Plasma progesterone concentrations, ovarian and endocrinological responses and sperm transport in ewes with synchronized oestrus

D. T. Pearce* and T. J. Robinson

Department of Animal Husbandry, University of Sydney, New South Wales 2006, Australia

Summary. Two experiments involving 24 and 54 Australian Merino ewes were conducted in which the establishment of a cervical population of spermatozoa and several endocrinological events were studied after several regimens for the synchronization of oestrus. Intravaginal sponges impregnated with 500 mg (Exp. 1) or 200, 400 or 600 mg (Exp. 2) progesterone resulted in the maintenance of plasma progesterone concentrations of 1.5–4.9 ng/ml over a 12-day insertion period compared with 1.9–6.9 ng/ml during dioestrus in control ewes. In Exp. 1 basal concentrations of ≤ 0.25 ng/ml plasma were attained by 4 h after sponge withdrawal and this decline was much more rapid than in normal luteolysis. This was associated with fewer spermatozoa recovered from the cervix 2 h after insemination, and PMSG had no significant effect. In Exp. 2 injection of a supplementary dose of progesterone at sponge withdrawal resulted in a rapid increase in plasma progesterone concentrations followed by an equally rapid decrease and an attenuation of the rise in plasma oestradiol-17 β , the LH surge, and the onset of oestrus. The numbers of spermatozoa recovered 4 h after insemination were not increased, and PMSG had no significant effect. Two factors were significant, namely the dose of progesterone in the sponge (600 mg > 400 or 200 mg, $P < 0.05$) and stage of oestrus when inseminated (mid- or late oestrus > early). The data demonstrated that an adequate dose of progesterone/progestagen incorporated into intravaginal sponges and accurate timing of insemination relative to the LH surge are the most important factors involved in penetration of the cervix by spermatozoa.

Introduction

It has been a common experience that fertility of ewes treated with intravaginal progestagens for the control of time of ovulation and oestrus is lower than that of ewes with a natural oestrus, particularly after artificial insemination (Robinson, Moore, Lindsay, Fletcher & Salamon, 1970). This has been attributed to an impairment of sperm transport through the cervix (Quinlivan & Robinson, 1969; Hawk & Conley, 1975; Hawk & Cooper, 1977). Dose of progestagen absorbed, which is affected by the method of impregnation of the sponge (Robinson, Quinlivan & Baxter, 1968), affects subsequent fertility and this is associated with the numbers of spermatozoa in the oviducts 24 h after insemination (Allison & Robinson, 1970). Subsequently, Croker, Robinson & Shelton (1975) showed that the numbers of spermatozoa recoverable from the cervix 2 h after insemination were highly correlated with the numbers in the oviducts at 24 h. They concluded that, largely because of the absence of zero values, counts of numbers of spermatozoa recoverable from the cervix was a more reliable estimate of the effectiveness of transport of spermatozoa and hence of fertilization.

*Present address: Department of Animal Science and Production, University of Western Australia, Nedlands, Western Australia 6009, Australia.

Quantitative and temporal relationships between several endocrinological events in cyclic and synchronized ewes have been studied in several laboratories (e.g. Goding *et al.*, 1969; Cumming *et al.*, 1970, 1973; Cognie & Pelletier, 1970; Cognie, Hernandez-Barreto & Saumande, 1975). However, data for plasma concentration of progesterone during and after sponge insertion and the relationship with subsequent endocrinological and other events, including the establishment of a cervical reservoir of spermatozoa, have been lacking.

This paper presents the results of two experiments, conducted in successive years, in which the numbers of spermatozoa recoverable from the cervix were related to different progestagen regimens. Because no radioimmunoassay was available for fluorogestone acetate (Cronolone: Searle; 17 α -acetoxy-9 α -fluoro-11 β -hydroxypregn-4-ene-3,20-dione), the synthetic steroid most widely used in intravaginal sponges, all information on rates of absorption, and presumptive concentrations in plasma have been based on data on residues in sponges estimated by gas-liquid chromatography (e.g. Morgan, Lack & Robinson, 1967). Sponges impregnated with 500 mg progesterone are as effective as are 30 mg fluorogestone acetate sponges, at least in some circumstances (see Gordon, 1983), and so progesterone-impregnated sponges were used in these experiments.

Materials and Methods

Experimental animals

Mature Australian Merino ewes were used in Exp. 1 (N = 24) and for Exp. 2 (N = 54). They were run in small paddocks at the University Farms, Camden, New South Wales, and the experiments were conducted in March–May, the breeding season in the southern hemisphere, in successive years. Three Merino rams provided the semen used for insemination.

