Oestrous behaviour and circulating progesterone and oestrogen levels during pseudopregnancy in the domestic rabbit

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Summary. Using a range of positive and negative sexual behaviour events, receptivity of 7 female rabbits was quantified from Day 4 to 21 of pseudopregnancy and related to colour of the vulva and patterns of circulating progesterone, oestrone and oestradiol. Females with a white vulva never accepted mating; the proportion of females with a red vulva submitting to mating varied from 13% on Days 6–9 to 80% on Days 14–21. In 3 females which were not receptive until Day 18, oestrogens were not detectable in peripheral serum; progesterone levels were maximal on Days 10–13 and gradually fell to levels <2 ng/ml on Day 17. In 4 females which were sporadically receptive during pseudopregnancy, oestrogen levels fluctuated between 15 and 140 pg/ml serum; the drop of progesterone began on Day 13 and was more rapid.

Introduction

Rabbit and hare females ovulate in response to copulation. Oestrous periods occur in various physiological situations: in non-pregnant non-pseudopregnant females, 6- or 7-day cycles of sexual attraction to the male have been described in the rabbit, Oryctolagus cuniculus (Myers & Poole, 1962) and in the brown hare, Lepus europaeus (Caillol & Martinet, 1979). In both species, sexual responsiveness to males decreases during pregnancy, but sporadic sexual chases and matings persist and increase just before parturition (Hediger, 1949; Beyer & Rivaud, 1969; Martinet, Llegouis & Moret, 1970). During pseudopregnancy, receptivity is maintained at a low level in the rabbit (Hammond & Marshall, 1925) and hare (Caillol & Martinet, 1979). In ovariectomized rabbits, as in many other species, oestradiol induces sexual behaviour while progesterone inhibits this behaviour (Beyer & McDonald, 1973). Nevertheless, hares and rabbits may display oestrous behaviour even when circulating progesterone concentrations are high.

In rabbits receptivity increases at the end of pseudopregnancy while progesterone levels decrease. We have therefore studied the pattern of sexual behaviour during pseudopregnancy in relation to circulating progesterone and oestrogen concentrations in domestic rabbits.

Materials and Methods

Animals. Seven virgin New Zealand female rabbits housed in individual cages and fed commercial pellets ad libitum were used when 4–5 months old. Pseudopregnancy was induced in females
displaying a red turgescent vulva suggesting sexual receptivity (Lefevre & Moret, 1978) by an i.v. injection of 50 i.u. hCG (day of injection = Day 0 of pseudopregnancy). Laparotomy was performed 24 h later under pentanyl citrate and fluanisone (Hypnorm, Janssen Pharmaceutica) anaesthesia and the number of corpora lutea (ovulations) on each ovary was recorded.

**Behavioural tests.** From Days 4 to 21 of pseudopregnancy, the females were presented every day to 3 different males for 90 sec each time. The doe was placed in the cage of the male; if she submitted to mating, she was promptly removed to prevent intromission and ovulation and was not presented to the other males that day. The occurrence of the observed behaviours was noted and their duration determined using a stopwatch. An accurate and quantitative description of receptivity criteria was made, resulting in the establishment of a scoring system similar to that proposed by Munro, Renton & Butcher (1979) for the mare. Before testing, the colour of the vulva was noted; after testing, 4–5 ml blood were collected by cardiac puncture and allowed to clot during 24 h at 4°C. Serum was separated by centrifugation and stored at −20°C until assay.

**Steroid assays.** Using specific radioimmunoassays, the concentrations of progesterone, oestrone and oestradiol-17β in each serum sample were determined. Non-radioactive steroids were purchased from Steraloids; radioactive steroids were obtained from the Radiochemical Centre (Amersham, U.K.). Merck analytical grade solvents were used without re-distillation. After diethyl ether extraction, progesterone, oestrone and oestradiol were purified on Sephadex LH-20 microcolumns (height 5 cm; benzene:ethanol, 90/10, v/v, as eluant), using a modification of the technique described by Castanier & Scholler (1970). The different fractions were then dried and steroids were dissolved in phosphate buffer (pH 7.25). Recovery was determined in an aliquot; two samples of 0.1 ml were incubated at 4°C for 3 h with the diluted antibody, then for another 2 h after addition of the radioactive steroid according to Caillol & Martinet (1976). Bound and free fractions were separated using a charcoal-dextran mixture. The blank values, run with each assay, were below assay sensitivity. The principal reliability criteria of these assays are shown in Table 1. The specificity of the oestrone assay was ensured by the chromatography on Sephadex LH 20.

