Determination of oestrous condition in female mice is dependent upon time of day

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Summary. Mice were maintained in rooms with constant ambient conditions and a 12 h light : 12 h dark daily light regimen with the lights on starting at 06:00 h. In Exp. I, detection of puberty, as measured by first vaginal oestrous, was recorded at significantly earlier ages when vaginal smears were taken at 04:00 or 08:00 h than at other times of the day. In Exp. II, adult females were detected as being in oestrus more frequently when vaginal smears were taken within 6 h before or after the onset of the light part of the cycle each day than at other times.

Introduction

Over a number of years the vaginal smear technique has been used on rodents as a means of assessing the stage of the oestrous cycle in females (Stockard & Papanicolaou, 1917; Schick, 1943). Various staining procedures and criteria for judging the stage of the oestrous cycle have been developed (e.g. Rugh, 1968; Dix & Billings, 1969; Hoglund, 1972; Davis et al., 1975). Changes in reproductive condition are related to hormone changes that are, in turn, reflected in changes in the vaginal epithelium. In many of the studies that have used the vaginal smear technique to assess reproductive condition, the time of day at which the smears were taken, in relation to the light cycle in use, is specified in the methods and procedures section of the report (Vandenbergh, 1967; Colby & Vandenbergh, 1974; Drickamer, 1984). In other studies the time of day at which smears are performed is not specified (Cowley & Wise, 1972; Bronson, 1975; Drickamer, 1981; Massey & Vandenbergh, 1981). Regardless of whether the time of day at which the measurements were made is specified or not, few investigators have taken this factor into account in the course of arriving at or explaining their conclusions. There are sufficient hormonal data to predict that, for mice (Bronson & Stetson, 1973; Stiff et al., 1974; Bronson, 1981) and many other rodent species, the females will attain oestrus at a predictable part of the daily cycle; for most species this is during the active phase (which may be diurnal or nocturnal) of the daily rhythm (see van Tienhoven, 1983). In two experiments reported here I tested whether the time of day at which the vaginal smears were made affected the results with respect to determining the age of puberty in young females or the frequency of oestrus in adult females.

Materials and General Methods

The mice used in these experiments were from a randomly bred closed colony of ICR/Alb house mice (Mus musculus). All colony and test mice were housed in shoe-box cages of polypropylene measuring 15 × 28 × 15 cm deep with opaque sides and fitted wire lids. Bedding of ground wood shavings was changed once each week. Purina Mouse Chow and water were supplied ad libitum throughout each experiment. All of the breeding and testing procedures were carried out in a suite of rooms maintained at 21–24°C and 30–60% relative humidity, on a 12 h light : 12 h dark daily regimen with lights on from 06:00 to 18:00 h. Daily vaginal smears were taken using a small glass pipette. The wet-mount smears were examined immediately using a light microscope and the cellular contents were judged to determine the stage of the oestrous cycle according to the criteria of Rugh (1968) and Vandenbergh (1969).
**Experiment I**

**Methods.** To assess whether taking vaginal smears at different times during the daily light cycle would influence the rate of detection of first vaginal oestrus in young female mice, pregnant female mice were isolated into individual cages during the last week before parturition. All cages were then checked daily and births were recorded. On the day after birth each litter was counted and all young were sexed. Each litter was reduced to exactly 10 young, 6 of which were females and 4 of which were males. Litters of fewer than 10 young were discarded. For this experiment, young females from 18 different litters were used and the split litter technique was employed. Mice were weaned at 21 days of age and were immediately assigned to one of six treatments by a random sequence. Mice in the 6 groups were monitored for vaginal introitus, and vaginal smears to detect first oestrus were taken daily at (1) 00:00 h, (2) 04:00 h, (3) 08:00 h, (4) 12:00 h, (5) 16:00 h, or (6) 20:00 h. There were therefore 6 test groups with 18 mice in each group, with one young from each litter distributed in each test group.

**Results.** A one-way analysis of variance revealed that there were significant differences in the age of puberty amongst the 6 test groups (Table 1). When vaginal smears were taken at 04:00 h and 08:00 h females were detected as having reached puberty significantly earlier than did mice from which vaginal smears were taken at any of the other four times. Mice tested at 00:00, 12:00 and 20:00 h did not differ in mean age at which puberty was detected, nor did the means differ for mice tested at 16:00 and 20:00 h.

