Aust. J. Biol. Sci., 1975, **28**, 279–90

Hormone Studies on Ewes Grazing an Oestrogenic (Yarloop Clover) Pasture During the Reproductive Cycle

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Abstract

The endocrine function of Merino and Corriedale ewes grazing an oestrogenic (Yarloop clover) pasture has been studied during the oestrous cycle, pregnancy and parturition, and the results compared with those from a study of similar ewes grazing a neighbouring grass pasture. Plasma progesterone, oestrogen and corticoids were measured using competitive protein binding assay procedures.

During the oestrous cycle clearly anomalous patterns in hormone content were evident in ewes grazing Yarloop, and this related to their significantly poorer ($P < 0 \cdot 001$) fertility.

The first mating, when ewes were $1\frac{1}{2}$ years of age, was particularly affected. Successful conception took place in only 27% of ewes mated on Yarloop, compared with 95% on grass. Evidence of disturbance in the normal patterns of both plasma oestrogen and progesterone was found in infertile ewes, including a shortened period of luteal function.

Disturbance of endocrine function caused by Yarloop clover ingestion was also found in pregnant ewes, with the mean plasma progesterone concentrations during the latter half of pregnancy reduced ($P < 0 \cdot 05$) and the plasma oestrogen and corticoid levels tending to be higher in these animals.

In detailed hormone studies in the periparturant period, both groups showed a similar fall in plasma progesterone and rise in plasma oestrogen prior to parturition. Where excessive time was taken for parturition (more than 30 min) this was reflected in higher plasma corticoid levels ($P < 0 \cdot 05$) within 8 h of birth.

Introduction

Reproductive difficulties in sheep grazing subterranean clover pastures were first described by Bennetts (1944) and Bennetts *et al.* (1946). Characteristic signs were ewe infertility, dystocia and uterine prolapse resulting in high ewe and lamb mortality. This syndrome of aberrant reproductive functions on clover pastures is now commonly referred to as 'clover disease' and its occurrence has been widely recognized in association with many different clover species throughout the world (Moule 1961; Moule *et al.* 1963; Bickoff 1968).

The results of extensive studies have revealed that the aetiology relates to oestrogenic isoflavones present in the plants (Batterham *et al.* 1965). However, the exact way in which these substances exert their adverse effects on reproductive function is as yet unresolved. In the present study, an investigation has been carried out to examine the possibility that the clover acts by causing disturbances in the endocrinology of reproduction.

Preliminary investigations (Obst *et al.* 1971*a*) suggested that good indices of reproductive endocrine function for grazing flock sheep can be obtained by using relatively simple radio-ligand assays for plasma hormones. Comparison of these indices throughout the reproductive cycle was, therefore, made between a group of sheep grazing an oestrogenic pasture (*Trifolium subterranean* cv. Yarloop) and a non-oestrogenic grass pasture.

Preliminary reports on this study have been published previously (Obst and Seamark 1970, 1972; Obst *et al.* 1971*b*; Obst 1972).

Materials and Methods

Merino ewes and wethers (Koonoona strain) were obtained from flocks of the Agricultural Research Station at Wanbi, S.A., and were born and reared on non-clover pastures. Corriedale ewes and wethers were obtained from flocks at the Kangaroo Island Research Centre (KIRC), maintained on mixed 50/50 pastures of perennial rye grass and clover (cvv. Mt. Barker, Yarloop and Woogenellup). The Merino rams (Bungaree strain) and Corriedale rams were also from KIRC flocks.

The Yarloop clover pasture of 17·2 ha (area A) was cleared and sown with Yarloop in 1959. Visual estimations of species composition of the pasture during the period of study (1969–1971) indicated that more than 70% of available feedstuff was Yarloop, with annual grasses and weeds constituting the remainder of the pasture. The grass pasture of 35 ha (area B) had a history similar to the Yarloop pasture until 1967 when a renovation program successfully established a pasture dominant in perennial rye grass. Contamination with Yarloop regrowth after renovation was less than 5% of the total pasture in 1969, but this increased to about 15 and 20% in 1970 and 1971 respectively.

Procedure

The investigation was initiated in February 1969 when ewes were 1½ years of age. Two groups of animals were formed each of 50 Merino and 50 Corriedale ewes and 40 wethers. One group was then maintained on the Yarloop clover pasture and the other on grass pasture throughout the period of the experiment. Ewes and wethers were weighed monthly. To maintain approximately equivalent liveweights in the two groups during and between years the stocking rates were adjusted with additional KIRC wethers. From January to April 1970, when dry Yarloop residues were limited, the Yarloop group was supplemented with Yarloop clover hay.