**Statistical analysis.** Differences between frequencies of observed behaviours and of the different colours of the vulva during pseudopregnancy were analysed by χ² tests. Mean ± s.e.m. values were calculated for durations of behavioural events and serum progesterone concentrations, and compared by variance analysis.

**Table 1. Reliability criteria of the progesterone, oestrone and oestradiol-17β radioimmunoassays**

| Steroid | Antibody | Cross-reaction | Recovery after extraction and chromatography | Sensitivity | Precision (%)
<table>
<thead>
<tr>
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</tr>
</thead>
<tbody>
<tr>
<td>Progesterone</td>
<td>Progesterone-11α hemisuccinate-BSA</td>
<td>5α-Pregnane-3,20-dione = 7-5% Deoxycorticosterone = 7-5%</td>
<td>64–97%</td>
<td>25</td>
<td>100</td>
</tr>
<tr>
<td>Oestrone</td>
<td>Oestrone-17 para-aminobenzoic-BSA</td>
<td>Oestradiol-17β = 20% Oestradiol-17α = 20% Oestriol = 13%</td>
<td>67–95%</td>
<td>5</td>
<td>15</td>
</tr>
<tr>
<td>Oestradiol-17β</td>
<td>Oestriol-17β 7-carboxy-methyl oxime-BSA</td>
<td>Oestradiol-17α = 0-5 Oestrone = 3-6 Oestriol = 3-6</td>
<td>62–85%</td>
<td>5</td>
<td>15</td>
</tr>
</tbody>
</table>

**Results**

**Sexual behaviour**

**Description of the behavioural events.** When the female was introduced into the cage of the male, he began to nuzzle her behind the ears and around the genital area. The female reacted to the male
in the following ways: (i) by flattening to the floor in a corner of the cage; (ii) by circling, i.e. the female ran away quickly from the male and circled around, sometimes jumping; (iii) by vocalizing during flattening or circling events; (iv) by trying to mount the male; (v) by standing motionless and submitting to mating: the male scratched the female's back with his forelegs, then tried to mount her and, after several pelvic thrusts, the female responded by taking the position of lordosis.

Classification of behavioural tests. A test was considered as negative when the female did not submit to mating and positive when she did.

A total of 296 tests were carried out; on 7 occasions the female submitted to mating as soon as she was introduced into the cage of the male and there were no other behavioural events. Each of the 289 remaining tests was grouped according to whether it was negative and both the other tests on the same day were negative (Group I), it was negative on a day when the last test was positive (Group II), or positive, i.e. with 0, 1 or 2 negative tests before it on that day (Group III).

Frequency and duration of the behavioural events. Flattening, circling and vocalizations were observed during positive as well as during negative tests (Table 2). Flattening throughout the whole test seemed to occur more often in Group I than in Group II (30 compared with 8%; \( P = 0.06 \)). Flattening and circling during the same test occurred in 64% of tests in Group I and in 84% in Group II \( (P = 0.08) \). When the females showed both behaviours, more time was spent in flattening than in circling in Group I \( (P < 0.01) \). During the positive tests (Group III), the time spent flattening or circling was nearly the same; circling was more often observed than in Groups I or II (28 compared with 6 and 8%, respectively; \( P = 0.1 \)). The mean latency time before lordosis was around 24 sec if the female was only flattening or only circling, but was significantly increased to 50 sec if she exhibited both behaviours \( (P < 0.005) \). Vocalization was more often heard during flattening \( (34\%) \) than during circling \( (6\%) \). However, the occurrence of vocalizations varied widely from one female to another; 2 females never vocalized while 2 others vocalized during 29 out of 49 and 32 out of 39 tests. The female mounted the male in 3 out of the 296 tests and this behaviour was always followed by lordosis during the same or the following test.

