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MATING, OVULATION AND CORPUS LUTEUM FUNCTION IN THE VOLE, *MICROTUS AGRESTIS*

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Summary. A study of the relationship between the mating behaviour of the vole and the induction of ovulation and CL function is described. A single intromission or an injection of LH-RF constitute stimuli which induce ovulation, but normally give rise to CL that degenerate soon after formation. More prolonged mating, or mechanical stimulation of the vagina and cervix given after a separate ovulatory stimulus, result in the maintenance of the CL. Mechanical genital stimulation is effective in inducing CL maintenance when given up to 48 hr after an LH-RF injection. Similarities, therefore, are apparent between the vole, an induced ovulator, and spontaneous ovulators such as the rat, mouse and hamster.

INTRODUCTION

The functional relationship between mating and aspects of reproduction other than the mere transference of semen is particularly obvious in those species which are normally dependent on coitus to stimulate either ovulation (e.g. rabbit, cat and ferret) or CL function (e.g. rat, mouse and hamster). The relationship has been investigated in a number of species (see review by Dewsbury, 1972) and these studies have indicated that the actual temporal pattern of copulation of a species may be of considerable significance to successful reproduction. The present study was undertaken to analyse the relationship between the mating behaviour of *Microtus agrestis* and the initiation of neuroendocrine mechanisms necessary for pregnancy. These voles seemed particularly suitable for such an analysis since they ovulate reflexly (Austin, 1957; Breed, 1967) and ovulation and CL maintenance can be dissociated (Milligan, 1974a).

MATERIALS AND METHODS

Laboratory bred *M. agrestis* were used in all experiments and were maintained as described by Breed (1969). Females were normally 2- to 3-month-old virgins judged to be mature by the appearance of vaginal smears consisting of predominantly cornified cells on 3 consecutive days (Milligan, 1974a). Females were caged singly for at least 2 days before treatment. Daily vaginal smears

were classified as described by Milligan (1974a). Males were fertile adults from the breeding colony.

For mating trials, the male was placed in a clean cage 5 min before introduction of the female. If mating had not begun within 5 min of pairing, the female was returned to her cage for a further week before being introduced to a new male. Females which failed to mate during three such trials were discarded. In Exps 1 and 2, involving 163 virgin females, mating began within 5 min of pairing in 135 out of 228 trials.

During copulation by the vole, *M. agrestis* (Milligan, 1974b), intromission (median duration 18 sec) is accompanied by pelvic thrusting. A series of one or more intromissions precedes ejaculation and each such series is referred to as an 'ejaculatory series'. A number of ejaculatory series occurs during a period of mating under laboratory conditions. The occurrence of ejaculation was confirmed by the male's behaviour and by the presence of a copulatory plug or spermatozoa in the vagina.

Experiment 1

Males were permitted to mount eleven females (five virgin and six parous) that had adhesive plaster over the vaginal aperture to prevent intromission. The pairs were separated 3 min after the first mount.

Forty-two other mating pairs were separated after one of the following: 5 sec from the start of the first intromission (i.e. incomplete intromission); 10 sec from the start of the first intromission (i.e. incomplete intromission); the first intromission, provided that this did not end in ejaculation; the first ejaculatory series (of one or more intromissions); the second ejaculatory series; the fourth ejaculatory series. All females were returned to their original cages after the separation and were later killed (see below).

Experiment 2

A regimen of mechanical stimulation was designed in an attempt to mimic the multiple intromission/multiple ejaculation mating pattern, and was modelled on the median values of behavioural measures obtained from observations of copulating laboratory voles (Milligan, 1974b). To simulate an intromission, a glass rod was moved within the vagina for a period of 20 sec. An ejaculatory series was simulated by giving three such periods of stimulation separated by approximately 8-sec pauses. Four of the simulated ejaculatory series were given, with an interval of 5½ min between each complete series. The instrument used was either a glass rod (2·5 mm in diam.), moved manually to and fro in the vagina, or a similar rod attached to the drive spindle of a 1·5-V electric toothbrush (Boots Ltd) run off a 3·0 V supply. The motor-driven rod ('vibrator') rotated rapidly through 45° at more than 30 Hz.

Mechanical stimulation of the vagina and cervix was applied to eleven virgin females. Other females were each given a separate ovulatory stimulus by pairing with a male and separating the pair immediately following the completion of the first intromission (see Exp. 1). Those with males that ejaculated were discarded, but the others were immediately given one of three treatments: genital stimulation using the hand-operated rod, genital stimulation with the vibrator,

or handling as in the previous two treatments but without any genital stimulation.

