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Effects of Oestradiol on Plasma Concentrations of Luteinizing Hormone in Ovariectomized Ewes with Clover Disease

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Abstract

The effects of oestradiol on plasma luteinizing hormone (LH) concentrations were examined in 15 ovariectomized control ewes and 15 similar ewes with permanent infertility after prolonged grazing on oestrogenic clover pasture ('clover disease'). Before treatment, the plasma concentrations of LH were similar in the control and affected ewes. After intravenous injection with 40 μg oestradiol-17β during the anoestrous season, the decline in LH concentration was greater in the clover-affected ewes and the subsequent elevation above original baseline levels was smaller. After intramuscular injection with 15 or 30 μg oestradiol benzoate during the normal breeding season, fewer clover-affected ewes showed a surge of LH, and the response was both reduced and retarded. This difference between the two groups has not been observed in studies on intact ewes, and it is suggested that in intact ewes the difference is masked by a greater tonic LH activity in affected ewes. The results of the present study are consistent with the hypothesis that prolonged exposure to oestrogenic pasture has a differentiating, or 'androgenizing' effect on the adult ewe.

Introduction

There is conflict in the literature as to whether ewes with permanent infertility after prolonged grazing on oestrogenic clover pasture (clover disease) have an impaired ability to produce a surge of luteinizing hormone (LH) after treatment with oestradiol. The initial report by Findlay *et al.* in 1973 suggested that affected ewes were less able to produce a surge of LH, but in subsequent studies (Rodgers *et al.* 1980; Chamley *et al.* 1981) this difference did not occur.

There are several possible explanations for this discrepancy. Findlay *et al.* (1973) were unable to obtain control ewes as old as the affected ewes studied (9–10 years old), and so the failure may have been due to the ewes' old age. Secondly, they studied ovariectomized ewes, while the subsequent studies were carried out on intact ewes during seasonal anoestrus, when endogenous levels of oestradiol are low. In intact animals, the affected ewes had higher initial concentrations of LH, and were therefore in a different physiological state from the controls. This difference may have masked any underlying disability of the surge mechanism.

Infertility in clover-affected ewes results mainly from differentiation of the cervix, which assumes a histological appearance similar to the uterus, and has an altered responsiveness to oestrogen (Adams 1981). There is little change in the ability of affected ewes to ovulate, suggesting that hypothalamic function is relatively normal (Adams *et al.* 1981). However, a mild change similar to sexual differentiation does

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occur in the hypothalamus of affected ewes so that male behaviour is slightly enhanced in affected ewes subsequently treated with testosterone (Adams 1981). The ability to give a surge of LH after oestrogen is also sexually dimorphic in the sheep (Karsch and Foster 1975), so that an altered ability to give a surge of LH might be expected in affected ewes. It is therefore worthwhile to clarify the discrepancy between the previous studies. The present work examines the effects of oestrogen on plasma LH concentrations in ovariectomized clover-affected ewes and comparable controls.

Materials and Methods

Sheep

Thirty 9-year-old Merino ewes were studied. Fifteen of these had been grazed on a highly oestrogenic pasture of subterranean clover (*Trifolium subterraneum* cv. Yarloop) for 3 years, and only 9% had lambed to their last mating. The control ewes had grazed non-oestrogenic pasture during this period and were of normal fertility. After treatment, the ewes were run together as a group on non-oestrogenic pasture for 5 years. All ewes were ovariectomized under general anaesthesia 1 year before the study commenced.

Experiment 1

In November, when sheep from this flock are normally in seasonal anoestrus (Adams 1979), the ewes were fitted with jugular cannulae. Three blood samples were collected at intervals of 2 h, and then 40 μg oestradiol-17β in 1 ml 10% (v/v) ethanol-saline was given rapidly to each ewe intravenously. Further blood samples were collected 4 and 7 h after injection to observe any negative feedback, then hourly from 10 to 24 h, and finally at 26 and 28 h.

