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Session 1

Chairman: Mr H. M. Radford

Effects of olfactory stimuli upon the unit activity of antidromically identified neurosecretory neurones in the paraventricular nuclei of the rat. P. M. Young, *Department of Anatomy, University of Adelaide, South Australia 5000.*

Olfactory afferents to the magnocellular neurosecretory nuclei have been demonstrated in several species (Hayward, 1973), and elevated plasma levels of oxytocin have been found in the male donkey before coitus (Walmsley, 1963). If the posterior pituitary hormones are indeed involved in the contractile activity of male sex organs during coitus (Melin, 1970) then it may be assumed, particularly in animals in which coitus is completed in very much less time than the latent period of the neuro-hormonal reflex (sheep and ox), that the stimuli initiating the reflex liberation of the hormones must originate during the courtship preceding active coitus. These observations in part prompted this investigation.

Neurosecretory neurones in the paraventricular nuclei of urethane-anaesthetized male rats were recorded with stainless-steel microelectrodes following their antidromic identification by delivering pulses to the neurohypophysis. In a separate series of experiments multiunit activity was recorded from a 50 μm electrode positioned in the pituitary stalk. The olfactory stimuli used were urine from individual females in oestrus, urine from ovariectomized females, and two analar grade chemicals, amyl acetate and cineole.

The existence of a unit response or response differences was determined as a significant deviation from resting activity with reference to the EEG changes compared to the variability of resting activity. Standard parametric statistics were used to assess borderline changes. Units responsive to odours were rarely found to respond to only one stimulus, although their pattern of discharge was found to be to some extent characteristic. Urine odour from females in oestrus was often found to produce a burst of activity of lower mean rate but of significantly longer duration than the activity produced by the other stimuli. An analysis of unit activity during EEG synchrony and desynchrony confirmed the characteristic of nonspecific activating effect of oestrous urine odour. The multiunit records from the axons in the pituitary stalk confirmed these effects in this, the last part of the final common pathway. The rate and duration of discharge provide a response spectrum compatible with the stimulation frequency necessary to release neurohypophysial hormones *in vivo* (Harris *et al.*, 1969) and *in vitro* (Ishida, 1970).

Whatever the role of neurohypophysial hormones in sexual function may be, and there is a wealth of observations involving gonadotrophin release, sexual behaviour, sperm transport and penile sensitivity, these findings demonstrate that at least in the male rat odour from the urine of a sexually receptive female is capable of activating a specific group of neurosecretory neurones in a characteristic

way that is compatible with the depolarization frequency necessary in these cells to release neuro-hypophysial hormones.

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Hypothalamic neurones involved in the neuroendocrine regulation of ovulation. J. Priedkalns and G. Sobels, *Harvard Medical School, and Department of Anatomy, University of Adelaide, Adelaide, South Australia 5001.*

Recent research (see Cross, 1973) has given new insight into the relationship of action potential activity in neurones and the discharge of hormonal products from their axonal terminations. To obtain further data on hypothalamic neurones which form the neurophysiological basis for ovulation, the ultrastructure and biogenic amine content of axon terminals were examined in perivascular parts of the external zone of the median eminence, and some electrophysiological properties of neurones determined in the anterior hypothalamus, on the 4 days of the oestrous cycle in the rat.

Linear scanning techniques were employed to determine quantitatively the % volumes of tissue occupied by different types of axon terminals. The % volumes of terminals containing mainly large (~120 nm) granules (dense-core vesicles) increased daily from oestrus ($6.2 \pm 1.5\%$ ($\pm$ S.E.)) to pro-oestrus ($8.9 \pm 1.4\%$), and of those containing mainly small (~40 nm) granules (resembling synaptic vesicles) decreased daily from oestrus ($26.1 \pm 2.6\%$) to pro-oestrus ($23.1 \pm 2.9\%$). Terminals with approximately equal representation of both types of granules were counted equally in the two groups. The increases and decreases were statistically not significant. The observations nevertheless suggest the possibility of utilization of the large granule content, and associated accumulation of small vesicles, during the period from pro-oestrus to oestrus, i.e. late on the day of pro-oestrus. Since the RF release related to the oestrous cycle and ovulation constitutes only one of the components of the hypothalamo-pituitary axis, only relatively small differences in the % volumes observed could be expected; morphological identification of the individual RFs and monoamines involved will be necessary in further work. By the assessment of monoamine fluorescence intensity, increased amounts of catecholamines were recorded during pro-oestrus and metoestrus in the external-zone axons.

In the anterior hypothalamus, we demonstrated increased electrical responsiveness of neurones to an iontophoretically applied progestagen in pro-oestrus. Initial data (Priedkalns *et al.*, 1975) showed that medroxyprogesterone acetate (U-21,687E) increased the firing rates of 41 % of anterior hypothalamic neurones studied in pro-oestrus, of 35 % in metoestrus, of 20 % in oestrus, and of none in dioestrus. These pro-oestrous results correlate with our previous work demonstrating facilitatory effects on ovulation of progesterone implants applied to the anterior hypothalamic area 2–4 hr before the 'critical period'.

The present observations (i) support the hypothesis that oestrous cyclicity and ovulation are at least partly determined by neurones in the anterior hypothalamus, and (ii) are consistent with hypotheses that some of the granules of axon terminals in the external zone of the median eminence contain Gn-RFs and that monoamines are important in their release at pro-oestrus.

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Sequential patterns of LH, FSH and prolactin in developing ram lambs: effects of castration and oestradiol-17 β treatment. V. W. K. Lee, I. A. Cumming, D. M. de Kretser and J. K. Findlay, *Medical Research Centre, Prince Henry's Hospital, Melbourne, Victoria 3004, Department of Agriculture and University of Melbourne, S. S. Cameron Laboratory, Werribee, Victoria 3030, Australia.*

Levels of LH, FSH and testosterone have been studied in rams from birth to sexual maturity (Lee

et al., 1975); the present study describes the effects of castration and oestradiol treatment on the pituitary-testicular relationships in developing rams. Twelve bleeds at 15-min intervals were taken by venepuncture from the jugular vein from 7 crossbred Merino–Corriedale lambs (bilaterally castrated within 24 hr of birth) and from 4 intact rams at the ages of 2, 5, 15, 21 and 28 weeks. LH, FSH and prolactin were measured by specific radioimmunoassays.

Plasma FSH levels were 35 ng/ml in the normal rams at the age of 2 weeks, 50 ng/ml at 5 weeks and at 15, 21 and 28 weeks ranged between 29.0 and 49.2 ng/ml. LH levels were <0.5 ng/ml at 2 and 5 weeks but increased to 1.4–3.5 ng/ml by the ages of 15, 21 and 28 weeks. Prolactin showed a mean value of 33 ng/ml at 2 weeks and increased to 245 ng/ml by the 5th postnatal week.

In castrated lambs, the mean FSH level (324 ng/ml) at the age of 2 weeks was significantly higher (4–10-fold) than that seen in intact ram lambs; this value was elevated to 803 ng/ml by the 5th week. At the ages of 15, 21 and 28 weeks, plasma FSH tended to decrease and varied between 389–855 ng/ml. In contrast, there was no significant difference in the mean LH values observed at the various times studied. Episodic secretion of LH was seen in most of the animals and values ranged between 8.2 ng/ml and 21.4 ng/ml. Prolactin levels exhibited a mean value of 43 ng/ml at 2 weeks after birth and increased to 226 ng/ml at the age of 5 weeks.

Preliminary results on the effects of oestradiol (5 µg i.v.) treatment in normal rams, wethers and ewe lambs suggests that only the latter group showed a positive response as early as at 2 weeks of age.

These results confirm that LH and FSH levels are elevated in ram lambs at 5–6 weeks of age, and demonstrate an increase in prolactin levels during this period. Elevated LH and FSH levels within 2 weeks of castration on the first neonatal day suggest that an inhibitory feedback relationship of the testis and pituitary had already been established. Despite the absence of the testis, there was an increase in FSH levels at 5 weeks of age suggesting a change in the hypothalamic control of FSH independent of testicular secretion.

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Effects of neonatal oestrogen treatment on pubertal development in bulls. J. J. Bass, A. J. Peterson, E. Payne, A. L. Sim and J. F. Smith, *Ruakura Animal Research Station, Private Bag, Hamilton, New Zealand*.

The presence or absence of testosterone during the neonatal development of rats has been shown to affect not only the central nervous system but also the sensitivity of androgen-dependent target tissues (Beach *et al.*, 1969). The present study was initiated to determine the effect of neonatally administered oestrogens on pubertal hormone levels in bull calves.

Five bull calves were injected i.m. with 6 mg oestradiol-17β-3-benzoate at birth and then daily doses of 3 mg were administered for the next 20 days. Two other male groups consisting of 5 normal calves and 4 castrates (steers) were injected with the carrier only. The bull calves were from 2 Friesian sires and each group consisted of at least 2 calves per sire. All the animals plus 5 Jersey heifer calves were run together until puberty. Blood samples were taken at regular intervals and plasma testosterone and LH concentrations were measured by radioimmunoassay.

During the first week of life testosterone levels were similar for all groups (0.12 ng/ml). The heifers and steers retained these low levels throughout the study. Normal bull calves showed a gradual rise in testosterone levels until 4 months of age at which time the plasma levels rose steeply to reach 6.7 ng/ml by the 5th month. The oestrogen-treated males had a similar rise in testosterone levels except that the rise was delayed by 2–3 weeks. When 500 i.u. HCG were given i.v. to all groups at 4 months of age, the plasma testosterone concentrations rose. The increase in the normal males was significantly greater than that of the oestrogen-treated males ($P < 0.05$) but this difference was decreased after HCG treatment at 5 months and had disappeared after similar treatment at 7 months of age.

The plasma LH concentrations decreased over the first 7 days after birth in all groups and then

gradually rose and there were no significant differences between the sexes even up to 4 months of age. The LH levels of the steers, however, rose rapidly from Day 30 and by 4 months the levels were higher than in all the other groups. The oestrogen-injected males showed no rise in LH until the end of the treatment and then the levels slowly rose, so that by 4 months there were no real differences between them and normal males.

These preliminary results indicate that neonatally administered oestrogen delays normal pubertal development of male calves.

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Session 2

Chairman: Dr D. M. de Kretser

Pituitary-testicular responses in rams to prolonged infusions of LH-RH. W. J. Bremner, J. K. Findlay, D. M. de Kretser, I. A. Cumming and B. Hudson, *Medical Research Centre, Prince Henry's Hospital, Melbourne, Victoria 3004, Department of Agriculture and Reproduction Research Section, S. S. Cameron Laboratory, University of Melbourne, Werribee, Victoria 3030, and Howard Florey Institute of Experimental Physiology and Medicine, University of Melbourne, Victoria 3052, Australia.*

The effect of constant, prolonged LH-RH stimulation on pituitary and gonadal function was studied in 7 Corriedale rams, aged 2–3 years. Synthetic LH-RH (Hoechst) was administered into a jugular vein for 4 hr. Blood was obtained from the opposite jugular vein at 15-min intervals for the determination of LH, FSH, testosterone and oestradiol-17 β by radioimmunoassay. Two rams received each of 4 dosages of LH-RH (1.0, 0.5, 0.1 and 0.05 μ g/min) in studies separated by at least 1 week. Five other rams received infusions of 0.5 μ g/min with blood sampling at 5-min intervals during the first hour as well as 15-min intervals during the rest of the study.

During the two higher dosage infusions (1.0 and 0.5 μ g/min), LH increased dramatically within 15 min from basal values of 1.0–2.4 ng/ml (NIH-S-8) to levels of 15–50.4 ng/ml, then remained stable or fell slightly until 60–75 min when a second sharp rise began, reaching peak values of 85–138 ng/ml at 105–150 min. After this time, LH values fell (23–57 ng/ml at 4 hr) in spite of continued LH-RH infusion and decreased further in the 2 hr after the infusion (5.4–12.6 ng/ml at 6 hr). The pattern of a rapid early increase in LH until 20–30 min, then a plateau until 60–75 min, followed by a second dramatic increase was confirmed in the 5 rams studied with 5-min blood sampling during the first hour of the infusions. During the 0.1 μ g/min studies, the early phase of LH release was markedly reduced (3.2–15.1 ng/ml at 15 min), but the later phase was similar to that found in the higher dosage infusions (83.2–112 ng/ml at 150 min). During administration of the lowest dosage (0.05 μ g/min), no early LH release was detected (1.5–3.6 ng/ml at 15 min) and the later phase was also reduced (39–42 ng/ml at 150 min). FSH values during infusions of all 4 dosages rose very slightly and very gradually from basal levels of 10–65 ng/ml (NIH-S-6) to maximal values of 22–82.3 ng/ml at 120–150 min after which they decreased to 10–80 at 4 hr and to 10–64 ng/ml at 2 hr after the infusions.

Testosterone levels began a dramatic increase 15–30 min after the LH increase, rising from control levels of 138–962 ng/100 ml to maximal levels of 650–2180 ng/100 ml at 90–180 min. The maximal increments in testosterone averaged 1180–1250 ng/100 ml during the infusions of 1.0, 0.5 and 0.1 μ g/min and 700 ng/100 ml in the 0.05 μ g/min studies. In spite of the marked increases in testosterone, no significant changes could be demonstrated in serum oestradiol levels (range < 15–21 pg/ml) during LH-RH infusions of 0.5 and 0.1 μ g/min.

These results demonstrate two phases of LH increase and only one phase of FSH increase during constant LH-RH administration to rams, similar to results in man (Bremner & Paulsen, 1974; de Kretser *et al.*, 1975). In addition, testosterone responses in these studies were much greater than

those found during similar infusions in men, suggesting that the ram testis may be more sensitive to endogenous gonadotrophin production than is the testis of man.

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The effect of Gn-RH on the LH response to oestradiol-17 β in the spayed ewe. R. D. Hooley, J. K. Findlay, L. D. Staples* and I. A. Cumming, * *Reproduction Research Section, University of Melbourne, and * Department of Agriculture, S. S. Cameron Laboratory, Werribee, 3030, Australia.*

It has been proposed that oestrogens may potentiate the LH response to Gn-RH (Reeves *et al.*, 1971) and that the positive feedback effect of oestradiol is directed at the hypothalamus where it stimulates Gn-RH release (Jonas *et al.*, 1973). This paper examines, (a) the effect of Gn-RH on oestradiol-induced LH release, and (b) the effect of oestradiol on Gn-RH-induced LH release. Four groups, each of 5 spayed ewes, received i.v. injections of either 40 μ g oestradiol, 20 μ g Gn-RH or saline as described in Table 1. Jugular blood samples were collected at 30-60 min intervals for 29 hr and the plasma assayed for LH. Plasma LH concentrations (LER 1374A) for selected time periods were subjected to an analysis of variance.

Table 1. Experimental design giving treatment times and the mean LH levels (ng/ml) within specified time periods

Group	Treatment time (hr)			Time period (hr)			
	0	5	9	0-5	5-9	9-13	14-24
I	Oestradiol	Saline	Saline	4.0 (b)*	3.2 (b)	3.4 (c)	11.8 (a)
II	Oestradiol	Gn-RH	Gn-RH	3.6 (b)	23.3 (a)	19.3 (a)	5.2 (b)
III	Oestradiol	Gn-RH	Saline	6.1 (b)	30.5 (a)	5.8 (b)	9.7 (a)
IV	Saline	Gn-RH	Gn-RH	8.1 (a)	21.3 (a)	15.0 (a)	2.6 (b)

* Within time periods: a > b > c ($P < 0.01$)

The results indicate that: oestradiol treatment lowered LH basal values (Period 0-5 hr); oestradiol did not potentiate or depress Gn-RH-induced LH release (Period 5-9 hr); in Groups II and IV, which received two Gn-RH injections, oestradiol did not significantly alter the magnitude of or the ratio between the responses to the first and second Gn-RH injection (Period 5-13 hr); the LH response to oestradiol in Group II, given two Gn-RH injections, was less ($P < 0.01$) than that in Groups I or III given no or only one Gn-RH injection (Period 14-24 hr).

These results confirm the negative and positive feedback of oestradiol on LH. Oestradiol had no effect on LH response to Gn-RH but Gn-RH overcame the negative feedback effect of oestradiol on LH and reduced the positive feedback effect of oestradiol, probably because of pituitary depletion of LH. Furthermore the increase in LH responsiveness to Gn-RH on Days 15 and 16 of the oestrous cycle is more likely to be due to the absence of progesterone (Hooley *et al.*, 1973) than to a potentiating effect of oestradiol.

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Effects of local testicular heating and rete testis fluid on plasma FSH and testosterone levels in male rats. J. K. Voglmayr, C. Roberson and A. Bartke, *Worcester Foundation for Experimental Biology, Shrewsbury, Massachusetts 01545, U.S.A.*

Selective destruction of the germinal epithelium causes plasma FSH levels to increase, presumably

by reducing the production in the testis of a substance ('inhibin') that specifically suppresses the secretion of FSH (Van Thiel *et al.*, 1972). Inhibin is thought to originate in the Sertoli cells or early germinal elements and its activity in rete testis fluid (RTF) has previously been demonstrated (Setchell & Jacks, 1974). In the present study, local application of heat was used to damage germinal elements in rats. Since an apparent, specific elevation in circulating FSH resulted, these heat-treated animals were used as models for testing differences of inhibin-like activity in RTF from rams exhibiting high or low spermatogenic activity.

The scrotum of each of 27 Charles River CDF inbred male Albino rats, 63 days old, was exposed to heat by immersion in water at 43°C for 15 min. Six other rats, left untreated, served as controls. Seven days after exposure, ram RTF or saline was injected i.p. twice daily, 0.66 ml at 09.00 hours and 1.33 ml at 17.00 hours, for 6 consecutive days. On the morning following the last day of injection, the rats were decapitated and blood was collected into heparinized receptacles and centrifuged. Plasma FSH and testosterone levels were determined by radioimmunoassay. Rete testis fluid, containing $>40 \times 10^6$ spermatozoa/ml (RTF-H) was compared with RTF from the same ram, but with a sperm concentration $<0.4 \times 10^6$ cells/ml (RTF-L). Samples of RTF, collected continuously at 15°C (Voglmayr *et al.*, 1967) from two rams, were tested.

Table 1. Mean plasma FSH and testosterone levels in male rats with locally heated testes and injected with ram RTF collected during periods of high (RTF-H) and low (RTF-L) spermatogenic activity

Treatment	No. of rats	FSH (ng/ml)	Testosterone (ng/ml)
Not heated/saline	6	381 $\pm$ 19	1.64 $\pm$ 0.23
Heated/saline	9	623 $\pm$ 13	1.13 $\pm$ 0.14
Heated/RTF-H	9	527 $\pm$ 31	1.45 $\pm$ 0.14
Heated/RTF-L	9	629 $\pm$ 39	1.63 $\pm$ 0.16

Table 1 shows that local heating of the testes caused a marked elevation ($P < 0.01$) in plasma FSH levels. The testicular weight of the heated rats was less than half that of the unheated controls, but plasma testosterone levels, presumed to parallel LH levels, were similar. Injection of RTF-H resulted in a significant decrease ($P < 0.025$) of the elevated plasma FSH levels in rats with locally heated testes, whereas RTF-L was ineffective. These results suggest that the inhibin-like activity in RTF may be related to sperm production rates.

