and six females in the experiment with the BALB mice. The control females in each strain were placed individually into clean, unused cages of the same type.

The soiled cages were replaced every 2 days by cages which had been soiled during the previous 4 days. This was done in such a manner that each experimental female received a cage soiled by each group of four or six females with equal frequency for the duration of the experiment. At the same time, the control cages were emptied of bedding and then refilled with fresh material.

The experiment with the C57BL females was begun in March. Each animal was placed in the appropriate housing condition and, after a 10-day adaptation period, vaginal smears were taken daily between 13.00 and 18.00 hours for 28 days. Four days later, the experimental animals were placed in clean cages, the control animals in soiled cages, and after 10 days, the vaginal smear observations were repeated.

The experiment with the BALB females was designed in essentially the same manner. It was begun in August, and the interval between housing in soiled or clean cages and the start of vaginal smears was 15 days rather than 10 days.

Data analysis

The data were subjected to analyses of variance and, where appropriate, were analysed by *t* tests and by regression. The 5% level of probability was accepted as significant in all cases.

RESULTS

The effects of various numbers of females

An analysis of variance of the data presented in Table 1 showed that housing condition had a significant effect on the number of complete oestrous cycles during the 28-day experimental period. There was also a significant effect of strain, and a significant interaction of housing condition and strain.

The oestrous cycle frequency of the C57BL females decreased with increasing cage density to the four per cage level, after which no significant differences were found. The BALB females showed a similar decrease in cycle frequency to about the level of six females per cage. The variance in cycle frequency was less in both strains for singly caged females than for any of the grouped conditions,

TABLE 1

EFFECT OF DIFFERENT DEGREES OF GROUPING OF FEMALE MICE ON THE NUMBER OF COMPLETE OESTROUS CYCLES IN 28 DAYS

Strain	No. of mice /cage	N	Mean no. of cycles $\pm$ S.E.	Two-tailed P values			
				2	4	6	8
C57BL/6	1	23*	4.43 $\pm$ 0.164	<0.01	<0.01	<0.01	<0.01
	2	24	3.46 $\pm$ 0.208		<0.01	<0.01	<0.01
	4	24	2.21 $\pm$ 0.217			N.S.	N.S.
	6	24	2.54 $\pm$ 0.248				N.S.
	8	24	2.00 $\pm$ 0.217				
BALB/Ci	1	24	3.92 $\pm$ 0.119	<0.01	<0.01	<0.01	<0.01
	2	24	2.96 $\pm$ 0.185		N.S.	<0.01	<0.01
	4	24	2.54 $\pm$ 0.190			<0.01	N.S.
	6	24	1.79 $\pm$ 0.170				N.S.
	8	24	2.13 $\pm$ 0.163				

N.S. means $P > 0.05$.

* One female, abnormal in size and cycling behaviour, was excluded from the data.

Analysis of variance

Housing condition: d.f. = 4/229; F = 42.80; $P < 0.001$
 Strain: d.f. = 1/229; F = 4.10; $P < 0.05$
 Interaction: d.f. = 4/229; F = 3.00; $P < 0.025$

TABLE 2

EFFECT OF DIFFERENT DEGREES OF GROUPING OF FEMALE MICE ON OESTROUS CYCLE COMPOSITION

	Strain	No. of mice /cage	Mean total no. of days $\pm$ S.E.	Two-tailed P values			
				2	4	6	8
Pro-oestrus-oestrus-metoeustrus	C57BL/6	1	20.83 $\pm$ 0.504	N.S.	<0.01	<0.01	<0.01
		2	19.71 $\pm$ 0.708		<0.01	<0.01	<0.01
		4	15.29 $\pm$ 1.14			N.S.	N.S.
		6	16.50 $\pm$ 0.882				N.S.
		8	14.33 $\pm$ 1.22				
Dioestrus	C57BL/6	1	7.17 $\pm$ 0.504	N.S.	<0.01	<0.01	<0.01
		2	8.29 $\pm$ 0.708		<0.01	<0.01	<0.01
		4	12.71 $\pm$ 1.14			N.S.	N.S.
		6	11.50 $\pm$ 0.882				N.S.
		8	13.67 $\pm$ 1.22				
Pro-oestrus-oestrus-metoeustrus	BALB/Ci	1	17.21 $\pm$ 0.345	<0.01	<0.01	<0.01	<0.01
		2	14.75 $\pm$ 0.596		N.S.	0.01	N.S.
		4	14.08 $\pm$ 0.704			N.S.	N.S.
		6	12.12 $\pm$ 0.702				<0.05
		8	14.08 $\pm$ 0.580				
Dioestrus	BALB/Ci	1	10.79 $\pm$ 0.345	<0.01	<0.01	<0.01	<0.01
		2	13.25 $\pm$ 0.596		N.S.	0.01	N.S.
		4	13.92 $\pm$ 0.704			N.S.	N.S.
		6	15.88 $\pm$ 0.702				<0.05
		8	13.92 $\pm$ 0.580				

Sample size for each housing condition is same as in Table 1.