Study I. Two fertile rams were introduced into the flock grazing Yarloop in February 1969 and the first six Merino and six Corriedale ewes mated were used for blood sampling and hormone measurement. During this period the pasture consisted mainly of green germinating Yarloop clover.

The ewes on the grass pasture were first joined with a vasectomized ram and the first six ewes mated were kept separately with the teaser ram for blood sampling and hormone determination. The remaining ewes in each group were mated to four fertile rams. Venous blood samples of 10 ml were collected into heparinized containers every second day commencing on the day of mating (day 0) or the first day after mating (day 1). Blood sampling continued for a period of 42 days.

Study II. Four vasectomized rams fitted with Sire-sine harnesses and crayons were introduced on 2 October 1969 to the ewes which had grazed the Yarloop pasture since its germination in February 1969. The identity of the animals mated was recorded daily. Of the ewes which did not produce a lamb in study I, the first 20 to mate (13 Merinos and 7 Corriedales) were confined to a smaller area (C) of green Yarloop clover within the same area (A) to aid the collection of blood samples.

Blood samples of 20 ml for hormone determination were collected daily at 0900 h from the 20 ewes commencing on 20 October until 21 November 1969. Plasma progesterone determinations were made on all samples collected, but plasma oestrogen concentrations were determined only on samples selected from ewes which exhibited specific patterns in the function of their corpora lutea. Corpus luteum function was classified on the basis of peripheral plasma progesterone concentrations as follows.

(1) Shortened—progesterone levels declined from day 12 to values less than 1·0 ng/ml on day 14 and remained below 1·0 ng/ml until oestrus.

(2) Normal—progesterone levels were maintained above 1·0 ng/ml from day 12 to day 14 inclusive and then declined to below 1·0 ng/ml by day 19.

(3) Prolonged—progesterone levels were maintained above 1·0 ng/ml from day 12 to beyond day 19.

Study III. Merino and Corriedale ewes on each pasture were mated separately for 6 weeks from mid-February to the end of March 1970 with two rams of the same breed per group. Ewes were then 2½ years old. Twenty ewes (5 Merino and 5 Corriedale from each pasture group) which did not return to service during the 6-week mating period were selected for hormone measurement.

To facilitate blood collection, the selected ewes were confined to smaller areas on the same pastures and jugular vein blood samples of 20 ml were taken daily at 0800 h from day 40 of gestation to 2 days post-partum. Additional blood samples were taken at 1600 and 2400 h from day 137 of gestation onwards to follow periparturant changes in more detail.

Lambing Observations

Lambing ewes (10) on the Yarloop pasture whose blood was being sampled were continuously observed with the aid of spotlights and binoculars to allow the recording of time of birth, duration of parturition and signs of dystocia. Duration of parturition (in minutes) was defined as the time from first appearance of the lamb's front feet at the vulva to complete delivery of the lamb. The 10 lambing ewes on grass pasture were observed every 30 min, and time of birth, incidence of dystocia and approximate length of parturition were recorded.

General lambing observations on all other ewes were performed daily at approximately 0800 h when each lamb born was identified with its mother, ear-tagged and weighed. The incidence of dystocia was recorded and a post-mortem examination made on ewes which died near term, or were unable to lamb even with manual assistance, to ascertain the number of dead lambs *in utero*.

Hormone Assay Procedures

(i) *Progesterone assay.* The competitive protein binding (CPB) procedure used was essentially the same as that described by Thorburn *et al.* (1969), employing light petroleum as the plasma extractant and dog or hen plasma as a source of corticosteroid binding globulin with [$^3H_2$]cortisol as the competing tracer. Free and bound steroids were separated on small Sephadex G25 (fine) columns (Bassett and Hinks 1969).

Progesterone standards containing 0, 0·2, 0·4, 1·0, 2·0 and 4·0 ng added to 0·2 or 0·5 ml of oophorectomized ewe plasma were run with each assay and results are expressed as progesterone equivalents.

(ii) *Oestrogen assay.* An index of the level of plasma oestrogen was determined using a CPB method based on that described by Korenman (1968, 1969). Uteri of 6-day-pregnant rabbits were used as a source of binding protein and the cytosol was prepared as described by Korenman (1968).

For assay, plasma (2–4 ml) was extracted with 2 volumes of ethyl acetate and the residue, after removal of the solvent, was reacted with the uterine cytosol. The competing tracer was [$^3H_2$]oestra-3,17β-diol. Competition for binding sites was allowed to occur for at least 6 h, usually overnight, at 4°C and the free and bound fractions separated by small Sephadex columns using 4 ml of 0·01M tris buffer (pH 8) as eluant.

Oestrogen standards containing 0, 20, 50, 100, 200 and 400 pg of oestra-3,17β-diol (E_2-17β) were run with each assay. As a check on procedure, samples of oophorectomized ewe plasma and water (2 or 4 ml) were also included as blanks.