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Melatonin in fetal and maternal ovine plasma. C. D. Matthews, Ingeborg C. Kowanko, D. Kennaway and R. F. Seamark, *Department of Obstetrics and Gynaecology, University of Adelaide, Adelaide, S.A. 5000 Australia.*

Melatonin (5-methoxyacetylserotonin) is generally regarded as the putative pineal hormone. However, few reliable and/or practical techniques are available for its assay in blood or tissues other than the pineal.

Our interest is in the function of the pineal in pregnancy in sheep and in this communication we report attempts to identify melatonin in the blood of pregnant sheep and within the fetal circulation using a sensitive and highly specific bioassay.

Pregnant Merino cross-bred ewes of known gestation (Day 120-147) were comfortably housed for several days in 12-hr light/12-hr dark. Before sampling they were sedated, without disturbance, with sodium pentobarbitone and a 200 ml sample of jugular vein blood taken. A hysterotomy was then

rapidly performed and a sample of fetal heart blood taken. Plasma was stored at -10°C until assayed within 7 days. For assay, the plasma was treated with 0.1 M-NaOH and extracted with chloroform. The extract was then taken to dryness *in vacuo* and the residue re-suspended in isotonic fish buffer so that a thousand-fold concentration of the original plasma volume was achieved. This was then injected intraperitoneally (10 μl /fish) into 8–10 pencil fish *Nannostomus beckfordi anomalus* according to the technique of Reed & Finnin (1973). This bioassay depends upon changes in melanosome distribution of the day band of the fish, and the definitive end point is judged visually. The assay is highly specific for melatonin, and related indoles do not significantly cross-react (Ruffin, Reed & Finnin, 1969). The useful range of the assay is 50–150 pg per injected sample and 80% recovery was achieved in the extraction procedure employed.

Twenty ewes and their fetuses were examined, 11 were sampled at about 12.00 hours and 9 at about 01.00 hours after 6 hr darkness. In daytime samples, melatonin was detected in one ewe sample (Day 146) and in one fetal sample (Day 120). Both results were at the lower limit of sensitivity of the assay i.e. 5 pg ml^{-1} . Melatonin was detectable (5–25 pg ml^{-1}) in 5 of the 9 samples of ewe blood taken at 01.00 hours, but was only found in 1 of the 8 fetal samples tested.

The levels of the hormone detected in sheep blood are such that they lie on the lower limits of the bioassay employed and consequently a confident recognition of a pattern was not possible, but the results did suggest that at least in ewes the hormone is more readily found during the hours of darkness. Preliminary data on melatonin in pineal tissues also indicate a day–night variation.

Early histological (Jordan, 1911) and more recent enzyme studies (Kennaway *et al.*, 1974) indicate that the pineal is functioning in fetal life and has the potential to synthesize methoxyindoles, especially towards the end of gestation.

The failure to obtain convincing evidence of melatonin in fetal blood may indicate that other alkylamines are important in mediating pineal endocrine functions before birth.

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Migration of stored human spermatozoa through cervical mucus in relation to the preovulatory LH surge.
 J. F. P. Kerin, C. D. Matthews, J. M. Svigos and M. Makin, *Department of Obstetrics and Gynaecology, University of Adelaide, Adelaide, S.A. 5000, Australia.*

The ability of cervical mucus to allow the penetration of preserved spermatozoa has been examined by an in-vitro test. The probable timing of ovulation has been inferred from gonadotrophin values, basal body temperature and the clinical assessment of changes in cervical function. The relationship between these parameters and sperm migration has been assessed.

Peripheral venous blood was obtained daily between 08.00 and 09.00 hours from patients about the periovular period attending for artificial insemination. All patients kept a basal body temperature chart.

LH was estimated by radioimmunoassay using anti-hLH and LER 960 as tracer and standard. The day of the highest LH value was designated Day 0.

Cervical function was assessed clinically by the degree of cervical dilatation, the amount of mucus, the presence of ferning, and the length of Spinnbarkeit, each parameter contributing equally to a cervical score (0–12).

Cervical mucus was aspirated daily from the endocervical canal into a graduated 50 μl pipette. One end of the pipette was placed within a small reservoir of 1–2 drops of thawed semen and incubated for 60 min at 37°C with humidification. The column of cervical mucus was then examined at centimetre intervals for the numbers of spermatozoa/high power field using light microscopy.

In-vitro tests (121) were performed on the mucus of 23 women over 47 menstrual cycles.

Maximum cervical scores appeared on Day –1 and Day 0 with a considerable diminution in

score at Day +1. The day of the thermal shift (greater than 0.2°C) occurred in 66% of cases at Day +1 and the remainder at Day +2. Sperm penetration was markedly increased during Days -1 and 0 compared with Days -2, +1 and +2. It is concluded that cervical mucus is most favourable to the penetration of spermatozoa on the day preceding and the day of the LH surge, and thereafter decreases rapidly. The value of such knowledge to the practice of artificial insemination is discussed.

Luteolytic properties of stallion semen. Manika Wodzicka-Tomaszewska,* A. Okólski, A. Bielański and W. Bielański, *Department of Physiology, University of New England, Armidale, N.S.W. 2351, Australia, and Agricultural Academy in Kraków, Institute of Applied Physiology of Animals, 30-059 Kraków, Al. Michiewicza 24/28, Poland.

Role of the morphogenetic hormones ecdysone and juvenile hormone in insect spermatogenesis. J. B. Dumser, Department of Entomology, Waite Agricultural Research Institute, Glen Osmond, S.A. 5064, Australia.

During larval life the insect testis develops slowly, never attaining a stage containing mature germ cells. Similarly, development ceases entirely during the long and often variable quiescent period termed diapause. In the final larval or pupal stage leading to the reproductive adult spermatocyte accumulation, meiosis, and spermiation occur. Early experimental work indicated that the testis was responding to the classic morphogenetic hormones of insects, ecdysone and juvenile hormone (Wigglesworth, 1936; Fukada, 1944; Williams, 1951). These findings have led to the hypothesis that juvenile hormone suppresses the meiotic division, resulting in the accumulation of meiocytes during larval instars. The absence of juvenile hormone in the pre-adult instar, together with the secretion of ecdysone results in a high level of meiosis and spermiation immediately before production of the adult (Takeuchi, 1969). Paradoxically, however, all elements of spermatogenesis occur both *in vitro* in the absence of insect hormones (Marks, 1970), and in the adults of many long-lived species (Phillips, 1970) which contain a theoretically unfavourable hormonal balance.

Quantitative histological analyses have recently demonstrated an absence of specific effects on meiosis by juvenile hormone or ecdysone in the bug *Rhodnius prolixus*. An alternative hypothesis has been put forward which states that the germ cell system is auto-differentiating and tied to a species-specific sequence of divisions. Ecdysone and juvenile hormone exert an influence on the rate of passage through the sequence but can neither halt the process nor alter its direction (Dumser & Davey, 1974). A series of experiments investigating the two main aspects of this hypothesis are reported here.

(1) Removal of the corpus allatum, source of the juvenile hormone in various larval stages of *Rhodnius*, results in the production of precocious adults. The level of differentiation of the germ cells is always consistent with the number of cells in the cyst, hence to the history of division of the germ cells and not to external morphological development of the insect. Precocious adults with mature or immature germ cell complements can thus be produced by selection of appropriately aged larvae, arguing against a role of juvenile hormone in direct suppression of meiocyte or spermatid differentiation, and in support of the autodifferential capacity of the testis.

(2) Surgical manipulation of endocrine structures and resupply of synthetic hormone analogues permits the demonstration of, firstly, an endogenous division rate in the absence of morphogenetic hormones; secondly an acceleration of this rate by ecdysone, the moulting hormone; and thirdly the suppression of the ecdysone stimulated division activity by juvenile hormone, without effect on the basal activity level in accordance with the aforementioned kinetic hypothesis of germ cell development.

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Ultrastructure of the mid-piece of the spermatozoon in the marsupial, *Trichosurus vulpecula*. H. R. Harding, F. N. Carrick and C. D. Shorey, *Department of General Studies and School of Zoology, University of New South Wales, P.O. Box 1, Kensington, N.S.W. 2033, and Department of Histology and Embryology, University of Sydney, N.S.W. 2006, Australia.*

This study reports the development of two unusual structures in the mid-piece of the spermatozoon of the brush-tailed possum, *Trichosurus vulpecula*, during epididymal transit.

Spermatozoa were collected separately from the caput, corpus and cauda epididymidis and the vasa deferentia of three possums and fixed for electron microscopy using a modification of Karnovsky's fixative with picric acid added, and the method of Jones (1973).

Transverse and longitudinal sections of the mid-piece of spermatozoa from the corpus epididymidis and more distal regions of the tract revealed a single layer of evenly spaced, rounded aggregates of electron-dense material, about 60 nm diameter, lying immediately beneath the plasma membrane. Superficial longitudinal sections showed that these formed regular helices around the posterior two-thirds of the mid-piece. The fibrous helices were always wound counter to the underlying mitochondrial helix. Beneath this fibre network, and separating it from the mitochondria, was a layer of unevenly condensed granular material.

An irregular array of vesicles lay between the plasma membrane and this granular layer, and interspersed between the fibrous helices. These vesicles are a later development than the fibre network, being found only in sperm from the cauda epididymidis and more distal regions of the tract. They resembled pinocytotic vesicles; some appeared as invaginations of the plasma membrane, and were open to the medium surrounding the spermatozoon by a narrow neck, while others were entirely enclosed within the spermatozoon and lay at various distances between the plasma membrane and the layer of granular material. To our knowledge this is the first report of such vesicles in the mid-piece of mammalian spermatozoa.

A mid-piece fibre network was recently reported by Olson (1975) in spermatozoa from the cauda epididymidis of *T. vulpecula*. The present investigation confirms his observations on this network, but in addition, shows that it develops during epididymal transit. No structure closely resembling the mid-piece fibre network has been reported for other mammals. However, freeze-fractures through the plasma membrane of the mid-piece of guinea-pig spermatozoa have revealed circumferentially orientated strands of 5–6 nm particles (Friend & Fawcett, 1973).

It is noticeable that the anterior region of the mid-piece, which lacks the fibrous helices, has a rather folded appearance when viewed by scanning electron microscopy, in contrast to the firm appearance of the posterior region. In addition, its helical nature and potential to counteract possible structural effects of the mitochondrial helix are suggestive of some role in the dynamics of flagellar movement, and the structural integrity of the spermatozoon of this species.

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Session 3

Chairman: Dr I. G. White

Metabolic and ultrastructural changes in ejaculated spermatozoa induced by heating the testes of rams. P. D. C. Brown-Woodman, H. Mohri, A. Darin-Bennett, C. D. Shorey and I. G. White, *Departments of Veterinary Physiology and Histology, University of Sydney, N.S.W. 2006, Australia.*

The harmful effects of a rise in temperature of the scrotal testis on spermatozoa are well known and the pachytene primary spermatocytes have been implicated as the main site of damage in the ram.

Voglmayr *et al.* (1971), however, found that the metabolic activity of testicular spermatozoa is depressed 1–5 days after heating the testes, indicating damage also during the later stages of spermatogenesis. This paper extends these observations to ejaculated spermatozoa using glycerol as substrate since Mohri *et al.* (1970) have found a decrease in the glycerol kinase activity of goat spermatozoa during summer.

Local heat (40–40.5°C) was applied to the scrotum of each of 5 rams for 3 hr. Spermatozoa were collected before, 1 day after and then at weekly intervals for 10 weeks. Metabolic activity, motility and morphology of the spermatozoa were examined at each collection; in addition, the total phospholipid–phosphorus/ejaculate and the phospholipid composition of the spermatozoa were determined. For metabolic studies, washed spermatozoa were incubated for 3 hr in Warburg flasks with 5 mM-glycerol.

There was an immediate reduction in oxygen uptake, with no effect on glycerol utilization or glycerol kinase activity, apparently indicating inhibition of endogenous respiration. One to 2 weeks later, glycerol breakdown and lactic acid production were severely inhibited. Each metabolic parameter returned to normal by 9–10 weeks after heating the testes. Glycerol kinase activity was slightly inhibited after 1 week; the most marked effect, however, was after 3 weeks when glycerol utilization was also severely inhibited. The motility of the spermatozoa was also reduced 2 weeks after heating, and recovery followed a similar pattern to glycerol breakdown.

Striking changes in the morphology of spermatozoa were also evident. From 2 weeks after heating the number of separate heads and tails increased, and there was also an increase in the number of spermatozoa with abnormal tails. Electron microscope studies have shown that 1 week after heating, the plasma membrane of the spermatozoa was rolled up; over the next 3 weeks it broke down, and by 5 weeks the membrane was absent from the nucleus, tail and mitochondrial region. Mitochondrial changes, particularly coiling of the cristae, were also evident at this time and considerable cell debris was apparent. The morphology of the spermatozoa was normal again 10 weeks after heating.

Local heating of the testis decreased the production of spermatozoa and, therefore, the total amount of phospholipid–phosphorus extracted/ejaculate. However, the phospholipid composition of the ejaculated spermatozoa was unaffected and the amount of phospholipid/cell showed only a slight decrease. This may reflect the loss of plasma membrane, but would not indicate more extensive breakdown of sperm structure.

These experiments show that local heat disrupts metabolic activity of the ejaculated spermatozoa, and the speed of onset suggests that the site of action is in the epididymis.

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Comparison of sperm numbers released in the urine and in ejaculates of the brush-tailed possum. J. C. Rodger and I. G. White, *Department of Veterinary Physiology, University of Sydney, N.S.W. 2006, Australia.*

Seminal plasma can be readily collected from a number of marsupial species by electroejaculation but only in the brush-tailed possum does the technique offer promise as a means of collecting spermatozoa (Rodger & White, 1975). In view of the continuous release of spermatozoa in the urine of marsupials (10^6 – 10^7 spermatozoa/day) and the long copulation time of some marsupials, Bolliger (1946) suggested that true ejaculation, as seen in eutherians and lower vertebrates may not occur. The data of Rodger & White (1975) were consistent with Bolliger's hypothesis that spermatozoa present in ejaculates may have been flushed from the urethra by electrically stimulated prostatic secretion, in a manner analogous to the spermatozoa found in urine.

In the present study not all possums produced ejaculates containing spermatozoa, but responsive animals gave ejaculates of consistent volume and total sperm numbers. Total spermatozoa/ejaculate had a mean of $4.6 \pm 1.07 \times 10^7$ for 19 ejaculates from the two responsive animals. The mean volume of

these ejaculates was 3.3 ± 0.44 ml. Loss of spermatozoa in the urine was of the order of approximately one-tenth of that observed in semen, i.e. 4×10^6 spermatozoa/day per possum. However, towards summer, following the second breeding period, the number of spermatozoa found in urine fell to around 10^4 spermatozoa/day. The numbers of spermatozoa obtained in ejaculates and in urine were much lower than those found in eutherian mammals, but, despite suggestions to the contrary in the literature, ejaculation in marsupials appears to be essentially similar to that in eutherians.

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Session 4

Chairman: Professor G. Sharman

Guest Lecture

Blood circulation in the testis: its control and significance. B. P. Setchell, *ARC Institute of Animal Physiology, Babraham, Cambridge, England.*

The summer solstice and the onset of breeding in the tammar wallaby C. H. Tyndale-Biscoe and Jennifer Hawkins, *Department of Zoology, Australian National University, Canberra, A.C.T.*

Pineal gland changes during the period of blastocyst activation in the tammar wallaby, *Macropus eugenii*. D. Kennaway and R. F. Seamark, *Department of Obstetrics and Gynaecology, University of Adelaide, Adelaide, S.A. 5000, Australia.*

The influence of the pineal gland on the seasonal breeding of rodents has been well established (see Reiter, 1973). Decreasing photoperiod stimulates the pineal gland to produce more methoxyindoles, resulting in a decrease in sexual activity. The tammar wallaby is a precise seasonal breeder. After mating the blastocyst develops to about 80 cells and then enters embryonic diapause. In this report we document changes in the pineal gland of the female tammar wallaby during the period in which the quiescent CL and blastocyst resume development,

Pineal glands from pregnant female tammar wallabies were obtained between 23.00 hours and 01.00 hours over 8 days during late December 1974 (courtesy of Dr C. H. Tyndale-Biscoe). The pineal glands were kept frozen on solid CO₂ until assayed. Pineal glands were randomized, weighed and homogenized in 0.154 M-KCl. Hydroxyindole-O-methyltransferase (HIOMT, EC 2.1.1.4) activity was assayed by the method of Axelrod *et al.* (1965). Monoamine oxidase (MAO, EC 1.4.3.4) was assayed by the method of Wurtman & Axelrod (1963). Soluble protein was measured after centrifugation of the homogenate by the method of Lowry *et al.* (1951). The coefficient of variation between assays measured on a single sheep pineal gland for HIOMT and MAO were 6.4% and 14.2% respectively. Enzyme results are expressed as units/gland per 60 min where 1 unit is equivalent to 1 pmol ¹⁴C-labelled product.

Results from other studies on the animals used here have shown that blastocyst activation occurred in the majority of animals around the time when pineal HIOMT activity was at its lowest. Berger (1970) has implicated photoperiod as a factor in the precise resumption of embryonic development in the tammar wallaby. These results further implicate the pineal gland and its methoxyindoles in the timing of blastocyst activation.

Table 1. Soluble protein (μg), HIOMT and MAO activity (units/gland per 60 min) in the pineal of the female tammar wallaby (nos in parentheses)

Date	Total soluble protein	HIOMT	MAO
21/22.12.74	275 $\pm$ 38 (5)	627 $\pm$ 166	2311 $\pm$ 164
22/23.12.74	296 $\pm$ 41 (6)	566 $\pm$ 98	2515 $\pm$ 248
23/24.12.74	377 $\pm$ 105 (5)	799 $\pm$ 164	2684 $\pm$ 413
24/25.12.74	315 $\pm$ 32 (5)	275 $\pm$ 42*	2839 $\pm$ 358
25/26.12.74	338 $\pm$ 35 (3)	603 $\pm$ 50	2750 $\pm$ 239
26/27.12.75	310 $\pm$ 21 (4)	810 $\pm$ 112	3194 $\pm$ 334
27/28.12.74	366 $\pm$ 70 (4)	426 $\pm$ 117	3057 $\pm$ 717
28/29.12.74	292 $\pm$ 16 (4)	673 $\pm$ 155	3312 $\pm$ 375

* Significantly different ($P < 0.05$) from preceding group.

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Reactivation of RNA synthesis in macropod embryos after diapause. G. P. M. Moore, *Department of Zoology, Australian National University, Canberra, A.C.T.*

The activation of the genome of dormant macropod blastocysts has been studied during growth stimulation. Changes in the transcriptional activity of the embryo cells were measured by assay for endogenous DNA-bound RNA polymerases. During assay, the polymerases catalyse the synthesis of RNA from the ribonucleoside triphosphates: ATP, GTP, CTP and tritiated UTP. The distribution and amount of polymerase activity in the nucleus may be correlated with the activity of different groups of genes. Nucleolar polymerases are involved in the synthesis of ribosomal RNA and nucleoplasmic polymerases are predominantly concerned with the production of messenger RNA.

