BIOLOGY OF REPRODUCTION 27, 1062–1075 (1982)

Photoperiodic Regulation of Reproductive Activity in the Ram: Evidence for the Involvement of Circadian Rhythms in Melatonin and Prolactin Secretion

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ABSTRACT

Adult Soay rams were housed under long days of 16L:8D for 16 weeks, and then exposed to a lighting regimen of either 8L:40D, 8L:28D, 8L:16D or maintained under 16L:8D. The diameter of the testes and the intensity of the sexual skin flush was recorded every 1-2 weeks, while on four occasions during the 8L:40D and 8L:28D regimens the concentrations of melatonin, prolactin and electrolytes (sodium and potassium) were measured in blood samples collected hourly for 2-4 days.

During the initial period of 16L:8D, the testes of all rams regressed in size and the sexual skin flush disappeared. In the animals maintained under 16L:8D for more than 16 weeks, the testes began to redevelop spontaneously and there was a reappearance of the skin flush after 23-29 weeks. Compared to this group, the rams switched to 8L:40D and 8L:16D after 16 weeks showed a significant acceleration in testicular development and an earlier skin flush. The rams exposed to 8L:28D failed to show this stimulated response and reactivation of the testes was similar to that under prolonged 16L:8D.

During the 16 weeks under 8L:40D there was a 3-fold increase in the 24-h mean plasma concentration of melatonin. A clearly defined 24-h rhythm in the levels of melatonin was evident during the treatment. The rhythm was synchronized in its timing for the different individuals in the group, while the peak levels of melatonin occurred earlier relative to the onset of the light period under 8L:40D compared to 16L:8D. Under the 8L:28D regimen there was an increase in the mean levels of melatonin as for 8L:40D; however, there was no consistent daily rhythm. Exposure to the two photoperiodic regimens also resulted in differences in the plasma profiles of prolactin, while no differences were evident for the levels of electrolytes.

The combined results are used to develop a model for the photoperiodic control of reproduction in sheep involving the control of circadian rhythms in hormone secretion; the control of the timing of the rhythm in secretion of melatonin is considered of central importance.

INTRODUCTION

Photoperiod acts as the major proximate factor dictating the timing of the breeding season in most birds and mammals from temperate and cold climates (Baker, 1938). Many species are stimulated into reproductive activity by increasing daily photoperiods, while some, such as sheep, goats and deer, are stimulated by decreasing photoperiods. We have previously made studies of the way photoperiod influences the endocrinology of reproduction in the semi-domesticated Soay sheep (Lincoln and Short, 1980), and this paper extends these observations to consider the mechanisms involved. Two main hypotheses exist to explain how organisms respond to changes in day length. In one, it is proposed that the response depends on the absolute number of hours of light or dark in each 24-h period ("hourglass" model). In the other, the response depends on the way the daily photoperiod influences circadian rhythms generated endogenously by the animal (circadian hypothesis). The hourglass model seems to hold for only a small number of insect species (Lees, 1966; Lees and Hardie 1981; Saunders, 1977) and possibly the lizard *Anolis carolinensis* (Underwood, 1981), while support for the circadian model comes from experiments on a wide range of insects (Saunders, 1970), birds (Hamner, 1963; 1964; Follett and Sharp, 1969) and mammals (Elliott et al., 1972; Morin et al., 1977; Grocock and

Accepted June 21, 1982.
Received July 28, 1981.

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Clarke, 1974; Rusak and Zucker, 1979; Nelson et al., 1982).

The importance of a circadian mechanism was first proposed by Bunning in 1936 (Bunning, 1973). He suggested that organisms possess an endogenous circadian rhythm in their sensitivity to light, consisting of two half cycles (12 h each), which he termed the *photophillic* and *scotophillic* phases. In long days (light period, 12 h), light impinges into the second half of the daily cycle which is sensitive to light, and in a long-day species this produces a photo-induced response. An equivalent mechanism, albeit opposite, can be envisaged to operate for a short-day species. Pittendrigh and Minis (1964) extended Bunning's hypothesis into an "external coincidence model" in which light has the dual role of inducer (or suppressor), and entraining agent for endogenous circadian rhythms. These authors also proposed an "internal coincidence model" in which light only served to entrain the endogenous rhythms, induction being the result of internal phase-relationships of at least two separate rhythms.

