Sexual behaviour responses of the ovariectomized ewe to oestradiol benzoate, and their persistent reduction after exposure to phyto-oestrogens

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Summary. Characteristics of oestrous behaviour were recorded for 14 control ovariectomized ewes treated with 15, 25 or 40 µg oestradiol benzoate (OB), and for 24 ovariectomized ewes, 11 of which were infertile after prolonged intake of oestrogenic clover, which were each treated successively with 0, 10, 15.6 and 24.3 µg OB.

Squatting by the ewe and Flehmen by a ram after sniffing the ewe were not associated with oestrogen treatment. 'Looking over the shoulder' was a sign exhibited by most ewes treated with 10 µg OB, but not by ewes that did not receive oestrogen. There were no differences between clover-affected and control ewes for these responses.

Active soliciting by the ewe, standing for mounting, and being mounted by the ram, were behavioural responses closely associated with doses of OB greater than 15 µg. Tail fanning and kicking by the ewe were less closely associated with higher doses of OB. All of these responses were slightly but significantly diminished in clover-affected ewes, either in incidence, duration, or by a later time to onset. The results support the hypothesis that prolonged exposure to plant oestrogens causes persistent desensitization of the hypothalamus of the ewe to oestrogen.

Introduction

Ewes which have grazed highly oestrogenic subterranean clover for several seasons develop a permanent infertility (Bennetts, Underwood & Shier, 1946) that is part of the syndrome called clover disease. The infertility is associated with a failure of the cervix to maintain a normal sperm reservoir (Lightfoot, Croker & Neil, 1967) because of abnormalities in the cervical mucus (Adams, 1976). It has been suggested that the central nervous system may also be affected because the infertility persists for years after the ewes are removed from the oestrogenic pasture (Underwood & Shier, 1951).

In ovariectomized ewes with clover disease, the hypothalamus has a reduced ability to stimulate the release of LH in response to treatment with oestradiol-17β (Findlay *et al.*, 1973). As well as the areas of hypothalamus which control gonadotrophin release, there are also sites within the hypothalamus which control sexual behaviour (Clegg & Ganong, 1960; Radford, 1967; Przekop & Domański, 1970). Several different behavioural characteristics have been associated with the occurrence of oestrus in ewes (Banks, 1964; Tomkins & Bryant, 1974) and the incidence, time to onset, and duration of behaviour such as standing to be mounted by the ram, are directly related to the amount of oestrogen to which the ewe is exposed (Scaramuzzi, Lindsay & Shelton, 1971). However, the duration of oestrous behaviour is reported to be unaffected in intact ewes with clover disease (Smith, 1971; Lightfoot *et al.*, 1974). This may be due either to a failure of the behavioural responses in the affected ewes to diminish, as does the LH response reported by Findlay *et al.* (1973), or to a greater supply of endogenous oestrogen in clover-affected ewes at oestrus. The present work was to examine the behavioural responses to oestrogen in ovariectomized ewes with and without clover infertility to determine which of these alternatives is correct.

Materials and Methods

The ewes were taken from the flock studied by Rossiter & Marshall (1974). Eleven (11) of the ewes had grazed the highly oestrogenic Dinninup cultivar of subterranean clover for 4 years, and only 1 had produced a lamb from the last mating. The 14 control ewes had grazed the only slightly oestrogenic Northam A cultivar of subterranean clover for 4 years, and 11 had produced a lamb from the previous mating.

The ewes had not been exposed to oestrogenic pasture for at least 18 months before the present experiment. All the ewes were ovariectomized 4 months before the behavioural observations, which were made between December and February. Each ewe was identified by a large symbol painted on her side. The oestrogen dosage and previous grazing history of each animal were unknown to the 3 trained observers, who worked in overlapping shifts to record behaviour. Eight rams known to have a high libido were used sequentially in pairs.