Embryos of the tammar wallaby, *Macropus eugenii*, usually enter dormancy as a normal part of the intrauterine phase. Females which are suckling a young animal in the pouch may carry a dormant embryo (lactational quiescence). Removal of the pouch young during lactation in the first half of the year will cause the embryo to resume development. However, the embryo will not reactivate if the pouch young is removed in the latter half of the year (seasonal quiescence). In this situation, reactivation occurs spontaneously at or near the summer solstice.

Resumption of development at the end of seasonal quiescence was investigated. Embryos were collected between 19–29 December 1974 from a wild population of wallabies on Kangaroo Island. Between 19–23 December all embryo cells showed relatively low levels of nuclear RNA polymerase activity. However, on the night of 23–24 December, a marked change occurred. The nucleolus-associated DNA polymerase activity of embryos was greatly enhanced relative to that in the nucleoplasm. The increased nucleolar activity was maintained throughout the remainder of the study period. By 28 December, 4 days after the activation of the nucleolus, the activity of the nucleoplasmic polymerases had also increased in a small number of embryos. The selective stimulation of ribosomal RNA synthesis in the quiescent embryo suggests that specific gene regulatory factors may be involved in the initiation of growth reactivation.

Preliminary observations on reproduction and embryonic development in the marsupial devil, *Sarcophilus harrisii*. R. L. Hughes, *School of Anatomy, University of Queensland, Lucia, Brisbane, Australia.*

The Tasmanian devils examined consisted of 15 females and 6 males. The animals were captured in the wild during either May 1974 or April 1975. The breeding season is restricted with the majority of

pregnancies occurring between March and May. The preovulatory period is characterized by a marked proliferation of the tissues of the oviduct and uterus. The ovaries are approximately equally functional with up to 23 eggs being ovulated in one set. As the pouch contains only 4 teats, considerable reproductive wastage is inevitable. The gametes are large. Spermatozoa have an overall length of about 213 μm and the flagellum diameter in the region of the mid-piece is about 2.6 μm . Ovulated eggs, including the mucoid coat and shell membrane, have an overall diameter of about 300 μm . The diameter of the vitellus of the egg is approximately 210 μm . Cleavage occurs asynchronously with the result that two-celled eggs and well-formed unilaminar blastocysts are present in the same uterus. The unilaminar blastocysts consist of a single-layered, hollow ball of cells without an inner cell mass and these blastocysts are formed directly from cleaving eggs without an intervening morula stage. A mucoid coat and an unruptured shell membrane are present at the unilaminar blastocyst stage.

Session 5

Chairman: Dr B. P. Setchell

Spermatogenesis in a passerine hybrid: phagocytosis by Sertoli cells after absence of synaptonemal complexes. M. Anne Denehy, *Department of Histology and Embryology, University of Sydney, N.S.W. 2006, Australia.*

The hybrids of canaries and goldfinches are usually sterile, although there have been reports of successful breeding from male hybrids (Willis, 1841). The aim of this study was to investigate the ultrastructure of spermatogenesis of the hybrid in the hope that it might provide insight into normal spermatogenesis, especially with regard to shaping of the sperm head. The goldfinch and the canary have spirally shaped sperm heads, but that of the goldfinch is smaller.

The testes of a male hybrid from a cross between a female canary and a male goldfinch were obtained in January (breeding season September–February). They were approximately four times smaller in diameter than those of a goldfinch and canary obtained in November and the seminiferous tubules were considerably narrower. All testes were fixed whole in Karnovsky's (1965) fixative. The goldfinch and canary testes were cut into four pieces after 30 min. No mature spermatozoa were produced by the hybrid and no sperm bundles were formed. In the goldfinch and canary the spermatids and nearly mature spermatozoa were enveloped by Sertoli cell cytoplasm and collected into long bundles which radiated towards the lumen of the tubule. These bundles were similar to the cysts of invertebrates and contained 32 sperm tails in a cross-section.

In the hybrid, no pachytene stage with synaptonemal complexes could be detected by electron microscopy, although this stage was prominent in goldfinch and canary due to its relatively long duration. This suggests that no crossing-over occurred in the hybrid. Spermatocyte stages were present in the hybrid; they contained sinuous arrangements of rough endoplasmic reticulum about the nucleus, and a Golgi apparatus (forming proacrosomal granules and vacuoles), with a pair of associated centrioles (as in the parent species). No acrosome formation ensued in the hybrid, and the nucleus did not condense or become shaped (unlike spermiogenesis in the parents, in which a large acrosome and helically shaped nucleus were formed). Many annulate lamellae, often of an unusual radiating crystalloid form, were present in the hybrid. Some lay within the perinuclear cisterna and demonstrated their origin from the nuclear envelope.

A few examples similar to the spermatid stage were seen in the hybrid; a simple flagellum was attached to the uncondensed but small and partly shaped nucleus. Although a few microtubules were present in the cytoplasm, the extensive helical arrangement around the nucleus found in the parent species was absent. As the spermatocytes or spermatids of the hybrid moved towards the lumen they were surrounded by Sertoli cells which then phagocytosed them. The breakdown products formed large residual bodies in the Sertoli cell cytoplasm—most were towards the tubule lumen, but some migrated towards the basement lamina.

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Decrease in the glycerylphosphorylcholine content of the epididymis after injecting α -chlorohydrin into rats. Suzanne Morris, Patricia D. C. Brown-Woodman and I. G. White, *Department of Veterinary Physiology, University of Sydney, N.S.W.* 2006, Australia.

Alpha-chlorohydrin, which is a simple mono-chlor derivative of glycerol, has been shown to produce temporary sterility in some species. The metabolism and motility of spermatozoa are inhibited strongly after injecting α -chlorohydrin into the rat and ram (Brown & White, 1973; Brown-Woodman & White, 1975; Mohri *et al.*, 1975). However, as α -chlorohydrin and glycerol are structurally similar, it seemed possible that α -chlorohydrin may interfere also with the synthesis of glycerylphosphorylcholine (GPC) in the epididymis.

In initial studies rats were injected with α -chlorohydrin at a dose of 90 mg/kg, causing a lesion in the caput epididymidis. GPC was isolated from the four segments of the epididymis (initial segment, caput, corpus and cauda) by paper chromatography and estimated by phosphorus analysis. There was a reduction in total GPC concentration of the epididymis 2 days after treatment with α -chlorohydrin and a further decrease until 20 days. However, similar results were obtained after ligation of the vasa efferentia, suggesting that the reduction in GPC may be due to the lesion in the epididymis blocking the duct rather than to a direct action of α -chlorohydrin on the epididymis.

Rats were given 6 daily injections of α -chlorohydrin at a dose (15 mg/kg) that does not cause lesions. The GPC in the 4 segments of the epididymis was isolated by cellulose TLC up to 8 days after the final injection. The GPC content of all segments was reduced significantly at all times measured when compared with control animals. This finding indicates that α -chlorohydrin can act directly on the epididymis to reduce the GPC content.

To investigate further the mode of action of α -chlorohydrin, its effect on the uptake of [32 P]-orthophosphate into epididymal GPC, was estimated. Rats were injected with [32 P]orthophosphate and 6 hr later with α -chlorohydrin; control animals received saline instead of α -chlorohydrin. After 48 hr the animals were killed and the radioactivity in the GPC of the caput, corpus and cauda epididymidis measured. Alpha-chlorohydrin appeared to have no effect on the uptake of 32 P into the corpus and cauda, but inhibited uptake into the caput to about 50% of the control level. Control values showed that the caput is the major site of GPC synthesis in the epididymis.

The decrease in GPC synthesis with α -chlorohydrin could well reflect a general decrease in synthetic activity of the epididymis, which could result in a significant change in the environment within the duct through which the spermatozoa pass over several days.

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The glycerylphosphorylcholine and phospholipid pattern of the genital duct and spermatozoa of the African elephant, *Loxodonta africana*. Annabelle Darin-Bennett, Suzanne Morris, R. C. Jones and I. G. White, *Department of Veterinary Physiology, University of Sydney, N.S.W.* 2006, and *Department of Biological Sciences, University of Newcastle, N.S.W.* 2308, Australia.

The elephant has no anatomically distinct epididymis and the comparable structure has been referred to, throughout its length, as the Wolffian duct (Short *et al.*, 1967). Previous studies by Jones *et al.* (1974) suggest that this duct performs the main functions of the epididymis in scrotal mammals. It was, therefore, of interest to investigate if the duct produces glycerylphosphorylcholine (GPC), a characteristic secretion of the epididymis (White, 1973), and to see if GPC levels through the duct

follow a similar pattern to that of other species. The opportunity was also taken to examine the phospholipid pattern of the spermatozoa from the storage end of the duct.

Material was collected in Kruger National Park, South Africa, as described by Jones *et al.* (1974). Genital ducts for analysis of GPC were frozen in the field within 30 min of collection and subsequently thawed, minced and deproteinized. Samples for phospholipid analysis were stored at 30°C and extracted with chloroform:methanol (1:2, v/v) within 80 min of collection. Phospholipid and GPC were estimated by thin-layer chromatography, followed by phosphorus analysis.

The GPC content, estimated from the vasa efferentia and along the length of the Wolffian duct, increased from the testis to the terminal segment. The largest increase was between the segments referred to by Glover & Nicander (1971) as the 'pre-initial' and 'initial' segments. This seems to indicate increased synthesis of GPC with maturation of the spermatozoa, and further points to the similarity of this duct in the elephant to the epididymis of other species.

The major phospholipids of the spermatozoa were, in order of quantitative importance: phosphatidyl choline, phosphatidyl ethanolamine, ethanolamine plasmalogen, choline plasmalogen, sphingomyelin, cardiolipin and phosphatidyl inositol. Sphingomyelin was quantitatively the most important phospholipid in the plasma, with appreciable levels of ethanolamine plasmalogen, phosphatidyl serine, phosphatidyl choline and phosphatidyl inositol, as well as a small amount of choline plasmalogen.

The phospholipid pattern of elephant spermatozoa obtained from the terminal segment, which is involved primarily in storage of spermatozoa, would seem to resemble most closely that found previously in the ejaculated spermatozoa of the boar (Darin-Bennett *et al.*, 1973). This is in contrast to the spermatozoa of the ruminants, ram and bull, on the one hand, and rabbit, dog and man on the other.

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Possible mechanisms involved in the reduction in motility of human spermatozoa by copper, zinc and silver. M. K. Holland, D. A. I. Suter and I. G. White, *Department of Veterinary Physiology, University of Sydney, N.S.W. 2006, Australia.*

The rise in popularity of the intrauterine device (IUD) as an alternative to oral contraceptives has led to the study of substances which can enhance IUD efficacy, and many heavy metals, particularly copper (Kesserü *et al.*, 1974), have been investigated in this regard.

In the present study, human spermatozoa were incubated with copper, silver and zinc wires (surface area 960 mm²) in 1 ml calcium-free Krebs–Ringer phosphate buffer, pH 7.4, containing [U-¹⁴C]glucose (3 mM). Oxygen consumption was measured in a micro-Warburg apparatus especially suited for the low oxygen consumption of human sperm. Motility was scored by microscopic examination using a points system ranging from 0 to 10.

Copper decreased sperm motility significantly within 1 hr and totally within 3 hr. However, oxygen consumption was stimulated in the presence of copper even after the spermatozoa had been rendered immotile. Glucose utilization, oxidation and lactate production were unaffected. It is suggested that the effect on motility is due to formation of cuprous ions which may readily cleave disulphide bonds in sperm proteins (Oster & Oster, 1974) and thus severely disrupt the protein's tertiary structure.

Incubation of spermatozoa with zinc significantly reduced motility over 3 hr, but not to the extent obtained with copper. Oxygen consumption was also stimulated but only to 50% of the level achieved with copper. However, glucose utilization, oxidation and lactate accumulation were unaffected. Thus it is suggested that zinc exerts its effect on sperm motility in a fashion similar to copper.

After 90 min of incubation silver significantly reduced sperm motility. However, in contrast to copper and zinc, silver reduced oxygen consumption and glucose utilization (by 50%) and oxidation (by 38%). Lactate accumulation was unaffected. This suggests that silver, unlike copper and zinc, interferes with enzymes of either the TCA cycle or electron transport chain.

All three metals exhibit significant effects on sperm motility after 3 hr, with copper the most effective. It is suggested that copper and zinc may seriously affect the structural integrity of the cell membrane while silver inhibits normal sperm energy metabolism.

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Production of prostaglandin-like substances *in vitro* by the ductus deferens of the mouse. P. B. Marley, N. R. Frith and F. E. Ickowicz, *Department of Pharmacology, School of Pharmacy, London University, U.K.*

A substantial quantity of a prostaglandin (PG)-like substance appears in the female tract when mice mate, and the most likely site of its synthesis is the ductus deferens (Marley & Smith, 1974, 1975). The following experiments examined whether or not the ductus has the capability to synthesize similar amounts of PG-like substances when it is incubated in the presence of the PG precursor, arachidonic acid, and attempted to determine whether the synthesis occurs in the ductus deferens itself or within the lumen due to the presence of spermatozoa.

Arachidonic acid was used as substrate throughout and was prepared as described by Palmer *et al.* (1973). Pairs of ductus deferentes were placed in flasks containing 3.5 ml ice-cold oxygenated Tyrode's solution. The various additions were then made and the tissues chopped *in situ*. The flasks were incubated, with agitation, at 37°C for 30 min in a water bath and then the reaction was stopped by acidification to pH 3. The incubations serving as blanks were acidified immediately before the incubation. PG-like substance was extracted into ethyl acetate and then measured by a bridging assay against authentic PG, or 'PGE' and 'PGF' were measured directly by radioimmunoassay (Clarke *et al.*, 1974).

There was comparatively little PG-like substance present in the absence of added substrate but the production increased with an increase in the amount of arachidonic acid added over a range of 2.5–50 µg/flask. Conversion rates ranged from 37% to 5% respectively and the amount of PGE-like to PGF-like substances synthesized was approximately 4:1 in the presence of 10 µg substrate. The addition of 50 µg noradrenaline bitartrate to the incubations nearly doubled the yield of PG-like substances.

When the contents of the ductus deferens were squeezed out and the lumen was flushed with Tyrode, before the incubation, the ductus deferens synthesized much less PG-like substance than untreated tissue whereas the squeezings synthesized as much as the untreated tissue. The squeezings consisted almost solely of spermatozoa and this suggested that the site of synthesis was in the spermatozoa. However, when spermatozoa were obtained by flushing without the initial squeezing, they had comparatively little ability to synthesize PGE or PGF. Subsequent squeezing of the flushed ductus deferens produced a small cellular component, probably from the luminal epithelium, with high synthesizing activity.

These results, together with previous evidence, strongly suggest that the ejaculated PG-like substance is synthesized in the ductus deferens.

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Inhibition of the oxidative metabolism of ram spermatozoa by α -chlorohydrin *in vitro*. I. G. White, P. D. C. Brown-Woodman, D. A. I. Suter and H. Mohri, *Department of Veterinary Physiology, University of Sydney, N.S.W. 2006, Australia.*

Alpha-chlorohydrin is an effective, reversible antifertility agent in the ram (Brown-Woodman *et al.*, 1974) and studies *in vivo* and *in vitro* in our laboratory have shown it to be a potent inhibitor of glycolysis (Brown & White, 1973; Mohri *et al.*, 1975). The enzymes glyceraldehyde-3-phosphate dehydrogenase, aldolase and triose phosphate isomerase are inhibited by α -chlorohydrin, and the following experiments were designed to determine the extent to which the metabolism of various sperm substrates is affected by α -chlorohydrin.

Washed ram spermatozoa were incubated for 3 hr in Warburg flasks in the presence of α -chlorohydrin (0, 0.1, 10 and 100 mM) with [U - ^{14}C]fructose (5 mM), [U - ^{14}C]glycerol (5 mM), [U - ^{14}C]lactate (5 mM), [U - ^{14}C]pyruvate (10 mM), [U - ^{14}C]acetate (10 mM) and [U - ^{14}C]palmitate (2 mM) (attached to 3% bovine serum albumin). In addition, endogenous respiration was measured in the presence of α -chlorohydrin.

With fructose as substrate, even at 0.1 mM- α -chlorohydrin, oxygen uptake of the spermatozoa was reduced by 40%, while at higher concentrations the inhibition was even more severe (85% at 100 mM); no lactate accumulated in the presence of α -chlorohydrin, even at 0.1 mM. When the spermatozoa metabolized glycerol, 10–100 mM- α -chlorohydrin was required to reduce (60–80%) oxygen uptake; lactate production was reduced at 1 mM- α -chlorohydrin but 100 mM was required to produce complete inhibition. With lactate as substrate, oxygen uptake was only reduced by 10% at 10 mM- α -chlorohydrin and 65% at 100 mM, while the oxygen uptake was slightly more affected using pyruvate (30% at 10 mM and 40% at 100 mM). There was greater inhibition of oxygen uptake when the spermatozoa metabolized acetate (20% inhibition at 1 mM α -chlorohydrin, 50% at 10 and 100 mM), while with palmitate there was less effect (40% at 10 and 100 mM- α -chlorohydrin). The endogenous oxygen uptake of spermatozoa was inhibited by 30% at 10 mM and 40% at 100 mM- α -chlorohydrin.

The experiments indicate that the α -chlorohydrin-induced block in metabolism mainly occurs high up in the glycolytic pathway since the metabolism of fructose was inhibited to the greatest extent. This view is supported by the fact that lactate and pyruvate oxidation were only inhibited at high concentrations of α -chlorohydrin when it is possibly acting as a less specific alkylating agent. However the considerable inhibition of oxygen uptake with acetate as substrate suggests an additional site of action of α -chlorohydrin in the Krebs tricarboxylic acid cycle, although palmitate, which is normally oxidized via acetate was not affected to such an extent.

Comparatively high oxygen uptake in the presence of α -chlorohydrin with glycerol as substrate may be due to oxidation of α -glycerophosphate to dihydroxyacetone phosphate in the mitochondria, a step which is presumably insensitive to α -chlorohydrin.

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Section 6

Chairman: Professor N. W. Moore

Endocytosis in the uterine epithelium of the mouse. Margaret B. Parr and E. L. Parr, *Department of Anatomy, University of Adelaide, Adelaide, South Australia 5001.*

Histological examination of the rat (Warren & Enders, 1964; Nilsson, 1966, 1972; Löff *et al.*, 1968;

Psychoyos & Mandon, 1971) and mouse (Nilsson, 1962; Bergström, 1972; Bergström & Nilsson, 1972, 1973) uterus during preimplantation stages or delayed implantation has shown that portions of the luminal epithelial cell surface often bulge or project into the uterine lumen. The projections, which have been described as sea-anemone-like formations or fungus-like protrusions, have recently been named pinopods by Enders & Nelson (1973). In the rat, the pinopods contain vacuoles measuring 0.5–3 µm in diameter. Most investigators suggested that the pinopods pinch off from the cells and release cellular materials into the lumen which nourish preimplantation embryos. However, in the rat, recent investigations showed that the pinopods are involved in endocytosis, not apocrine secretion (Enders & Nelson, 1973; Parr & Parr, 1974). Exogenous tracers could be detected in the pinopod vacuoles shortly after their instillation into the uterine lumen and later in multivesicular bodies and dense bodies in the cells.