(iii) *Corticoid assay.* Plasma corticoid concentrations were determined by the method of Bassett and Hinks (1969). Results expressed as cortisol equivalents (ng/ml) were corrected to allow for interference by progesterone present in the ethanol extract.

Assessment of Oestrogenicity of the Pastures

(i) *Isoflavone content.* Samples from Yarloop pasture taken in July–August 1970 were kindly analysed for the isoflavones, genistein, formononetin and biochanin A by Dr R. C. Rossiter of the Division of Land Resources Management, CSIRO, Wembley, Perth, W. A.

(ii) *Bioassay.* At various times during 1969 and 1970, the size of the bulbo-urethral gland of wethers grazing the two pastures was assessed by digital palpation through the rectum, and the size and state of the glands were scored as follows to assess any accumulative effects of the oestrogenic activity of the pasture:

Score	Diameter (mm)	Description
1	5	Normal, soft palpable
2	5–15	Slightly enlarged, hard
3	15–25	Enlarged, hard
4	25–35	Very enlarged, hard
5	35	Grossly enlarged with formation of a *cul de sac*

(iii) *Analysis of results.* Comparison of reproductive performance between pastures were made using the χ^2 test (Snedecor and Cochran 1968) and the significance of differences in the mean plasma hormone concentrations assessed using Student's *t*-test (Snedecor and Cochran 1968). Attempts were also made to relate individual ewe hormone levels with lambing observations.

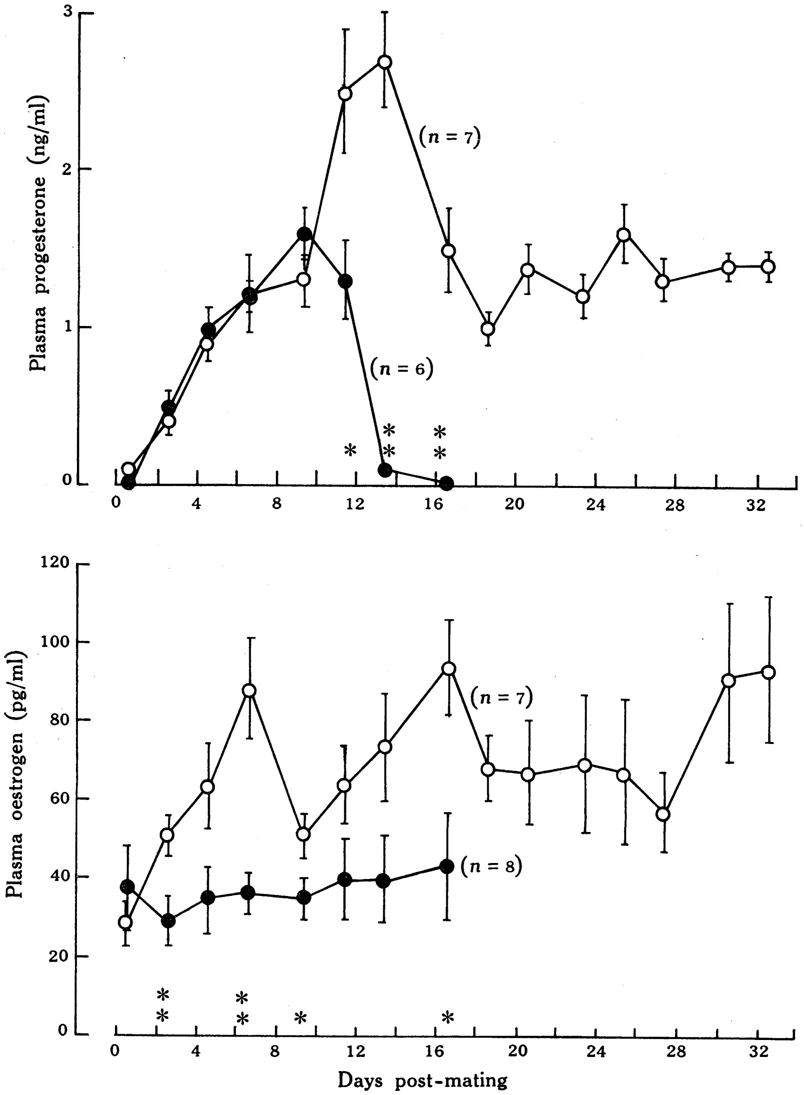

Fig. 1. (*a*) Plasma progesterone and (*b*) oestrogen following mating in ewes grazing green Yarloop clover (study I). Vertical bars represent ±S.E.M. for fertile (○) and infertile (●) ewes. The numbers of ewes in each group are shown in brackets. Statistically significant differences are denoted thus: * $P < 0{\cdot}05$, ** $P < 0{\cdot}01$.