Nanda and Hamner (1958), studying flower formation in the short-day soybean *(Glycine max)*, devised what is still considered to be the best test for the involvement of circadian rhythms in the photoperiodic response. Their protocol consisted of measuring the photoperiodic response under a range of light/dark (LD) cycles in which a fixed period of light was coupled to dark periods of varying duration (e.g., 6L:6D, 6L:12D, 6L:18D, 6L:24D... 6L:66D). Nanda and Hamner (1958) observed that maximal responses were obtained when the LD cycle lengths equaled 24 h, 48 h and 72 h, and that cycle lengths of 18 h, 36 h and 60 h produced minimal responses. These schedules have been used widely in the study of photoperiodism in birds (Hamner, 1963, 1964; Follett and Sharp, 1969; Follett, Mattocks and Farner, 1974), but the only mammalian species subjected to resonance photoperiods so far have been the golden hamster (Elliott et al., 1972; Stetson et al., 1975), vole (Grocock and Clarke, 1974) and laboratory rat (Nelson et al., 1982).

In the present study we designed a simple resonance photoperiod experiment for Soay sheep. Because of the large size of the animals it was only practicable to use two groups of 8 rams. Initially, we studied the effect of exposing rams to a change from long days of 16L:8D to either 8L:40D or 8L:28D. At a later date, we recorded the effect of a change from 16L:8D to 8L:16D and the effect of maintaining animals for a prolonged period under 16L:8D. The reproductive response was indicated by the changes in the size of the testes and the intensity of the sexual skin flush; the latter develops under the influence of testosterone (Lincoln and Davidson, 1977).

To provide information on the central mechanisms controlling the secretion of the gonadotrophic hormones, we chose to measure the hourly fluctuations in the blood plasma concentrations of melatonin, prolactin and electrolytes (sodium and potassium). Melatonin originates largely from the pineal gland and is thought to be involved in the photoperiodic control of testicular activity in rams (Barrell and Lapwood, 1979a,b; Lincoln and Almeida, 1981; Lincoln et al., 1982). Prolactin is secreted by the anterior pituitary gland under the influence of regulatory factors from the hypothalamus, and its release is markedly influenced by the photoperiod (Ravault and Ortavant, 1977; Lincoln et al., 1978). The concentration of electrolytes in the plasma is influenced by a variety of factors, including the intake of food and the release of adrenocorticotrophic hormone from the anterior pituitary gland (Kreiger, 1979).

MATERIALS AND METHODS

Animals

Sixteen sexually mature rams of the Soay breed (aged 1-2 years) which had been living outdoors near Edinburgh (56°N) since birth, were brought into two adjacent light-proof sheds in November, 1978. The animals were penned individually in two equal groups and fed a standard diet (AA6, Animal Breeding Research Organization, Edinburgh) given once daily at 0900 h. The lighting was provided by white fluorescent strip lights providing approximately 160 Lux at floor level. The temperature in the sheds was not controlled, although heaters were used at night to reduce the daily fluctuations. The lighting for the two groups of rams was adjusted as follows: One group received a photoperiod of 16L:8D for 16 weeks, followed by 8L:40D for 16 weeks. The photoperiod was then changed back to 16L:8D for 16 weeks before being switched to 8L:16D for 16 weeks. Finally it was changed back to 16L:8D and maintained for 36 weeks. The time of "lights on" was 0800 h throughout, and the changes in photoperiod were achieved by altering the time of "lights out." The second group of rams received a photoperiod of 16L:8D for 16 weeks followed by 8L:28D for 16 weeks. The photoperiod was then changed to 8L:16D and the experiment ended after 10 weeks. The timing of "lights on" was 0800 h throughout except during the 8L:28D regimen when it was 0800 h and 2000 h on alternate days.