Analyses of variance for housing condition

C57BL (Both cycle components) d.f. = 4/114; F = 8.95; $P < 0.005$
 BALB/Ci (Both cycle components) d.f. = 4/115; F = 9.28; $P < 0.005$

indicating that group housing affected the oestrous cycle of only some of the females.

An examination of the distribution of cycle lengths for each housing condition (Text-figs. 1 and 2) shows a major peak at 5 to 6 days in each case for the C57BL females and a peak at 5 to 8 days for the BALB animals which appears to shift from 5 days toward 8 days as the number of females per cage

TEXT-FIG. 1. C57BL/6 cycle length distribution by caging conditions.

increases. All degrees of grouping result in a wider distribution of cycle lengths than is seen in the singly caged animals, giving a further demonstration of the greater variability of the grouped animals in their oestrous response. There is an indication of a small secondary peak at 9 to 12 days in the distribution for each of the C57BL grouped conditions and one at 13 days for the BALB females housed six and eight per cage. However, the size of these peaks and the number of cycles involved makes their reality rather doubtful.

The effects of different degrees of grouping on the combined pro-oestrous-oestrous-metoeostrous and the dioestrous components of the cycle are shown in

Table 2. Analyses of variance showed that housing condition had a significant effect on the relative duration of both components in both strains. Paired group comparisons within each strain demonstrate that the decrease in cycle frequency which accompanies grouping is closely paralleled, and can probably be accounted for, by an increase in the relative length of the dioestrous component of the cycle.

TEXT-FIG. 2. BALB/Ci cycle length distribution by caging conditions.

Regression equations of cycle length on density, log density and density + log density were computed using the data obtained from both strains. In each case, the slope of the regression line was positive and significantly different from zero ($P \leq 0.025$). However, the value of the coefficient of determination (r^2) for each regression equation revealed that only about 17% and 25% of the variability in the C57BL and BALB cycle length data, respectively, was attributable to the independent variable, density. The values for r^2 varied only slightly in both strains whether the regression equation took the form of cycle length on density,

log density or density+log density (r^2 for C57BL = 0.1496–0.1767; r^2 for BALB = 0.2363–0.2679). It therefore appears that cycle length in these two strains is not very predictable on the basis of density. The high degree of variability in cycle length (Text-figs. 1 and 2), particularly under grouped conditions, is no doubt responsible for this lack of predictability.

An examination into the nature of the stimulus

The number of cycles observed for each animal in clean and soiled cages was

TABLE 3
NUMBER OF COMPLETE OESTROUS CYCLES OCCURRING IN 28 DAYS IN INDIVIDUAL C57BL AND BALB/Ci FEMALES IN CLEAN AND SOILED CAGES

C57BL/6			BALB/Ci			
Clean	Soiled		Clean	Soiled		
First	Second	Δ	First	Second	Δ	
5	1	-4	4	4	0	
4	5	+1	5	5	0	
3	3	0	5	3	-2	
4	3	-1	4	2	-2	
4	2	-2	3	4	+1	
6	4	-2	4	3	-1	
Mean ₁	4.3	3.0	-1.3	4.2	3.5	-0.7
Second	First	Δ	Second	First	Δ	
2	1	-1	4	4	0	
6	3	-3	4	3	-1	
1	0	-1	5	4	-1	
4	4	0	5	3	-2	
4	3	-1	4	4	0	
5	3	-2	4	3	-1	
Mean ₂	3.7	2.3	-1.3	4.3	3.5	-0.8
Mean ₁₊₂	4.0	2.7	-1.3	4.3	3.5	-0.8

Analyses of variance

	C57BL/6			BALB/Ci		
	d.f.	F	P	d.f.	F	P
Soiled versus clean	1/10	10.32	<0.01	1/10	6.86	<0.05
Inter-animal	11/10	3.20	<0.05	11/10	1.16	N.S.
Cage order	1/10	0.00	N.S.	1/10	0.09	N.S.

recorded and is presented in Table 3. Analyses of variance showed that both strains had significantly fewer cycles when housed in soiled cages than when housed in clean cages. The analyses also revealed that there was a significant amount of inter-animal variation in the C57BL females, but not in the BALB females. No significant difference was found for either strain, whether the animals were first housed in clean and then in soiled cages, or the reverse. There

was no significant strain difference (by *t* test) in the number of cycles observed in clean cages, in soiled cages or in the magnitude of the oestrous response to the soiled cages (Δ).

DISCUSSION

The data from the first experiment clearly indicate that grouping of C57BL/6 and BALB/Ci females may lengthen the oestrous cycle, and that even grouping in pairs can have a significant effect on the length of the cycle. In the C57BL strain, four females per cage appear to be the grouping maximum, beyond which there are no additional effects on the frequency of oestrous cycles up to and including the eight per cage level. The BALB females present a slightly different and more confusing picture. There was no obvious grouping maximum found for this strain. The data suggest a maximum effect at six females per cage, but this is open to other interpretations. The response of both strains, however, is similar in that the decreased oestrous frequency which accompanies grouping can be accounted for by a relative lengthening of the dioestrous component of the cycle.