Results

Pasture Oestrogenicity

The mean content of the isoflavones formononetin, genistein and biochanin A in the Yarloop clover during July and August 1970 was 1·2, 2·5, and 0·54%, respectively, of the dry leaf weight.

The bulbo-urethral glands of the wethers were palpated at intervals of approximately 2 months from February 1969 to July 1970, and during this time 34 out of 40 wethers grazing Yarloop pasture and 13 out of 40 wethers grazing grass showed evidence of gland enlargement. On Yarloop, 24 of the 34 were given a score of 3 or more for at least two of the six palpations, whereas only 5 of the 13 on grass pastures received a score of 3 or more.

Fertility and Reproductive Performance

The overall fertility of ewes grazing Yarloop pastures (Table 1) was severely reduced compared with ewes on grass ($P < 0{\cdot}001$). The largest difference in fertility between groups of animals on the different pastures occurred at the first mating in 1969. Following this mating only 14% of the Corriedales and 40% of the Merino ewes produced lambs while grazing green Yarloop pasture compared with over 90% of Corriedales and Merinos grazing the grass pasture. The percentages of Merino and Corriedale ewes which lambed 0, 1, 2 or 3 times during the 3 years whilst grazing on Yarloop were 22·2, 37·4, 28·3 and 12·1%, respectively, whereas the comparable values for ewes on the grass pastures were 3·1, 9·5, 34·7, and 52·6%.

Lamb survival was lower ($P < 0{\cdot}01$) on the Yarloop pasture (55%) than on the grass pasture (71%). The differences between pastures in numbers of lambs born and in lamb and ewe mortality were most marked in the Corriedales. Uterine prolapse following parturition was observed only on the Yarloop pasture.

Endocrine Investigations: Assay Assessment

For the progesterone assay, estimates of the coefficient of variation at different points on the standard curve ranged from 2·6 to 6·5%. The mean recovery±S.E.M. of added amounts of progesterone (0·2–8·0 ng) was 90±2% ($n = 12$) with no plasma and 86±2·5% ($n = 12$) with oophorectomized ewe plasma.

For the oestrogen assay the coefficient of variation of determinations of various amounts of E_2-17β over the useful range (20–400 pg) of the standard curve varied from 2·9 to 6·1%. Recovery estimates of non-labelled E_2-17β (0–200 pg) added to ewe plasma were 93–96%.

Of the isoflavones tested, genistein in 10-ng amounts assayed as equivalent to 35 pg of E_2-17β. However, 200 ng or more of diadzein, biochanin A or formononetin was required to be present in samples before significant interference was recorded from these compounds. No equol was available during these investigations but the results of Shutt and Cox (1972) indicate that equol may react similarly to genistein under the conditions of assay employed.

Study I. All of the ewes on Yarloop selected for hormone assessment remated at least once during the 42-day period of study (17 matings total) and five lambs resulted, two to the second mating. The mean ±S.E.M. plasma progesterone and oestrogen concentrations following these infertile and fertile matings of animals on Yarloop are presented in Fig. 1. Plasma progesterone concentrations were similar up to days 9–10 in both groups. At days 11–12, however, progesterone concentrations of the infertile group began to fall to reach oestrous levels at days 13–14, indicating a shortened period of corpus luteum (CL) function. Infertile matings were also characterized by lower plasma oestrogen levels than were found in fertile matings (Fig. 1).

Study II. Following 9 months of grazing on green Yarloop pastures, nearly all ewes which were infertile at their first mating and showed evidence of shortened periods of CL function had normal luteal phases. Of the 32 luteal phases studied, 24 were of normal length and were followed by oestrus, three had normal levels of plasma progesterone but there was no subsequent oestrus in the period of study, two luteal phases were shortened and the remaining three were prolonged. Patterns of plasma progesterone and oestrogen that are representative of each type of CL function are presented in Figs 2 and 3. Plasma progesterone concentrations (Fig. 4) on days 6–14 post-oestrus were significantly ($P < 0\cdot05$) higher in ewes grazing Yarloop in October 1969 (study II), than in those grazing Yarloop or grass in February –March 1969 (study I).