Experimental design

Experiment 1. This was a 3 $\times$ 2 factorial design (N = 24; *n* = 4) incorporating (a) 3 types of cycle, 2 controlled and 1 normal, and (b) 2 PMSG treatments, 0 and 400 i.u.

Experiment 2. This was a 3 $\times$ 3 $\times$ 3 factorial design (N = 54; *n* = 2) incorporating (a) dose of progesterone in the sponge (200, 400 or 600 mg), (b) dose of supplementary progesterone (0, 5 or 10 mg), and (c) dose of PMSG (0, 300 or 600 i.u.).

Preparation of sponges

Experiment 1. Progesterone (Searle, Mexico; Batch V-48), 500 mg, was dissolved in 5 or 10 ml ethanol, the latter to give more even dispersion, and then pipetted on to the sponges which were allowed to dry in air.

Experiment 2. Progesterone (200, 400 or 600 mg) was dissolved in 10 ml ethanol and the solution was pipetted on to the sponges which were dried in air.

Accuracy was checked by comparing the dry weights of loaded and blank sponges.

PMSG

The preparations used were Folligon (Intervet, Sydney, Australia) in Exp. 1 and, because this became unavailable, frozen-thawed mare serum in Exp. 2. PMSG was injected i.m. at the time of sponge withdrawal. Both preparations were assayed by Dr A. Gidley-Baird of the Department of Veterinary Physiology, University of Sydney. The procedure was essentially that of Steelman & Pohley (1953) except that hypophysectomized mice were used in a 5-point assay with 5 replicates,

with ovarian weight as the end point. The standard was the W.H.O. Second International Reference preparation of PMSG (Bangham & Woodward, 1966).

Supplementary progesterone

This was injected i.m. in propylene glycol at the time of sponge withdrawal in Exp. 2.

Experimental procedures

The times of oestrus and insemination were manipulated so that normal and synchronized oestrus coincided over a 2-day period. Ewes were synchronized with intravaginal sponges impregnated with 30 mg fluorogestone acetate inserted for 12 or 14 days followed by 250 i.u. PMSG at withdrawal. Ewes were in oestrus 2 days later. After another 14 days the experimental progesterone-impregnated sponges were inserted and were left in place for 12 days. At withdrawal, ewes were allocated at random to treatment groups and PMSG and supplementary progesterone were injected as appropriate. Control ewes of Exp. 1 were similarly synchronized as shown in the following schedule.

	Days before insemination					
	Fluorogestone acetate sponges		Oestrus	Progesterone sponges		Oestrus and A.I.
	Inserted	Removed		Inserted	Removed	
Exp. 1						
Controls	30	18	16	—	—	0
Treated	42	30	28	14	2	0
Exp. 2						
All ewes	44	30	28	14	2	0
	Synchronization period			Cycle studied		

Semen was collected by artificial vagina from 3 rams and, if of high quality, was pooled. Concentration was estimated using a photocolormeter (Salamon, 1976) and dilution was to 2000×10^6 spermatozoa/ml in a Tris-based diluent (Tris, 1.81 g; citric acid, 1.00 g; fructose, 0.25 g; made up to 50 ml with distilled water). There was one insemination period each day, 11:00–12:00 h, using 200×10^6 spermatozoa per ewe. Treated ewes in Exp. 1 were inseminated on the 2nd day after sponge withdrawal, i.e. at 54 h, while those ewes in Exp. 1 with a normal cycle and all ewes in Exp. 2 were inseminated at the first insemination period after detection of oestrus.

On the day before the withdrawal of sponges, vasectomized rams fitted with Sire-sine harnesses and crayons were introduced. Ewes were inspected at intervals of 4 h, up to 2 h before the scheduled time of insemination.

The ewes with a normal cycle in Exp. 1 were bled by jugular venepuncture daily from Day 2 to Day 14 of the cycle and 5 times per day thereafter, at intervals of 4 or 5 h, until oestrus. The treated ewes were bled daily from sponge insertion until withdrawal and thereafter at 0, 2, 4, 7 and 10 h.

All ewes of Exp. 2 were bled on Days 1, 3, 5, 7, 9, 11 and 12 after sponge insertion (Day 0). After withdrawal they were bled at 4-h intervals until the detection of oestrus, followed by 2-h bleeds for 12 h or until the time of insemination. It was recognized that this schedule could result in the LH peak being missed in some ewes inseminated shortly after coming into oestrus; regular bleeding could not be continued after insemination for fear of an effect of stress.

