

## Role of melatonin and circadian rhythms in seasonal reproduction in rams

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**Summary.** In the ram, changes in daylength influence testicular activity by modifying the release of luteinizing hormone-releasing hormone (LH-RH) and thus the gonadotrophins. To investigate the nature of this response the hourly fluctuations in the circulating levels of prolactin, luteinizing hormone (LH) and melatonin were measured in rams kept under various artificial lighting conditions.

In Exp. 1, 8 Soay rams (4 control and 4 from which the superior cervical ganglia had been removed) were exposed to alternating 16-week periods of short days (8L:16D) and long days (16L:8D) for over 2 years, and blood samples were collected hourly for 25 h on two occasions. The lighting regimen resulted in marked testicular and endocrine changes in the controls but not in the ganglionectomized rams which had low or undetectable levels of melatonin ( $\leq 33$  pg/ml) and an unusual diurnal rhythm in prolactin.

In Exp. 2, 8 intact Soay rams were exposed to an ahemeral lighting regimen of 8L:28D for 16 weeks; at the end of this period blood samples were collected hourly for 52 h and assayed for prolactin. During the pretreatment period of long days (16L:8D), the testes became fully regressed. During the 16 weeks of 8L:28D, redevelopment occurred, but the growth of the testes was slow compared to that normally occurring under short days of 8L:16D. The prolactin profiles showed evidence of circadian rhythm in hormone secretion, with a correlation between the timing and duration of the rhythm and the degree of testicular development.

These combined results support the idea that the photoperiodic response in the ram involves an interplay between the secretory activity of the pineal gland, and a light/dark entrained circadian mechanism in the brain.

### Introduction

Changes in the secretion of the gonadotrophic hormones and prolactin occur in rams exposed to alterations in daylength (Ortavant, Mauléon & Thibault, 1964; Lincoln & Short, 1980). This response to changing photoperiod is thought to be governed by the hypothalamus through the secretion of neurohormones such as luteinizing hormone-releasing hormone (LH-RH) and dopamine released locally into the pituitary portal blood system. The question to be considered in the present paper is: 'how does the ram measure changes in daylength and translate this information into an alteration in the neurosecretory activity of the hypothalamus?'

One hypothesis is that the response to a change in daylength is dependent on photoreception by the eyes, and is dictated by the combined activities of two discrete areas in the brain, namely the suprachiasmatic nucleus (SCN) and the pineal gland (Rollag, O'Callaghan & Niswender,

1978a; Rusak & Zucker, 1979; Turek & Campbell, 1979). The SCN is believed to be the circadian rhythm generator of the brain, and is necessary for entrainment to the daily light cycle. It is believed that the pineal secretes a hormone, which if released at the appropriate time relative to a daily rhythm of sensitivity of the hypothalamus (dictated by the SCN) results in reduced 'neurosecretory' activity. Melatonin is the principal indole amine secreted by the pineal gland and could possibly be the hormone involved. Recent studies on sheep have shown that the pineal gland is involved in mediating effects of changing daylength on the secretory activity of the anterior pituitary gland (Barrell & Lapwood, 1978, 1979a, b, c; Lincoln, 1979a, b; Brown & Forbes, 1980), and Domanski, Przekop & Polkowska (1980) have indicated the involvement of the SCN in the regulation of seasonal breeding.

## Materials and Methods

### *Experiment 1*

Eight adult Soay rams living out of doors near Edinburgh were taken into a light-proof building in July 1976 and exposed to an artificial lighting regimen consisting of alternating 16-week periods of long days (16 h light:8 h darkness, 16L:8D) and short days (8 h light:16 h darkness, 8L:16D) for more than 2 years (Lincoln, 1979a). Four of the rams (SCG rams) had been cranially sympathectomized 4–6 months before the study by removal of the superior cervical ganglion from both sides of the neck (Appleton & Waites, 1955) while the other 4 served as controls; one of the latter group received a sham operation. Details of the long-term changes in the size of the testes, and the plasma levels of prolactin, LH, FSH, testosterone, triiodothyronine and thyroxine in these rams have been reported previously (Lincoln, 1979a, b; Lincoln, Klandorf & Anderson, 1980).