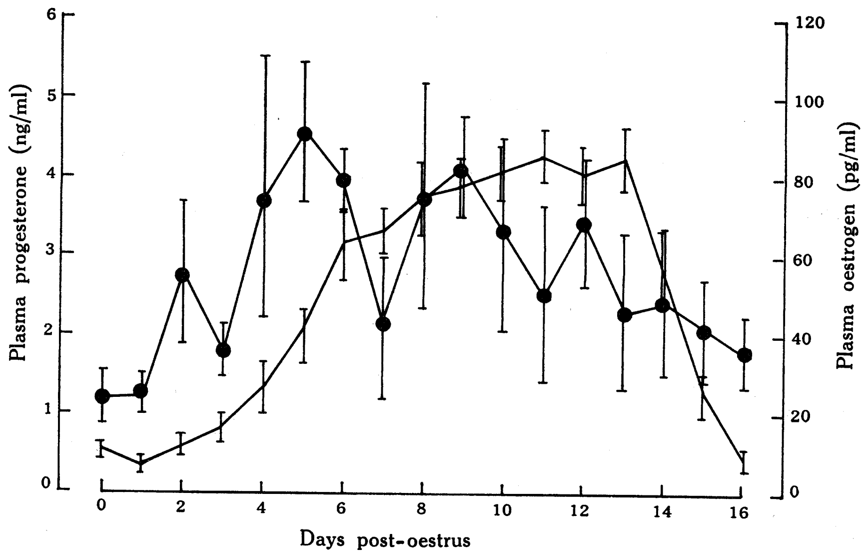

Fig. 2. Plasma progesterone (—) and oestrogen (●) in five ewes exhibiting normal CL function while grazing Yarloop clover pastures (study II). Vertical bars represent ±S.E.M.

The higher percentage (84%) of normal luteal phases among the selected ewes in study I compared with study II is reflected in the improved fertility of the experimental flock during the mating in February and March 1970 (Table 1).

Study III. The mean plasma progesterone concentration of pregnant ewes grazing Yarloop was significantly ($P < 0\cdot05$) lower than in those grazing grass pasture from 90 days gestation to term (Fig. 5*a*). Plasma corticoid concentrations for ewes on both pastures increased significantly ($P < 0\cdot001$) from 40 days gestation to 100–120 days gestation (Fig. 5*a*). Thereafter, concentrations declined towards term but the mean values for ewes on Yarloop remained higher ($P < 0\cdot05$) from 120 to 140 days gestation than the mean values for those on grass pasture.

Significantly ($P < 0\cdot05$) higher concentrations of plasma oestrogen were observed from 110 to 120 days and at 140 and 145 days gestation (Fig. 5*a*) in the ewes grazing Yarloop pasture compared with the ewes grazing grass pasture throughout pregnancy. No differences, however, were apparent between the two groups in the characteristic rise in plasma oestrogen occurring within 24 h of birth (Fig. 5*b*).