In this report, we present evidence that the pinopods of the mouse uterine luminal epithelial cells, during luteal stages, rapidly incorporate exogenous tracer from the uterine lumen into pinopod vacuoles and similar vacuoles in the apical portion of the cells.

The apical surface of the uterine luminal epithelium of 15 mice during preimplantation and delayed implantation stages was studied with light and electron microscopy. Some cells had irregular protrusions or pinopods which frequently contained vacuoles up to 3 µm in diameter. Similar vacuoles were also present in the apical cytoplasm of some cells lacking pinopods. The pinopods were found on Day 4 of pregnancy and Day 10 of delayed implantation, but were absent on Days 2, 3 and 5 of pregnancy.

A study of the absorption of ferritin from the uterine lumen in 4 mice during delayed implantation showed that the pinopods were involved in the formation of endocytic vacuoles. At 15 min after injection of ferritin into the uterine lumen, all of the pinopod vacuoles that were examined and many vacuoles in the apical cytoplasm contained the tracer. In addition to the pinopod vacuoles, endocytosis was infrequently carried out by coated vesicles.

There was no evidence that pinopods of mouse luminal epithelial cells constitute an apocrine secretion which might be involved in the maintenance of preimplantation blastocysts.

The demonstration of ferritin particles in the pinopod vacuoles in mouse and rat uterine epithelial cells clearly shows that they carry on endocytosis at a time when unimplanted embryos are present in the lumen. The process seems to be terminated as implantation begins. Although several suggestions have been put forward for the possible role of endocytosis during this period (Enders & Nelson, 1973; Parr & Parr, 1974), its function remains unknown.

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Preliminary investigation into uterine epithelial cell types using cluster analysis. J. H. Stanton, *School of Anatomy, University of New South Wales, Kensington, N.S.W. 2033, Australia.*

Differences appear in staining intensity and ultrastructural appearance of the uterine epithelial cells of the normal cyclic mouse at dioestrus. Furthermore, differences in the method of ferritin uptake from the uterine lumen by these cells has been demonstrated (Stanton & Smith, 1975). Because no distinguishable feature of the cell types was immediately evident, the cell characteristics had to be more objectively analysed. The statistical test used on the cells and their characteristics was a cluster analysis.

Classification of the different epithelial cells was done using low-power electron micrographs, noting the position of a cell within the epithelium, the relative size and shape and its nuclear and cytoplasmic constituents. A total of 125 cell morphological properties were used and degrees of similarity or dissimilarity were calculated by comparing two cells at a time. The similarity coefficients obtained were then used in a cluster analysis (CLUSTAN package programme on a CYBER 76 computer). A combination of 4 similarity and 5 hierarchical clustering methods was used.

In all of these methods, two cell types could be distinguished. There remained, however, cells which did not enter into these clusters and remained relatively distinct from these two groups. The uniqueness of the groups was cross-checked by clustering the cell characters. Those cell characters which showed a high binary frequency ratio (% occurrence in cluster/% occurrence overall) for each of the cell clusters appeared independent of each other, confirming the presence of at least two distinct groups of cells, each with its own characters.

The appearance of more than one cell type within this epithelium, as suggested in preliminary morphological studies, has been confirmed using a statistical analysis new to qualitative cell morphology.

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Uptake and metabolism of [³H]progesterone by the uterus of the ovariectomized mouse. L. C. Murphy and G. M. Stone, *Department of Veterinary Physiology, University of Sydney, N.S.W. 2006, Australia.*

It is well established that the expression of the physiological effect of progesterone in the uterus depends upon its initial exposure to an oestrogenic stimulation (Courrier, 1950). The reasons for this dependence, however, and the mechanism of action of progesterone in the uterus are not clearly understood.

These experiments have examined the effect of prior oestrogenic stimulation on the uptake of [³H]progesterone following local application to the uterine lumen, the subcellular distribution of radioactivity, and the nature of radiometabolites in the uterus.

Subcutaneous pretreatment with oestradiol-17 β (0.1 μ g/day for 2 days) did not change the initial uptake of radioactivity at 1 min after injection but slightly decreased the retention of radioactivity at 60 min. This decrease was significant ($P < 0.05$) following the injection of 10^{-4} μ g but not 10^{-2} μ g [³H]progesterone. A rapid uptake of radioactivity was indicated by the low levels of radioactivity remaining in saline washings after 1 min.

There was a pretreatment effect in the relative levels of radioactivity in the soluble and 'nuclear/myofibrillar' fractions of uterine homogenates prepared from tissue 1 and 60 min after [³H]progesterone administration. At the later time a greater proportion of the tissue radioactivity was found in the 'nuclear/myofibrillar' fraction. No such change was found with animals which had received no pretreatment.

The nature of the radioactive compounds in the tissue fractions also changed with pretreatment and with time. In control animals at 1 min, more than 80 % and at 60 min, more than 40 % of the radioactivity in all fractions was associated with [³H]progesterone. In treated animals at 1 min, less than 25 % of the radioactivity in all fractions was associated with [³H]progesterone, while at 60 min the % of progesterone in all fractions increased, due in part to a declining level of tissue radioactivity. Irrespective of pretreatment or time of killing, little or no radioactivity was associated with 5 α -pregnan-3,20-dione.

In controls, radioactivity not associated with progesterone was mainly associated with 5 α -pregnan-diols and more polar metabolites. In the treated animals at 1 min in each fraction more than 50 % of the radioactivity was associated with 20 α - or 20 β -dihydroprogesterone, with minor levels of the 5 α -reduced products. At 60 min the proportion of the former was reduced.

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Interactions between steroids and spasmogens on the myometrium of the ewe. M. J. Carrick and P. T. Cupps, *Department of Animal Science and Production, University of W.A., Nedlands, W.A. 6009, Australia, and Department of Animal Science, University of California, Davis, U.S.A.*

Although the hormonal changes occurring shortly before and during natural and induced parturition in the ewe have been measured extensively, theories concerning the regulation of parturition in the ewe are lacking in detail because there is little information on the direct effects of these hormones on the myometrium. To study these effects in greater detail oestradiol benzoate, progesterone, dexamethasone, oxytocin and PGF-2 α were administered to ewes which had been prepared surgically to provide information on the actions and interactions of these substances in the myometrium of the ewe.

Six ewes, each of which was equipped with an intrauterine pressure balloon, six extracellular electrodes placed in the myometrium, and an intrauterine infusion catheter were used. The variables measured were mean pressure, maximum pressure, minimum pressure (tonus), rate of contraction, following rate of electrical spike production and spike potential.

A preliminary experiment revealed that oestrogen treatment increased spontaneous myometrial activity by 2 to 4-fold, as compared with the castrate or following other steroid treatments. Responsiveness to oxytocin paralleled the level of spontaneous activity. The response to PGF-2 α was highest following oestrogen treatment and declined during the other steroid treatments.

The main experiment was of factorial design with factors arranged as a split-split-plot. Factors were sheep, steroids (oestradiol benzoate (E), E plus progesterone (E+P), E plus dexamethasone (E+D), or (E+P+D), duration of steroid treatment (Days 1, 2, 3, 4) and spasmogen treatment (oxytocin or PGF-2 α).

Progesterone (E+P) modified the effects of oestrogen by increasing tonus (18%) and reducing rate of contraction (30%), rate of spike production (29%) and spike potential (47%). Mean pressure was not modified significantly by progesterone. Changes produced by progesterone suggest desynchronization of cellular activity in the myometrium. Sensitivity to oxytocin was decreased by E+P treatment and there was little effect on the response to PGF-2 α . The mean pressure during oestrogen treatment alone rose with increasing duration of treatment whereas during E+P treatment mean pressure declined slightly ($P<0.05$).

Dexamethasone (E+D) inhibited the spontaneous activity (49%) and oxytocin responsiveness (46%) compared to that during treatment with oestrogen only. During the E+D treatment the decline in the response to PGF-2 α was relatively small (23%). The change in spontaneous myometrial activity and response to spasmogens during E+D treatment occurred mainly on the first day of treatment and there was little change thereafter. The combination of all these steroids (E+P+D) produced results that were similar to those found during E+D treatment.

These experiments revealed that oxytocin and PGF-2 α interact with the steroids in producing their effects on the myometrium. Oxytocin sensitivity is highest during treatment with oestrogen alone and declines markedly under the influence of any combination of the steroids studied. PGF-2 α sensitivity was highest during E and E+P treatment and declined slightly during treatment with E+D or E+P+D. It appears that the action of glucocorticoids in initiating parturition may occur in spite of an inhibitory effect on myometrial activity.

Progesterone and induction of lactation in ewes. W. J. Fulkerson, R. D. Hooley, G. H. McDowell and L. R. Fell, *University Farms, Camden, 2570, S. S. Cameron Laboratories, Werribee, Victoria 3030, and Dairy Research Centre, Richmond, 2753, Australia.*

The non-pregnant ewe induced to lactate artificially appears to be a useful model for defining the mechanisms of initiation of lactation (Fulkerson & McDowell, 1974, 1975; Fulkerson *et al.*, 1975). The present studies were conducted, using this model, to evaluate the involvement of progesterone in the lactogenic response in the ewe.

Thirty-six nulliparous ewes, ovariectomized 3 weeks previously, received 10 s.c. injections of 240 μ g oestradiol benzoate (OB) plus 60 mg progesterone at intervals of 3 days. Commencing 3 days

after the last injection, groups of 4 ewes then were treated for 5 days (trigger phase) as follows: Groups 1 and 2, 40 µg OB s.c. twice daily; Groups 3 and 4, 2.5 mg OB s.c. twice daily; Groups 5 and 6, 10 mg dexamethasone trimethylacetate s.c. once daily; Groups 7 and 8, 1 i.u. syntocinon i.v. 4 times daily; Group 9, no hormone. Ewes in Groups 2, 4, 6 and 8 also received 20 mg progesterone/day. All ewes were milked by hand from the first day of the trigger phase.

Milk yields were recorded and lactose contents determined. Blood samples, collected at frequent intervals before, during and after the trigger phase, were analysed for prolactin (Fell *et al.*, 1972).

After 21 days of milking, milk yields from ewes in Groups 3–6 (about 200 ml/day) were significantly higher ($P < 0.05$) than those from ewes in Groups 1, 2 and 7 (about 75 ml/day) which in turn were significantly greater ($P < 0.05$) than the trivial yields from ewes in Groups 8 and 9. Lactose concentrations in milk from ewes in Groups 1 and 3–7 (about 5.3 %) were substantially higher than those for ewes in Groups 2 and 8.

Before the trigger phase, levels of prolactin for all ewes were low (about 20 ng/ml). Within 4 hr of injecting OB (Groups 1–4) or syntocinon without progesterone (Group 7), levels of prolactin increased to 350–400 ng/ml and about 200 ng/ml respectively then fell to low levels (about 40 ng/ml) over the next 24 hr despite further injections. In contrast, levels of prolactin in serum of all other ewes remained low throughout the experiment.

The results show that the restraining influence of progesterone on lactogenesis in the ewe can be overcome. Injections of progesterone inhibited the lactogenic response to syntocinon, suppressed the response to low doses of OB, but failed to influence the response to dexamethasone or high doses of OB. Moreover, these results are consistent with the earlier conclusions (Fulkerson *et al.*, 1975) that OB and possibly syntocinon appear to be lactogenic by virtue of their effect on prolactin secretion whereas dexamethasone appears to act independently of prolactin.

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Session 7

Chairman: Dr J. K. Findlay

Effect of differing environments on the oestrous cycle and ovulation rates in several species of native Australian rats. W. G. Breed, *Department of Anatomy, University of Adelaide, S.A. 5001, Australia.*

Species of rodents living in different environments show different population dynamics and in Australia there are a number of species of *Rattus* occurring in very diverse ecological niches. The aim of the present study was to determine whether these species have diverged in their reproductive potential, and especially in age of puberty, oestrous cycle length, and ovulation rate. *Rattus leucopus leucopus*, *R. leucopus cooktownensis*, and *R. fuscipes coracius* occur in rain forests; *Rattus villosissimus* occurs in central Australia where plague numbers occur spasmodically; and other species live in swamplands (*R. lutreolus*); southern woodlands (*R. fuscipes*); and canefields (*R. sordidus*). To date, apart from incidental observations of Taylor & Horner (1973) only the reproductive biology of the last two species has been investigated in any detail (McDougall, 1946; Taylor & Horner, 1972; Warneke, 1971).

In the present study the following parameters have been investigated: (1) onset of oestrus as indicated by first occurrence of a cornified vaginal smear, (2) length of oestrous cycle as shown by vaginal smears, and (3) number of healthy CL, determined by scanning serial sections of ovaries which usually contain 2–4 sets of CL, representing 2–4 successive ovulations.

The results are from animals caught in the wild and caged singly or from progeny of pregnant females caught in the wild and born in captivity.

First oestrus occurred at a mean of 94 ± 0 (S.E.M.) days in *R. leucopus cooktownensis*, 100 ± 20 days in *R. f. coracius*, whereas that of *R. villosissimus* was at 70 ± 5 days.

All 9 oestrous cycles of *R. leucopus cooktownensis* were of either 4 or 5 days, and 13/16 cycles of *R. leucopus leucopus* were <10 days and had a mean length of 4.0 ± 0.5 days ($N=2$), whereas 34/37 oestrous cycles recorded from *R. f. coracius* were <10 days and had a mean length of 5.3 ± 0.2 days ($N=5$). Similarly 44/46 cycles of *R. villosissimus* were <10 days and had a mean length of 5.2 ± 0.2 days ($N=6$). Identical results were also obtained for *R. lutreolus* ($N=4$).

By contrast, the following numbers of recently formed CL were observed; *R. leucopus cooktownensis* had 5.0 ± 0 , and *R. f. coracius* had 5.7 ± 0.6 . However, *R. villosissimus* had significantly more CL with a mean of 9.5 ± 0.7 ($P < 0.01$) and *R. lutreolus* had 7.3 ± 1.2 . These differences no doubt reflect larger numbers of oocytes being released at each ovulation in *R. villosissimus* compared to other species, and thus indicate considerable variation in ovulation rate between closely related species of rats living in different habitats, even though oestrous cycle length, and the age of puberty remain similar.

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Primordial follicles in the ovary of the neonatal ewe lamb. Carol Worthington and J. P. Kennedy, *School of Wool & Pastoral Sciences, University of New South Wales, Kensington, N.S.W.* 2033, Australia.

Mauleon (1969) stated that oogonial mitoses in the sheep ceased well before birth but that a decrease in numbers of primordial follicles was difficult to demonstrate in the few months after birth. However, Trounson *et al.* (1974) found that there was an increase in numbers of primordial follicles between 7 days and 5 months of age when paired ovaries of hemispayed lambs were compared. Additional data on changes in numbers of primordial follicles have been obtained from the lambs described by Kennedy *et al.* (1974).

Merino lambs were slaughtered at 0, 4, 8, 12, 16½, 20, 24 and 33 weeks after birth. Ovaries were serially sectioned at 8 µm. Primordial follicles were counted in every 40th section and the total count per ovary was estimated by multiplying the count per section by 40. Results are given in Table 1.

Table 1. Mean no. ($\pm$ S.E.M.) of primordial follicles in ovaries of lambs of different ages

Age (weeks)	0	4	8	12	16½	20	24	33
No. of lambs	4	4	2	3	4	4	4	4
Follicles ($\times 10^{-3}$)	47.8 ± 9.2	65.6 ± 8.2	78.3 ± 18.2	96.5 ± 6.0	42.5 ± 5.3	92.6 ± 7.9	84.0 ± 34.4	39.7 ± 9.7

Because variances were heterogeneous (Bartlett's test) the data were analysed by the method of weighted mean squares (Snedecor, 1956). Age was found to be a significant main effect ($P < 0.05$). The mean of the means of follicle numbers between 4 and 20 weeks was significantly greater than the mean at birth ($P < 0.05$) using the *t* test with weighted variances.

These data provide further evidence that primordial follicles increase after birth in lambs. However, the evidence for postnatal oogenesis is inconclusive because this was a cross-sectional study and mitosis was not observed in primordial oocytes.

Follicle counts were made independently in both ovaries of each lamb and the intraclass correlation of follicle numbers was calculated to be 0.92. The small variation between each pair of ovaries gives confidence in the method of estimating follicle numbers and also indicates that in further work it is not necessary to examine both ovaries.

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Gonadotrophins in the neonatal female lamb. R. Tassell, W. A. Chamley* and J. P. Kennedy, *School of Wool & Pastoral Sciences, University of New South Wales, Kensington, N.S.W. 2033*, and **Department of Physiology, University of Melbourne, S. S. Cameron Laboratory, Werribee, Victoria 3030, Australia.*

Kennedy *et al.* (1974) showed that ovarian weight in lambs increased significantly between birth and 8 weeks of age, due to the growth and proliferation of small and vesicular follicles. The endocrine control of these events was investigated in this study.

Merino lambs were randomly allocated to 6 groups, of 4–6 animals, to be slaughtered at birth or at 2, 4, 6, 8 and 10 weeks of age. Blood samples were taken at hourly intervals for 6 hr before slaughter. One ovary from each lamb was homogenized and progesterone and oestradiol-17 β levels determined by competitive protein-binding assay. The other ovary was serially sectioned and examined microscopically. Peripheral plasma levels and pituitary content of LH and FSH were determined by radioimmunoassay.

The mean ovarian weights at 4 weeks and 8 weeks were approximately 5 and 10 times greater than at birth (121 ± 4 (S.E.M.) mg). From 4 weeks onwards the ovaries contained numerous follicles of 1–2 mm diameter and from 6 weeks each ovary contained at least one follicle >2 mm in diameter.

Plasma FSH levels ranged between 20 and 51 ng/ml at birth and did not change with increasing age. However, pituitary concentration of FSH rose from 161 ± 26 μ g/g at birth to 284 ± 70 μ g/g at 4 weeks and to 346 ± 59 μ g/g at 10 weeks. Mean levels of LH in plasma did not change significantly between birth (1.2 ± 0.4 ng/ml) and 10 weeks (1.4 ± 0.1 ng/ml). Small peaks of LH (>4.5 ng/ml) were observed in 2/6, 1/5, 2/4 and 2/4 lambs at 4, 6, 8 and 10 weeks, respectively. The pituitary concentration of LH at 6 weeks (305 ± 86 μ g/g) was greater than at younger ages.

Ovarian concentration of oestradiol-17 β fluctuated widely between lambs of the same group and bore no obvious relationship to age, to follicular development or to plasma levels of gonadotrophins. Ovarian concentration of progesterone fell sharply between 2 and 4 weeks of age.

These data do not provide an obvious explanation for the ovarian development which occurs shortly after birth in the young lamb.