Experiment 3

Seventy-two females were anaesthetized with ether and a saline solution of LH-RF (the synthetic decapeptide) was injected into the external jugular vein. The dose of LH-RF varied between 25 and 100 ng. At various times afterwards (15 min, 6 hr, 24 hr or 48 hr), comparable females were either stimulated with the vibrator ('stimulated'), or given a similar amount of handling but with no genital stimulation ('handled'). Additional 'stimulated' females were used to test the ability of the CL to support a decidual reaction (see below). Five other females were injected with saline and stimulated 15 min later.

Functional activity of CL

The ability of the CL to support a decidual reaction was tested in some females. Using ether anaesthesia, the left uterine horn was subjected to trauma 3½ days after mating (Exp. 2) or injection of LH-RF (Exp. 3). The horn was scratched three or four times along the anti-mesometrial surface with a burred needle inserted at the oviducal end through small incisions in the skin, body wall and uterus. These females were killed 3 days later.

Autopsy

The reproductive state of each female was assessed by examining the vaginal smears during, and the ovarian structure at the end of, treatment. Following ovulation, the cornified smears typical of virgin females (Austin, 1957; Breed, 1967; Milligan, 1974a) were usually interrupted by a period of thin or leucocytic smears. Females that showed such an interruption were normally killed when cornified smears returned; those that continued to show cornified smears after treatment were killed within 4 days.

Fresh ovaries were examined for luteinized structures: these can be easily and reliably identified as pink or white opaque bodies which contrast with the stroma and translucent follicles. Paraffin wax-embedded sections of ovaries possessing luteinized bodies, and of uteri subjected to trauma, were prepared and stained with Ehrlich's haematoxylin and eosin. The diameters of luteinized structures, except those containing ova, were measured.

RESULTS

Three reproductive states were recognized in females following ovulation induced by coitus or injection of LH-RF: females (a) showed only a transient interruption of the cornified vaginal smears and possessed degenerating CL ('short-lived' CL) when killed 3 or 4 days after coitus or injection, (b) became pseudopregnant, possessing either histologically healthy CL when killed 3 or 4 days after coitus or injection, or degenerating CL on the return of cornified smears after 9 to 10 days, or (c) became pregnant, showing thin or leucocytic smears until parturition.

Effect of various amounts of copulation

The results of Exp. 1 (Table 1) indicate that the response of females to mating (with respect to ovulation and the development of the CL) is dependent on the amount of mating experienced. Repeated mounts without intromission were not sufficient to induce ovulation in the five virgin females, but three of the six parous females ovulated and became pseudopregnant. Only a small proportion of females ovulated when they experienced less than one complete intromission, but the majority ovulated when they experienced one or more complete intromissions.

Table 1. Induction of ovulation and luteal function in voles after different amounts of mating

*Treatment**	*No. of females*			
	Total	*Ovulating*	*With short-lived CL*	*Pregnant or pseudopregnant*
Mounting only				
Parous	6	3	0	3
Virgin	5	0	—	—
Intromission for 5 sec	4	1	0	1
Intromission for 10 sec	4	1	1	0
One complete intromission (mean duration ± S.E. = 22 ± 3·5 sec)	12	11	8	3
One ejaculatory series	6	6	3	3
Two ejaculatory series	6	6	2	4
Four ejaculatory series	10	10	0	10

* See text for further explanation.

The CL resulting from ovulation were either 'functional', i.e. characteristic of pregnancy or pseudopregnancy, or short-lived. The proportion of ovulating females that developed functional CL after experiencing one or more complete intromissions increased with the amount of mating experienced.

Effect of mechanical stimulation

The eleven virgin females subjected to mechanical stimulation only did not ovulate.

There were no significant differences between the other three treatments of Exp. 2 (Table 2) in either the proportion of females ovulating ($\chi^2 = 3{\cdot}64$; $P > 0{\cdot}1$), or in the number of CL/ovulating female ($\chi^2 = 1{\cdot}82$; $P > 0{\cdot}1$). The duration of the intromissions (the ovulatory stimuli) was not significantly different between the treatments. Most of the females which were only handled following the intromission developed short-lived CL and only a few became pseudopregnant. A significantly higher proportion of females became pseudopregnant when the limited mating stimulus was followed by genital stimulation using either the hand-operated rod ($\chi^2 = 6{\cdot}44$; $P < 0{\cdot}01$) or the vibrator ($\chi^2 = 17{\cdot}65$; $P < 0{\cdot}001$). Although a higher proportion of ovulating females became pseudopregnant after stimulation with the vibrator than with the hand-operated glass rod, this difference was not significant ($\chi^2 = 2{\cdot}81$; $P > 0{\cdot}05$).