For the observations the 2 rams were placed with the ewes in a large pen, 36 m², for a 45-min period every 4 h for 56 h, starting immediately after an injection of oestradiol benzoate (OB). During the trial in which ewes were not injected with OB, observations were carried out for only 32 h. The rams normally circulated well throughout the group of ewes, and did not spend a lot of their time with any particular ewe. The sheep were observed from seclusion as much as possible, but once mated, ewes were removed from the group for the remainder of the observation period.

Experiment 1

This experiment was carried out to determine the relationships between the dose of OB and the duration and time to onset of the various behavioural responses in the 14 control ewes. The ewes were given a daily i.m. injection of 10 mg progesterone in arachis oil for 10 days, and then randomly allocated to groups and injected i.m. with 15 (N = 5), 25 (N = 4) or 40 (N = 5) µg OB in arachis oil 72 h after the last progesterone injection.

Experiment 2

The doses in this experiment were those suggested by Reardon & Robinson (1961). Differences in the incidence, time to onset, and duration of behaviour characteristics were estimated. The 11 clover-affected ewes and 13 control ewes were injected i.m. with 10 mg progesterone in arachis oil daily for 10 days, and 72 h after the last progesterone injection each ewe was given an i.m. injection of 10.0, 15.6 or 24.3 µg OB in arachis oil. The progesterone pretreatment and trial was repeated 3 times, so that each ewe received each dose of oestrogen. A recovery period of 1 week was allowed between each trial. A fourth trial was conducted 2 weeks after the third: the ewes received progesterone pretreatment, followed by arachis oil only and were observed at 4-h intervals for 32 h after the arachis oil dose.

Statistical analysis

The results from Exp. 1 were examined by regression analysis, but this method could not be used for Exp. 2 because the small proportion of ewes responding made it impossible to plot an accurate regression. Results from Exp. 2 were compared statistically by Student's *t* test, or, when variances were not homogeneous or in accord with normal distribution, with Wilcoxon's Ranking Test. Differences between the number of ewes showing behavioural patterns were compared with the paired χ^2 test.

Behaviour recorded

The descriptions of Banks (1964) and Tomkins & Bryant (1974) of the behavioural manifestations of oestrus in sheep were used as the basis for the observations recorded in the present study. Ewes in oestrus also may make slow, rhythmic kicking movements with a hind limb at the belly and opposing forelimb (M. R. Sanders, personal communication) and this behaviour was also recorded. The signs

recorded for each ewe in each observation period were: (1) squatting, frequently accompanied by urination, by the ewe in response to approach by a ram; (2) Flehmen by a ram after sniffing at the ewe's perineal region; (3) the ewe looking back over her shoulder at a ram; (4) active soliciting of a ram by the ewe; (5) fanning of the tail by the ewe, when associating with a ram; (6) kicking at the belly by the ewe (not recorded in Exp. 1); (7) the ewe standing still for service by a ram, so that the ram could achieve intromission and ejaculation; (8) attempted and/or successful mounting of the ewe by a ram. An attempted mount was recorded regardless of whether the ewe stood sufficiently still for the ram to achieve intromission and these attempts were grouped with mounts at which intromission or ejaculation was achieved for analysis of mounting behaviour. No attempt was made to record the intensity of frequency of signs within each observation period.

Results

Oestrogen dose and behaviour

Experiment 1. Increasing doses of OB resulted in linear increases in the mean number of periods in which a ewe showed standing, soliciting, looking over the shoulder, or was mounted successfully or otherwise by the ram (Table 1). The time to first appearance of soliciting or being mounted decreased linearly with increasing dose of OB (Table 1). Neither the amount nor the time to onset of squatting, tail fanning or stimulation of Flehmen appeared to be related to the dose of OB (Table 1).