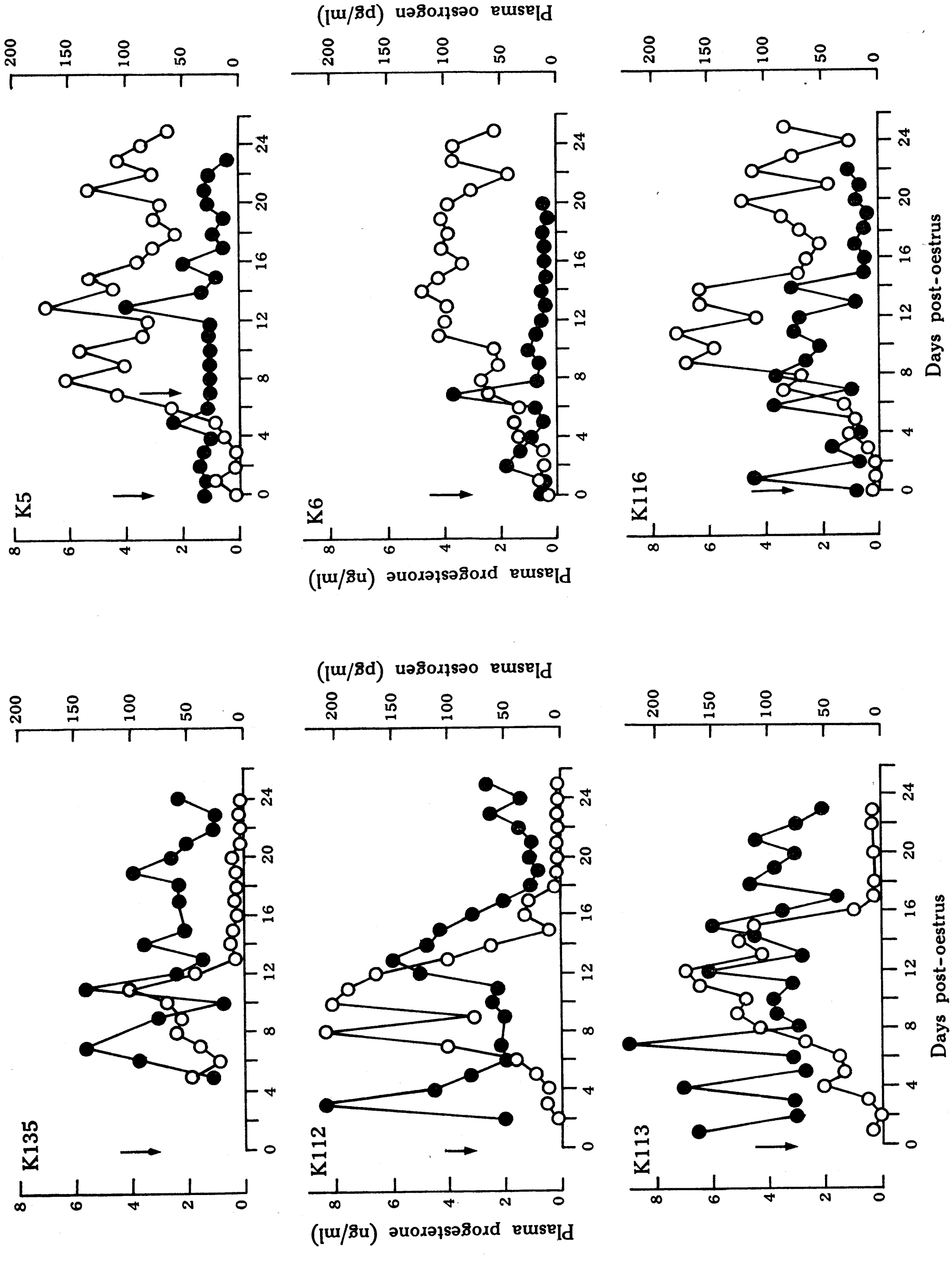

Fig. 3. Plasma progesterone (○) and oestrogen (●) in individual ewes exhibiting irregular oestrus or CL function or both while grazing Yarloop clover (study II). Arrows indicate the occurrence of oestrus.

The mean (±s.e.m.) gestation lengths and birth weights of lambs were similar between the groups selected from Yarloop ($151 \cdot 4 \pm 1 \cdot 70$ days, $4 \cdot 15 \pm 0 \cdot 25$ kg) and grass ($150 \pm 1 \cdot 06$ days, $4 \cdot 05 \pm 0 \cdot 15$ kg) pastures. However, four ewes on the Yarloop pasture experienced lambing difficulties compared with only one on grass. In these ewes the mean plasma corticoid concentration within 8 h of birth was higher ($P < 0 \cdot 05$) than in the other ewes where parturition took less than 30 min. No relationship was apparent, however, between plasma progesterone or oestrogen concentration and duration of parturition.

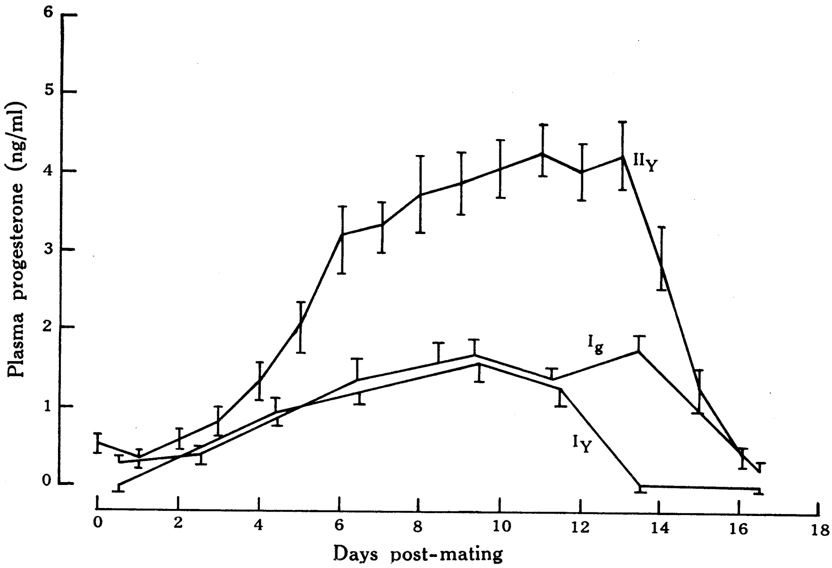

Fig. 4. Plasma progesterone in ewes grazing Yarloop clover in study I (I_Y) and study II (II_Y) compared with ewes grazing grass pasture in study I (I_g). Vertical bars represent ±s.e.m. $n = 5$. Progesterone concentrations in study II were significantly higher ($P < 0 \cdot 05$) from day 6 to day 14 post-mating.