Experiment 2

In February, the beginning of the normal breeding season for these ewes (Adams 1979), they were again fitted with jugular cannulae. Six ewes from each group were bled every 15 min for 8 h, and the remaining ewes were sampled each hour; 4 h after the commencement of sampling, eight ewes from each group were injected (i.m.) with 15 μg oestradiol benzoate (ODB) in 1 ml oil, and the remaining seven ewes from each group were injected with 30 μg ODB. In each group, three of the six ewes being bled every 15 min received 15 μg ODB, and the remaining three received 30 μg ODB. After this 8-h period, all the ewes were sampled at hourly intervals for 28 h after injection with ODB.

LH Assay

Plasma was separated from the blood samples and stored at -20°C until assayed for LH by the method described by Martin *et al.* (1980). The limit of detection of the standard curve was 38 pg per tube, and non-specific binding was less than 3·7%. Two pooled samples, containing 2·75$\pm$0·07 and 11·27$\pm$0·15 ng/ml (mean$\pm$s.e.) were assayed in each of the seven assays, and used to assess assay variation. The mean$\pm$s.e. within-assay coefficients of variation were 5·73$\pm$0·76 and 3·82$\pm$0·52%, and the between-assay coefficients of variation were 6·8 and 3·5% respectively.

Differences between groups were compared using the *t*-test at the times indicated.

Results

Experiment 1

The mean concentration of LH in the plasma was similar in affected and control ewes before treatment with oestradiol-17β, but after injection the LH concentration was generally lower in the affected ewes (Fig. 1). Suppression of LH (negative feedback) occurred in both groups at 4, 7 and 10 h after injection (Fig. 1).

Analysis of variance of these samples suggested that the depression was greater in the clover-affected ewes ($F_{1,28} = 4{\cdot}27$, $P < 0{\cdot}05$). The concentrations of LH then rose, reaching a maximum 16 h after injection. Only five ewes (four controls and one affected ewe) showed clear positive feedback of LH during this period (i.e. a sustained rise for 6 h or more above 10 ng/ml and a peak of at least 15 ng/ml), although all ewes showed some increase in concentration of LH (Fig. 1). The increase was greater in the control ewes, so that *t*-tests indicated significant ($P < 0{\cdot}05$) differences between the groups at 19, 20 and 22 h after injection.