Table 1. The relationship between treatment with 15, 25 or 40 µg oestradiol benzoate (OB) and behavioural responses of ovariectomized ewes

Behaviour of ewe	Regression equations of behaviour (y) in periods on dose (µg) of OB (x), and the standard deviation from regression in periods ($sy.x$)	
	Duration	Onset
Standing	$y = 5.64^{**}x - 5.21; 0.95$	$y = 11.36 - 3.06x; 1.92$
Being mounted	$y = 9.44^{**}x - 9.07; 2.01$	$y = 15.85 - 6.84^{*}x; 1.51$
Soliciting	$y = 6.09^{**}x - 7.04; 1.10$	$y = 17.86 - 8.70^{*}x; 1.38$
Looking over shoulder	$y = 10.30^{***}x - 10.83; 1.51$	$y = 10.82 - 3.90x; 2.80$
Tail fanning	$y = 1.50x + 0.06; 2.10$	$y = 8.87 - 1.56x; 1.30$
Squatting	$y = 3.35 - 1.33x; 1.22$	$y = 5.82 - 0.33x; 3.60$
Flehmen	$y = 5.39 - 2.74x; 1.42$	$y = 6.40 - 0.98x; 3.01$

Values of the regression coefficient significantly different from zero: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

Experiment 2. No significant differences were detected between the 3 repetitions of Exp. 2, or between results recorded by the three observers. The behavioural signs recorded fell into three groups; those unrelated to oestrogen dosage, those seen in ewes treated with the low dose of oestrogen, and those appearing only in ewes treated with more than 15 µg OB. There was again no relationship between treatment with OB and squatting by the ewe or stimulation by the ewe of Flehmen in the ram: the mean ($\pm$ s.e.m.) number of ewes squatting in any observation period was 5.0 ± 0.3 , and of ewes stimulating Flehmen was 3.5 ± 0.3 , regardless of the dose of OB.

Apart from squatting and Flehmen, the only behavioural sign recorded consistently in ewes treated with 10 µg OB was that of the ewes looking over their shoulder at the ram (Table 2). There was a significant increase in the number of ewes showing this sign in any observation period when treated with 10 µg OB than when no oestrogen was given (Wilcoxon's Test, $P < 0.05$). There was, however, no significant increase in the number of ewes looking over their shoulder as the dose of OB increased from

10 to 24 µg. The mean number of observation periods in which ewes were seen to look over their shoulder increased significantly with the higher dose of OB in this experiment (1.5 ± 0.3 and 5.1 ± 0.5 periods at 10 and 24 µg OB, respectively; paired $t_{16} = 5.8$, $P < 0.001$). However, the time to onset of this behaviour did not vary with the OB dose (Table 2).

Table 2. The mean ($\pm$ s.e.m.) incidence, duration and time to onset of behavioural signs of oestrus shown by ovariectomized control and clover-affected ewes treated with different doses of oestradiol benzoate (OB)

Behaviour shown by ewe	10 µg OB		15.6 µg OB		24.3 µg OB	
	Control	Clover-affected	Control	Clover-affected	Control	Clover-affected
No. of ewes	13	11	13	11	13	11
<i>Standing for mounting</i>						
No. of ewes	0	1	5	5	11	8
Duration†	—	2	2.6 ± 0.7	2.6 ± 0.7	4.7 ± 0.8	4.4 ± 1.1
Time to onset (h)	—	26	24.4 ± 1.6	$30.8 \pm 0.8^*$	20.9 ± 1.3	25.0 ± 2.6
<i>Being mounted</i>						
No. of ewes	0	3	7	6	12	9
Duration†	—	1.3 ± 0.3	2.9 ± 0.9	2.7 ± 0.7	5.6 ± 0.6	4.4 ± 1.0
Time to onset (h)	—	34 ± 6	23.7 ± 1.5	$30.7 \pm 1.2^{**}$	20.7 ± 1.5	26.0 ± 2.5
<i>Active soliciting</i>						
No. of ewes	1	3	6	7	11	7
Duration†	1	1.3 ± 0.3	1.5 ± 0.3	1.3 ± 0.2	2.7 ± 0.4	2.0 ± 0.4
Time to onset (h)	14	16.0 ± 7.0	28.7 ± 8.0	28.9 ± 3.0	17.6 ± 2.3	$31.1 \pm 5.6^*$
<i>Tail fanning</i>						
No. of ewes	1	0	6	2	9	2*
Duration†	3	—	1.7 ± 0.3	1	3.6 ± 0.6	1.5
Time to onset (h)	3	—	22.0 ± 3.2	36	23.3 ± 3.0	26
<i>Kicking</i>						
No. of ewes	1	0	6	3	6	6
Duration†	1	—	1	1.3 ± 0.3	2.8 ± 0.7	$1.2 \pm 0.2^*$
Time to onset (h)	54	—	34 ± 3	32.7 ± 3.5	28 ± 3	30 ± 3
<i>Looking over shoulder</i>						
No. of ewes	10	7	9	9	12	11
Duration†	1.3 ± 0.2	1.9 ± 0.6	3.1 ± 0.7	3.2 ± 0.8	5.6 ± 0.6	4.5 ± 0.9
Time to onset (h)	28.4 ± 5.7	17.7 ± 3.9	27.3 ± 4.6	23.8 ± 2.8	19.6 ± 3.0	23.6 ± 3.1