On one day during a period of exposure to short days (Day 50 of the 16-week period) and on one day during the long days (Day 65 of the 16-week period) samples of blood were taken from the jugular vein of all rams at hourly intervals for 25 h. An indwelling cannula was inserted into the vein one day before sampling and on the day of sampling special care was taken not to disturb the normal routine of the animals. Plasma was separated immediately after blood collection, and frozen at  $-20^{\circ}\text{C}$  until the concentrations of LH, prolactin and melatonin were measured by radioimmunoassay. The validations of the assay procedures have been reported previously for LH (Scaramuzzi, Caldwell & Moor, 1970), prolactin (McNeilly & Andrews, 1974) and melatonin (Arendt, Wetterberg, Heyden, Sizonenko & Paunier, 1977), and the limits of detection are shown in Text-fig. 1. At weekly intervals throughout the study the size of the testes of each ram was measured with calipers (Lincoln, 1979a).

### *Experiment 2*

Eight adult Soay rams living out of doors near Edinburgh were taken into a light-proof building in October 1978, and exposed to long days (16L:8D) for 16 weeks. The lighting was then changed to an ahemeral (cycle not equal to 24 h) regimen consisting of 8L:28D for 16 weeks, before being changed back to long days (16L:8D). At weekly intervals throughout the study the diameter of the testes of each ram was measured; a summary of the changes has been published (Lincoln & Short, 1980). On a single occasion at the end of the 16-week period of 8L:28D blood samples were collected from the jugular vein at hourly intervals for 52 h using an indwelling cannula as in Exp. 1. The blood plasma was frozen immediately after collection, and the concentration of prolactin was measured using the radioimmunoassay method of McNeilly & Andrews (1974). The plasma profiles of prolactin were scanned to identify the peak values which were taken as the highest levels of prolactin during any 24-h period commencing at 08:00 h at the beginning of the blood sampling.

PLATE 1



**Fig. 1.** Control ram kept in short days; the testes are large, the scrotum is covered in dense wool, and there is a conspicuous 'sexual flush' of the exposed skin in the inguinal region.

**Fig. 2.** Control ram kept in long days; the testes are small, the wool is moulting from the scrotum, and there is no 'sexual flush' of the exposed skin in the inguinal region.

**Fig. 3.** Superior cervical ganglionectomized ram kept in long days; the testes are large, there is a sparse covering of wool on the scrotum, and only a slight 'sexual flush' of the skin in the inguinal region.

(Facing p. 24)



## Results

### Experiment 1

*Control rams.* On Day 50, under the short-day photoperiod (8L:16D), the mean  $\pm$  s.e.m. diameter of the testes of the control rams was  $52.0 \pm 1.2$  mm. On Day 65 of the long-day photoperiod, the diameter was significantly smaller at  $43.5 \pm 0.8$  mm ( $P = <0.01$ , Student's paired *t* test; see Pl. 1, Figs 1 and 2). These differences were correlated with the endocrine patterns of the animals (Text-fig. 1a). The plasma levels of melatonin and prolactin were lower during the short days, while the reverse was the case for the levels of LH. All the hormones varied in concentration from hour to hour, with maximum values of melatonin occurring during darkness in both photoperiods. The profile of prolactin during long days showed evidence of peaks during the early parts of both the light and dark phases, while the levels of LH were particularly variable (Text-fig. 1a).