At 2-week intervals during the treatments the

diameter of the testes within the scrotum was measured for each ram using calipers, and the inguinal sexual skin flush was recorded using an arbitrary scale of 0–5 (Lincoln and Davidson, 1977). On 4 occasions during the 8L:40D and 8L:28D photoperiods, blood samples were collected at hourly intervals for 2-4 days (Days 0-4, 28-29, 78-79, 111-112 after the change from 16L:8D). For this an indwelling cannula was inserted into the jugular vein of each ram during one of the 8-h periods of light, at least 24 hours before the beginning of the blood collections. On a number of occassions the cannulae became blocked or broken and were replaced, using a point source of low-intensity light from a hand-held torch when necessary, taking care to shade the animal's face. The blood was collected into heparinized tubes and centrifuged within 30 min of collection. The blood plasma was stored at -20°C until required for the measurement of the concentration of melatonin, prolactin, sodium and potassium.

Assays

The concentrations of melatonin and prolactin in the blood plasma were measured using radioimmunoassays. Details of the methods with validation for use with plasma from sheep are described elsewhere for melatonin by Rollag and Niswender (1976), and for prolactin by McNeilly and Andrews (1974). The reference standard for prolactin was NIH-P-S9. The lower limit of detection for the melatonin assay was 20 pg/ml, and the intra- and inter-assay coefficients of variation were 15% and 25%, respectively. The lower limit of detection for the prolactin assay was 1.5 ng/ml, and the intra- and inter-assay coefficients of variation were 4% and 10%, respectively. For both assays the values for the coefficients of variation were calculated using mean values for quality controls showing approximately 20, 50 and 80% displacement of labeled hormone. Where possible, all samples from an individual animal were assayed together.

The concentrations of sodium and potassium in the blood plasma samples were measured by flame photometry. The precision of the sodium estimations was ±1 mmol/1 and of the potassium estimations, ±0.1 mmol/1. These measurements were performed on samples from only 4 of the rams from each group.

Statistical Analysis

The rate of growth of the testes and the timing of the initial reappearance of the sexual skin flush under the different lighting regimens was compared using the Mann-Whitney *U* test. The mean plasma concentrations of melatonin and prolactin were compared using Student's *t* test, while the correlations for individual rams under the 8L:28D photoperiod were assessed using the Spearman rank correlation test.

The hourly hormone data for individual animals were analyzed for peaks using a combination of the nonparametric "sign" test and Grubbs-Type statistics for the detection of outliers (Tietjen and Moore, 1972). The analysis consisted of the following steps: *a)* the value of the median hormone concentration for each 24-h period was computed for the results from the individual rams, *b)* each hourly value was assigned as being above, below or equal to the median value for the 24-h period in which it occurred, and *c)* the high values which represented significant outliers for the values for the entire 24 h were determined as described by Tietjen and Moore (1972). From these results a significant peak in the hormonal profile was taken as any period with at least 4 consecutive hourly values above the 24-h median which included at least one significant outlier.

The nature of the results for the electrolyte concentrations (gradual build-up and decline during 24 h) did not allow the detection of peaks by the outlier method. However, the consistency of the results allowed the use of the "sign test" alone: a peak being defined as a period when at least 4 consecutive values were above the 24-h median for the period in which they occurred.