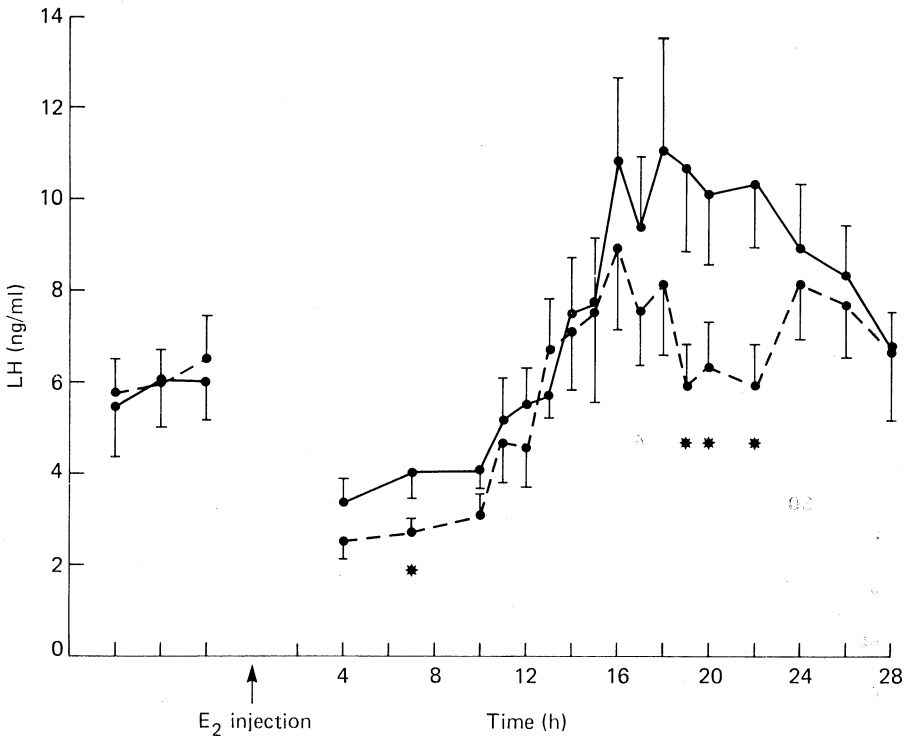


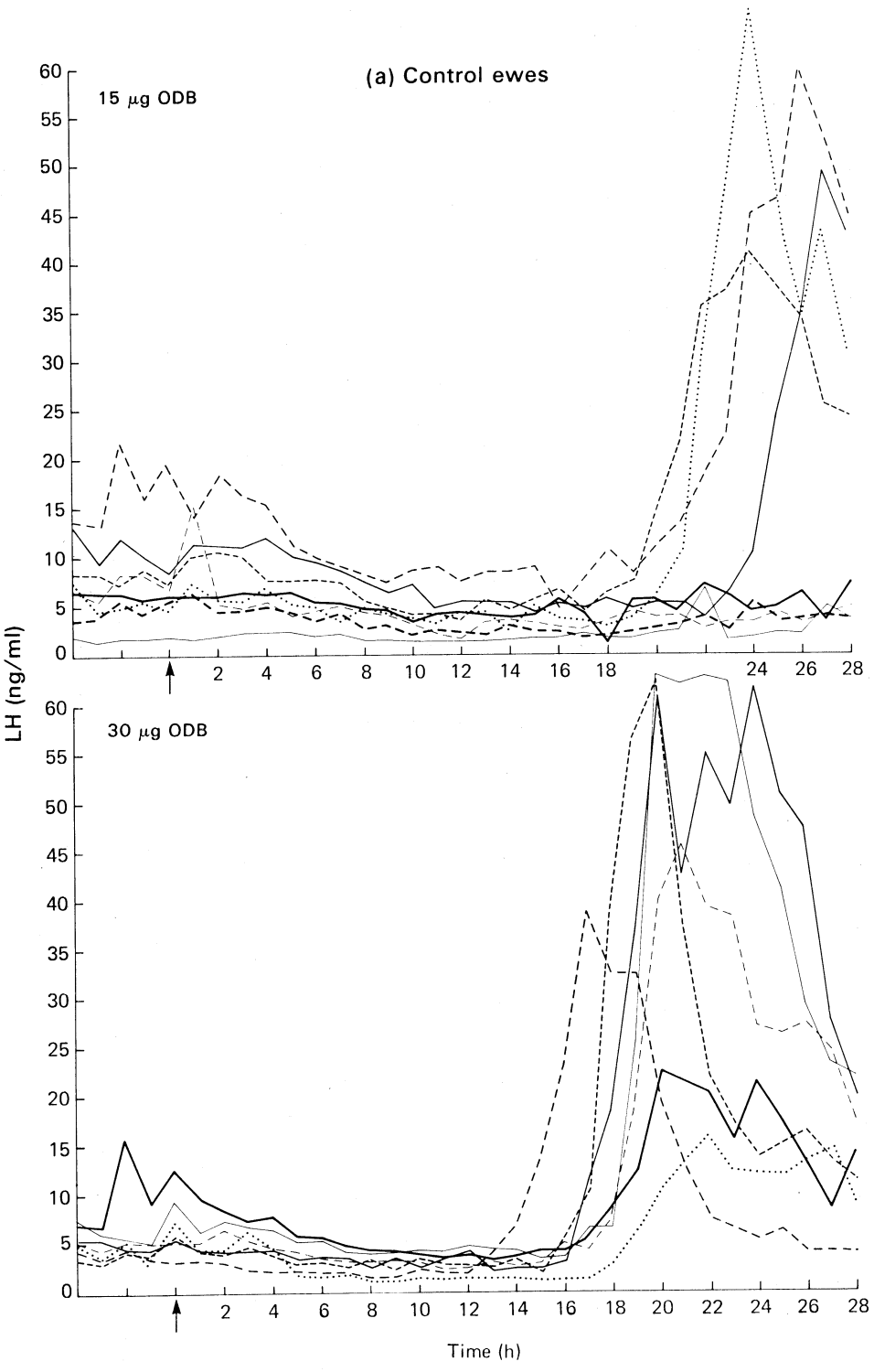
Fig. 1. Experiment 1: mean (with s.e.) plasma levels of LH in 15 control (●——●) and 15 affected (●- - -●) ovariectomized ewes before and after injection (i.v.) of 40 oestradiol-17β. * Groups significantly different ($P < 0{\cdot}05$) at this time.