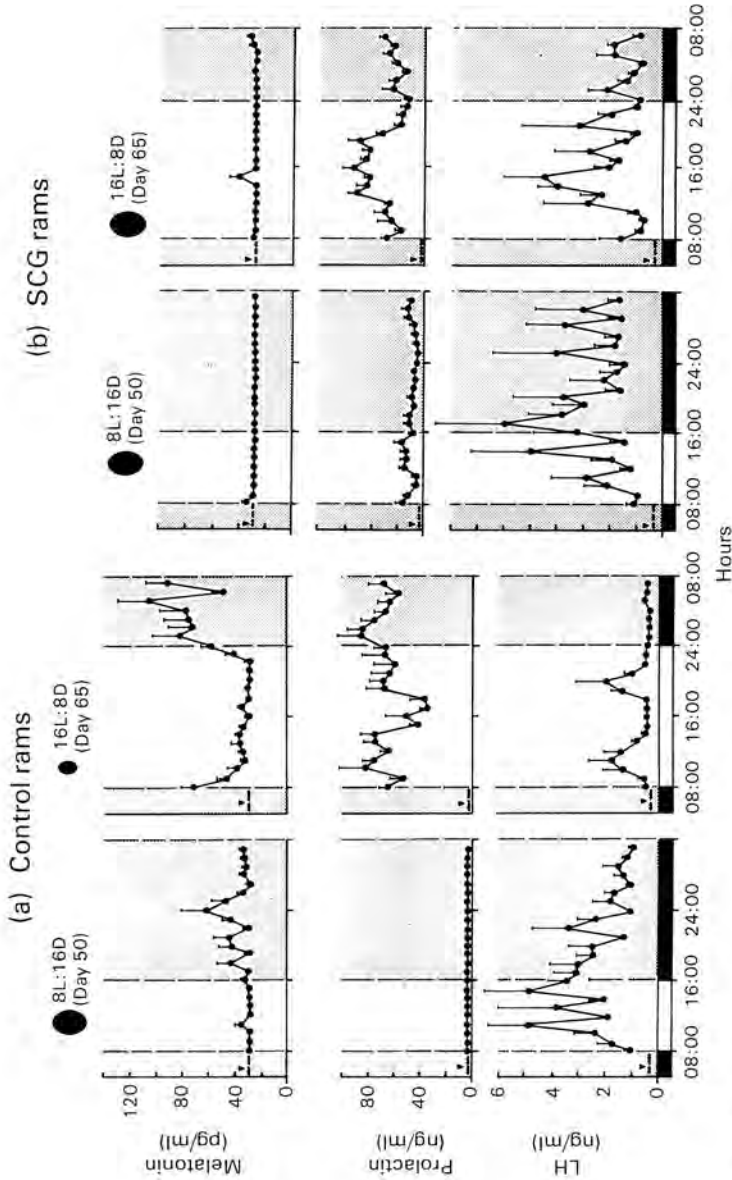
*Superior cervical ganglionectomized rams.* On Day 50 during the short-day photoperiod, the mean  $\pm$  s.e.m. diameter of the testes was similar to that on Day 65 during long days ( $54.0 \pm 1.0$  and  $54.5 \pm 1.2$  mm, respectively; see Pl. 1, Fig. 3). There were relatively minor differences in the plasma concentrations of prolactin, LH and melatonin (Text-fig. 1b), but there were notable differences in the plasma levels of these three hormones when compared to those of the control rams. The plasma levels of melatonin were low or undetectable ( $<33$  pg/ml) in the SCG rams under both photoperiods (Text-figs 1a and 1b). The levels of prolactin were of an intermediate range, with a possible diurnal rhythm showing maximum values in the late light phase. The plasma LH concentrations were high under both photoperiods with a diurnal pattern similar to that observed in the controls kept in short days (Text-figs 1a and 1b).

### Experiment 2

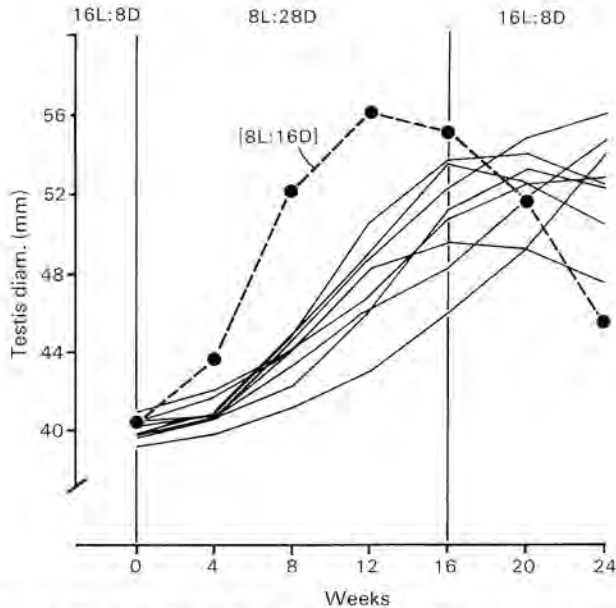
*Testis growth during 8L:28D.* As shown in Text-fig. 2, under the initial period of long days (16L:8D) the testes had become fully regressed, and during the 16 weeks of 8L:28D lighting redevelopment occurred in all animals. However, there was considerable variation in the degree of testicular growth. The most 'advanced' animals showed full growth of the testes during the 16-week period while in the most 'retarded' ram the diameter was only 65% of the maximum achieved at the time of full development later in the sexual cycle. The testes of the slow responders continued to enlarge for several weeks after the 8L:28D treatment was ended while those of the advanced animals began to regress. In all the rams the rate of testicular growth during the 16 weeks of 8L:28D lighting was slow compared to that previously recorded during a 16-week period of short days (8L:16D) following long days (16L:8D) (Exp. 1, see Text-fig. 2).