An estimate of the interval between peaks in the concentration of melatonin, prolactin and electrolytes was obtained for individual animals using the starting value for each peak. The average interval for the groups was expressed as the median and range (Table 3). In addition, autocorrelation and spectral time

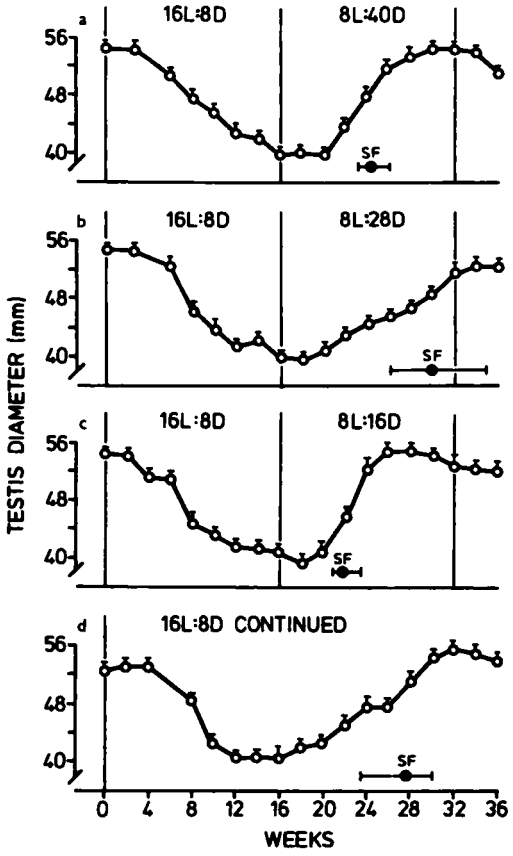

FIG. 1. Changes in the diameter of the testes (mean ± SEM) and the timing of the reappearance of the sexual skin flush (mean and range) in groups of 8 adult Soay rams exposed to 16L:8D for 16 weeks, followed by: *a)* 8L:40D, *b)* 8L:28D, *c)* 8L:16D, or *d)* maintained under 16L:8D. Note the more rapid sexual redevelopment under the 8L:40D and 8L:16D regimens compared to the other two treatments.

series analysis techniques were used to further investigate periodicity in the melatonin results, using the method of Howles et al. (1982). The results are shown in Fig. 8.

Preliminary results from a small part of this study have been published previously (Lincoln and Short, 1980; Lincoln et al., 1981).

RESULTS

Testicular Size and Sexual Flush

The changes in the diameter of the testes and the intensity of the sexual skin flush during the various lighting regimens are summarized in Figs. 1 and 2 and Table 1. During exposure to the initial period of 16L:8D, the testes of all rams became regressed and the sexual flush disappeared. In the animals maintained under 16L:8D for more than 16 weeks, the testes began to redevelop spontaneously and there was a reappearance of the sexual flush after 23–29 weeks. Compared to this pattern, the rams switched to 8L:40D and 8L:16D after 16 weeks under 16L:8D showed a significant acceleration in testicular redevelopment and an earlier sexual flush (Table 1). There was no significant difference between the 8L:40D and 8L:16D in this response. The rams exposed to 8L:28D showed no stimulation of testicular development relative to the changes occurring under 8L:40D or 8L:16D, and the reproductive changes were similar to those observed in the animals under prolonged 16L:8D (Fig. 1 and Table 1). However, when these rams were transferred from 8L:28D to 8L:16D the sexual flush became intensified in all animals after 5-8 weeks and the testes were maintained in size (Fig. 2).

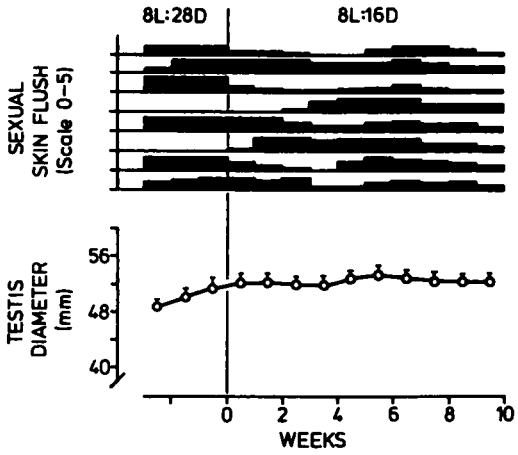

FIG. 2. Changes in the diameter of the testes (mean ± SEM) and the intensity of the sexual skin flush (values for individuals) in a group of 8 adult Soay rams exposed to 8L:16D for 10 weeks after a period under 8L:28D lasting 16 weeks. Note that most of the animals showed reactivation of the testes and development of the sexual flush during the period under 8L:28D (see Fig. 1); exposure to 8L:16D led to maintenance of the testes and a synchronous intensification of the sexual flush after 5-8 weeks.