Experiment 2

No differences were seen between groups or doses of ODB in the number of pulses of LH detected in the 12 ewes sampled every 15 min. A mean$\pm$s.e. of $9{\cdot}0\pm1{\cdot}2$ pulses per ewe occurred in the 8 h of study.

Mean values of the hourly bleeds before injection with ODB were $6{\cdot}7\pm1{\cdot}3$ *v.* $6{\cdot}1\pm1{\cdot}1$ ng/ml in control and affected ewes respectively. Levels of LH declined after treatment with ODB, and the minimal values of $3{\cdot}5\pm0{\cdot}7$ *v.* $3{\cdot}1\pm0{\cdot}6$ ng/ml occurred in control and affected ewes respectively 12 h after injection with ODB (Fig. 2).

Differences in the surge of LH are presented in Table 1. Half of the ewes (four control and three affected ewes) treated with 15 μg ODB gave an identifiable surge


(a) Control ewes
15 μg ODB
30 μg ODB
LH (ng/ml)
Time (h)
0
5
10
15
20
25
30
35
40
45
50
55
60
2
4
6
8
10
12
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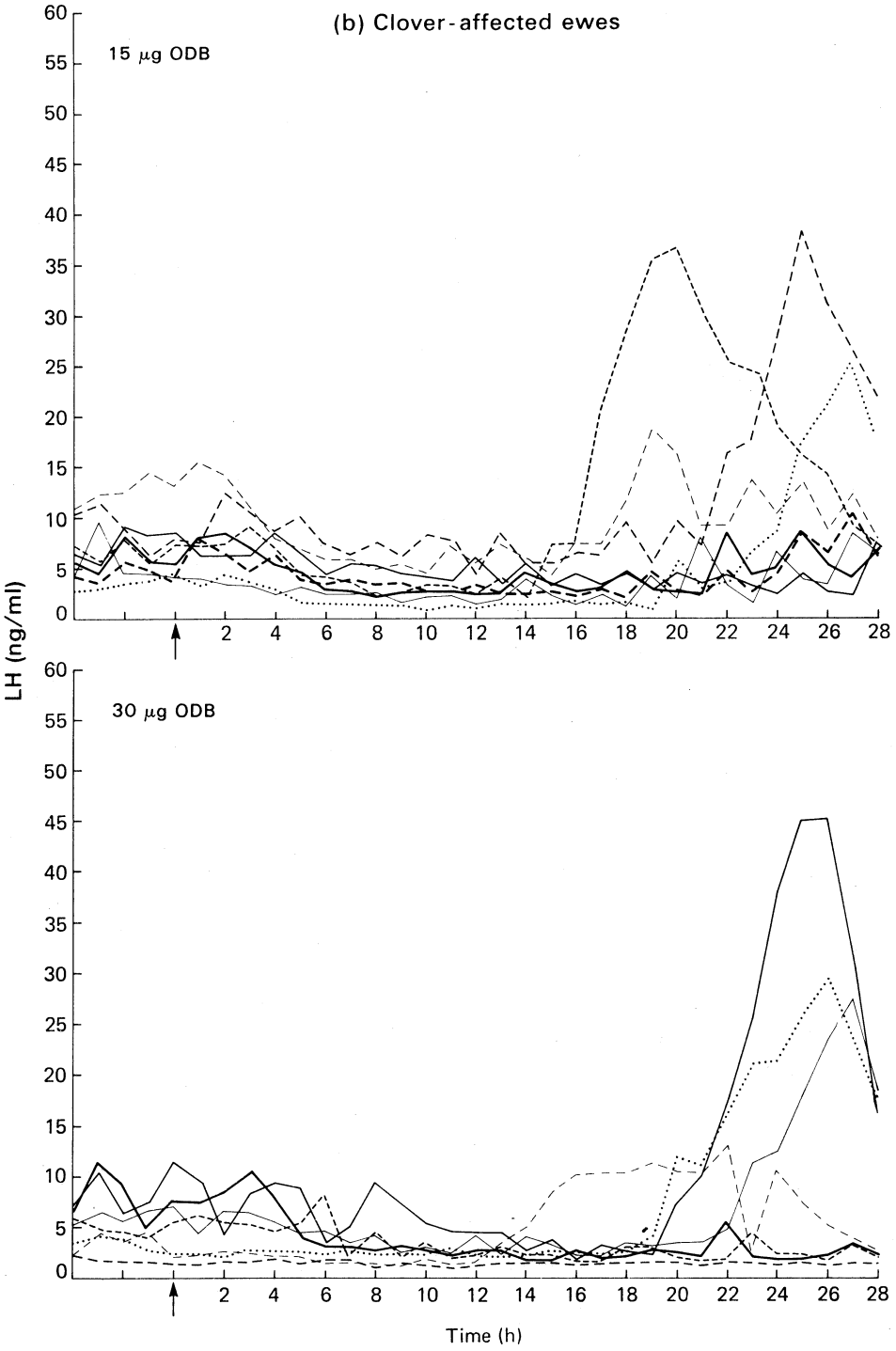