Changes in sexual behaviour during pseudopregnancy. The proportion of tests in which the does flattened for 90 sec (Text-fig. 1a) decreased significantly at the end of pseudopregnancy (Days 18–21) compared to that during Days 4 to 17 \( (P < 0.025) \). Circling or vocalization did not appear to vary throughout pseudopregnancy (Text-fig. 1a). The number of females displaying lordosis

### Table 2. Frequency and duration of sexual behaviour events in 7 pseudopregant rabbits tested 1–3 times/day with different males on Days 4–21 of pseudopregnancy

<table>
<thead>
<tr>
<th>Behavioural events</th>
<th>Group I ( (n = 219) )</th>
<th>Group II ( (n = 24) )</th>
<th>Group III ( (n = 46) )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of tests (%)</td>
<td>Mean ± s.e.m. duration (sec)</td>
<td>No. of tests (%)</td>
</tr>
<tr>
<td>Flattening</td>
<td>41 (19)</td>
<td>( F = 90 )</td>
<td>1 (4)</td>
</tr>
<tr>
<td>Flattening + vocalization</td>
<td>23 (11)</td>
<td>( F = 90 )</td>
<td>1 (4)</td>
</tr>
<tr>
<td>Circling</td>
<td>14 (6)</td>
<td>( C = 90 )</td>
<td>2 (8)</td>
</tr>
<tr>
<td>Circling + vocalization</td>
<td>2 (0.01)</td>
<td>( C = 90 )</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Flattening + circling</td>
<td>113 (52)</td>
<td>( F = 55.5 ± 1.9* )</td>
<td>16 (67)</td>
</tr>
<tr>
<td>Flattening + circling + vocalization</td>
<td>26 (12)</td>
<td>( C = 33.4 ± 1.8 )</td>
<td>4 (17)</td>
</tr>
</tbody>
</table>

\( F = \) flattening; \( C = \) circling.

\* \( F \) significantly higher than \( C \) \( (P < 0.01) \).
Text-fig. 1. Behaviour of female rabbits tested with males during pseudopregnancy.
(a) Occurrence of behaviours: F = flattening during 90 sec; C = circling during 90 sec; F + C = flattening and circling; black bars = vocalizations; S = total number of vocalizations. (b) Order of the male to which the female submits. (c) Latency before lordosis. The numbers of observations are given in parentheses.

increased after Day 18. However, lordosis was observed sporadically in 4 does (D, E, F, G) throughout pseudopregnancy; in the other 3 does (A, B, C) lordosis was observed first on Day 14, then from Days 18 to 21 (Text-fig. 2a). The proportion of females which submitted to mating with the first male decreased from Days 4–5 to Days 10–13, then increased again until Days 18–21 (Days 18–21 compared to Days 4–17, \( P < 0.01 \)). The proportion of females showing lordosis with the third male was highest on Days 10–13 (Text-fig. 1b). At the end of pseudopregnancy the proportion of females showing lordosis in a 10-sec period was significantly higher than between Days 4 and 17 (\( P < 0.01 \)) (Text-fig. 1c).

Determination of the sexual behaviour score. Females that did not submit to mating were considered to be dioestrous, and those that did were noted as oestrous. Although flattening, circling and vocalizations were observed during positive as well as negative tests, flattening during the whole test or during part of the test was more frequent in Group I than in Group II tests; circling might be a step towards submission to the male, but mounting was observed only in females displaying lordosis on the same day. We therefore devised a system of scoring the sexual behaviour: a flattening female scored \(-1\); a flattening and circling female scored 0 and a circling female \(+1\); mounting scored \(+2\); and lordosis \(+3\). Vocalization did not seem to be a criterion of dioestrus or oestrus and was therefore not included in the scoring system. The sexual behaviour score was the mean of the points scored on each day during 1, 2 or 3 tests. In Females A, B and C, which displayed lordosis only at the end of pseudopregnancy, the mean score fluctuated between \(-1\) and 0 until Day 17, then rose to the maximum from Day 18 to 20. In the other 4 females, the mean score fluctuated
Females A, B, C

From 0 to 3 during the whole of pseudopregnancy; however, this score was higher from Day 13 than during mid-pseudopregnancy (Text-fig. 2b).