Values significantly different from that for corresponding control ewes, * $P < 0.05$, ** $P < 0.01$.

† Indicates the average number of periods in which ewes were recorded as showing this sign.

The other behavioural signs which were seen mainly in ewes treated with 15.6 or 24.3 µg OB are given in Table 2. The numbers of ewes showing the different behavioural signs in the 10 and 24.3 µg OB groups were compared by paired χ^2 tests. The increase with increasing dose was greatest for the number of ewes standing (1 versus 19, $\chi^2_1 = 16.1$, $P < 0.005$), followed by ewes being mounted by the ram (3 versus 21, $\chi^2_1 = 15.1$, $P < 0.005$), soliciting (4 versus 18, $\chi^2_1 = 9.4$, $P < 0.005$), kicking (1 versus 12, $\chi^2 = 9.1$, $P < 0.005$), and tail fanning (1 versus 11, $\chi^2_1 = 7.1$, $P < 0.01$).

Behaviour of ewes not injected with oestradiol

There were no differences in behaviour between the clover-affected and control ewes in the fourth trial of Exp. 2. There was considerable exploratory activity by the rams, resulting in squatting by the ewe and Flehmen. These encounters occurred with a frequency similar to that when the ewes had been injected with OB, the mean ($\pm$ s.e.m.) numbers of ewes in any observation period squatting and stimulating Flehmen being 4.9 ± 0.5 and 3.6 ± 0.6 , respectively. Few other signs were observed in the absence of OB. One ewe was recorded as tail fanning, and 3 ewes were each recorded once during the 8 observation periods as looking over the shoulder at the ram.

Differences between clover-affected and control ewes

It is apparent from Table 2 that, when treated with 24.3 µg OB, fewer clover-affected than control ewes showed those signs associated with higher levels of oestrogen treatment, including being mounted, standing, soliciting, tail fanning and kicking. In the clover-affected ewes the mean number of periods in which these signs were recorded for active ewes was also fewer and the time to onset was longer.

When ewes were treated with 15 µg OB, clover-affected ewes were mounted later than controls ($P < 0.01$), and clover-affected ewes took significantly longer to stand for the ram to mount ($P < 0.05$). There were no differences between groups of ewes treated with 10 µg OB. No differences were observed at any dose for those behavioural signs which were not associated with high oestrogen doses (squatting, Flehmen or looking over the shoulder).

Discussion

There were no differences between clover-affected and control ewes in those signs which were independent of oestrogen treatment (squatting by the ewe, or Flehmen by a ram after sniffing the ewe). Clover-affected ewes did show a significant depression of the incidence, duration, or time to onset of all the behavioural signs which were displayed only after a dose of oestrogen sufficient to stimulate normal standing oestrus (standing, being mounted, active soliciting, tail fanning, and kicking by the ewe). Since the degree of response is related to the dose of OB, there is little doubt that the clover-affected ewes were less sensitive to the oestrogen treatment.