Plasma samples were stored at -15°C until thawing before radioimmunoassay.

Slaughter of ewes and recovery of spermatozoa

Ewes of Exp. 1 were slaughtered 2 h after insemination and those of Exp. 2 at 4 h, in the order in which they were inseminated, and the reproductive tract was removed. The cervix was ligated at the utero-cervical and vagino-cervical junctions, severed from the remainder of the tract, snap frozen in liquid nitrogen and stored at -15°C .

Spermatozoa were recovered by the methods described by Allison (1972) and Lightfoot & Salamon (1970) and counted by the methods of Mattner & Braden (1963) and Allison & Robinson (1970). Each cervix was cut, while frozen, into caudal, mid and cranial thirds and each third was divided into 6–8 longitudinal strips. The longitudinal strips were placed in a vial containing 14.8 ml sterile 0.9% (w/v) NaCl and allowed to thaw for 30 min. Eosin stain (5% in H_2O , 0.2 ml) was added and the vials were shaken vigorously for 30 sec and then left for at least 1 h. Two strips of Vaseline were placed about 35 mm apart on a microscope slide and 0.2 ml of the thoroughly mixed flushing solution was placed between them. A 20×40 mm coverslip was then placed over the Vaseline strips and gently pushed down until the flushing solution occupied the area under the coverslip and all air bubbles had been expressed. The slide was then allowed to settle for 30 min. The numbers of spermatozoa were counted in a single lengthwise traverse under $\times 450$ magnification through a green filter. The area of the traverse represented 0.33% of the total area under the slide (i.e. 0.0044% of the total volume of 15 ml). Six slides were prepared for each third of the cervix and the means of the numbers of spermatozoa counted in the 6 traverses were used for analysis. The actual numbers present in the 15 ml of washings were estimated by application of the factor of 22 500.

The numbers of mature and recently ovulated follicles were recorded for each ovary. Mature follicles were ≥ 7 mm in diameter, translucent and turgid.

Hormone assays

Progesterone. The radioimmunoassay used the method of Thorneycroft & Stone (1972) with minor modifications. The antiserum was raised in a male rabbit by injection of a progesterone-11-bovine serum albumin conjugate. Cross-reaction with 17α -hydroxyprogesterone was 3.4% and with other steroids was 1% or less.

Progesterone (Batch V-48, Searle, Coapa, Mexico City, Mexico) was used to prepare standards in charcoal-stripped plasma of spayed ewes. Samples or standards of 0.2 ml plasma were extracted with 2 ml hexane. After freezing the aqueous phase the hexane was decanted and evaporated under nitrogen at 45°C . All tubes received 0.2 ml phosphate buffer (0.1 M, pH 7.0) to redissolve the extract. Antiserum (0.1 ml, 1:16 000 final dilution) and $[1,2,6,7\text{-}^3\text{H}]$ progesterone (10 000 d.p.m./0.1 ml; 18 pg) diluted in buffer were added. The contents were mixed on a vortex mixer and incubated overnight at 5°C .

Dextran-coated charcoal (0.4% in phosphate buffer, 0.8 ml) was used to separate bound and free fractions. The supernatant containing the antibody-bound fraction was added to 10 ml of a scintillant consisting of two parts toluene with 0.35% PPO (2,5-diphenyloxazole) and 0.01% POPOP (1,4-bis(5-phenyloxazol-2-yl)benzene) and one part wetting agent (Teric X-10, I.C.I.). The radioactivity was counted in a liquid scintillation counter (Packard).

The bindings of buffer blanks and stripped plasma were not significantly different. The limit of detection of the assay was 50 pg/tube and the coefficients of variation for between- and within-assay variation were 9.6% and $5.3 \pm 0.7\%$ for Exp. 1. and 7.6% and $7.1 \pm 0.8\%$ for Exp. 2.

Oestradiol- 17β . The method used was as described by Evans & Robinson (1980a). The limit of detection of the assay was 5 pg tube. Three pooled plasma samples were run in each assay with oestradiol- 17β concentrations of 20 ± 2 , 34 ± 3 and 61 ± 10 pg. The coefficient of variation between assays was 15.1% and within assays was $6.1 \pm 0.8\%$ ($n = 8$).

LH. The solid-phase assay was as described by Evans & Robinson (1980b). The procedure was identical except that all incubations took place at 37°C , and the antibody-coating incubation was

2 h using a 1:40 000 dilution and label diluted to 220 d.p.m./ml (0.1 ml, 0.3 ng LH). All samples were run in a single assay and within-assay variation was 10.8%.