*Prolactin rhythms during 8L:28D.* The changes in the concentration of prolactin in the plasma of the 8 rams over 52 h at the end of the 16 weeks of exposure to 8L:28D are shown in Text-fig. 3. All animals had low circulating levels of prolactin at this time; the mean value being  $<20.0$  ng/ml and in some rams the level was  $<2.0$  ng/ml. The plasma profiles for all animals showed hour-to-hour variations with at least two principal peaks in the prolactin concentration during the 52-h period (Text-fig. 3). The timing of these peaks varied between the different rams; however, 4 of the animals (Group 1) had very similar profiles with two principal peaks during the 52 h, the first occurring 5–7 h after the beginning of the study during darkness and the second occurring 23–26 h later ( $25.0 \pm 0.6$  h, s.e.m.), also during darkness (Text-fig. 3, rams 1, 2, 3 and 5). The remaining 4 rams (Group 2) had more variable prolactin profiles. In 3 of the animals, the initial peak occurred 20–24 h after the beginning of the study, i.e. during or shortly after the onset of the light period, and there was a second peak 20–22 h later ( $21.0 \pm 0.5$  h, s.e.m.) during darkness (Text-fig. 3, rams 4, 6 and 8).

The 4 rams described as Group 1, on the basis of the hourly variations in prolactin concentration, were all animals which showed good growth of the testes during the 16 weeks of



**Text-fig. 1.** Changes in the concentration of melatonin, prolactin and luteinizing hormone in the blood plasma of (a) 4 control Soay rams and (b) 4 rams from which the superior cervical ganglia had been removed. The samples were taken hourly for 25 h during a 16-week period of short days (Day 50 of short days, 8L:16D) and for 25 h during a 16-week period of long days (Day 65 of long days, 16L:8D). The lower limit of detection for each hormone is indicated (▼) along with the duration of the daily period of daylight (open) and darkness (shade). The upper solid symbol depicts diagrammatically the relative size of the testes of the rams on the two occasions.



**Text-fig. 2.** Changes in the diameter of the testes of 8 intact Soay rams during exposure to a 16-week period of an ahemeral lighting regimen of 8L:28D. The animals had been initially exposed to long days (16L:8D) for 4 months before the experiment, and after the 16 weeks of 8L:28D, the long day (16L:8D) regimen was resumed. The broken line (—•—) shows changes in the mean diameter of the testes of 4 control Soay rams during a 16-week period of short days (8L:16D) following long days (16L:8D) based on the data from Lincoln (1979a).