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Plasma FSH in Merino lambs selected for fecundity. J. K. Findlay and B. M. Bindon, *Reproduction Research Section, University of Melbourne, Werribee, Victoria 3030*, and *C.S.I.R.O. Animal Genetics, P.O. Box 90, Epping, N.S.W. 2121, Australia.*

Plasma FSH was measured by radioimmunoassay (Salamonsen *et al.*, 1973) in samples collected at 09.00, 12.00, 15.00 and 18.00 hours on the 30th day of life from male and female lambs of the C, O, T and B flocks of the CSIRO fecundity selection experiment. These flocks are respectively the control, and flocks selected for low, medium and high litter size (Bindon & Turner, 1974).

In male lambs FSH values rarely exceeded the lower limit of sensitivity of the assay (15 ng/ml) and there was little variability in sequential samples from the same animal. Between-flock differences were not significant. There were substantial differences in the mean FSH values for female lambs although

Table 1. Mean ($\pm$ S.E.M.) plasma FSH concentrations in 30-day-old Merino lambs (nos in parentheses)

Flock	Incidence of multiple births (%)	Plasma FSH (ng/ml)	
		Male lambs	Female lambs
C	0-10	17.8 $\pm$ 2.8 (6)	42.0 $\pm$ 16.6 (4)
O	0-10	17.0 $\pm$ 1.3 (5)	26.8 $\pm$ 2.2 (8)
T	50-60	17.7 $\pm$ 1.4 (5)	111.0 $\pm$ 73.5 (7)
B	70-80	22.2 $\pm$ 4.8 (4)	159.1 $\pm$ 77.6 (7)

the S.E. terms indicate large differences between lambs in the same flock, especially in T and B. However, when the results are compared on the basis of the number of lambs with mean FSH greater than 40 ng/ml, the 14 T and B lambs had significantly higher FSH levels than the 12 C+O lambs ($\chi^2(1)=6.68$; $P<0.01$).

The LH levels in these samples have been described previously (Bindon & Turner, 1974). There was a small but significant correlation ($r=+0.62$, $P<0.05$) between the mean LH and FSH values from the T and B female lambs. When elevated values of FSH and LH occurred these tended to be in the same animal. More importantly, sequential FSH values from the same lamb were less variable than the corresponding LH levels. For example, in female lambs the mean coefficient of variation for 4 sequential samples was 0.25 for FSH measurements but 0.46 for LH measurements. The interpretation of FSH levels may therefore be less complicated by the episodic release patterns characteristic of LH in such animals.

These preliminary data suggest that FSH levels, like LH, are higher in young female lambs from flocks that have been selected for high litter size. It is not yet known if FSH concentration is a useful early indicator of fecundity.

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FSH levels and unilateral ovariectomy in the ewe. I. A. Cumming and J. K. Findlay, *Department of Agriculture, Victoria and Reproduction Research Section, University of Melbourne, S. S. Cameron Laboratory, Werribee, Victoria 3030, Australia.*

We have used the phenomenon of ovarian compensatory hypertrophy (Mallampati & Casida, 1970; Land, 1973) to examine a possible role of FSH in determining ovulation rate in the ewe. Forty-two ewes were allocated at random within live-weight ranges to 4 groups. FSH concentrations were measured in peripheral plasma by radioimmunoassay (Salamonsen *et al.*, 1973) in half the ewes of each group for up to 36 hr after unilateral ovariectomy (leaving the ovary with a CL) on Days 14 or 16 (Groups U14 and U16). Control animals (Groups C14 and C16) underwent a sham operation and were otherwise treated similarly. Blood samples were taken at 30 min intervals for 3-5 hr before operation (Period 1) and then after operation every 15 min for 2 hr (Period 2), 30 min from 2-5½ hr (Period 3), hourly from 5½-12 hr (Period 4) and continued 2 hourly from 12-36 hr (Period 5). Data on FSH were subjected to analysis of variance. All ewes were slaughtered 12-14 days after the post-operative oestrus to determine ovulation rate and ovarian and CL weights. The results are shown in Table 1.

Although ovarian weights had fully compensated at slaughter in both treatment groups, there was a suggestion that compensation of ovulation rate in Group U16 was incomplete (U14 versus U16, $P<0.10$). FSH values showed a significant increase in Group U14 at 5½-12 hr (Period 4) after unilateral ovariectomy and then declined during the next 24 hr. A similar trend ($P<0.10$) occurred in Group U16, whereas no changes occurred up to Period 4 in either control group. There was a decline in FSH during Period 5 in Group C16 which was probably associated with the fall in FSH before oestrus

Table 1. The effects of unilateral ovariectomy of ewes at Days 14 or 16 of the cycle

Parameter	Group C14	Group U14	Group C16	Group U16
Total no. of ewes	10	11	10	11
No. in oestrus after operation	9	8	9	9
Ovulation rate per ewe	1.70	2.00	1.90	1.46
Ovarian weight (g)	1.53	2.18	1.52	1.90
Weight of CL (g)	0.53	0.47	0.49	0.46
FSH (% change	Period 1	100	100	100
relative to	2	92.7	106.1	101.7
Period 1)	3	91.7	107.2	110.3
	4	100.8	131.2*	95.3
	5	94.8	114.6	69.2
				125.0†
				109.9

* Period 1 versus Period 4, $P < 0.05$; † $P < 0.10$ within treatment groups.

(Salamonsen *et al.*, 1973). These results suggest that an increase in FSH could be involved in the increase in ovulation rate per ovary following unilateral ovariectomy.

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Changes in total ovarian blood flow during anaesthesia and cannulation of utero-ovarian veins. P. E. Mattner, B. D. Stacy and B. W. Brown, *C.S.I.R.O., Division of Animal Physiology, Prospect, N.S.W. 2149, Australia.*

In each of two ewes, Doppler ultrasonic flow probes (White *et al.*, 1974) were implanted around both ovarian arteries. They were used to monitor changes in total blood flow to the ovaries during anaesthesia and during the collection of ovarian venous outflow via catheters (Mattner & Thorburn, 1969). During successive oestrous cycles, sodium pentobarbitone, sodium thiopentone or halothane was administered on Day 1 and Day 9 of the cycle (oestrus=Day 0). Collection of ovarian venous outflow via catheters was performed with the ewes under sodium pentobarbitone anaesthesia on Day 14 or 15 of the cycle.

At Day 9, prolonged anaesthesia (1.5–2 hr) with sodium pentobarbitone or sodium thiopentone had no material effect on total ovarian blood flows. At this stage of the cycle, halothane given alone or after prolonged anaesthesia with the above anaesthetics had a marked effect within 15–20 min. The total blood flow rose by 46% (range 20–90%) for the ovary bearing a CL and by 128% (90–190%) in the contralateral ovary without a CL, with the result that the initial, large disparity between the flows to the two ovaries was markedly reduced.

At Day 1 when total blood flow to each ovary was low, anaesthesia with halothane elevated the flow to a similar extent in each ovary (mean 60%, range 40–80%).

During collection of ovarian venous outflow, the total ovarian blood flow, as monitored with the ultrasonic probes, increased by 30% in ovaries bearing a CL and by 70% in the contralateral ovary without a CL.

It appears that, in sheep, some anaesthetics (e.g. halothane) and drainage of ovarian venous blood through cannulae may interfere with local control of the ovarian vasculature and cause an increase in the blood supply to the ovary. The effects which were most pronounced in ovaries not bearing a CL could markedly influence estimates of total ovarian blood flow based on timed collections of ovarian venous outflow.

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Session 8

Chairman: Professor C. W. Emmens

JAMES GODING MEMORIAL LECTURE

The role of follicles in luteolysis. W. Hansel, *Department of Animal Science, Cornell University, Ithaca, New York, U.S.A.*

Effects of lupin grain supplementation on ovulation rate and fertility of Merino ewes. R. J. Lightfoot and T. Marshall, *Department of Agriculture, South Perth, Western Australia 6151.*

Two experiments were conducted to examine the effects of supplementary feeding with sweet lupin grain (*L. angustifolius*; 33 % crude protein) on ovulation rate and fertility of Merino ewes grazing various pastures in South Western Australia.

Experiment 1 (Lightfoot & Marshall, 1974) was of factorial design (2×5; n=50; N=500) and examined effects of rate of lupin grain supplementation and base pasture type (dry subterranean clover based pasture versus wheat stubble; 7.5 ewes/ha). Lupins were fed thrice weekly commencing 35 days before, and continuing for 18 days after, the start of mating. Ovulation rates were determined by laparotomy during the first 21 days of mating.

Table 1. The effects of feeding lupin grain supplement to ewes

	Ovulation rate	% ewes lambing	% ewes twinning
1. Lupin supplement (g/ewe/day)	(n = 47-49)	(n = 90-96)	(n = 90-96)
0	1.17	69	10
62.5	1.09	71	7
125	1.21	81	13
250	1.37	81	21
500	1.55	85	20
χ^2 lin. lupins	$P < 0.01$	$P < 0.05$	$P < 0.01$
2. Base pasture	(n = 119-120)	(n = 226-240)	(n = 226-240)
Sub. clover	1.34	79	17
Wheat stubble	1.22	76	12
χ^2	$P < 0.05$	N.S.	$0.05 < P < 0.1$

The results (Table 1) showed that lupin grain supplements significantly increased ovulation, lambing and twinning rates. Twinning rates in ewes conceiving after Day 18 of mating (i.e. after lupin feeding ceased) fell to control levels.

In Experiment 2 (Marshall & Lightfoot, 1974) control ewes grazing dry subterranean clover based pasture were compared with ewes grazing the same pastures but given lupin grain supplement at 500 g/ewe/day for 14 or 35 days before mating or for 18 days after mating started. Ovulation rates were 1.24, 1.48 and 1.48 and the % of ewes twinning was 10, 21 and 23, respectively.

The results indicate that ovulation rate responds rapidly to lupin feeding and that the effect is not necessarily related to liveweight changes.

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Effect of lupin grain supplementation on ovulation rate in Border Leicester×Merino ewes. D. J. Rizzoli, R. Baxter, J. L. Reeve and I. A. Cumming, *Department of Agriculture, S. S. Cameron Laboratory, Werribee, Victoria 3030, and Rutherglen Research Station, Rutherglen, Victoria 3685, Australia.*

Recent reports from Western Australia describe marked increases in the number of ovulations occur-

ring in ewes fed a supplement of lupin grain at February matings (Lightfoot & Marshall, 1974; Marshall & Lightfoot, 1974). As most sheep in Victoria are mated from October to March it was decided to explore the value of lupin grain as a supplement over this period. In an attempt to understand more fully the reasons why lupin grain in particular was so effective as a supplement, two iso-energy supplements (0.5 kg/ewe/day) of either lupin grain or wheat (protein content, 32 and 11 % of dry matter respectively) were fed for 14 days before mating and for the following 20 days of a mating period to 600 ewes aged 7½ years at the Rutherglen Research Station. Ewes were allotted at random to the lupin or wheat treatments and to six mating groups. At monthly intervals from October to March one group from both the lupin and wheat treatments were paired with fertile rams and, after a 20-day mating period ovulation rates for all ewes were determined by counting CL, either at slaughter or by surgical examination. Mean liveweight of ewes did not differ significantly between wheat or lupin treatments. The mean liveweights of the groups mated from October to March were 65, 67, 64, 63, 61 and 58 kg. During all mating periods, ewes declined in liveweight (rate 0.3–2.5 kg) even though supplemented, except the ewes in lupin groups of October and March which gained 0.8 kg. There was no significant difference in ovulation rate between the lupin and wheat groups from October to January (1). However, ovulation rates increased in February and March ($P<0.05$) and in these months alone lupin grain supplementation resulted in an increase in ovulation rate above that of ewes given wheat supplementation ($P<0.01$).

Text-fig. 1. Mean ovulation rates in ewes fed supplements of lupin grain (solid columns) or wheat (open columns). Figures give nos of ewes in groups.

This experiment demonstrates the need to recognize the seasonal factor which is so integrally involved in determining ovulation rate in ewes. Why lupin grain failed to stimulate ovulation rates from October to January is unknown but it is interesting to speculate that ewes during this period may already be expressing their maximum ovulation rates and only in February and March were the pastures deficient in some nutritional requirement for ovulation rate potential to be realized. In February–March, lupin grain was more effective than the iso-energy supplementation of wheat in increasing ovulation rates.

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Effect of lupin grain, wheat and lucerne supplements on ovulation rate in maiden Border Leicester× Merino ewes. J. L. Reeve, P. A. Kenney, R. Baxter and I. A. Cumming, *Department of Agriculture, Rutherglen Research Station, Rutherglen, Victoria 3685, and S. S. Cameron Laboratory, Werribee, Victoria 3030, Australia.*

Low ovulation rate is a major factor limiting fecundity of young ewes in the commercial sheep flocks of Australia (Cahill & Blockey, 1974). Recent publications report considerable increases in ovulation rate in mature ewes mated in February and March fed lupin grain at 0.5 kg/ewe/day for 2 weeks before mating with continued feeding through the mating period (Rizzoli *et al.*, 1976). No such dramatic increase in ovulation rate has been reported for ewes fed other supplements for such

restricted periods. The present study was designed to define the effect of lupins and other supplements on ovulation rate in young ewes mated in February and March. Twelve hundred Border Leicester× Merino ewes, aged 18 months, were allotted at random within liveweight ranges to one of five groups and ewes of each group to three subgroups. The first group was not fed a supplement. The remaining four groups were fed one of the supplements listed. Supplements were fed for 14 days before mating and throughout the mating period. Supplements were fed to the three subgroups at 0·17, 0·34 or 0·51 kg/ewe/day. Ovulation rate was determined in all ewes by laparoscopy at which the ovaries were examined and the number of CL counted. Throughout the experiment ewes grazed pastures where available dry matter was greater than 4000 kg/ha and crude protein ranged from 6 to 16% of the dry matter. As feed quantity was not limiting, no assessment can be made of the effect of diet selection on crude protein intake from pasutre.

Table 1. Mean ovulation rates of ewes within subgroups

Type	Supplement Crude protein (% dry matter)	Supplement feeding level* (kg/ewe/day)		
		0·17	0·34	0·51
Green lucerne	15	1·13 (41)†	1·22 (43)	1·15 (43)
Wheat	11	1·14 (43)	1·12 (44)	1·24 (43)
Wheat and 3 % urea	—	1·16 (43)	1·22 (43)	1·08 (43)
Lupin grain	28	1·23 (43)	1·25 (45)	1·47 (48)
LSD $P = 0·05$		0·16	0·16	0·16

* Mean ovulation rate of ewes in 3 subgroups not fed supplement was 1·13 (42).

† Figures in parentheses indicate liveweight (kg) 13 days after introduction of rams.

At the highest level of supplementation lupin grain resulted in a dramatic increase in ovulation rate (Table 1). The relatively low ovulation rate in the green lucerne subgroup supplemented at 0·51 kg/ewe/day suggests that the increase in protein intake supplied by the lucerne was not sufficient to increase ovulation rates markedly. The low mean ovulation rate of the wheat and wheat and urea subgroup fed 0·51 kg/ewe/day suggests that energy and energy and nitrogen respectively do not in themselves increase ovulation rate. The increase in ovulation rates in ewes fed the lupin grain above that in ewes fed the alternative diets suggests that either the lupins contain a compound/s of considerable physiological significance or the lupin grain supplies a highly digestible and balanced diet, this being reflected in liveweight and ovulation rate increases.

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Session 9

Chairman: Dr R. I. Cox

cAMP formation in the sheep ovarian follicle in response to gonadotrophins. T. J. Weiss, R. F. Seamark, R. M. Moor and J. E. A. McIntosh, *Department of Obstetrics and Gynaecology, University of Adelaide, Adelaide, S.A. 5000, Australia, and A.R.C. Unit of Reproductive Physiology and Biochemistry, University of Cambridge, Cambridge, CB3 0JQ, U.K.*

Changes in the sterol content of sheep ovarian follicles in culture: relationship to steroidogenesis and effects of gonadotrophins. R. F. Seamark, R. P. Hamilton and R. M. Moor, *Department of Obstetrics and Gynaecology, University of Adelaide, Adelaide, S.A. 5000, Australia.*

This study concerns an investigation of sterol metabolism in intact sheep ovarian follicles in culture following exposure to HCG. Ovarian follicles 4–6 mm in diameter were obtained from ewes on Days 4–14 of the oestrous cycle, and set up in tissue culture as described by Moor (1973). Theca and granulosa tissues were separated by microdissection. Lipids were analysed by gas-liquid chromatography as trimethyl silyl or methyl esters following initial isolation of the major lipid groups by thin-layer chromatography. Steroid hormones were assayed by radio ligand binding procedures (Seamark *et al.*, 1974).

Oestrogen secretion by untreated follicles remained at about 0.1 nmol mg⁻¹ per day throughout the period of culture: no progesterone was detected. Treatment with HCG (20 i.u. ml⁻¹) did not affect oestrogen secretion, but caused a dramatic rise in progesterone production to about 0.2 nmol mg⁻¹ per day by Day 3.

The sterol content of theca and granulosa cells separated from follicles cultured in the presence and absence of HCG is shown in Table 1. No effects of HCG were evident.

Table 1. Sterols in cultured sheep ovarian follicles (mean $\pm$ S.E.M., no. of observations in parentheses)

Days in culture	Cholesterol (nmol mg ⁻¹)		Cholesterol ester (nmol mg ⁻¹)	
	Theca	Granulosa	Theca	Granulosa
1	13.7 $\pm$ 1.0 (19)	3.0 $\pm$ 0.2 (16)	3.1 $\pm$ 0.4 (19)	3.1 $\pm$ 0.4 (19)
2	9.6 $\pm$ 2.0 (4)	4.6 $\pm$ 0.5 (4)	1.3 $\pm$ 0.3 (4)	2.3 $\pm$ 1.0 (4)
+ HCG (24 hr)	7.2 $\pm$ 0.5 (4)	4.1 $\pm$ 0.5 (4)	1.5 $\pm$ 0.3 (4)	1.8 $\pm$ 0.2 (4)
3	5.4 $\pm$ 0.5 (5)	6.4 $\pm$ 0.5 (5)	1.0 $\pm$ 0.3 (4)	1.8 $\pm$ 0.1 (5)
+ HCG (48 hr)	5.4 $\pm$ 1.0 (5)	4.9 $\pm$ 0.5 (5)	0.8 $\pm$ 0.1 (4)	1.6 $\pm$ 0.3 (5)

The fatty acid composition of the cholesterol ester fraction (relative molar ratios, C16=1) was C14, 0.42; C16, 1; C16:1, 0.6; C18:1, 1.89; C18:2, 0.14; C18:3, 0.77. No evidence of selective de-esterification with HCG treatment was found. A portion of the non-esterified cholesterol pool in the theca and, to a lesser extent in the granulosa, was shown to exchange with isotopically labelled cholesterol added to the medium. However, this exchange was not facilitated by the addition of HCG, and no evidence of cholesterol ester synthesis was found. It was concluded that HCG has little demonstrable effect on cholesterol and cholesterol ester metabolism in isolated ovarian follicles in culture.