Statistical methods

To achieve homogeneity of variance, the data for declining progesterone concentrations after sponge withdrawal and for numbers of spermatozoa recovered were transformed to $\log_{10}$. The numbers of developed follicles and recent ovulations were transformed using a square root transformation. Differences in the interval from detection of oestrus to insemination in Exp. 2 were taken into account in analysing the sperm recovery data by use of covariance.

Results

One ewe of Exp. 2 died before sponge withdrawal. One ewe with a 200 mg progesterone sponge became oestrous on the day before sponge removal and another early when the intravaginal sponge was lost. Endocrinological data pertaining to oestrus, ovulation and sperm transport were lost for these 3 ewes. The data for plasma oestradiol concentration were unavailable for one other ewe.

Progesterone concentration during the insertion of intravaginal sponges

Experiment 1. The plasma progesterone profiles were indistinguishable during the period of insertion of sponges impregnated with 5 or 10 ml ethanol, with mean values of 2.9 and 3.4 ng/ml. These were significantly lower than the mean of 4.1 ng/ml assayed during the luteal phase of the 8 ewes with a normal cycle ($P < 0.05$). Text-figure 1 shows the profiles to be biphasic ($P < 0.001$).

Experiment 2. The mean plasma progesterone concentrations resulting from the 3 intravaginal sponge treatments were not significantly different. There was a linear decline with time ($P < 0.001$) and a biphasic pattern ($P < 0.01$) with elevated concentrations midway through the period of insertion (Text-fig. 2). Mean values of 1.0 ng/ml occurred only on Day 12. The overall mean was 3.5 ng/ml.

Progesterone concentrations after the withdrawal of intravaginal sponges

Experiment 1. Plasma progesterone concentrations fell rapidly, basal concentrations of < 0.25 ng/ml being reached within 4–10 h, with no measurable difference between the two methods of preparation of sponges. Plasma progesterone concentrations in the ewes with a normal cycle fell more slowly ($P < 0.001$), starting on Day 14 and continuing until basal concentrations were attained 54 h later (Text-fig. 1).

Experiment 2. The interval between sponge withdrawal and the attainment of basal progesterone concentrations increased linearly with dose of supplementary progesterone ($P < 0.001$; Table 1). Other treatments had no significant effect. There was an interaction between dose of progesterone in the sponge and dose of supplementary progesterone, the effect of which was more pronounced in ewes receiving the lowest dose in the sponge ($P < 0.01$). After the dose-dependent surge of injected progesterone, plasma concentrations decreased rapidly until basal values were reached, and the shape of the decline curve did not differ between treatments (Text-fig. 3).

Secretion of oestradiol-17 β

Experiment 2. Rising concentrations of oestradiol which began, on average, 24.7 h after sponge withdrawal were generally rapid but the resulting profiles were variable. In many ewes, concentrations rose from basal values of ≤ 1 pg/ml to a peak of 5–10 pg/ml within 4 h and then fell, only

Text-fig. 1. Mean $\pm$ s.e.m. plasma progesterone concentrations in Exp. 1 for 8 ewes during the luteal phase (●) and for 16 during the insertion of progesterone-impregnated intravaginal sponges (○).

Text-fig. 2. Mean plasma progesterone concentrations in Exp. 2 for all 51 ewes (●—●) during insertion of intravaginal sponges and for the 16–18 ewes in each of the 200, 400 and 600 mg progesterone treatments.

to rise again at subsequent bleedings. These variations in the nature of profiles could not be related to treatment or to ovarian response and so posed a problem as to interpretation and method of analysis of the data. Consequently, the time of the first rapid surge in oestradiol-17 β concentration was used as a datum point for oestrogen secretion.

Table 1. Effects of supplementary progesterone on the intervals between sponge withdrawal and subsequent events in ewes in Exp. 2

Supplementary progesterone (mg)	No. of ewes	Sponge withdrawal to basal progesterone (h)	Basal progesterone to oestradiol surge (h)	Oestradiol surge to oestrus (h)	Sponge withdrawal to oestrus (h)
0	16	3.8 ± 0.8	11.4 ± 1.2	12.0 ± 2.0	27.2 ± 2.7
5	18	9.8 ± 0.9	17.4 ± 1.8	13.9 ± 1.5	41.1 ± 2.1
10	17	14.0 ± 0.8	17.8 ± 2.1	17.7 ± 2.3	49.5 ± 2.4
Significance (mean)	51	$P < 0.001$	$P < 0.05$	$P < 0.05$	$P < 0.001$

Text-fig. 3. Mean $\pm$ s.e.m. plasma progesterone concentrations in Exp. 2 after withdrawal of intravaginal sponges and injection of 0, 5 or 10 mg supplementary progesterone. Data are for 16, 18 and 17 ewes.