Discussion

The study reveals that the poor reproductive performance of ewes grazing Yarloop is associated with marked disturbances in reproductive endocrine function. That hormonal disturbances may occur in ewes grazing oestrogenic pasture was suggested by the anomalies in both the incidence of oestrous and inter-oestrous intervals observed by previous workers (Underwood and Shier 1952; Turnbull *et al.* 1966; Fels and Neil 1968).

The cause of the particularly low fertility of ewes grazing Yarloop in 1969 remains unresolved. It appears to have been a temporary effect, as the majority of the ewes had improved fertility in study II despite continued grazing of the Yarloop in the intervening 9-month period. The improvement in fertility was accompanied by a higher incidence of normal periods of luteal function and, interestingly, higher plasma progesterone levels than found in study I. These changes probably relate to age of the ewe and seasonal factors, but it is possible, as suggested by Morely *et al.* (1969), that some ewes adapted to adverse effects of ingesting the large amounts of oestrogenic isoflavones present in the pasture. Certainly, the pastures remained oestrogenic as indicated by the effects on the bulbo-urethral gland of the wethers.

Table 1. Reproductive performance of Merino and Corriedale ewes grazing Yarloop or grass pasture during the years 1969, 1970 and 1971

	Merinos			Total or	Corriedales			Total or	Merinos and Corriedales
	1969	1970	1971	mean	1969	1970	1971	mean	1969–1971
				Yarloop pasture					
Ewes mated	50	43	44	137	50	44	45	139	276
% Ewes fertile	40	67	59	55	14	50	44	38	46
Lambs born									
Singles	20	24	21	65	7	17	20	44	109
Multiples	0	10	10	20	0	10	8	18	38
Total (% of total ewes)	40	79	70	62	14	61	62	45	53
Lamb deaths									
Singles	11	7	7	25	3	11	9	23	48
Multiples	0	5	3	8	0	7	3	10	18
Total (% of lambs born)	55	35	32	39	43	67	43	53	45
Ewe deaths	0	3	5[A]	8	0	4	6[B]	10	18
Lamb deaths *in utero*	0	4	4	8	0	6	5	11	19
Lambs marked (% of ewes mated)	18	51	48	38	8	20	36	21	29
				Grass pasture					
Ewes mated	52	46	47	145	48	38	43	129	274
% Ewes fertile	90	74	74	80	100	87	74	78	84
Lambs born									
Singles	44	24	21	89	47	25	24	96	185
Multiples	6	20	28	54	2	16	17	35	89
Total (% of total ewes)	96	96	104	99	102	108	95	102	100
Lamb deaths									
Single	12	4	2	18	11	5	4	20	38
Multiples	4	13	17	34	2	2	3	7	41
Total (% of lambs born)	32	39	39	36	27	17	17	21	29
Ewe deaths	0	2	2	4	0	0	0	0	4
Lamb deaths *in utero*	0	4	3	7	0	0	0	0	7
Lambs marked (% of ewes mated)	65	59	64	63	75	89	79	81	71

[A] Includes two ewes with prolapsed uterus. [B] Includes one ewe with prolapsed uterus.

The basis for the endocrine disturbances caused by Yarloop ingestion during pregnancy is also not understood. Whether the different amounts of plasma hormones in ewes grazing Yarloop reflect changes in the rates of hormone synthesis or metabolic clearance or both is unknown.

Recent studies on the origin of plasma progesterone in sheep have shown that up to 110 days gestation the CL is a major contributor, but between 130 days and birth the ovarian contribution is small (Edgar and Ronaldson 1958; Mattner and Thorburn 1971). Albeit, bilateral oophorectomy at 110–124 days gestation resulted in a 50% reduction of peripheral plasma progesterone concentrations in late pregnancy (Fylling 1970), a difference similar to that seen between the ewes on Yarloop and those on grass pasture.