Relationship between sexual behaviour and the colour of the vulva. Females with a white vulva were only noted between Days 6 and 13. The proportion of females with a red vulva was lowest on Days 10 to 13 and highest on Days 18 to 21 ($P < 0.01$) (Table 3). Females with a white vulva never displayed lordosis, whereas 28 or 57% of those with a pink or a red vulva, respectively, submitted to mating; the percentage of lordosis in females with a red vulva increased from 13% on Days 6–9 to 80% on Days 14–21 ($P < 0.01$) (Table 3).

Serum progesterone and oestrogen concentrations during pseudopregnancy

Progesterone concentrations rose from Days 4–5 of pseudopregnancy to a maximum on Day 11. Then, they decreased slowly and reached basal levels on Days 19–20. For Females D, E and F, the drop was more rapid and the mean level was lower on Days 14 and 15 ($3.45 \pm 0.49$ ng/ml) than for Females A, B and C ($7.14 \pm 0.64$ ng/ml) ($P < 0.005$). Female G showed very high levels of circulating progesterone from Days 4 to 15 (Text-fig. 2c). Oestrogen levels varied widely from one female to another; they remained below 15 pg/ml throughout pseudopregnancy in 4 females (A, B,
Table 3. Vulval coloration of female rabbits during pseudopregnancy and the numbers submitting to males

<table>
<thead>
<tr>
<th>Days</th>
<th>No. of females</th>
<th>Vulval coloration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>White</td>
</tr>
<tr>
<td>4-5</td>
<td>In group</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Submitting to ♂</td>
<td>0</td>
</tr>
<tr>
<td>6-9</td>
<td>In group</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Submitting to ♂</td>
<td>0</td>
</tr>
<tr>
<td>10-13</td>
<td>In group</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Submitting to ♂</td>
<td>0</td>
</tr>
<tr>
<td>14-17</td>
<td>In group</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Submitting to ♂</td>
<td>0</td>
</tr>
<tr>
<td>18-21</td>
<td>In group</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Submitting to ♂</td>
<td>0</td>
</tr>
<tr>
<td>4-21</td>
<td>In group</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Submitting to ♂</td>
<td>0</td>
</tr>
</tbody>
</table>

C and F). A single peak of oestradiol (110 pg/ml) was observed on Day 5 in Female D. Oestrone levels in Female G and oestrone and oestradiol levels in Female E were detectable and variable; Peaks of oestrone or oestradiol did not coincide with the behaviour score of the female that day or the day after (Text-fig. 3).

Text-fig. 3. Oestrone and oestradiol patterns and sexual behaviour scores during pseudopregnancy in Females E and G.

Discussion

These results show that the difference between dioestrous and oestrous behaviour is not easy to establish in the rabbit. When introduced into the cage of a male, females may display 3 different behavioural patterns: flattening, circling away from the male or standing still ready to mate. Flattening, running or circling have been considered as dioestrous behaviour in the wild (Southern, 1947; Rowley & Morrison, 1955) as in the domestic rabbit (Heath, 1972). Our findings, however, show that flattening and circling very often precede lordosis and submission to mating; nevertheless flattening can be considered as an indication of dioestrus rather than oestrus. Mounting the male which is always followed by submission to mating seems to be a sign of "intense heat" as noted by Hammond & Marshall (1925) and Yachine, Mena & Beyer (1967).
The pattern of sexual behaviour during pseudopregnancy changes from day to day and varies widely between females. From Days 4 to 14, a deep dioestrous behaviour was observed in 3 does; when lordosis was observed in the other 4 does, it generally occurred only with the second or even the third male and with a latency exceeding 10 sec. From Day 14, oestrous behaviour was increasing and on Day 20 all the does submitted to mating; lordosis then usually occurred with the first male and within 10 sec after the doe was put into the male's cage. These results confirm the observations of Hammond & Marshall (1925), Hughes & Myers (1966) and Rubin & Azrin (1967) who all reported irregular and unpredictable matings during pseudopregnancy with an increased incidence of matings at the end of this period. We did not, however, observe a 6-day cycle of "sexually orientated activities" as described by Hughes & Myers (1966) for pseudopregnant domestic rabbits in large enclosures.