<table>
<thead>
<tr>
<th>Time of daily vaginal smear*</th>
<th>No. of mice</th>
<th>Age of first oestrus (days)†</th>
</tr>
</thead>
<tbody>
<tr>
<td>00:00 h</td>
<td>18</td>
<td>36.1 (0.9)bc</td>
</tr>
<tr>
<td>04:00 h</td>
<td>18</td>
<td>33.3 (0.8)*</td>
</tr>
<tr>
<td>08:00 h</td>
<td>18</td>
<td>34.3 (0.8)*</td>
</tr>
<tr>
<td>12:00 h</td>
<td>18</td>
<td>36.7 (0.8)bc,c</td>
</tr>
<tr>
<td>16:00 h</td>
<td>18</td>
<td>38.7 (0.8)c</td>
</tr>
<tr>
<td>20:00 h</td>
<td>18</td>
<td>37.9 (0.7)bc,c</td>
</tr>
</tbody>
</table>

*Lights on 06:00–18:00 h.
†Those means not marked by the same superscript letter are significantly different at \( P < 0.02 \) (Duncan's New Multiple Range Test).

**Experiment II**

**Methods.** To determine whether the time of day at which the vaginal smears were taken from adult female mice would influence the rate of detection of oestrus, adult (aged 110 days at the start of the test), virgin females derived from 45 different litters were used. Females were assigned at random to one of six treatments, involving daily vaginal smears at (1) 00:00 h, (2) 04:00 h, (3) 08:00 h, (4) 12:00 h, (5) 16:00 h, or (6) 20:00 h and there were 30 females for each time period. Each test female was caged alone for 10 days and then, maintaining the single-cage housing condition, daily vaginal smears were taken at the specified time for 21 consecutive days.

**Results.** The results were analysed using the total number of days each female was in oestrus as independent datum points. A one-way analysis of variance indicated that there were significant
Table 2. Mean number (±1 s.e.m.) of days in oestrus for adult female mice from which vaginal smears were taken for 21 consecutive days

<table>
<thead>
<tr>
<th>Time of daily vaginal smear*</th>
<th>No. of mice</th>
<th>No. of days in oestrus†</th>
</tr>
</thead>
<tbody>
<tr>
<td>00:00 h</td>
<td>30</td>
<td>6.7 (0.3)</td>
</tr>
<tr>
<td>04:00 h</td>
<td>30</td>
<td>7.2 (0.3)</td>
</tr>
<tr>
<td>08:00 h</td>
<td>30</td>
<td>7.6 (0.4)</td>
</tr>
<tr>
<td>12:00 h</td>
<td>30</td>
<td>7.0 (0.3)</td>
</tr>
<tr>
<td>16:00 h</td>
<td>30</td>
<td>5.9 (0.3)</td>
</tr>
<tr>
<td>20:00 h</td>
<td>30</td>
<td>6.0 (0.4)</td>
</tr>
</tbody>
</table>

*Lights on 06:00–18:00 h.
†Means not marked by the same superscript letter are significantly different at $P<0.02$ (Duncan’s New Multiple Range Test).

differences in the rates of detection of vaginal smears indicative of oestrus dependent upon the time of day at which the smears were taken. Post-hoc analyses revealed an overlapping pattern of significant differences amongst the 6 treatment means (Table 2). In general, smears taken near the time of transition between the dark and light phases of the daily regimen were more likely to be considered indicative of oestrus than were smears taken at other times of the day. The fewest number of ‘oestrous’ smears was detected at smearing times during the last part of the light phase of the daily cycle.

Discussion

The results of these experiments can be used to support two conclusions. First, the period just before and after the onset of the light portion of the daily cycle is the best time of day for detection of oestrus in young and adult female mice. That is, for the largely nocturnal house mouse the occurrence of oestrus is confined primarily to the dark portion of the daily cycle, being initiated by late evening and lasting for some hours during the night. This is reflected in the vaginal cornification that occurs, with a delay period of several hours, at the end of the dark phase of the cycle and lasting into the early portion of the light phase. This first finding is in substantial agreement with the reports to date regarding the patterns of hormone concentrations in blood in mice (Bronson & Stetson, 1973; Bronson, 1981). Normally, oestradiol acts via a negative feedback loop to suppress LH secretion. Near the time of ovulation the negative feedback control of LH secretion is overridden. Oestradiol then increases in a two-step process, remaining stable for a period at moderate levels and then surging upward during the light phase of the cycle (Bronson, 1981). That surge of oestradiol concentration results in (a) ovulation, (b) behavioural oestrus during the ensuing dark phase of the daily cycle, and (c) cornification of the epithelium lining the vagina. It is this last effect that is measured by the vaginal smear technique as a measure of ovulation and oestrus. If the results of the two experiments are compared it appears that the vaginal cornification associated with the oestrous condition persists for a few more hours in adult females than in juvenile females.

Second, these findings have some consequences for the methodology used in studies involving measurements of oestrus, or studies in which detection of the timing of oestrus is a factor. All investigators using vaginal smears and studying related reproductive biology phenomena should report both the light cycle regimen used in their animal rooms and the time of day at which vaginal smears were taken relative to the light cycle.

This research was supported in part by U.S. Public Health Service Grant Award No. HD-08585 and by National Science Foundation Award No. BNS-8516331.
References


Received 21 October 1986