Table 2. The effect on voles of mechanical stimulation of the vagina and cervix after a single intromission

*Treatment**	*No. of females*				*Proportion of ovulating females becoming pseudopregnant*	*Mean (±S.E.) no. of CL/ ovulating female*	*Mean (±S.E.) duration of intromission (sec)*
	Total	*Ovulating*	*With short-lived CL*	*Pseudo-pregnant*			
Manual glass rod	19	15	4	11	73%	5·33±0·50	18·1±2·9
Electric vibrator	16	16	0	16	100%	4·75±0·48	18·1±3·1
Handled only	18	15	12	3	20%	4·00±0·50	18·2±3·0

* See text for further details.

Females possessing functional CL as a result of mechanical stimulation following an intromission showed a marked decidual response to uterine trauma.

Effect of LH-RF and mechanical stimulation

None of the five females in Exp. 3 that were treated with saline and given artificial stimulation ovulated. There was no significant difference in either the proportion of females ovulating ($\chi^2 = 2{\cdot}56$; $P > 0{\cdot}1$) or the number of CL/ovulating female ($t = 1{\cdot}46$; $P > 0{\cdot}1$) between the 'stimulated' and 'handled' voles treated with LH-RF (Table 3). All 'handled' females that ovulated developed short-lived CL, but one female killed 4 days after injection possessed a single histologically healthy CL in addition to four other degenerating ones. By contrast, many of the females given genital stimulation between 15 min and 48 hr after LH-RF injection became pseudopregnant. In the females given this treatment (the 'stimulated' females of Table 3 and the additional females used to test the functional activity of the CL), stimulation at 15 min, 6 hr, 24 hr and 48 hr after the LH-RF injection was effective in maintaining the CL in 7/10

Table 3. The effect on voles of mechanical stimulation or handling after LH-RF injection

Treatment	*No. of females*			
	Total	*Ovulating*	*With short-lived CL*	*Pseudopregnant or with histologically healthy CL*
Stimulated	38	30	11	19
Handled	34	21	21*	1*

* One female with both types of CL (see text).

(70%), 8/10 (80%), 8/15 (53%) and 6/18 (33%) of the ovulating females, respectively. Females which developed histologically healthy CL after stimulation at 6 or 24 hr showed a marked decidual reaction in response to uterine trauma. Of the females in which the CL were maintained following stimulation at 48 hr, only one had a characteristic pseudopregnancy. The others had histologically healthy CL but little change in the cornified smear pattern occurred before they were killed at 4 days (three females) or 6½ days (two females) after the LH-RF injection. The uteri of the latter two females were subjected to trauma, but no decidual reaction was evoked.

DISCUSSION

Microtus agrestis, in common with other microtine rodents (Richmond & Conaway, 1969; Clarke, Clulow & Greig, 1970; Clulow & Mallory, 1970; Kirkpatrick & Valentine, 1970; Hasler & Banks, 1973), is an induced or reflex ovulator (Austin, 1957; Breed, 1967; Breed & Clarke, 1970a). Irrespective of the ovulatory stimulus, induced ovulators such as the rabbit, cat and ferret normally develop functional CL and become pseudopregnant or pregnant following ovulation (Everett, 1961; Conaway, 1971). *M. agrestis* differs from these 'typical' induced ovulators in that, in some circumstances, ovulation may

give rise to CL that begin to degenerate (Table 4) within about 2 days of the inducing stimulus (i.e. within about 1½ days of ovulation, as ovulation occurs 9 to 12 hr after an inducing stimulus: Austin, 1957; Breed & Clarke, 1970a). Short-lived CL may occur after ovulations induced by 'remote' male stimuli (Milligan, 1974a), limited amounts of mating (Exps 1 and 2) and injections of LH-RF (Exp. 3) or LH (Milligan, 1974b). The life-span of these CL is in marked contrast to that of the CL associated with pregnancy or pseudopregnancy (Breed & Clarke, 1970b; see also Table 4, first line) and they are unable to support a decidual reaction (Milligan, 1974b).

Table 4. The diameter of CL in female voles killed at various times after an ovulation-inducing stimulus

Nature of inducing stimulus	*Days after inducing stimulus* 1	2	3	4	5
Unrestricted mating* (2 days with male)	599 ± 24·1 (3)	744 ± 5·8 (3)	749 ± 25·9 (4)	820 ± 27·3 (4)	740 ± 35·9 (4)
Close contact with male; no copulation†		726 ± 49·9 (3)	436 ± 57 (3)	265 ± 20·7 (3)	175 ± 18·5 (3)
Single intromission only‡		709 ± 5·3 (3)	309 ± 66·4 (2)	229 ± 6·4 (2)	
LH-RF intravenously (100 ng)*		605 ± 61·5 (2)	300 ± 6·9 (2)	169 ± 1·9 (2)	
LH-RF subcutaneously (200 ng)*		666 ± 10·1 (2)		214 ± 9·4 (2)	127 ± 0·9 (2)
LH subcutaneously (20 μg)*		668 ± 65·8 (2)		280 ± 31·0 (2)	158 ± 4·5 (2)

Luteal diameters (± S.E.) are in μm. Figures in parentheses represent the number of females.