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FSH and LH activities of PMSG fractions isolated from serum and from fetal trophoblast cells maintained in culture. J. E. A. McIntosh,* M. Kaethner, Francesca Stewart, W. R. Allen and R. M. Moor, *ARC Unit of Reproductive Physiology and Biochemistry, University of Cambridge, Cambridge CB3 0JQ, U.K.* (*Present address: *Department of Obstetrics and Gynaecology, University of Adelaide, Adelaide, S.A. 5000, Australia.*)

Equine gonadotrophin (PMSG) is a sialic acid-containing glycoprotein which exhibits FSH and LH activities. It is widely used in animal production and veterinary medicine for increasing ovulation rate and for the treatment of infertility. Unfortunately, the wide variation in response to injections of PMSG limits its usefulness.

As a first step in clarifying the relationship between the composition of PMSG and its biological

activity, with the aim of attempting to standardize and improve its gonadotrophic quality, we have purified it from the sera of individual pregnant mares by an improved method. This product was compared with PMSG which had not been released into the maternal circulation but was isolated from medium in which had been cultured specialized fetal trophoblast cells recovered from pregnant mares (Hamilton *et al.*, 1973). PMSG was determined using the haemagglutination-inhibition assay (Allen, 1969).

Isolation from serum was achieved either by the method of Gospodarowicz & Papkoff (1967) or, under milder conditions taking advantage of the low isoelectric point of PMSG, by dialysis against 50 mM-ammonium acetate, pH 4.5, followed by centrifugation. The supernatant or dialysed fetal culture medium was adsorbed onto a column of Sephadex A-25 (50 mM-ammonium acetate, pH 4.5) and PMSG was eluted with a gradient of NaCl (0–200 mM). After gel filtration (Sephadex G-100; 50 mM-ammonium acetate, pH 4.5) tubes containing activity were loaded onto a column of Sephadex SP-25 equilibrated with the same buffer. The pH was increased by gradient to 8.5 and then the ionic strength was varied to 300 mM in a gradient of ammonium acetate, pH 8.5.

Between one and four PMSG fractions were revealed by the two gradients, or by isoelectric focusing of material from the A-25 step. The isoelectric points of the major serum fractions were 2.8 and 3.2. The chief component produced in culture had an isoelectric point of 3.4. Multiple forms of PMSG have not previously been reported and would not have been detected by the isolation method used before.

Rat testis receptor preparations were used to determine the ratio of FSH-like to LH-like activity in each fraction in terms of ^{125}I -labelled hFSH and hLH (Leidenberger & Reichert, 1972; Reichert & Bhalla, 1974). The FSH/LH ratios of different fractions varied between 0.7 and 4, those from culture being low. Furthermore, in whole serum from individual mares this ratio remained relatively constant between 40 and 90 days of gestation.

The origin and biological significance of the different forms of PMSG have yet to be determined. In structural terms, they may reflect variations in sialic acid content or perhaps different combinations of the subunits reported by Gospodarowicz (1972) and Papkoff (1974).

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Session 10

Chairman: Dr P. E. Mattner

Role of progesterone in regulating the length of the oestrous cycle in sheep. A. R. Bray, J. F. Hecker and M. Wodzicka-Tomaszewska, *Department of Physiology, University of New England, Armidale, N.S.W. 2351, Australia.*

Ginther (1968) and Thwaites (1971) have shown that exogenous progesterone injected into ewes may affect the length of the oestrous cycle. We investigated this finding in more detail. In these experiments the progesterone, dissolved in peanut oil, was injected i.m. into ewes at the dosages and times shown. All treatment groups contained 6 ewes. Oestrus was detected using vasectomized or entire rams carrying marking harnesses.

In the first experiment the effect of time of progesterone treatment was studied. Progesterone was administered at 40 mg/day for a period of 3 days and the following results were obtained:—Treatments started on Day 0 (day of oestrus) produced a mean cycle length of 12.9 days; on Day 1, 14.0;

on Day 2, 15.2; on Day 3, 16.2; on Day 4, 17.3; on Day 5, 18.0; on Day 8, 17.3; and on Day 11, 18.3. The mean cycle length of a control group of ewes treated with peanut oil only was 17.4 days. Shortening of cycle length occurred in all groups treated before Day 4 with the mean interval from first injection to oestrus of 13 days.

The minimum dosage of progesterone required to cause shortening of the cycle was investigated in a second experiment, in which ewes were treated with various progesterone dosages from Day 0 for a period of 4 days with the following results:

Dosage (mg)	0	2.5	5	10	20	40
Mean inter-oestrus interval (days)	17.7	17.2	16.5	15.0	12.0	11.7

Luteolysis has been shown to start 4 days before oestrus (Deane *et al.*, 1966). On the basis of this finding and the data reported we postulate that the ability of the uterus to effect luteolysis is dependent on prior exposure to a finite amount of progesterone. In the untreated non-pregnant ewe this priming period extends from Day 4 to about Day 13.

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Effect of oestradiol-17 β , progesterone and prostaglandin F-2 α antiplasma on luteal function in the ewe.
 R. J. Fairclough, J. F. Smith, A. J. Peterson and L. McGowan, *Ruakura Agricultural Research Station, Hamilton, New Zealand.*

In an attempt to elucidate the role of progesterone, oestradiol-17 β and prostaglandin F-2 α (PGF-2 α) in the regulation of the oestrous cycle we passively immunized ewes with oestradiol, progesterone and PGF-2 α antiplasma during the late luteal phase of the cycle. Twenty Romney ewes were treated as follows.

- Group 1. Oestradiol antiplasma given on Days 9-17 of the cycle (5 ewes).
- Group 2. Progesterone antiplasma given on Days 9-12 of the cycle (2 ewes).
- Group 3. Progesterone and oestradiol antiplasma given on Days 9-17 of the cycle (2 ewes).
- Group 4. Progesterone and PGF-2 α antiplasma given on Days 9-12 of the cycle (1 ewe).
- Group 5. PGF-2 α antiplasma given on Days 12-14 of the cycle (1 ewe).
- Group 6. Ovariectomized ewe plasma given on Days 9-17 of the cycle (6 ewes).
- Group 7. Three untreated control ewes.

The ewes were run with a harnessed vasectomized ram to detect behavioural oestrus. Blood was collected from the ewes throughout the trial and the total levels of oestradiol, progesterone and PGF-2 α were determined by radioimmunoassay. The free and bound fractions were estimated by equilibrium dialysis at 37°C. Twelve days after the start of treatment the ewes were subjected to laparotomy and the ovaries examined. The hormone levels during and following treatment and luteal function at laparotomy were as follows.

Group 1. (i) Free oestradiol levels were reduced by around 95%. (ii) The ewes failed to exhibit oestrus and did not ovulate. (iii) Changes in progesterone concentrations indicated that luteolysis occurred at the expected time. (iv) Increased follicular activity was noted at laparotomy.

Group 2. (i) Total progesterone concentrations rose for 4 days, then decreased sharply. (ii) Free progesterone concentrations were reduced by 50-75%. (iii) The ewes ovulated, exhibited oestrus and the CL regressed at the normal time.

Group 3. Results were similar to those found for Group 1. However, one ewe exhibited oestrus on Day 16 of the cycle but failed to ovulate.

Groups 4 and 5. Plasma progesterone levels did not fall at the time of expected luteolysis, indicating that the life of the CL was prolonged. However, the ewe receiving anti PGF-2 α and anti-progesterone showed a rise in total progesterone levels as found for the Group 2 ewes.

Groups 6 and 7. (i) These ewes exhibited oestrus, ovulated and the CL regressed at the normal time.

(ii) Hormone levels were similar to those previously reported (Thorburn *et al.*, 1969; Obst *et al.*, 1971).

We conclude that: (1) substantial reductions in free oestradiol concentrations from Days 9–17 of the cycle block ovulation and behavioural oestrus but do not appear to prevent luteolysis; (2) reduced levels of free plasma progesterone concentrations over the last half of the oestrous cycle do not appear to affect luteolysis or the length of the cycle; (3) passive immunization against PGF-2 α blocked CL regression, thus providing direct evidence that PGF-2 α is the ovine luteolytic factor.

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Effects of immunizing sheep to steroid oestrogens and phyto-oestrogens. R. I. Cox and Patricia A. Wilson, *C.S.I.R.O., Division of Animal Physiology, Prospect, P.O. Box 239, N.S.W. 2148, Australia.*

Immunophysiological studies are being carried out on the effects of active immunization using antigenic oestrogen–protein conjugates, particularly in relation to animal responses to endogenous or ingested oestrogens. Immunization to steroid oestrogens can alter hypothalamus–hypophysis–ovary interactions; whether immunization of sheep to phyto-oestrogens may protect against such compounds when ingested, and hence control clover infertility, is under investigation.

Merino ewes over 2 years of age were used as experimental animals. Steroid- and phyto-oestrogen derivatives were conjugated to bovine serum albumin. These antigens in solution or suspension were emulsified with Freund's complete adjuvant, followed by re-emulsification in 1% Tween 80. Such antigen preparations were injected about monthly to develop and maintain antibody titres using 1 mg antigen/sheep over two i.m. and 6–8 s.c. sites.

Immunization with oestradiol-17 β -6-CMO-BSA (number of sheep treated, N=18) and with oestrone-6-CMO-BSA (N=12) resulted in an absence of oestrous behaviour. After immunization with oestradiol-17 β -bis-diazobenzidine-protein, very low titres of antibodies were obtained and some behavioural oestrus was recorded (N=12). In contrast, animals immunized with antigens containing phyto-oestrogenic haptens showed a normal incidence of oestrus. The main groups studied were those with high titres of antibodies selective for genistein (N=24) and equol (N=13). These differences between ewes immunized to steroid or phyto-oestrogens indicate that phyto-oestrogen selective antibodies do not cross react *in vivo* with steroids sufficiently to disturb the appearance of regular oestrus. Specificity tests *in vitro* on antisera showed that genistein or equol selective antisera had negligible cross-reactions of below 0.01% with oestradiol-17 β or oestrone. Ewes immunized to genistein in mid-1972, when compared with control ewes, showed normal oestrus, mating and lambing in the 1973 and 1974 seasons.

Recent work has indicated the effectiveness of DEAE-dextran as an adjuvant for immunization (Wittmann, 1970). This compound was compared with Freund's complete adjuvant using 6 ewes in each group and oestrone-6-CMO-BSA as antigen. Good titres were obtained where DEAE-dextran was used, with a rapid build-up of antibody titres and a more even response between animals. Titres were similar in both groups; they tended to decline more rapidly with DEAE-dextran adjuvant if injections of antigen were not continued monthly.

The data obtained show that an absence of oestrous behaviour results where ewes are immunized with antigens containing oestrone or oestradiol derivatives as haptenic groups, but not with phyto-oestrogen derivatives as haptens. The specificity of the phyto-oestrogen reactive antisera is sufficiently great that oestrous behaviour is not disturbed.

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Predicting the number of corpora lutea in Merino ewes. D. G. Fowler, *N.S.W. Department of Agriculture, P.O. Box 239, Blacktown, N.S.W. 2148, Australia.*

An attempt has been made to predict the number of CL from the mating activity of two different groups of Merino ewes. One group (high) consisted of the heavier, 47.7 ± 0.35 kg, and genetically more fertile and fecund ewes and the other (low) consisted of the lighter, 38.9 ± 0.23 kg, and genetically less fertile and fecund ewes. The prediction equation used was: no. of CL = $19.64 (T/N) - 243.9$; $r = 0.81$. The theoretical basis leading to the derivation of this equation has been described (Fowler, 1975).

Each group of 153 ewes was run with 3 rams for 34 days in autumn 1975. All sheep were individually identified and rams were interchanged between groups regularly. During the first 17 days of mating, each group was observed on six separate occasions for 9 hr each time. The ram and ewe involved in each mount and service was identified and the time was recorded. The ewes were always in view of at least two observers who were located in a mobile observation tower from which a clear and uninterrupted view of the flock could be maintained.

The following measurements were obtained: (1) T = the sum of times from first to last observed mount for each ewe; (2) N = the total number of mounts observed during 9 hr.

The ewes were slaughtered at a local abattoir soon after rams were removed and the total number of CL were counted. For the high group, T/N, was 24.80 ± 2.09 and for the low group, 23.07 ± 2.11 . In the high group, 145 ewes were slaughtered; 230 CL were expected and 226 were found. In the low group, 149 ewes were slaughtered; 204 CL were expected and 199 were found.

The ratio T/N has been suggested to be directly related to fertility and fecundity in Merinos (Fowler, 1975).

Ovulation rate in Merinos is influenced by genotype, age and liveweight (Braden & Baker, 1973). The groups which provided the prediction equation and those used in the present study were consciously selected to differ in these aspects. Accurate prediction of the number of CL is possible only if the total effect of all interacting influences that can operate in any flock, can be described objectively and the ratio T/N, offers this possibility. However, the work is at a very early stage and more successful predictions are necessary before acceptance could be considered.

The new prediction equation is: no. of CL = $256.9 - 2.64 (T/N)$.

This equation describes the relationship as it has been found for 5 different flocks in which genotype, age, liveweight and year of mating vary.

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Effects of colchicine on the structure and function of the corpus luteum in the sheep. R. T. Gemmell and B. D. Stacy, *C.S.I.R.O., Division of Animal Physiology, Prospect, N.S.W. 2149, Australia.*

A previous ultrastructural study has drawn attention to the presence of secretory granules in the CL (Gemmell *et al.*, 1974); the formation and secretion of granules were related to the known pattern of progesterone secretion in the cyclic ewe. To gain further information on the role of the granules in the mechanism of progesterone secretion, the effects of colchicine on the CL have been investigated. Insulin is secreted in granule form and colchicine inhibits this process by disrupting the microtubules that transfer granules from the interior to the periphery of the pancreatic β cell (Malaisse, 1973). Microtubules are also present in the luteal cell and it was of interest to know whether secretory granules and hormone secretion in the two different types of endocrine cell would exhibit comparable responses to colchicine.

Ewes close to Day 10 of the cycle were given an i.v. injection (1 mg/kg) of colchicine; at intervals up to 4 hr after treatment animals were anaesthetized and the ovaries fixed by vascular perfusion with glutaraldehyde. Samples of luteal tissue were prepared for examination in the electron microscope; blood samples for progesterone determination were taken from the jugular vein.

The morphology of the luteal cells had changed 45 min after the injection, since the number of densely staining granules in the cytoplasm and in the intercellular space had decreased. By 2 hr there was no sign of the granules being secreted nor were they readily detectable within the cell. With the disappearance of the granules, densely staining vesicles began to appear near the Golgi complexes, and these abnormal organelles became more prominent 4 hr after injection of colchicine. Absence of microtubules and proliferation of agranular endoplasmic reticulum were also features of the response to the drug. The concentration of progesterone in peripheral plasma fell to 86% of control values at 1 hr, 62% at 2 hr, 51% at 3 hr and 48% at 4 hr after treatment.

These results indicate that colchicine disrupts the microtubular system in the luteal cell and thereby inhibits the intracellular transport of granules. The decline in progesterone values accompanying the interference to granule transfer and secretion provides further evidence that progesterone is secreted by the luteal cell in granule form (Gemmell *et al.*, 1974).

The increase in agranular endoplasmic reticulum indicated that the cells were still able to synthesize steroids after colchicine treatment. Evidence of this was also seen in tissue levels of progesterone; 4 hr after colchicine luteal tissue contained twice the normal amount of progesterone. As in the pancreas, the action of colchicine appeared to be directed towards hormone secretion and not biosynthesis.

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Morphological and functional changes in the corpus luteum after restriction of ovarian blood flow.
 B. D. Stacy and R. T. Gemmell, *C.S.I.R.O., Division of Animal Physiology, Prospect, N.S.W.* 2149, Australia.

Pharriss & Wyngarden (1969) originally suggested that the demise of the CL is brought about by the veno-constrictive action of prostaglandin (PG) F-2 α on the ovarian vasculature. Subsequently little supporting evidence has emerged and some results have tended to discount the suggestion (Labhsetwar, 1974). Certain experiments in sheep have, however, given a definite indication of selective reduction in blood flow to the CL following administration of PGF-2 α via a uterine vein (Thorburn & Hales, 1972). The morphological effects of PGF-2 α on the ovine CL have been reported (Stacy *et al.*, 1975); speculation arose that it might be possible to induce these regressive changes artificially by reducing the flow of blood to the CL.

To test this possibility a cuff was placed surgically round the ovarian artery of ewes near the mid-point of the luteal phase of the cycle. The cuff was adjusted so that blood flow to the CL was visibly reduced as judged by blanching of the superficial tissue. The animals were allowed to recover and 48 hr later were re-anaesthetized and the ovaries fixed by vascular perfusion with glutaraldehyde. Luteal tissue was taken for electron microscopic examination; blood samples for the determination of progesterone were taken from the jugular vein.

In several animals the treatment resulted in a marked fall in plasma progesterone concentration and this was accompanied by morphological changes in the CL. The luteal cells bore unmistakable signs of degeneration typical of the changes seen in normal or in PGF-2 α -induced regression (Stacy *et al.*, 1976). Granule secretion had decreased, autophagocytic bodies were present and lipid droplets were profusely distributed throughout the cytoplasm.

These experiments were crude because there was no quantitative assessment of the impairment to blood flow following partial occlusion of the ovarian artery. Nevertheless the results are consistent with the concept that the luteolytic action of PGF-2 α is mediated by its effects on the ovarian vasculature.

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Session 11

Chairman: Dr G. M. Stone

Plasma progesterone levels in non-pregnant sows showing delayed return to oestrus following mating.
D. P. Hennessy and P. Williamson, *Attwood, Veterinary Research Laboratory, Department of Agriculture, Westmeadows, Victoria 3047, Australia.*

An analysis of breeding records from several commercial piggeries revealed that more than 10% of breeding sows and gilts showed a delayed return to oestrus (>42 days) following an unsuccessful mating. A method of early pregnancy diagnosis was needed to identify those sows affected. Vaginal biopsies, taken 20–22 days after mating are inaccurate (Williamson & Hennessy, 1975); the vaginal epithelium of non-pregnant sows was similar to that of pregnant sows, indicating that at the time biopsies were taken the sows were either pregnant and later suffered embryonic or fetal death or that an endocrine imbalance due to cystic or quiescent ovaries was present.

The present study was undertaken to assess the accuracy of plasma progesterone concentrations 18 days after mating as a method of pregnancy diagnosis in commercial piggeries in which delayed return to oestrus occurs.

Sows and gilts were bled from a marginal ear vein 18 days after mating. Plasma progesterone was assayed using a modification of the method described by Cain *et al.* (1972). Free [³H]progesterone was separated from that bound to binding protein obtained from guinea-pig plasma, by incubating with 1 ml of 10 mg charcoal (Norit A)/100 ml for 15 min at 4°C and then centrifuging for 10 min at 2000 g.

Sows with plasma progesterone concentrations lower than 5 ng/ml were classified non-pregnant. Farrowing records for all sows sampled were used to assess the accuracy of pregnancy diagnosis.