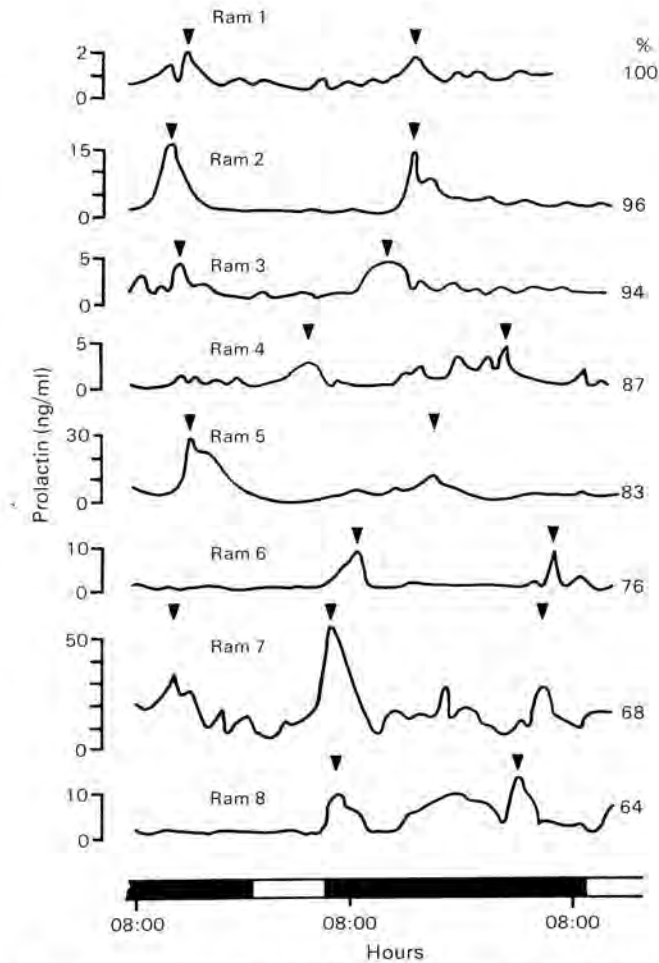
8L:28D (83–100% redevelopment compared to the maximum achieved during the sexual cycle; Text-fig. 3), while 3 of the remaining rams were more retarded. Taking the results for all 8 animals and selecting peak values of prolactin, there was a positive correlation between the duration of the prolactin rhythm (interval between peaks) and the degree of testicular growth which had occurred ( $r = 0.762$ , Spearman's rank correlation,  $P = 0.05$ ).

## Discussion

### *Role of the pineal gland*

It is well established from experimental studies of the hamster and ferret that the pineal gland is involved in the control of the reproductive changes that are induced by alterations in daylength (Reiter, 1972; Herbert, Stacey & Thorpe, 1978; Turek & Campbell, 1979). For the sheep the same appears to be true. Both pinealectomy (Brown, Jones & Forbes, 1977; Barrell & Lapwood, 1979a, b; Brown & Forbes, 1980) and superior cervical ganglionectomy (Lincoln, 1979a, b) interfere with the normal responses to changing photoperiod. The most likely hormone involved is melatonin since this is secreted in large amounts by the pineal, its release varies markedly from day to night, and when injected at the appropriate time it can mimic the effects normally induced by a change in daylength (Wurtman & Moskowitz, 1977; Turek & Campbell, 1979).

In the ewe melatonin is present in the peripheral circulation, and high levels persist throughout the dark phase of the 24-h cycle (Rollag *et al.*, 1978a; Rollag, Morgan & Niswender, 1978b). The present results confirm that a similar pattern of secretion occurs in the Soay ram. However, in this case there is not a simple relationship between the plasma levels of melatonin and the daily light-dark cycle. For example, in the rams living under the long-day photoperiod



**Text-fig. 3.** Prolactin concentrations in 8 adult Soay rams sampled hourly for 52 h at the end of a 16-week period of the ahermal lighting regimen of 8L:28D. The timing of daylight (open bar) and darkness (shaded bar) is shown below, and to illustrate the rhythmic nature of the prolactin values, the two principal peaks in the hormone concentration are arrowed (in one ambiguous case 3 peaks have been selected). The animals are ranked in order of the degree of testicular development which occurred during the 16 weeks of exposure to the 8L:28D regimen (testis size shown on right as %, at the time of study, of fully active size achieved later in the sexual cycle). Note that the scale used to depict the prolactin concentration varies between animals.

(16L:8D) the levels of melatonin were increased during darkness for at least as many hours as under the short-day photoperiod (8L:16D), and the maximum concentrations achieved at night were highest under long days. Therefore, the pattern of melatonin secretion does not reflect the duration of darkness. The increase in the nocturnal secretion of melatonin during long days occurs in association with a decline in the plasma levels of LH and regression of the testes (Text-fig. 1a). The possibility that this is a causal relationship is supported by the results from the SCG rams, since these animals did not show a similar response to long days, and there was no nocturnal increase in the circulating levels of melatonin.