* Data from Milligan (1974b).
† Data from Milligan (1974a); time from inducing stimulus an approximation.
‡ Data from Experiment 2 and additional females.

Although the existence of these short-lived CL indicates that ovulation may occur independently of the development of CL function, stimulation of both processes must occur if mating is to result in successful pregnancy. Limited amounts of mating (e.g. a single intromission) readily induce ovulation but only short-lived CL are usually formed, and more prolonged mating is normally necessary to induce the development of functional CL (Exp. 1). The vole's multiple intromission/multiple ejaculation mating pattern meets the stimulus requirements for both processes. The results from the virgin and parous females that had been mounted only (Exp. 1) suggest that there may be a difference in the response to copulatory stimuli of females of different reproductive status.

Mechanical stimulation of the vagina and cervix can mimic mating by inducing ovulation in some other reflex ovulators (cat—Greulich, 1934; mink—Enders, 1952; rabbit—Carlyle & Williams, 1961), but previous attempts to induce ovulation by this technique in *M. californicus* (Greenwald, 1956), *M. agrestis* and *Clethrionomys glareolus* (Clarke & Clulow, 1973) have been unsuccessful. The failure of mechanical genital stimulation to induce ovulation in the present study (Exps 2 and 3) is in contrast to the response even to limited mating (Exp. 1). This suggests that the mechanical stimulation may have failed

to mimic some essential feature of the penile stimulation (e.g. penile spines) and/or that other stimuli associated with the male may be important. The fact that mounting alone (Exp. 1) or the presence of males nearby (Milligan, 1974a) are both able to induce ovulation suggests that other stimuli may be involved.

Genital stimulation was able to mimic, however, that part of the mating process that causes the development of functional CL. A single intromission or an injection of LH-RF normally gave rise only to short-lived CL, but if either of these ovulatory stimuli was followed by mechanical stimulation, a high proportion of females developed functional CL (Exps 2 and 3). Simple artificial mechanical stimulation also may induce pseudopregnancy in the rat, mouse, hamster and Mongolian gerbil (De Feo, 1966; Diamond & Yanagimachi, 1968; Diamond, 1970; Wu, 1974). In the mouse, the ejaculatory reflex is the effective copulatory stimulus (McGill & Coughlin, 1970; McGill, 1972), and in the hamster, mating provides a more effective stimulus than mechanical stimulation (Diamond & Yanagimachi, 1968). To what extent the regimen of stimulation used in the present study provided the same effective stimuli as copulation is uncertain, but ejaculation was neither necessary, nor necessarily sufficient, to induce the maintenance of the CL (Exp. 1). Cornified epithelial spines, similar to those described in rats (Beach & Levinson, 1950), occur on the glans penis of microtine rodents (Hooper & Hart, 1962), including the male voles of our laboratory colony (Milligan, 1974b). These spines may be involved in the induction of neuroendocrine mechanisms leading to ovulation and luteal maintenance (Zarrow & Clark, 1968).

The present results indicate that ovulation and the development of CL function in *M. agrestis* are dependent on reflex mechanisms that appear to have different stimulus requirements for their activation. Ovulation is readily induced by very limited mating but not by artificial genital stimulation, but the development of functional CL is induced either by more prolonged mating or by artificial stimulation. Although the effective stimuli for the induction of both processes are normally provided within a fairly limited period by a single unrestricted session of mating, the results of Exp. 3 indicated that the mechanical stimulation could still induce functional CL in some voles even when given many hours after an ovulatory stimulus. The maintenance of the CL resulting from artificial stimulation 48 hr after LH-RF administration did not, however, appear to be accompanied by the development of full secretory function.

It is apparent that there are similarities between the vole, an induced ovulator, and spontaneously ovulating animals such as the rat, mouse and hamster. In these latter animals, mating also stimulates the development of functional CL (Everett, 1961), and the CL of the rat and the hamster, like those of the vole, retain the capacity for the development of full secretory function for some time after ovulation (Nikitovitch-Winer & Everett, 1958; Greenwald, 1963; Staples, 1965). In addition, the distinction between the nature of ovulation in the vole and these other animals is not absolute, as induced ovulations can also occur in rats (e.g. Zarrow & Clark, 1968; Aron, Roos & Asch, 1970; Brown-Grant, Davidson & Greig, 1973) and possibly in mice (Allen, 1922; Togari, 1927).

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