Melatonin and Prolactin Concentrations

Changes in the mean concentration of melatonin and prolactin in the blood plasma of rams on five occasions during exposure to 8L:40D or 8L:28D following a 16-week period of 16L:8D are summarized in Table 2. The 24-h mean concentration of melatonin increased during both the 8L:40D and 8L:28D regimens while the mean concentration of prolactin decreased markedly. The only significant

TABLE 1. Summary of the changes in the diameter of the testis and time of reappearance of the sexual skin flush (mean ± SEM) in adult Soay rams exposed to four different photoperiodic regimens.

Photoperiodic regimens	Testis diameter (mm)[a]	Sexual skin flush (weeks)[b]
8L:40D	13.8 ± 0.8[c]	8.1 ± 0.4[c]
8L:28D	5.3 ± 0.9	13.9 ± 1.1
8L:16D	13.9 ± 0.8[c]	6.4 ± 0.3[c]
16L: 8D	8.6 ± 0.8	11.4 ± 0.7

[a]Increase in diameter of testes from Weeks 4-12 from beginning of lighting treatment or corresponding period under 16L:8D (See Fig. 1).

[b]Weeks to first appearance of sexual skin flush from beginning of lighting treatment or corresponding time under 16L:8D (See Fig. 1).

[c]Significantly different from corresponding value for 16L:8D treatment; $P=<0.05$ Mann-Whitney U test.

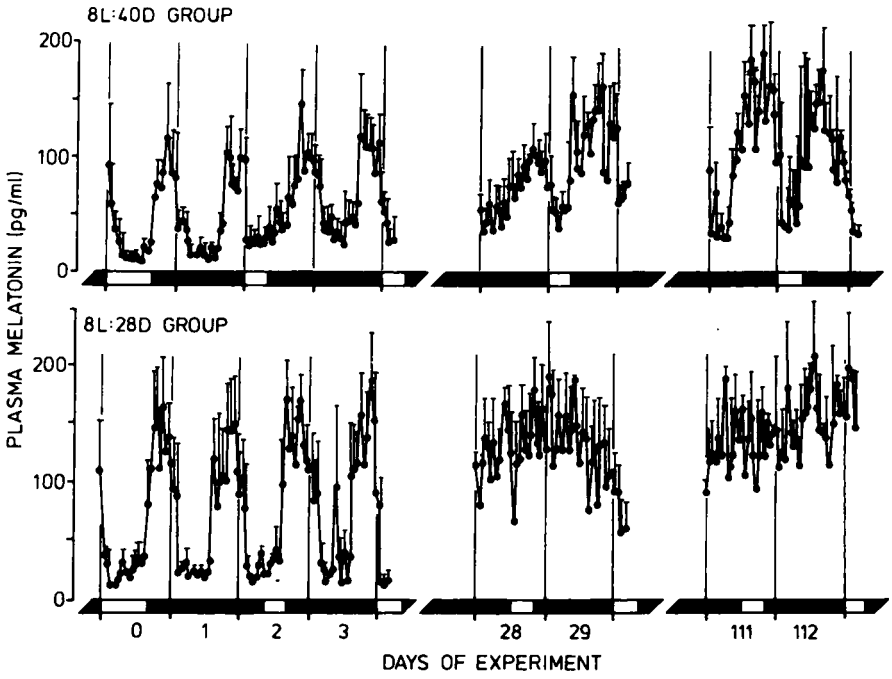

FIG. 3. Changes in the concentration of melatonin (mean ± SEM) in the blood plasma from 8 adult Soay rams sampled at hourly intervals for 2-4 days on 3 occasions during exposure to 16 weeks of either 8L:40D *(upper panel)* or 8L:28D *(lower panel)*, following 16 weeks of long days (16L:8D). *Day 0* is the last day of long days. The timing of the periods of light *(open bar)* and darkness *(closed bar)* is shown at the bottom of each panel.

difference between the two groups was in the timing of the decline in the mean concentration of prolactin; the levels were significantly lower by Days 28-29 under the 8L:40D regimen compared to Days 111-112 under the 8L:28D regimen.