The changes in the secretion of LH and prolactin which occur in response to alterations in daylength are presumably controlled by the hypothalamus. For example, the control of LH is dictated by the release of LH-RH, with a change in frequency of pulsatile release being of



particular significance (Lincoln, 1978; Lincoln & Short, 1980). If melatonin mediates the effects of changes in photoperiod then this hormone must affect the activity of the neurones in the hypothalamus; whether this is a direct or indirect effect is unknown. Studies on the uptake of radioactive melatonin made in other species indicate that the hormone binds to various areas of the brain including the medial basal and suprachiasmatic areas of the hypothalamus (Anton-Tay & Wurtman, 1969; Cardinali, Vacas & Boyer, 1979; J. Joss, personal communication). In the present study, the SCG rams showed an atypical diurnal rhythm in the plasma level of prolactin compared to the controls, which could indicate that melatonin influences the rhythm as well as the overall level of hormone secretion by the anterior pituitary gland (see also Lincoln, 1979b).

### *Role of circadian rhythms*

In considering the effect of a change in daylength on testicular activity in the ram it is tempting to assume that it is the change in the total amount of light or darkness per day which causes the response. However, studies with the hamster and various species of birds have shown that daylength *per se* is unimportant and that there is some form of circadian mechanism underlying the photoperiodic response (Follett, 1973, 1978; Elliott, 1976; Rusak & Zucker, 1979; Turek & Campbell, 1979). In the model proposed for the sheep by Rollag *et al.* (1978a), the assumption is that the suprachiasmatic nucleus acts as a biological clock and governs circadian rhythms, including that of the pineal gland, and therefore is of central importance in the control of the response to photoperiod.

The possible involvement of circadian rhythms was considered in the second part of the present study. The ahemeral regimen of 8L:28D was used, because in absolute terms it is a short day (5.3 h light/24 h). However, since the total cycle takes 36 h, rather than 24 h or a multiple thereof, it can be used to test the circadian system. According to the formula proposed by Morris (1978), such a photoperiod could be interpreted as a long day (20L:4D), a feature confirmed in studies of hamsters (Elliott, Stetson & Menaker, 1972; Stetson, Elliott & Menaker, 1975). With the Soay rams, growth of the testes occurred during the 16-weeks period of exposure to 8L:28D lighting, following the long days (16L:8D). This result may be taken to indicate that the rams interpreted the ahemeral regimen of 8L:28D as a stimulatory short day. However, the rate of growth of the testes under 8L:28D was sluggish compared to that previously observed in rams exposed to short days (8L:16D) or 'extra' short days (8L:40D) following previous treatment with 16 weeks of long days (Lincoln, 1979a; Lincoln & Short, 1980). This emphasizes that the stimulatory nature of the photoperiod is not simply related to absolute amounts of light and darkness. It is also important to remember that regrowth of the testes would be expected to occur if the rams were simply maintained on long days for more than 16 weeks although the actual rate of regrowth has not been determined (Lincoln & Davidson, 1977).

In the experiment using the 8L:28D regimen there was a relationship between the degree of testicular growth and the rhythm in the plasma concentration of prolactin. All the rams had peaks in prolactin at intervals of about 24 h ( $23.0 \pm 0.86$  h, mean  $\pm$  s.e.m.), although the timing of the peaks varied between the animals. The group of 8 rams could be divided into 4 which had good testicular growth during the 16 weeks of 8L:28D and had very similar patterns in prolactin, and 4 rams which were generally slower in the testicular response and had more variable rhythms in prolactin. In the former group the duration of the prolactin rhythm ( $25.0 \pm 0.6$  h) was longer than in the latter ( $21.0 \pm 0.5$  h). One interpretation of these results is that the patterns in secretion of prolactin provide an index of hypothalamic activity, and reveal that a circadian rhythm occurs during exposure to 8L:28D. Since there was synchrony in the timing of the peaks in plasma prolactin in some of the rams it is probable that the lighting regimen was entraining the rhythm in these animals. The difference between the group of 4 rams with similar prolactin patterns and the other animals is evidence that not all the animals adjusted their

circadian rhythms in the same manner, and it may be significant that these differences were correlated with the degree of testicular growth which occurred.

In conclusion, the results from the two experiments provide support for the model proposed by Rollag *et al.* (1978a) for the photoperiodic control of seasonal breeding in sheep, involving an interaction between the pineal gland and the generation of a circadian rhythm in the brain, regulated in its timing by the daily cycle of light and darkness.

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