The mean peak concentration of plasma oestradiol-17 β was 6.9 pg/ml (Table 2). There was no effect of dose of progesterone in the sponge but the concentrations increased linearly with dose of supplementary progesterone ($P \leq 0.01$). Both doses of PMSG resulted in an increase of similar magnitude ($P < 0.01$). No relationship could be demonstrated with numbers of developed follicles or recent ovulations.

Time relationships from sponge withdrawal

Experiment 2. The only experimental factor which affected the time relationships between sponge withdrawal and the subsequent events of attainment of basal concentrations of plasma progesterone, the oestradiol surge, and onset of oestrus was the dose of supplementary progesterone. The mean time to basal plasma progesterone values increased linearly and the intervals

Table 2. Mean peak concentrations (pg/ml) of oestradiol-17 β in relation to treatments in Exp. 2

	Dose of progesterone in sponge			Dose of supplementary progesterone			Dose of PMSG		
	200 mg	400 mg	600 mg	0	5 mg	10 mg	0	300 i.u.	600 i.u.
No. of ewes	16	17	17	15	18	17	16	18	16
Mean	6.5	7.5	6.9	5.8	6.8	8.2	5.3	8.0	7.6
$\pm$ s.e.m.	± 0.7	± 0.7	± 0.6	± 0.6	± 0.5	± 0.7	± 0.4	± 0.7	± 0.6
Significance									
Linear		N.S.			$P < 0.01$			$P < 0.01$	
Quadratic		N.S.			N.S.			$P < 0.05$	

to subsequent events were also increased (Table 1). The final result was a linear increase from 27.2 to 49.5 h in the mean interval to detection of oestrus ($P < 0.001$).

The interval from detection of oestrus to insemination ranged from 2 to 22 h, by 4-h intervals, which were classed as 'early' (< 6 h, 16 ewes), 'mid' (6 to < 14 h, 18 ewes) and 'late' (≥ 14 h, 17 ewes). There was no covariance of this interval on any of the treatment effects.

Secretion of LH

Experiment 2. A rise in plasma LH concentration was detected in 22 of the 51 ewes in oestrus; 2 of the 16 inseminated 'early', 8 of 18 inseminated in mid-oestrus and 12 of 17 inseminated 'late'. The mean $\pm$ s.e.m. interval from detection of oestrus to the LH peak in these 22 ewes was 5.3 ± 0.6 h. Of the ewes in which no LH peak was detected, 1 of 14 inseminated early, 5 of 10 inseminated in mid-oestrus and 5 of 5 inseminated late had ovulated by the time of slaughter ($P < 0.05$, Table 3).

LH concentrations rose from a range of 5–10 ng/ml to a mean peak of 40.6 ± 2.4 ng/ml. The treatments variance for time of the LH surge after sponge withdrawal was significant ($P < 0.05$) but because of the incomplete data it was not possible to partition this between treatments. The mean time ($\pm$ s.e.m.) to the LH surge from basal progesterone concentration was 33.2 ± 1.8 h. Other time relationships are shown in Table 4. The mean intervals from the surge of oestradiol and time of detection of oestrus to the release of LH, 19.8 ± 1.9 and 5.3 ± 0.6 h, were unaffected by the previous progesterone treatment.

Ovarian response

Experiment 1. The effect of PMSG on the sum of mature follicles and ovulations was significant ($P < 0.01$) while the apparent interaction between type of sponge and the number of ewes which had ovulated was not (Table 5).

Experiment 2. All 51 ewes showed evidence of ovarian activity (Table 6), with mature follicles, recent ovulations, or both; 14 had ovulated. The proportions which had ovulated were highest at the lowest dose of progesterone in the sponges ($P < 0.05$) and at the highest dose of PMSG ($P < 0.01$). Dose of supplementary progesterone had no significant effect, and there were no interactions.

The overall mean sum of mature follicles and recent ovulations was 1.5, and there was a linear increase with dose of PMSG (0, 1.1; 300 i.u., 1.2; 600 i.u., 2.4; $P < 0.001$).