Melatonin, Prolactin and Electrolyte Rhythms

The hour-to-hour fluctuations in the plasma concentrations of melatonin, prolactin and electrolytes (sodium and potassium) on various occasions during exposure to the 8L:40D and 8L:28D lighting regimens are shown in Figs.

TABLE 2. Summary of the changes in the concentration of melatonin and prolactin (mean ± SEM of 24-h median hormone value) in blood plasma from 8 rams sampled at hourly intervals for 1-2 days on 5 occasions during exposure to either 8L:40D or 8L:28D after a period of 16 weeks on 16L:8D. Day 0 is the last day of the 16L:8D regimen.

	Day 0 (16L:8D)		Day 1-2		Day 28-29		Day 78-79		Day 111-112	
Melatonin (pg/ml)										
8L:40D group	25.3	8.3	32.7	5.6	71.8	10.8[a]	.....		79.8	16.5[a]
8L:28D group	38.3	13.9	38.1	6.1	109.2	24.5[a]	101.2	22.2[a]	127.0	25.1[a]
Prolactin (ng/ml)										
8L:40D group	67.6	11.6	66.7	8.4	20.8	10.4[ab]	1.5	0.8[ab]	1.5	0.8[a]
8L:28D group	71.9	10.6	66.9	8.7	77.0	14.2	67.6	12.3	3.7	1.8[a]

[a]Significantly different from corresponding values for Day 0; Student's *t* test, $P<0.05$.

[b]Significantly different from corresponding values for 8L:28D Group; Student's *t* test, $P<0.05$.

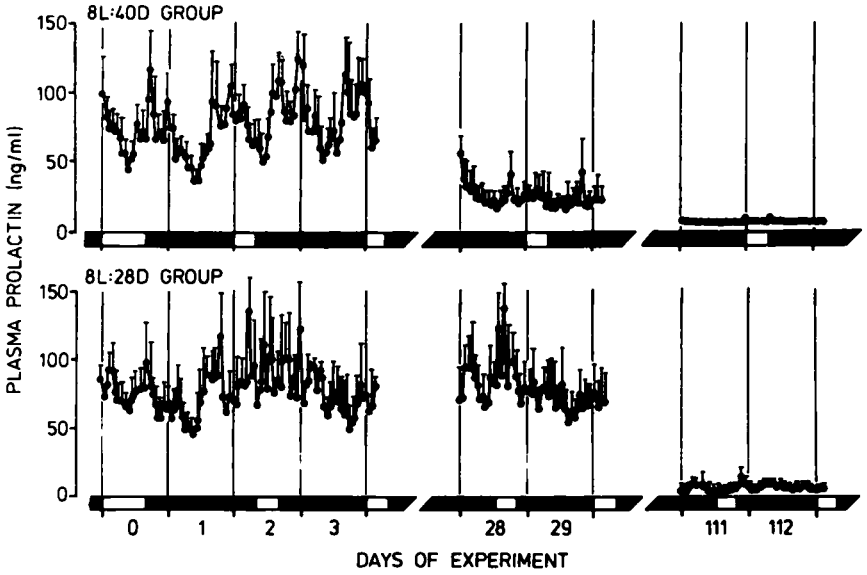

FIG. 4. Changes in the concentration of prolactin (mean ± SEM) in the blood plasma from 8 adult Soay rams sampled at hourly intervals for 2-4 days on 3 occasions during exposure to 16 weeks of either 8L:40D *(upper panel)* or 8L:28D *(lower panel)*, following 16 weeks of long days (16L:8D). *Day 0* is the last day of long days. The timing of the periods of light *(open bar)* and darkness *(closed bar)* is shown at the bottom of each panel.