Table 1. Accuracy of pregnancy diagnosis based on plasma progesterone concentration

	No. of sows tested	No. correctly diagnosed	% correct
Pregnant sows	66	66	100
Non-pregnant sows	19	10	53
Delayed return to oestrus (> 42 days)	15	7	47
Normal return to oestrus (18–24 days)	4	3	75
Overall	85	76	89

Approximately half of the non-pregnant sows showing a delayed return to oestrus were classified as being pregnant on the basis of their plasma progesterone concentrations. These results indicate that, as in the vaginal biopsy trial, these sows suffered embryonic or fetal death after sampling, or an endocrine imbalance after mating.

Oestrogen concentrations are presently being measured in the plasma samples to establish if an hormonal imbalance is involved in the delayed return to oestrus.

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Steroid and prostaglandin F concentrations in human corpora lutea in relation to luteolysis. D. A. Shutt, R. P. Shearman and R. C. Lyneham, *Department of Obstetrics and Gynaecology, University of Sydney, Sydney, N.S.W. 2006, Australia.*

A uterine luteolysin, prostaglandin F-2 α , is secreted from the uterus in some mammalian species and is responsible for the regression of the CL in those species. This mechanism has not been demonstrated in women (Lyneham *et al.*, 1975). Prostaglandin F-2 α or oestradiol-17 β may be luteolytic in women when injected directly into the CL or into the ovary containing the CL (Knobil, 1973; Korda *et al.*, 1975). To provide further information on the mechanism of luteolysis in man, changes in concentration of progesterone, 17-hydroxyprogesterone, oestradiol-17 β and prostaglandin F within the CL during the luteal phase of the menstrual cycle and in early pregnancy have now been examined.

Corpora lutea were freshly obtained from women admitted to the hospital for elective gynaecological surgery. The CL were snap-frozen in liquid air and stored at -10°C until assayed. After extraction and chromatography of the steroids and prostaglandin F in the tissue, the compounds were assayed by radioimmunoassay (Shutt *et al.*, 1975).

Steroid concentrations (mean $\pm$ S.E.) of progesterone, 17-hydroxyprogesterone and oestradiol-17 β in CL ($\mu\text{g/g}$ tissue) from 14 non-pregnant women were 8.5 ± 1.8 , 0.41 ± 0.07 , and 0.09 ± 0.01 respectively, and in CL from 7 pregnant women were 9.9 ± 1.1 , 0.82 ± 0.16 and 0.13 ± 0.04 , respectively. Prostaglandin F concentrations (mean $\pm$ S.E.) were 14 ± 4 ng/g in the non-pregnant patients and 3 ± 1 ng/g in the pregnant patients. Mid-luteal levels of the three steroids in luteal tissue were comparable to those found in CL from women in early pregnancy. In contrast the highest prostaglandin F levels in the range 13–46 ng/g tissue occurred in 6 CL from non-pregnant women, and significantly four of these were on Days 23–25 of the menstrual cycle—a time when luteolysis would be commencing.

The results indicate that relatively higher prostaglandin F levels are associated with some CL from non-pregnant women in comparison with the lower levels found in CL from women in early pregnancy. The relationship of the observed changes in steroid and prostaglandin F concentrations in the CL to the mechanism of luteolysis in women has still to be determined.

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Induction of parturition in sheep with a single dose of oestradiol benzoate. L. P. Cahill, B. W. Knee and R. A. S. Lawson, *Department of Agriculture, S. S. Cameron Laboratory, Werribee, Victoria 3030, Australia.*

The rise in the level of oestrogen in maternal ewe plasma is well documented (Challis, 1971; Obst & Seamark, 1972). This study investigated whether a single dose of oestradiol benzoate (OB) administered to ewes during the last week of gestation could induce parturition.

One hundred and eighty-eight Merino ewes which had been mated and had not subsequently returned to oestrus were separated into weekly mating groups. Within groups the ewes were allocated at random to either a treatment (84 ewes) or a control (104 ewes) subgroup. The treated ewes received a single i.m. injection of 20 μg OB in 2 ml peanut oil, when the ewes in the group were at 142–148 days of gestation. The control ewes were allowed to lamb normally under the same conditions as the treated ewes.

By 48 hr and 96 hr after treatment 65% and 84%, respectively, of the treated group had lambed whilst only 12.5% and 34.6% of the control group had lambed by this time. The incidence of dystocia and the birth-weight, survival and growth rate of lambs born was similar in the control group and in the treated ewes which lambed within 96 hr or OB treatment. However, the 16% of treated ewes which did not lamb until 5–8 days after treatment showed a significantly higher incidence of dystocia ($P<0.05$) than the control ewes or remaining treated ewes, and significantly more of the resultant lambs 16/18) were still-born or died soon after birth.

A single dose of exogenous OB administered after 142 days of gestation can cause parturition to be initiated within 96 hr in the majority of ewes. The malfunction at parturition in 16% of the treated ewes suggests that in these ewes a premature or insufficient increase in the level of circulating oestrogen may disrupt the normal physiology of parturition. The high incidence of lamb losses in ewes which did not respond to treatment would not permit the use of OB to control parturition in commercial flocks at this stage.

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Comparison of dexamethasone trimethylacetate and prostaglandin F-2 α as abortifacients in the cow.
C. A. Sloan, *Department of Agriculture, S. S. Cameron Laboratory, Werribee, Victoria 3030, Australia.*

A reliable technique for causing abortion in cows in the middle and last thirds of pregnancy was required to obtain non-pregnant cows for training technicians for artificial insemination procedures. The technique also has application in cases of mismating. Fifty-one cows were used in experiments designed to assess the abortifacient potential and safety of several drugs. Dexamethasone trimethylacetate (Opticortenol: Ciba Geigy) was used alone by single or repeated i.m. injections, or in association with stilboestrol dipropionate (Stilboestrol: May and Baker) or oxytocin (Oxytocin: Intervet) (Table 1). Abortion occurred in cows given dexamethasone at a 6-day interval, but the treatment-abortion interval was longer than with a 4-day treatment interval or the i.m. injection of 25 mg over multiple sites (5 $\times$ 5 mg). By comparison the stilboestrol dipropionate and oxytocin produced a considerable delaying effect on dexamethasone-induced abortion (Table 1). Studies with PGF-2 α (PGF-2 α in ethanol: Fuji) are reported for a further 10 cows, where dosages administered directly into the fetal fluids caused rapid expulsion of the fetus with no complications to the cow (Table 1). The jugular plasma progesterone levels from pretreatment to abortion were monitored. In general, the mean progesterone concentrations in venous blood had decreased within 2 days of dexamethasone administration and showed less variation in cows treated at 4- rather than 6-day intervals.

Table 1. Abortifacient activity of dexamethasone trimethylacetate and PGF-2 α in the cow

Treatment					Treatment to abortion interval (days)			
Initial		Subsequent			No. of cows		Mean	Range
Compound	Dose (mg)	Compound	Dose	Days*	Treated	Aborted		
Dexamethasone	25	—	—	—	5	4 (1)†	12	11-13
Dexamethasone	25	Dexamethasone	25 mg	4	16	12‡	12.1	9-23
Dexamethasone	25	Dexamethasone	25 mg	6	11	10 (1)†	15.6	8-22
Dexamethasone	25	Stilboestrol	250-500 mg	6	5	5	23.2	13-39
Dexamethasone	25	Oxytocin	100 i.u.	6	5	5	21.4	16-30
PGF-2 α	30	—	—	—	3	3	1.3	0.5-2
PGF-2 α	20	—	—	—	5	2	2.5	1-4
PGF-2 α	15	—	—	—	1	1	2	

* Days between initial and subsequent treatments.

† Figures in parentheses represent number of cows with a retained dead fetus.

‡ Fetal membranes retained in two cows.

Dexamethasone was found to be a safe and efficient abortifacient in the middle and last thirds of pregnancy, provided the time taken to induce abortion is not critical. The initial results with PGF-2 α administered to the fetal fluids suggest this procedure may be more satisfactory if rapid abortion or removal of fetal debris is required.

Hormonal changes in cow and calf about normal and induced calving. J. P. Drinan, M. S. F. Wong and R. I. Cox, *C.S.I.R.O. Division of Animal Physiology, Prospect, P.O. Box 239, N.S.W. 2148, and School of Biological Sciences, Macquarie University, North Ryde, N.S.W. 2113, Australia.*

The use of synthetic corticosteroids to induce calving suggested the desirability of comparing the hormonal events about normal and induced calving. It was also considered that this induced parturition might provide a further insight into the sequence of hormonal changes preceding birth.

The hormonal changes around parturition were studied in 11 cows and 3 of their calves. With two of these cows, Hereford-Friesian crosses, it was possible to contrast the data from consecutive calvings, normal in 1972 and induced in 1973. Flumethasone (10 mg) was given i.m. on Days 267 and 272 of gestation; calving occurred 58 and 45 hr later. Jugular blood samples were taken from the cows with increasing frequency as parturition approached. During the 24 hr after birth, frequent concurrent samples were taken from cows and calves. Competitive protein binding assays were used to measure oestrone, oestradiol, progestins and corticosteroids. Oestrogen sulphates were measured as free oestrogens after solvolysis.

Many hormonal changes observed at normal parturition confirmed and extended earlier observations. Thus progestins declined over the last 30 days of gestation, over the last 15 days oestrogens rose and an oestrone peak was observed in the last 10 days and before the peak at parturition. Of particular interest was the observation that oestrone sulphate reached a maximum concentration of about 10 ng/ml some 10 days before the unconjugated oestrone peak. Post-partum progestin concentrations remained close to baseline until after onset of oestrus for 3 cows studied.

The sequence of events following flumethasone administration included an immediate drop in corticosteroids in cow plasma and a decline in progestins, 14 and 12 hr later. The other endocrine events preceding induced parturitions were similar to normal: oestrone and oestradiol rose to peaks immediately about birth and progestins remained at low levels over at least 30 hr before birth. The oestrone peak occurring 2-5 days before the normal birth was absent.

After induced birth, corticosteroids remained low in cow but as high as normal in calf plasma, remaining high for at least as long a time. Maternal oestrone concentrations (400 and 1600 pg/ml) and oestradiol (40 and 200 pg/ml), oestrone sulphate (14 and 42 ng/ml) and oestradiol sulphate (0.5 and 1.0 ng/ml) were initially similar to those for cows with normal births. The clearance of these oestrogens was, however, slower in the cows with induced parturition. Instead of falling to baseline within 48 hr of calving, oestrone remained about 50 pg/ml until placental expulsion about 100 and 115 hr later.

Immediately after the induced births, calf oestrone (30 and 80 pg/ml) and oestradiol (40 and 60 pg/ml) were at similar concentrations to those observed with normal birth, but the sulphates were at higher concentrations (oestrone sulphate: normal, 31 and 5, induced; 50 and 26 ng/ml; oestradiol sulphate: normal, 14 and 6; induced, 45 and 22 ng/ml). The clearance of these oestrogens was slower in the induced calves.

Apart from the expected suppression of maternal corticosteroid concentrations, hormonal patterns before parturition were similar for normal and induced parturition; however, post-partum differences were evident.

Effects of medroxyprogesterone acetate and oestradiol-17 β on pregnancy maintenance in ovariectomized rats. N. W. Bruce and R. K. Bartholomeusz, *Department of Anatomy and Human Biology, University of Western Australia, Nedlands, W.A. 6009, Australia.*

Rabbits, ovariectomized early in pregnancy and treated only with medroxyprogesterone acetate (MPA) produce heavier fetuses and placentae than normal (Bruce, 1973). However, in preliminary trials with rats, ovariectomy with MPA supplementation alone failed to maintain pregnancy. These trials were extended to provide a firmer basis for comparison.

Eighteen rats were injected i.m. with 1 mg medroxyprogesterone acetate (Depo-Provera: Upjohn) on Day 11 and were ovariectomized on Day 12 of gestation (Day 1 is the day that spermatozoa are first found in a vaginal smear). In addition, 8 of the 18 rats were injected daily with 0.5 or 1.0 μ g

oestradiol-17 β starting on Day 12. On Day 22, all rats were killed and their uterine contents were examined and compared with the number of implantation sites at the time of ovariectomy.

Of the 10 ovariectomized rats treated with MPA alone, only 2 live fetuses were found at term; one in each of 2 rats. Both fetuses appeared to be malformed due to high intrauterine pressures. About 30% of the original implantation sites were still represented by almost normal sized placentae at Day 22. However, these placentae had a characteristic conical rather than hemispherical shape.

Seven of the 8 ovariectomized rats injected with MPA and oestradiol-17 β had live fetuses at Day 22. The mean number of fetuses surviving was 5.2 out of 12.6 implantation sites. Fetal and placental weights were slightly lower than in control rats. However, no other abnormalities were evident.

Pregnancy can be maintained in ovariectomized rabbits treated only with MPA. However, ovariectomized rats appear to require oestrogen treatment as well.

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Session 12

Chairman: Dr R. F. Seamark

Uptake and incorporation of [U-¹⁴C]glucose during culture of mouse blastocysts previously undergoing normal implantation and induced delay of implantation. I. L. Pike and R. G. Wales*, *Department of Veterinary Physiology, University of Sydney, N.S.W. 2006, and *School of Veterinary Studies, Murdoch University, W.A. 6153, Australia.*

Implantation may be delayed in the mouse during lactation or following ovariectomy early in pregnancy. Few experiments have examined the metabolism of energy substrates by the blastocyst at this time and, in the present studies, the uptake and utilization of glucose by mouse blastocysts at implantation and during the delay of implantation resulting from ovariectomy or administration of antiserum raised against LH (anti-LH) have been investigated.

Embryos were obtained from random-bred mice on (i) Day 5 of pregnancy where Day 1=the day of finding a vaginal plug ('implanting' blastocysts); (ii) Day 8 of pregnancy following ovariectomy on Day 3 ('ovariectomy delayed' blastocysts); and (iii) Day 8 of pregnancy following s.c. injection of 0.2 ml rabbit anti-LH serum at 09.00 hours on either Day 2 or Day 3 ('anti-LH delayed' blastocysts). The embryos were washed and cultured in medium containing 5.56 mM-[U-¹⁴C]glucose (sp. act. 3 μ Ci/ μ mol) as sole energy substrate for either 0.5 or 5 hr. Following culture, the embryos were recovered, washed free from exogenous radioactivity and the total uptake of glucose and incorporation of glucose into various biochemical components were estimated using standard techniques.

During incubation for 0.5 hr in radioactive glucose, no significant difference was found in the uptake of label by 'implanting' and 'ovariectomy delayed' blastocysts, but the uptake by 'anti-LH delayed' blastocysts was less than 50% of that of the other blastocysts. In a further experiment, the short-term (0.5 hr) uptake of glucose by 'implanting' blastocysts decreased rapidly during culture in non-radioactive glucose medium but the uptake by 'anti-LH delayed' embryos remained unaltered.

The level of label accumulated by 'ovariectomy delayed' blastocysts during culture for 5 hr was markedly reduced when compared with 'implanting' blastocysts. More than 70% of the glucose carbon incorporated by these blastocysts over this time was extracted in the acid-soluble fraction. Using the 'implanting' blastocysts as a control, less label was found in both the non-glycogen and glycogen portions of the acid-soluble fraction of the delayed blastocysts. The amount of label in the protein fraction was also reduced during the delay. Little difference was observed in the incorporation of label into the remaining fraction (lipid and nucleic acids) of the blastocysts.

Previous studies have suggested that the mouse embryo enters a state of relative metabolic

quiescence during the delay of implantation. The results of the present experiments are in agreement with this hypothesis. Furthermore, the administration of anti-LH during early pregnancy inhibits the uptake of glucose by the mouse embryo, perhaps due to hindrance to glucose entry by the binding of the antibody to LH-like antigenic sites around the surface of the blastocyst, as uptake by these embryos was low from the time of embryo collection.

Proteins of the uterine secretion in sheep. L. D. Staples, I. A. Cumming, R. A. S. Lawson and F. J. Morgan, *St. Vincent's School of Medical Research, Fitzroy, Victoria 3065, and Department of Agriculture, S. S. Cameron Laboratory, Werribee, Victoria 3030, Australia.*

The uterine environment is considered to be an important factor affecting blastocyst development before implantation. In the rabbit a uterine-specific protein (blastokinin or uteroglobin) which can account for up to half of the secreted protein has been shown to influence blastocyst development *in vitro* (Gulyas *et al.*, 1969; El-Banna & Daniel, 1972). Secretion of this protein is influenced by progesterone. For embryo survival in the ewe synchronous development of the blastocyst with the uterine environment is required (Moore & Shelton, 1964). This relationship is influenced by the level and duration of progesterone influence (R. A. S. Lawson, unpublished). Together, these findings suggest that specific uterine secretions may influence blastocyst development in the ewe.

Uterine protein secretions were examined throughout the oestrous cycle in Border Leicester× Merino ewes, and in spayed ewes injected with 7 or 15 mg progesterone/day for periods of 0 to 16 days. Uterine protein samples were obtained by flushing the lumen of the uterus with 5 ml 0.15 M-NaCl. After dialysis of flushings against water the secretions were examined by total protein analysis (ninhydrin method), polyacrylamide gel electrophoresis (7% gels, tris-glycine buffer, pH 8.28) and for ability to specifically bind progesterone (competitive-binding assay). Total protein recoveries ranged between 2–9 mg, with no differences between early or late phases of the oestrous cycle or between entire ewes and progesterone-treated spayed animals.

The pattern of protein secretion separated as described was dominated by albumin with fewer bands in the macroglobulin area than serum. In some animals an additional protein band, not seen in serum, was observed in the post-albumin region from Days 10 to 16 of the oestrous cycle. At no time was this a major component of the secretion. This fraction was not apparent in flushings from spayed or progesterone-treated spayed ewes, and although the constancy of its appearance remains to be confirmed, it has similar electrophoretic mobility to a uterine specific protein recently reported to appear after Day 14 in the sheep (Menezo, 1973). No significant differences in progesterone binding by uterine secretions could be observed between the groups of animals.

We have observed no dominant uterine secretory protein analogous to rabbit blastokinin in sheep under the conditions described above, and it seems that any cyclic changes in uterine protein secretion may be more subtle in this species.

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An immunological test for pregnancy in the ewe. R. A. S. Lawson, Mildred Cerini and J. K. Findlay, *Department of Agriculture, Victoria and Reproduction Research Section, University of Melbourne, S. S. Cameron Laboratory, Werribee, Victoria 3030, Australia.*

Antigen(s) specific to pregnancy have been detected by immunofluorescence in the embryo, uterus, CL and maternal circulation of pregnant ewes from as early as Day 6 after mating (Cerini *et al.*, 1976). Apart from any active role played by these antigens in the establishment of pregnancy, their presence offers the opportunity to develop immunological tests for pregnancy in sheep and other domestic animals.

Simple haemagglutination, using one drop of a 1/16 dilution of rabbit anti-sheep embryo serum added to a 5% (v/v) dilution of whole blood, was used to detect antigen adsorbed onto maternal erythrocytes. The diagnoses, confirmed by slaughter, on 330 ewes which had been mated up to 55 days before testing, and on an unmated control flock tested at monthly intervals for 3 months are shown in Table 1. The accuracy of pregnancy diagnosis relative to the stage of gestation at testing in ewes subsequently confirmed pregnant is shown in Table 2.