Spermatozoa recovered from the cervix

Experiment 1. Table 5 shows that significantly more spermatozoa were recovered after a normal oestrus than at a controlled oestrus ($P < 0.02$). There was no effect of PMSG but there was an

Table 3. Relationship between stage of oestrus when inseminated, LH release, ewes ovulated and estimated numbers of spermatozoa in the cervix in Exp. 2 (data are for reconverted means following analysis using a log₁₀ transformation)

Stage of oestrus	LH release detected				LH release not detected				Total	
	No. of ewes	Ewes ovulated	Spermatozoa ($\times 10^{-6}$)	No. of ewes	Ewes ovulated	Spermatozoa ($\times 10^{-6}$)	No. of ewes	Ewes ovulated	No. of ewes	Spermatozoa ($\times 10^{-6}$)
Early (<6 h)	2	0	1.2	14	1	1.5	16	1	16	1.5
Mid- (6- <14 h)	8	2	8.6	10	5	1.7	18	7	18	3.4
Late (≤ 14 h)	12	1	2.8	5	5	1.9	17	6	17	2.6
Total	22	3	5.7	29	11	1.6	51	14	51	2.4

Significance: Ewes ovulated: early vs mid- and late oestrus ($P = 0.05$); LH release detected, early, mid-, late oestrus ($P < 0.01$). Spermatozoa recovered: early vs mid- + late oestrus ($P < 0.05$); mid-oestrus, LH detected vs not detected ($P < 0.05$); mid-oestrus, LH detected vs remainder ($P < 0.02$); all stages oestrus, LH detected vs not detected ($P < 0.05$).

Table 4. Times between LH release, sponge withdrawal, the surge of oestradiol-17 β and detection of oestrus in 22 ewes in which LH release was detected in Exp. 2

Supplementary progesterone (mg)	No. of ewes	Interval (h) to LH release from		
		Sponge withdrawal	Surge of oestradiol	Oestrus
0	8	33.8 $\pm$ 3.8	18.3 $\pm$ 3.0	5.8 $\pm$ 1.2
5	8	42.3 $\pm$ 2.2	17.8 $\pm$ 2.3	5.5 $\pm$ 0.7
10	6	51.7 $\pm$ 5.0	24.7 $\pm$ 4.8	4.3 $\pm$ 1.0
Mean	—	—	19.8 $\pm$ 1.9	5.3 $\pm$ 0.6

Values are mean $\pm$ s.e.m.

Table 5. Ovarian response and ewes that had ovulated 2 h after insemination and numbers of spermatozoa counted from 3 regions of the cervix in Exp. 1

Treatment	Ewes treated	Ovarian response			Ewes ovulated	Spermatozoa counted*			
		Mature follicles	Ovulations	Total†		Caudal	Mid-	Cranial	Total‡
No PMSG									
Sponges, 5 ml EtOH	4	4	1	5	1	255	39	7	301
Sponges, 10 ml EtOH	4	4	0	4	0	27	17	1	343
Normal cycle	4	4	0	4	0	713	244	52	1009
400 i.u. PMSG									
Sponges, 5 ml EtOH	4	10	0	10	0	334	8	1	343
Sponges, 10 ml EtOH	4	1	5	6	3	641	467	49	1157
Normal cycle	4	4	8	0	0	2232	480	12	2724

*Mean of 6 traverses of slide.

Significance of differences: †PMSG vs no PMSG, $P < 0.01$;

‡sponge-treated vs normal cycle, $P < 0.02$.

Interaction: PMSG $\times$ sponge preparation, $P < 0.02$.

Table 6. Distribution of ewes that had or had not ovulated at time of slaughter in Exp. 2

	Dose of progesterone in sponges (mg)			Dose of supplementary progesterone (mg)			Dose of PMSG (i.u.)		
	200	400	600	0	5	10	0	300	600
Ovulated	8	3	3	4	3	7	2	3	9
Not ovulated	9	14	14	12	15	10	14	15	8
χ^2_{21}	4.92 ($P < 0.1$)			2.65 ($0.2 < P < 0.3$)			8.40 ($P < 0.02$)		
	200 vs $\sum$ 400, 600; 4.92 ($P < 0.05$)			10 vs $\sum$ 0, 5; 2.55 ($P \approx 0.1$)			600 vs $\sum$ 0, 300; 9.00 ($P < 0.01$)		
	400 vs 600; 0.00			0 vs 5; 0.10			0 vs 300; 0.03		

interaction between PMSG and type of sponge preparation ($P < 0.02$), relatively larger numbers being counted in the PMSG-treated ewes treated with sponges impregnated with progesterone in 10 ml ethanol. The decrease in numbers of spermatozoa recovered from the treated ewes applied to

each region of the cervix, the retransformed mean numbers for the normal cycle being 2200, 309 and $53 (\times 10^3)$ and for the treated ewes 440, 100 and $21 (\times 10^3)$ for the caudal, mid- and cranial regions, respectively.