3-5. On Day 0 (last day of long days of 16L: 8D), there were clearly defined 24-h rhythms in the plasma levels of melatonin, prolactin and the electrolytes, with peak values occurring late in the light phase or during darkness. During each period of blood sampling under the 8L:40D photoperiod there was also evidence of consistent 24-h rhythms in the plasma levels of melatonin and the electrolytes, with peak values occurring during darkness following the 8-h light period, and during darkness approximately 24 h later (Figs. 3 and 5). The plasma profiles of prolactin showed evidence of a 24-h rhythm at the beginning of the exposure to 8L:40D but the levels soon became too low to record any patterns (Fig. 4). Under the 8L:28D

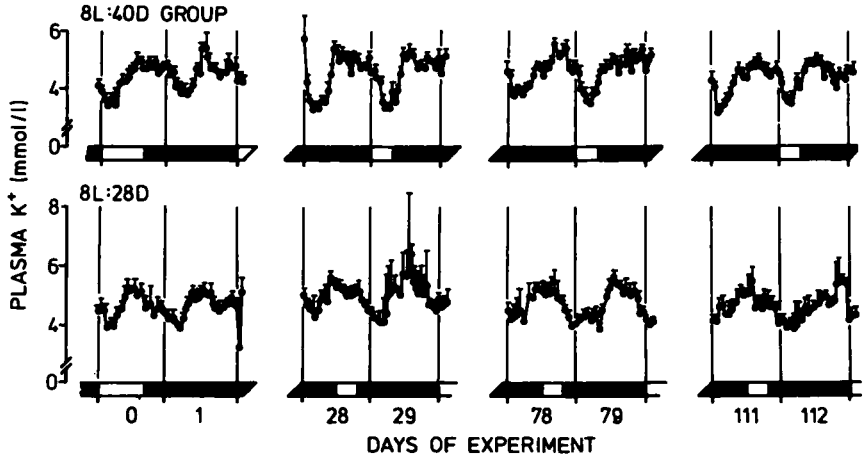

FIG. 5. Changes in the concentration of potassium (mean ± SEM) in the blood plasma from 4 adult Soay rams sampled at hourly intervals for 2 days on 4 occasions during exposure to 16 weeks of 8L:40D *(upper panel)* or 8L:28D *(lower panel)*, following 16 weeks of 16L:8D. *Day 0* is the last day of 16L:8D. The timing of the periods of light *(open bar)* and darkness *(closed bar)* is shown at the bottom of each panel.