The ewes had not been exposed to oestrogenic pasture for 18 months, and it is therefore most unlikely that there was any pharmacological effect on the metabolic clearance of oestrogen. This conclusion is supported by the failure to detect any difference in the clearance of oestradiol from the circulation between clover-affected and control ewes (B. Y. Tang & N. R. Adams, unpublished observations). It is therefore probable that circulating levels of oestrogen were similar in both groups of ewes during the present study. Because oestrous behaviour is mediated by the hypothalamus in the ewe (Radford, 1967; Przekop & Domański, 1970), as in most other species (see Ciaccio & Lisk, 1973; Everitt & Herbert, 1975), the present findings support the hypothesis of Findlay *et al.* (1973) that the hypothalamus of ewes with clover infertility is less sensitive to oestrogen.

Findlay *et al.* (1973) were unable to detect a positive feedback effect of oestradiol on LH levels in 4 of 5 clover-affected ewes injected intravenously with 40 µg oestradiol-17β, although the LH response of the pituitary to Gn-RH was unimpaired. In contrast, the desensitization of the hypothalamus of clover-affected ewes in the present study was much less clear-cut. This discrepancy may be due to differences between the sheep used in the two studies, or to a greater sensitivity of the neurones responsible for the positive feedback of oestrogen on LH to previous prolonged exposure to plant oestrogens. Another explanation of the difference is that a depot injection of OB in oil was given intramuscularly in the present study, whereas Findlay *et al.* (1973) infused oestradiol-17β in saline into the jugular vein. The more prolonged exposure to oestrogen in the present work may have diminished the effects of hypothalamic insensitivity, as suggested by Powers (1975). It is likely that the duration of exposure to oestrogen at natural oestrus would also be sufficient to diminish the effects of reduced sensitivity to oestrogen for LH release by the hypothalamus. This could explain why the regularity of the duration of the oestrous cycle is not greatly affected in ewes with clover disease (Adams, Lightfoot & Charlick, 1975).

Smith (1971) and Lightfoot *et al.* (1974) observed a similar duration of standing behaviour at natural oestrus in clover-affected and control ewes and Smith (1971) concluded from observations of the duration of natural oestrus that there was no substantial difference between clover-affected and control ewes in circulating oestrogen levels at oestrus. Although the responses of the clover-affected ewes in the present experiments were depressed, the differences were not very large, e.g. 1.6 h in the ewes treated with 25 µg OB.

The results obtained in any behavioural experiment depend to some extent on the methods used. The present study was modelled on previous work in which only standing behaviour was measured (Fletcher & Lindsay, 1971; Scaramuzzi *et al.*, 1971) and the results of the studies are comparable. The ram-ewe interactions were observed in groups of animals and some competition between ewes must have existed, perhaps preventing the weaker levels of ewe response from being expressed. Furthermore the method did not allow measurement of the intensity of response by individual ewes within observation periods. More sensitive methods for studying individual ewes might amplify differences between clover-affected and control ewes, and increase knowledge of the responses of sheep to oestrogen. Nevertheless, under the conditions which have previously been used to measure responses to oestrogen (Reardon & Robinson, 1961; Scaramuzzi *et al.*, 1971; Fletcher & Lindsay, 1971) the differences between the clover-affected and control ewes were relatively minor in the present experiments. Standing, being mounted, soliciting, looking over the shoulder, tail fanning and kicking, all appeared to be expressed to a degree dependent on the dose of oestrogen, but only standing behaviour has been used previously in assaying doses of oestrogen. 'Looking over the shoulder' may have value in future studies on responses to low doses of oestrogen, since its expression is related to oestrogen dose, and it can be observed in ewes treated with low doses of oestrogen that do not induce other signs of behaviour.

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