Fig. 2. Experiment 2: individual LH concentrations for (*a*) control and (*b*) clover-affected ewes (opposite) injected with either 15 or 30 μg ODB. Arrow indicates the time of oestradiol-17β injection.

of LH, defined as a sustained rise in values exceeding 10 ng/ml with a peak of at least 15 ng/ml (Fig. 2). All the controls, and three out of seven ewes treated with 30 μg ODB had a surge of LH. Fisher's one-tailed exact probability technique gives a value of $P = 0{\cdot}035$ that this difference was due to chance. The higher dose of ODB caused the LH surge to begin earlier in the control ewes ($P < 0{\cdot}01$), but it did not do so in the affected ewes. Thus, the surge began earlier in the control ewes than in the affected ewes in the groups treated with 30 μg ODB ($P < 0{\cdot}01$).

Table 1. Characteristics of the LH surge in clover-affected and control ewes

Values are means ± s.e. * $P < 0{\cdot}05$, ** $P < 0{\cdot}01$, significantly different from control ewes at that dose

Dose of ODB (μg)	No. of ewes	No. with surge	Time to onset (h)	Mean value of surge (ng/ml)	Peak value (ng/ml)
			Control ewes		
15	8	4	21·5±0·9	34·2±1·8	54·5±5·6
30	7	7	17·9±0·6	30·6±6·3	56·4±15·4
			Clover-affected ewes		
15	8	3	21·3±2·3	22·7±1·7**	33·4±4·1*
30	7	3	21·7±0·8**	23·3±4·0	34·2±5·6

The amount of LH released was independent of dose of ODB, when estimated either as the peak value attained during the surge, or the mean value during the surge. As shown in Table 1, clover-affected ewes released less LH than control ewes treated with 15 μg ODB ($P < 0{\cdot}01$). The variation in response was greater in ewes treated with 30 μg ODB, and so the difference was not statistically significant. The mean ± s.e. peak value obtained was greater in the control ewes than in affected ewes (55·7±9·7 *v.* 33·8±3·1 ng/ml, $t = 2{\cdot}14$, $P < 0{\cdot}05$).

The means ± s.e. plasma concentrations of LH for animals classified as not giving a surge during the period 15–28 h were 3·67±0·50 and 5·56±1·33 ng/ml for control and affected ewes respectively treated with 15 μg ODB, and 3·74±1·61 ng/ml for affected ewes treated with 30 μg ODB.