Vulva coloration varied during pseudopregnancy, a white vulva occurring only on Days 6–13, the proportion of rabbits with a red vulva rising on Days 18–21. Females with a white vulva never displayed any oestrous behaviour whatever the stage of pseudopregnancy while more than 50% of those with red vulva submitted to mating. However, the relationship between the colour of the vulva and the incidence of lordosis varied according to the physiological stage since the later the stage of pseudopregnancy the greater the number of females with a red vulva that displayed oestrous behaviour. The same relationship was observed in nulliparous females (Lefevre & Moret, 1978) or after pregnancy or pseudopregnancy (Friedman, 1938).

The mean peripheral serum levels of progesterone recorded in the present study are similar to those reported previously for pseudopregnant rabbits (Fuchs & Beling, 1974; Thau & Lanman, 1975; Holt, Heise, Wilson & Landis-Keyes, 1976; Spilman & Wilks, 1976; Thorbert, Batra, Owman, Rosengren & Sjöberg, 1976; Harrington & Rothermel, 1977; Browning, Landis-Keyes & Wolf, 1980; Richardson & Oliphant, 1981). Among the 7 females studied, one exhibited very high levels of progesterone which could not be explained.

Serum oestradiol and oestrone levels fluctuated between 0 and 140 pg/ml throughout pseudopregnancy, the mean level of oestradiol being slightly higher than that of oestrone; there was no definite change in the oestrogen levels during pseudopregnancy. Few data are available concerning the patterns of peripheral serum oestrogen levels during that period. Batra, Owman, Sjöberg & Thorbert (1979) reported oestradiol levels fluctuating between 32 and 49 pg/ml and increasing slightly on Day 18 of pseudopregnancy. The same pattern was described by Browning et al. (1980), but with levels varying below 10 pg/ml. Richardson & Oliphant (1981) found oestradiol levels between 20 and 50 pg/ml and no variation at the end of pseudopregnancy. The pseudopregnant females in our study could be divided into two groups according to sexual behaviour, and there were some differences in their circulating steroid levels. The progesterone levels of does in deep dioestrous until Day 18 exceeded 5 ng/ml until Day 15, and their oestrogen levels were always undetectable. However, for females submitting to mating whatever the stage of pseudopregnancy, progesterone levels began to decrease on Day 13 and the oestrogen levels were detectable and variable, but, in one doe rabbit, very high levels of circulating progesterone did not inhibit oestrous behaviour. These findings agree with Beyer & McDonald (1973) who reported that oestrogen injections promoted oestrous behaviour in ovariectomized rabbits whereas progesterone injections in oestrogen-treated ovariectomized animals inhibited this behaviour. Using techniques of active immunization against sex steroids, Elsaesser (1980) showed that oestrogens are necessary for receptivity in the rabbit and that progesterone inactivation does not interfere with receptivity. The role of aromatizable androgens in the appearance of receptivity in the rabbit has also been demonstrated (McDonald, Vidal & Beyer, 1970; Elsaesser, 1980); but no data are available concerning the circulating levels of these steroids during pseudopregnancy. The relationship between testosterone and androstenedione concentrations and sexual behaviour would provide valuable information on the hormonal initiation of receptivity in rabbits: during pregnancy, circulating levels of testosterone are higher than those of oestradiol (Lau, Saksena & Salmons, 1982) and, in the ovarian vein, the testosterone/oestradiol ratio is about 10 (Hilliard, Scaramuzzi, Pang, Penardi & Sawyer, 1974).
We thank Dr Fevre (INRA, Jouy-en-Josas) for the gift of progesterone antibody; Dr Terqui (INRA, Nouzilly) for the gift of oestrone antibody; Roussel-Uclaf (France) for that of the oestradiol-17β antibody; Mrs Bernadette Rossano for technical assistance; and Miss Aline Solari for help with statistics.

References


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