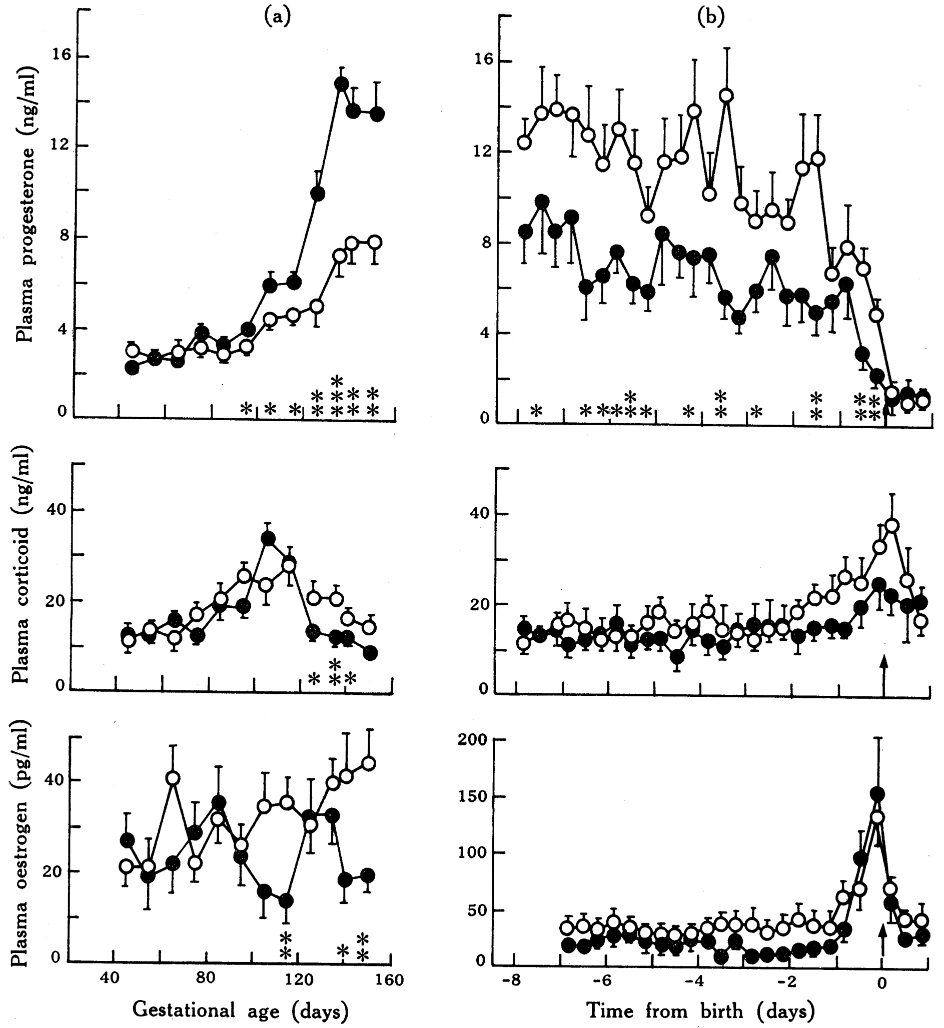

Fig. 5. Plasma progesterone, corticoid and oestrogen (*a*) during pregnancy and (*b*) about parturition in nine ewes grazing grass (●) and nine ewes grazing Yarloop clover (○). Vertical bars represent ±S.E.M. Statistically significant differences are denoted thus: * $P < 0 \cdot 05$, ** $P < 0 \cdot 01$, *** $P < 0 \cdot 001$.

The pattern of plasma corticoid found in ewes on grass pastures is very similar to that reported by Basson *et al.* (1969). The reduction in plasma corticoid concentration occurring after 120 days gestation has been attributed to an expansion in plasma volume or, alternatively, to increase in the metabolic clearance rate of cortisol (Paterson and Harrison 1968). Thus the higher than normal concentration of maternal corticoid in the Yarloop group may reflect disturbances in the factors regulating these functions.

Undernourishment combined with cold stress can also cause higher than normal maternal plasma corticoid concentrations in the latter part of pregnancy (Lindner 1959; Saba 1965). However, because both the ewes on Yarloop and those on grass produced lambs with similar birth weights, and because both groups were exposed to the same climate and handling regimen, it is unlikely that these factors would explain the higher plasma corticoid levels in ewes grazing Yarloop pasture.

The apparent association between duration of parturition and plasma corticoid concentrations in the 8 h prior to birth may simply relate to the fact that the ewes with dystocia were stressed. It is known, however, that a functioning foetal pituitary–adrenal axis is necessary for successful parturition (Liggins *et al.* 1967; Drost and Holm 1968), and it is feasible that on account of high maternal corticoid concentrations some corticoid could cross the placenta (Dixon *et al.* 1970) and affect the development of this axis resulting in dystocia and the poor post-partum survival.

The pattern of plasma oestrogen concentration during pregnancy was similar to that reported by Challis (1971). No evidence was obtained in the present study to suggest that the characteristic pre-partum oestrogen rise was related to the length of parturition. Oestrogen peaks were observed in ewes which showed signs of dystocia as well as in ewes which were able to deliver their lambs unaided, both on Yarloop and on grass pasture. However, it is possible that in ewes exhibiting dystocia, the normal response by tissues was mollified by competition or previous effects of the high levels of circulating isoflavones in the plasma. Alternatively, the lambing difficulties may have been due to disturbances in the oestrogen/progesterone ratio seen in the ewes grazing Yarloop. Such an imbalance may have prevented not only normal myometrial activity, but also the normal preparation or relaxation of the uterus, cervix and vulva (Hindson *et al.* 1967; Csapo 1969).

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Manuscript received 14 March 1975