Discussion

Ovariectomized clover-affected ewes were less able to give an increase in LH after treatment with oestrogen, both in the normal breeding season and in the anoestrous season. This confirms the findings of Findlay *et al.* (1973), and suggests that Rodgers *et al.* (1980) and Chamley *et al.* (1981) did not find this effect because they used entire ewes. Basal levels of LH are higher in entire affected ewes than in controls during anoestrus (Rodgers *et al.* 1980; Chamley *et al.* 1981), but not in ovariectomized ewes. The higher basal values indicate an altered physiological state in the entire affected ewes, which may explain why they give a greater LH response to oestradiol than occurs when both groups have a similar basal level of LH. For example, the higher levels of LH in entire affected ewes result at least partly from more frequent LH peaks (Rodgers *et al.* 1980), indicating that the release of gonadotrophin-releasing hormone (GnRH) is more frequent in these ewes (Clark

and Cummins 1982). If the small releases of GnRH seen by Clark and Cummins (1982) also occur more frequently in affected ewes, these may prime the pituitary to respond, as has been reported in women treated with GnRH (Hoff *et al.* 1979). An increased responsiveness of the pituitary is also indicated by the more rapid surge of LH reported in entire affected ewes by Chamley *et al.* (1981). When the difference in basal levels of LH is removed by ovariectomy, it can be seen that, in fact, affected ewes are less likely to give an LH surge.

A reduced ability to give an LH surge, and a decreased size of the surge, is consistent with a more 'masculinized' pituitary and hypothalamus in the affected ewes (Karsch and Foster 1975; Clarke and Scaramuzzi 1978). It is not clear, however, whether this is why the negative feedback of oestradiol on LH appeared to be greater in affected ewes during anoestrus. Negative feedback is normally reduced in castrated males when compared with castrated females (Karsch and Foster 1975), although a normal or enhanced negative feedback has been observed in both ewes and female rats which have been masculinized by treatment with testosterone during organogenesis (Turgeon and Barraclough 1974; Clarke and Scaramuzzi 1978). The changes in oestrogen receptors in the pituitary and hypothalamus of affected ewes (Tang and Adams 1978) may also play a role in the altered responsiveness to oestrogen.

It is unlikely that the differences between affected and control ewes were due to different rates of oestrogen metabolism. The amount of LH released in a surge was independent of the dose of ODB, but was reduced in ewes with clover disease. The time interval between treatment and the LH surge was dependent on the dose of ODB in the control ewes and was longer in affected ewes, but factors other than dose, such as the stage of the breeding season, can also affect this characteristic (Howland *et al.* 1978).

Both Rodgers *et al.* (1980) and Chamley *et al.* (1981) have reported that basal levels of LH are increased in entire, affected ewes. It is difficult to understand the mechanism for this. The increase must reflect a difference in the feedback of the ovary on the hypothalamo-hypophyseal system, because the difference was not present in the ovariectomized ewes in the present study. However, Chamley *et al.* (1981) found that the negative feedback induced by oestradiol is enhanced in affected ewes, and the present study on ovariectomized ewes suggests that it is at least as great in affected ewes as in controls. Furthermore, it is most likely that entire affected ewes produce more oestrogen than controls (Adams *et al.* 1979). Thus, the elevated LH in entire affected ewes cannot be the result of inadequate feedback by oestradiol. We must therefore conclude that some other factor which controls the level of LH during anoestrus is lacking in ewes with clover disease. Such a factor must be produced by the ovary, or be oestradiol-dependent for the expression of its biological activity.

The 'organizational' effects of oestrogen or aromatizable androgen on the central nervous system of the neonatal rat include a loss of the LH surge response, and diminished female sexual behaviour and enhanced male behaviour in response to sex steroids in later life (Gorski 1973). Ovariectomized ewes permanently affected with clover disease show similar changes in sexual behaviour (Adams 1981) and in positive feedback of oestradiol on LH. The main differences between 'androgenization' in neonatal rats and sheep with clover disease are that the changes in

sheep can be brought about in adult life, and are less marked in the sheep. Failure of positive feedback on LH is the primary cause of the permanent infertility in the 'androgenized' rat. In contrast, the entire clover-affected ewe can fully compensate for the defects in both behavioural responsiveness and the sensitivity of positive feedback, so that the duration of oestrus is normal in affected ewes (Lightfoot *et al.* 1974) and the surge of LH occurs normally (Rodgers *et al.* 1980; Chamley *et al.* 1981). As discussed above, the method by which the entire ewe compensates is unclear.

Acknowledgments

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