Experiment 2. The distribution between treatments of stage of oestrus when ewes were inseminated did not differ from random. Analysis of covariance taking this factor into account yielded a result indistinguishable from simple analysis of variance.

More spermatozoa were recovered from each region of the cervix of ewes treated with sponges containing 600 mg progesterone than from those treated with 400 or 200 mg ($P < 0.05$). The retransformed mean numbers for the caudal, mid and cranial regions were 2680, 500 and $57 (\times 10^3)$ for 600 mg, 1020, 250 and $31 (\times 10^3)$ for 400 mg, and 1385, 245 and $33 (\times 10^3)$ for 200 mg progesterone. Other treatments had no significant effects. There were significant differences between the regions of the cervix ($P < 0.001$), but no significant interactions with any of the main treatments.

The random distribution between treatments rendered possible an analysis of the effect of stage of oestrus. Ewes inseminated early yielded fewer spermatozoa than did those inseminated later ($P < 0.05$, Table 3) with the suggestion of a quadratic effect favouring ewes inseminated in mid-oestrus ($P < 0.1$). This was associated with a significant relationship between category of LH release and spermatozoa recovered. The group of 29 ewes in which the LH surge was not detected yielded fewer spermatozoa than did the 22 in which the peak was measured before oestrus ($P < 0.05$, Table 3). Associated with this was a higher incidence of ovulation (not detected 11/29, detected 3/22; $P < 0.05$).

Discussion

Progesterone-impregnated sponges maintained mean plasma progesterone concentrations at levels which generally are accepted as normal for sheep (e.g. Thorburn, Bassett & Smith, 1969) throughout most of the period of insertion in both experiments, for all doses of progesterone impregnation and volume of solvent used. The mean values were slightly lower than for the normally cyclic ewes of Exp. 1, the difference being most evident towards the end of the treatment period, and there were significant effects of dose. A dose of 200 mg was marginal as shown by an increased proportion of ewes which had ovulated at slaughter and by one ewe which displayed oestrus towards the end of the period of sponge insertion.

The profile of plasma progesterone concentrations was uniformly biphasic, a phenomenon which cannot readily be explained. The pattern in the sponge-implanted ewes is explicable on the basis of an additive effect of endogenous and exogenous progesterone during the early days of sponge insertion—sponges were inserted on Day 14 when, as shown by the cyclic ewes of Exp. 1, the corpus luteum was still active. Efficiency of release of progesterone from the sponges, as indicated by elevated plasma progesterone concentrations, increased from Day 6 to Day 10 and thereafter declined. The rate of decline in plasma progesterone concentration was much more rapid after sponge withdrawal than in normal luteolysis, basal concentrations being reached by 4 h. The transitory rise at 7 h in Exp. 1 may not have been an artefact but could have resulted from compression of the sponge as it was withdrawn with consequent squeezing out of fluid containing residual progesterone, so providing a reservoir for rapid intake (Robinson *et al.*, 1970).

Injection of supplementary progesterone at the time of sponge withdrawal failed to alter the rate of decline. There was a rise to high concentrations by 4 h after injection, followed by a precipitate fall at a rate indistinguishable from that following sponge withdrawal alone. Surprisingly, this had the effect of attenuating the sequence of subsequent events, a phenomenon which needs further investigation. It was not confined to delaying the time to reach basal plasma progesterone concentrations: all events were spaced out. Thus, the mean time to basal progesterone concentration was increased as were those from basal progesterone to the surge of oestradiol and from this surge

to oestrus. Despite the apparent transitory effect of the injected progesterone (an increase of only 10 h to basal progesterone concentrations) there was a cumulative increase of 22 h in the interval from sponge withdrawal to oestrus. Supplementary progesterone injection also increased oestrogen secretion and the proportion of ewes which had ovulated at slaughter. It therefore seems that the surge of progesterone 4 h after intramuscular injection had a pharmacological effect unlike that obtained with gradual release from the intravaginal sponge.