It cannot be conclusively stated whether or not spontaneous pseudopregnancy occurred in any of the subjects from either strain as none of the physiological criteria for pseudopregnancy were examined in this study. If the small secondary peaks which appear in the cycle length distributions of the grouped C57BL females and the more densely grouped BALB females are indeed true peaks, pseudopregnancy may be suspected. These small peaks are approximately the same size as those found by van der Lee & Boot (1955) in pseudopregnant F_1 females from a cross of C57BL $\times$ DBA. However, Lamond (1959) has reported for grouped females a similar, but much larger, peak at approximately twice the length of the cycle seen in singly caged females, which did not represent pseudopregnancy but, rather, a prolonged dioestrus.

It is clear from the results of this study that strain differences in the response of the oestrous cycle to grouping do exist. Identical environmental conditions and experimental procedures were used in obtaining the data for both strains, and strain differences in oestrous cycle frequency were found. This is evident from the significant interaction between strains and housing conditions. Comparison of the results of previous investigations has suggested that strain differences did exist, but such comparisons were hampered by the differences in experimental procedures and conditions employed in the various studies.

The results of the second experiment demonstrate that the effects of female grouping on the oestrous cycle can be imitated by placing a single female in a cage which has been soiled by other females and suggest the involvement of a pheromone. In view of the procedures employed in this study and what is known about other pheromones implicated in the control of reproductive cycles in mice, it seems likely that an excretory product may be the source of the stimulus producing the suppression response. This possibility is being investigated. It also seems likely that olfaction may be at least partially involved in the reception of the stimulus.

The results fail to support the suggestion that oestrous suppression is induced

by male-like mating behaviour. The fact that suppression of oestrus can be induced in the absence of other females would appear to rule out this possibility, at least to the extent that male-like behaviour among females is responsible for all types of oestrous suppression. As the design of this study did not permit the experimenter to classify the type of suppression shown by the females in soiled cages as either pseudopregnancy or an extension of dioestrus, Dewar's suggestion may, in fact, be valid, but limited to the induction of pseudopregnancy only. If this were indeed the case, it might at least partly explain the difference between the pseudopregnant, anoestrous and extension of dioestrous responses to grouping which have been reported by other investigators.

ACKNOWLEDGMENTS

I wish to thank Dr E. W. Caspari for his valuable advice throughout the course of this study, and Dr W. K. Whitten for a critical reading and discussion of the manuscript. The work reported here is part of a dissertation submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy at The University of Rochester, Rochester, New York, and was supported by Graduate Training Grant 5 T1 GM 658 from the National Institutes of Health. Part of the data analysis and the preparation of the manuscript was supported by NIH Training Grant T01 CA 05013 from the National Cancer Institute to The Jackson Laboratory.

REFERENCES

- ANDERVONT, H. B. (1944) Influence of environment on mammary cancer in mice. *J. natn. Cancer Inst.* **4**, 579.
- BRONSON, F. H. (1967) *Effects of social stimulation on adrenals and reproductive physiology of rodents*. In: Husbandry of Laboratory Animals. Ed. M. L. Conalty. Academic Press, London.
- BRONSON, F. H. (1968) *Pheromonal influences on mammalian reproduction*. In: Perspectives in Reproduction and Sexual Behavior: A Memorial to William C. Young. Ed. Milton Diamond. Indiana University Press.
- DEWAR, A. D. (1959) Observations on pseudopregnancy in the mouse. *J. Endocr.* **18**, 186.
- LAMOND, D. R. (1958) Spontaneous anoestrus in mice. *Proc. Aust. Soc. Anim. Prod.* **2**, 97.
- LAMOND, D. R. (1959) Effect of stimulation derived from other animals of the same species on oestrous cycles in mice. *J. Endocr.* **18**, 343.
- MODY, J. K. (1963) Structural changes in the ovaries of IF mice due to age and various other states: demonstration of spontaneous pseudopregnancy in grouped virgins. *Anat. Rec.* **145**, 439.
- SNELL, G. D. (1941) *Reproduction*. In: Biology of the Laboratory Mouse. Ed. G. D. Snell. Blakiston, New York.
- VAN DER LEE, S. & BOOT, L. M. (1955) Spontaneous pseudopregnancy in mice. *Acta physiol. pharmac. néerl.* **4**, 442.
- VAN DER LEE, S. & BOOT, L. M. (1956) Spontaneous pseudopregnancy in mice. II. *Acta physiol. pharmac. néerl.* **5**, 213.
- WHITTEN, W. K. (1959) Occurrence of anoestrus in mice caged in groups. *J. Endocr.* **18**, 102.
- WHITTEN, W. K. (1966) *Pheromones and mammalian reproduction*. In: Advances in Reproductive Physiology, Vol. 1. Ed. Anne McLaren. Logos Press, London.
- WHITTEN, W. K. (1969) *Mammalian pheromones*. In: Olfaction and Taste, Proceedings of the Third International Symposium. Ed. Carl Pfaffmann. Rockefeller University Press.
- WHITTEN, W. K. & BRONSON, F. H. (1970) *Role of pheromones in mammalian reproduction*. In: Advances in Chemoreception. Eds. J. W. Johnston, Jr, D. C. Moulton and A. Turk. Appleton-Century-Crofts, New York.