Table 1. The accuracy of pregnancy diagnosis between Days 6 and 55 by haemagglutination in mated and unmated ewes

Flock	No. of ewes	Correct		False		% Correct
		Positive	Negative	Positive	Negative	
Mated	330	125	87	63	55	64
Unmated	99	—	91	8	—	92
control	98	—	79	19	—	81
	95	—	95	0	—	100

Table 2. The accuracy of pregnancy diagnosis relative to stage of gestation in ewes

Stage of gestation (days)	6-12	13-20	21-30	31-40	41-50
No. of pregnant ewes	51	67	18	47	25
Correct diagnoses (%)	61	72	78	85	44

Fifty-five percent of false positive tests were attributed to ewes in which embryo death and resorption was considered to have occurred. The remaining 45% of false positive tests may be accounted for by operator error in embryo recovery from slaughter material and reading the immunological test. The incidence of false positive tests due to non-specific haemagglutination was low in the unmated control flock. The high incidence of false negatives which increased in the 41-50 day period in the mated flock suggested that the presence of excess unbound antigen in the plasma may have interfered with haemagglutination. This was confirmed using haemagglutination-inhibition and passive haemagglutination tests. It was concluded that immunological methods could be used to diagnose pregnancy from an early stage in ewes. The inability of the simple haemagglutination test to distinguish between high and low titres of antigen indicated the necessity of using an alternative haemagglutination procedure to improve the accuracy of diagnosis.

Reference

- CERINI, M., FINDLAY, J.K. & LAWSON, R.A.S. (1976)
J. Reprod. Fert. **46**, 65-69.

Distribution of prostaglandin F, progesterone and pregnancy-specific antigen in unilaterally pregnant ewes. J. C. Cerini, Mildred Cerini, J. K. Findlay and R. A. S. Lawson, *Reproduction Research Section, University of Melbourne, and Department of Agriculture, Victoria, S. S. Cameron Laboratory, Werribee, Victoria 3030, Australia.*

Pregnancy-specific antigen(s) has been shown by immunofluorescent protein tracing to be present from Day 8 in the uteri, CL, embryonic membranes and circulation of pregnant ewes (Cerini *et al.*, 1976). The antigen(s) was not detected in non-pregnant ewes or in other tissues of pregnant ewes.

The distribution of this antigen(s) relative to progesterone and prostaglandin F in pregnant and non-pregnant ewes has been investigated. Unilaterally pregnant ewes (18) were prepared by surgically dividing the uterine horns of ewes with bilateral ovulations and transferring a blastocyst into one horn. At laparotomy on Days 14 or 17, jugular, uterine and ovarian venous blood samples were taken. The uteri were flushed with saline to recover embryos which had developed. Tissue samples from each uterine horn and both ovaries were fixed in phosphate-buffered 10% formalin for subsequent immunofluorescence studies (Cerini *et al.*, 1976).

Immunofluorescence studies revealed that in the non-pregnant uterine horns PGF was distributed on the surface of the columnar epithelium, in cells of the endometrium and in the walls of blood vessels in the myometrium. By contrast, in pregnant uterine horns PGF was detected only in the lamina propria and myometrium. PGF was also localized in the walls of blood vessels in CL of both ovaries. In both non-pregnant and pregnant uterine horns, progesterone was distributed throughout the endometrium and myometrium. Progesterone was present in about 90% of luteal cells in CL ipsilateral to the pregnant uterine horn but only in about 20% of cells in regressing contralateral CL. The pregnancy-specific antigen(s) was present in the lumina of pregnant uterine horns, in the myometrium and blood vessels of both the pregnant and non-pregnant horns, in the ipsi- and contralateral CL and in the peripheral circulation. Using a haemagglutination-inhibition assay, the concentrations of antigen in peripheral and venous blood from both uterine horns was found to be identical. This study shows (a) a different distribution of PGF in the tissues of pregnant and non-pregnant uterine horns; (b) the intrauterine distribution of progesterone was not influenced by pregnancy, whereas there was less in the regressing CL; and (c) pregnancy-specific antigen(s) was found in regressing and maintained CL.

Reference

- CERINI, M., FINDLAY, J.K. & LAWSON, R.S.A. (1976)
J. Reprod. Fert. **46**, 65-69.

Preliminary characterization of pregnancy specific antigen(s) in the ewe. Mildred Cerini, J. C. Cerini, J. K. Findlay and R. A. S. Lawson, *Reproduction Research Section, University of Melbourne, and Department of Agriculture, Victoria, S. S. Cameron Laboratory, Werribee, Victoria 3030, Australia.*

Antigen(s) specific to pregnancy have been detected by haemagglutination in the plasma and on the erythrocytes of pregnant ewes as early as Day 6 after mating (Cerini *et al.*, 1976). Preliminary characterization of the antigen(s) has been carried out using uterine vein plasma of Day 10 pregnant ewes as a source of antigen and a passive haemagglutination test to monitor antigen titres.

The activity in plasma is stable at 4°C for at least 1 week and at -15°C for at least 12 weeks. The activity was slightly reduced after heating at 56°C for 30 min and was destroyed after heating at 80°C for 30 min. Following dialysis (mol. wt cut off 6000-8000) against phosphate-buffered saline (pH 7.2) overnight at 4°C, the titre of antigen in dialysed plasma remained unchanged. Ammonium sulphate precipitation revealed that 45% saturation was the lowest effective concentration which removed the antigen from solution. Electrophoresis on cellulose acetate membranes at pH 9.5 indicated that the antigen(s) had an electrophoretic mobility in the post-albumin region, similar to α -globulins and acid-glycoproteins. Treatment of pregnancy plasma with M-trichloroacetic acid completely removed the antigen(s) from solution, indicating that a protein component is necessary for expression of antigenicity. Sodium meta-periodate at 0.20 M to 0.05 M was effective in destroying the activity of the pregnancy-specific antigen in plasma whereas after treatment with 0.025 M meta-periodate, the activity was unchanged. This suggests that the antigen contains a carbohydrate component. Filtration of pregnancy plasma on Sephadex G-25 (fractionation range 100-5000 mol. wt) showed that the antigen was present in the main protein fraction collected in the exclusion volume of the column.

These preliminary studies of pregnancy-specific antigen(s) detected immunologically in the plasma of pregnant ewes indicate that they are of a molecular weight greater than 8000 and have protein and carbohydrate components.

Reference

- CERINI, M., FINDLAY, J.K. & LAWSON, R.A.S. (1976)
J. Reprod. Fert. **46**, 65-69.

The function of anterior pituitary hormones in the initiation of implantation in mice. A. A. Gidley-Baird and C. W. Emmens, *Department of Veterinary Physiology, University of Sydney, N.S.W. 2006, Australia.*

The work reported here is an extension of that presented last year (Gidley-Baird & Emmens, 1975),

which demonstrated the capacity of various combinations of anterior pituitary hormones to initiate implantation in hypophysectomized mice. The aim of this work was to determine the function of these hormones in achieving their effects.

Exp. 1. Four groups, each of 15 female QS mice hypophysectomized at 11.00 hours on Day 2 of pregnancy (Day 1 being the day on which the copulatory plug is found), were injected s.c. at 14.00 hours on Day 2 as follows. Group 1, 0.1 ml of 15% gelatin vehicle; Group 2, 50 µg prolactin (NIAMD-Rat-RP1); Group 3, 50 µg LH (NIH-LH-S16; Group 4, 50 µg rat prolactin+50 µg LH. Five mice from each group were killed at 8, 16 and 32 hr after the injection. The plasma was collected and stored at -20°C until assayed for progesterone content by a protein binding method.

Exp. 2. Female QS mice were hypophysectomized on Day 2 of pregnancy and given progesterone replacement therapy in conjunction with either NIH-LH-S16, rat prolactin or FSH (NIAMD-Rat-FSH-B1) until Day 8, when they were killed and examined for implanted embryos.

Exp. 3. Female QS mice were hypophysectomized on Day 2 of pregnancy and the capacity of NIH-Prolactin-S11 and oestradiol-17β to initiate implantation was tested in a 2×3 factorially designed experiment. Mice were killed and examined for implanted embryos on Day 8.

Exp. 4. Mice which showed post-partum copulatory plugs and each suckling 8 young were allocated into 4 groups of 7 and on Day 5 of pregnancy had their litters removed and received the following treatments: Group 1, 0.2 ml normal rabbit serum (NRS) at 06.00 hours on Day 5; Group 2, 0.2 ml NRS at 12.00 hours on Day 5; Group 3, 0.2 ml anti-NIH-LH-S16 serum at 06.00 hours on Day 5; Group 4, 0.2 ml anti-LH serum at 12.00 hours on Day 5.

Both LH and prolactin individually and in combination were capable of significantly increasing plasma progesterone levels.

All the hypophysectomized mice receiving progesterone+FSH had implanted embryos. Groups receiving progesterone+LH or prolactin showed no implantation.

Only hypophysectomized mice receiving prolactin+oestradiol had implanted embryos. The group injected with prolactin+a low dose of oestradiol contained 8/9 mice with implantations.

Anti-LH serum failed to block implantation in mice after litter removal.

The results of Exps 1-3 together with the work of Gidley-Baird & Emmens (1975) suggest that LH and prolactin are responsible for the progesterone production while FSH is primarily responsible for the oestrogen production required for implantation in the mouse. The result of Exp. 4, together with the fact that implantation can occur in the absence of pituitary LH, suggests that anti-LH may also block implantation at a site other than that concerned with the inhibition of progesterone production. It is possible that this site might involve cross-reaction with an LH-like substance found on the surface of preimplantation mouse embryos (Wiley, 1974).

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Session 13

Chairman: Dr R. A. S. Lawson

Effects of progesterone and oestradiol on endometrial metabolism in the ovariectomized ewe. B. G. Miller and N. W. Moore, *Department of Animal Husbandry, University of Sydney, Camden, N.S.W. 2570, Australia.*

The hormonal regulation of the environment in the uterus in which early embryonic development occurs is poorly understood. The effects of progesterone secreted before oestrus, and of oestradiol secreted at about the time of oestrus on endometrial metabolism during early pregnancy were investigated, using ovariectomized ewes. At the start of the experiment (Day 0) all ewes received

25 µg oestradiol, and they were then placed under one of five oestradiol/progesterone regimens, which have been fully described (Moore & Miller, 1976).

Three ewes from each of the 5 treatment regimens were killed around 08.00 hours on each of Days 18, 21 and 24. Slices of endometrium were prepared from dissected caruncles. RNA and DNA were extracted from endometrial homogenates by incubation at 90°C for 20 min in 0.5 M-HClO₄, and assayed colorimetrically. Rates of protein synthesis were determined from the incorporation of [methyl-³H]L-methionine into protein during 2 hr incubations of slices in Eagle's HeLa medium.

Table 1. RNA:DNA ratios in the endometrium of ewes

Day of experiment				18	21	24
Days after oestrus*				1	4	7
Group		Treatment regimen				
1	Progesterone†	Oestrogen	Progesterone‡	0.913	0.786	0.649
2	Progesterone†	Oestrogen	—	0.865	0.676	0.530
3	Progesterone†	—	Progesterone‡	0.585	0.512	0.558
4	—	Oestrogen	Progesterone‡	0.742	0.667	0.723
5	—	—	Progesterone‡	0.410	0.423	0.544

* In ewes receiving oestrogen, † Priming progesterone; ‡ Maintenance progesterone.

When maintenance progesterone was omitted (Group 2 versus Group 1) there was a small decrease ($P<0.005$) in RNA:DNA ratios on Days 21 and 24 (Table 1). In ewes which received maintenance progesterone the omission of oestrogen (Groups 3 and 5 versus Groups 1 and 4) markedly decreased RNA:DNA ratios and rates of synthesis of protein ($P<0.005$). By Day 24 the effect on protein synthesis had disappeared, and that on RNA:DNA ratios was reduced. Omitting priming progesterone had no effect on rates of protein synthesis, but did reduce RNA:DNA ratios on Days 18 and 21 ($P<0.005$). There were no interactions between oestrogen and priming progesterone on RNA:DNA ratios. In ewes which received maintenance progesterone the effects of priming progesterone and of oestrogen on RNA:DNA ratios in the endometrium followed the same pattern as their effects on survival of embryos (Moore & Miller, 1976). It is suggested that changes in the RNA:DNA ratio reflected the effects of priming progesterone and of oestrogen on the potential of the endometrium for glandular secretion under the subsequent influence of maintenance progesterone.

Reference

MOORE, N.W. & MILLER, B.G. (1976) *J. Reprod. Fert.* **46**, 536–537.

Progesterone and oestrogen requirements for the survival of embryos in the ovariectomized ewe. N. W. Moore and B. G. Miller, *Department of Animal Husbandry, University of Sydney, Camden, N.S.W. 2570, Australia.*

Studies on the hormone requirements during early pregnancy in the ewe have been confined to animals ovariectomized a few days after oestrus or to ovariectomized ewes to which embryos were transferred after the induction of oestrus by oestrogen given after a period of progesterone treatment. Thus, little is known of the role that progesterone secreted before oestrus or oestrogen secreted around oestrus might play in survival of embryos.

In the present experiment ewes that had been ovariectomized several months previously were used and at the start of the experiment (Day 0) they were given 25 µg oestradiol and then placed on one of five oestradiol/progesterone regimens (Table 1).

Table 1. Treatment regimens of ovariectomized ewes

Group	Priming progesterone*	Oestradiol†	Maintenance progesterone‡	Ewes with normal embryos
1	+	+	+	8/13
2	+	+	—	0/11
3	+	—	+	1/13
4	—	+	+	2/11
5	—	—	+	0/11

* 5 mg twice daily on Days 3–14 (incl.).

† Total of 35 µg given over Days 15 and 16.

‡ Doses (mg) given a.m. and p.m. respectively on Day 18, 0.5 and 0.6; Day 19, 0.75 and 1.0; Day 20, 1.25 and 1.5; Day 21, 2.0 and 2.5; Day 22, 3.0 and 4.0; Day 23, 5.0 and 6.0; Days 24–25, 8.0 and 8.0; Days 26–42, 12.0 (a.m. only).

The hormones were given s.c. and each component of the regimen attempted to simulate a phase of ovarian secretion during the oestrous cycle. A single embryo collected from donors 4 days after mating was transferred to each ewe on Day 21, 4 days after induced oestrus in ewes which received oestradiol. Treatment with maintenance progesterone continued to Day 42 when all animals were autopsied. At autopsy normal embryos were recovered from 11 animals (Table 1).

Clearly progesterone given after oestrus was necessary for survival, and equally apparent is the need for both oestradiol and progesterone given before oestrus. When either priming progesterone or oestradiol were omitted (Groups 3 and 4) few embryos survived. When both were omitted (Group 5) none survived.

The results suggest that in the entire ewe progesterone secreted before oestrus and oestrogen secreted around the time of oestrus are involved in survival and development of embryos.

Storage of cattle embryos. R. J. Bilton and N. W. Moore, *Department of Animal Husbandry, University of Sydney, Camden, N.S.W. 2570, Australia.*

The long-term storage of embryos has been an area of continued investigation, but to date reliable methods for frozen storage have been reported for only one species—the mouse (Whittingham *et al.*, 1972; Wilmut, 1972), although there is a report of a single pregnancy following the transfer of some 22 frozen cattle embryos to recipient cows (Wilmut & Rowson, 1973).

In an initial experiment in the present study, 21 cattle embryos collected as morulae and early blastocysts from donors 7 and 8 days after oestrus were cooled to 6°C (0.7°C/min), held at 6°C for 1, 2 or 4 days, and then warmed to 37°C (0.7°C/min) and cultured for 36 hr. Thirteen embryos developed during culture to expanded or hatched blastocysts and 11 of the 13 were transferred to 9 recipients, of which 3 were diagnosed as pregnant, by rectal palpation, 8 weeks after transfer. Duration of storage had no effect upon development in culture, but none of four recipients which received embryos stored for 4 days was diagnosed as pregnant.

Subsequently, 39 embryos of similar age were cooled and stored in liquid nitrogen (–196°C) for 2–7 weeks. They were then warmed and cultured for 12 hr. Cooling, storage, warming and culture were carried out in pyrex glass tubes containing Dulbecco phosphate buffer enriched with 25% heterologous bovine serum (DB+25% S). Before cooling dimethylsulphoxide (DMSO) in DB+25% S was added slowly over 20 min to give a final concentration of 1.5 M-DMSO. The tubes were then cooled to 0°C at a rate of 0.7°C/min. Further cooling to –55°C (0.13–0.15°C/min) was carried out in a solid CO₂/ethanol bath. The tubes were then transferred to a liquid nitrogen container and slowly lowered through the vapour into storage in liquid nitrogen.

Following storage the tubes were warmed to 0°C at rates of 1.2 or 2.2°C/min (measured over the range –50 to 0°C) and from 0°C to 30°C at a rate of 0.7°C/min. DMSO was removed by washing in DB+25% S containing decreasing concentrations of DMSO and the washed embryos were then cultured for 12 hr at 37°C. During culture 16 of the 39 embryos developed to expanded or hatched

blastocysts while the remaining 23 showed no, or limited development. Eleven of the 16 and 12 of the 23 were transferred to 17 recipients. Recipients received either one embryo that had developed, or two which had shown no, or limited development in culture. Rectal palpation 8 weeks after transfer showed that 6 recipients were pregnant and all had received embryos which developed during culture.

The results show that cattle embryos can be stored in either the liquid or frozen state, but liquid storage is probably limited to a few days.

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Fertility of boar semen frozen-stored for 2½ years. S. Salamon and G. N. Pearse, *Department of Animal Husbandry, University of Sydney, N.S.W.* 2006, Australia.

There seems to be no information on fertility of boar semen frozen-stored over a prolonged period. To examine the effect of long-term storage on fertility, a semen bank has been laid down, and this communication presents the results for the first stage of the fertility test in which boar semen stored for 2½ years was used for insemination.

The semen was collected by the manual method, and the sperm-rich fraction of ejaculates was diluted at 30°C (1:2) with a tris-based diluent (250 mM-tris–111 mM-fructose–15 mM-EDTA (disodium salt)–79.5 mM-citric acid). The diluted semen containing 15% (v/v) egg yolk and 2% (v/v) glycerol was cooled to 5°C in 1.5 hr, then frozen in pellet form (0.25 ml) on solid CO₂ and subsequently stored in liquid nitrogen.

Oestrus was detected by teaser boars twice daily, and surgical inseminations were performed 19–28 hr after detection. For insemination, 2–3 pellets were thawed in a water-bath at 37°C. The inseminating doses were 0.10 and 0.20 ml, containing 6.7×10^6 and 13.4×10^6 motile spermatozoa. After exposing the reproductive tract by mid-ventral laparotomy, the thawed semen was introduced into the oviduct via the utero-tubal junction.

A total of 22 animals was inseminated, of which 20 (90.9%) farrowed 122 pigs. The average litter size was 6.1. Farrowing numbers for 0.10 and 0.20 ml inseminate volumes were 10/11 and 10/11. All 14 animals inseminated before ovulation and 6/8 animals inseminated after ovulation farrowed.

Surgical insemination with semen frozen-stored for 2½ years yielded high fertility. Semen stored for longer than this period will be used in future fertility tests.