Treatment with PMSG increased the ovarian response, as shown by the numbers of developed follicles in Exps 1 and 2, and the concentration of plasma oestradiol in Exp. 2 as shown earlier by Evans & Robinson (1980a). Although there was no measurable effect upon the time of onset of oestrus, time of ovulation was advanced as indicated by the numbers of follicles which had ovulated. There was no significant effect upon numbers of spermatozoa recovered, an observation in accord with that of Allison & Robinson (1970).

The estimated mean numbers of spermatozoa recovered from each region of the cervix at 2 and 4 h after insemination were similar to those presented by Croker *et al.* (1975) who used the same method of presentation of the data, namely log transformation, calculation of means, and reconversion. This statistical technique results in a substantial reduction in the arithmetic means and in the s.e.m. and probably accounts for the lower values than those cited by Hawk & Conley (1975) and Hawk & Cooper (1977) for control and sponge-treated ewes. The relative differences between the two classes of ewes are identical.

The increase, in Exp. 2, of the interval from insemination to sperm recovery from 2 to 4 h was justified by the larger numbers of spermatozoa recovered from the cervixes of treated ewes than in Exp. 1 and by increases from 62 to 81% and from 31 to 57% in the percentage of ewes from which spermatozoa were recovered in the mid- and caudal thirds of the cervix.

The relatively low numbers of spermatozoa recovered from the progesterone sponge-treated ewes could not be related to abnormally low plasma progesterone concentrations during the artificial luteal phase. The conclusion is that the primary factor involved is the rapid decline in plasma progesterone concentration after sponge withdrawal and the consequent prolonged interval between progesterone influence and oestrus and insemination. This conclusion is supported by the observation of Hawk, Cooper & Pursel (1981) that removal of the corpus luteum in a normal cycle impaired transport of spermatozoa at the ensuing oestrus due to decreased viability. Use of supplementary progesterone after sponge removal failed to ameliorate the situation, which fits the observation of Gordon (1971) that conception rate was unchanged by the injection of 5 mg progesterone at the time of sponge withdrawal.

An important secondary factor is the timing of endocrinological events and in particular the time of release of LH. The quantitative and temporal relationships of these events were in general agreement with those published previously for cyclic and progestagen-synchronized ewes, with the exception of those treated with supplementary progesterone in which events were spaced out. The overall mean plasma concentration of oestradiol of 7.0 ± 0.5 pg/ml was indistinguishable from the values of 21.8 ± 8.7 and 6.8 ± 2.2 pg/ml for non-lactating and suckling anoestrous ewes reported by Cognie *et al.* (1975). The mean interval from peak oestradiol to the LH peak in those 22 ewes in which a peak was detected was 19.8 ± 1.9 h compared with intervals of 11 ± 3.4 h and 13 ± 7 h for the non-lactating and lactating ewes of Cognie *et al.* (1975), while that of 5.3 ± 0.6 h from the onset of oestrus to the LH peak fell within the limits of 4 to 16 h observed by Goding *et al.* (1969). Cumming *et al.* (1970) reported a similar time relationship in cyclic ewes but found greater variability in ewes in which oestrus had been synchronized, 7 of 12 ewes having ovulated before oestrus, a factor which may have contributed to the failure to detect LH release in 29 of the 51 ewes of Exp. 2.

Despite the apparent irregularities in timing of oestrus and LH release in synchronized ewes, Cumming *et al.* (1973) found the time between LH release and ovulation to be remarkably uniform in young or old Merino or crossbred ewes whether cyclic or synchronized, with ovulation occurring 21–26 h (mean 24 h) after the LH peak. They concluded that knowledge of the time of the LH peak

had a greater predictive value of the time of ovulation than had the onset of oestrus. Given the 24-h interval to ovulation and the time needed to develop a large population of spermatozoa in the oviducts, it follows that insemination a few hours after LH release should provide the optimum interval for fertilization to occur. The results of Exp. 2 show that this is also the optimum time of insemination for the establishment of a cervical reservoir of spermatozoa, in that more were recovered from ewes in which a preovulatory surge of LH was detected before insemination than from those in which no surge was detected. This was not an artefact: 14 of the 29 ewes in which no LH surge was detected were inseminated within 6 h of detection of oestrus and so their LH surge probably was missed; they were inseminated relatively early while 9 of the 15 inseminated in mid- to late oestrus had ovulated by the time of slaughter, presumably experiencing an early release of LH relative to the time of detection of oestrus (Cumming *et al.*, 1970). This illustrates the importance of timing of insemination relative to that of endocrinological events as distinct from behavioural manifestations which are subject to variable social and management factors (Lindsay, 1966).

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