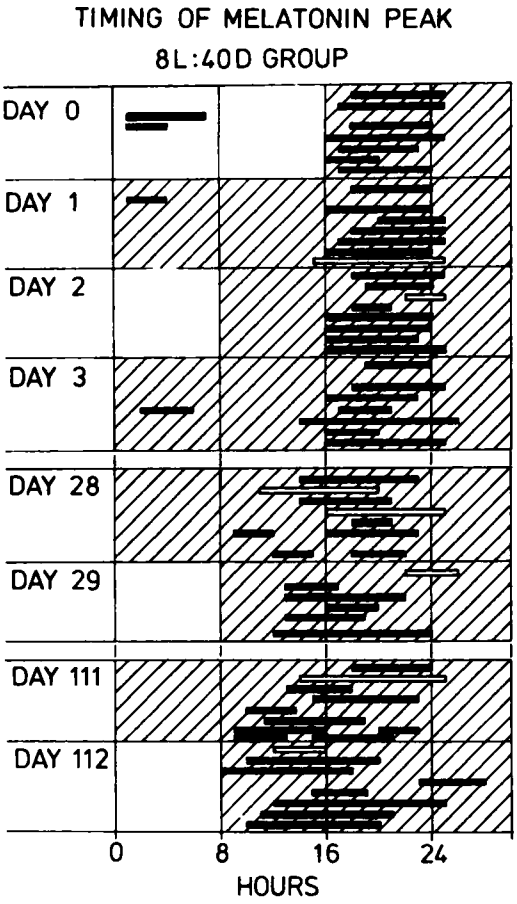

FIG. 6. Timing of peaks in the plasma concentration of melatonin in 8 adult Soay rams based on blood samples collected at hourly intervals for 2-4 days on 3 separate occasions during exposure to 16 weeks of 8L:40D, following a period of 16 weeks 16L:8D. *Day 0* is the last day 16L:8D. Each *solid horizontal bar* represents the duration of a significant peak for an individual animal (see text for definition) while an *open bar* represents a peak which does not include a significant outlier and is shown for completeness. Periods of darkness are indicated by *diagonal shading*.

regimen no regular pattern in the hourly fluctuations in the plasma levels of melatonin and prolactin was apparent, although there was a consistent 24-h rhythm in the plasma concentration of the electrolytes throughout (Fig. 5).

A detailed analysis of the rhythms in the plasma levels of melatonin is shown in Figs. 6-8 and in Table 3 which compares the results for both the 8L:40D and 8L:28D treatments. Under 8L:28D the peaks of melatonin occurred irregularly (Fig. 7), and the period between the peaks was seldom close to 24 h (Fig. 8). This is in contrast to the data for 8L:40D which revealed peaks in melatonin occurring at roughly the same time of day, albeit earlier than in the initial period under 16L:8D. The interval between peaks for the 8L:40D rams was close to 24 h (Figs. 6 and 8). The analysis of rhythms in prolactin secretion also revealed differences between the two groups, while no differences were apparent for the rhythmical changes in the plasma levels of sodium and potassium (Table 3).

Individual Variation in the 8L:28D Group

Under the 8L:28D regimen there was considerable variation between the individual rams in the timing of the testicular changes and

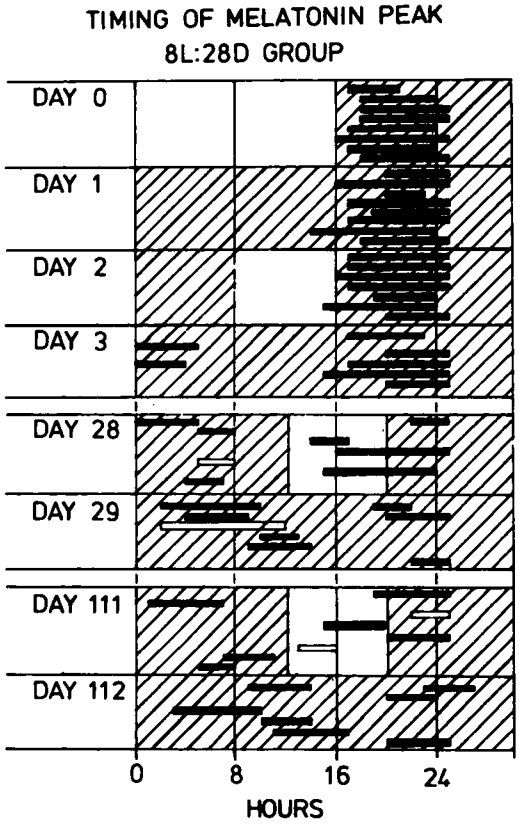

FIG. 7. Timing of peaks in the plasma concentration of melatonin in 8 adult Soay rams based on blood samples collected at hourly intervals for 2-4 days on 3 separate occasions during exposure to 16 weeks of 8L:28D, following a period of 16 weeks of 16L:8D. *Day 0* is the last day of 16L:8D. Each *solid horizontal bar* represents the duration of a significant peak for an individual animal (see text for definition), while an *open bar* represents a peak which does not include a significant outlier and is shown for completeness. Periods of darkness are indicated by *diagonal shading*.

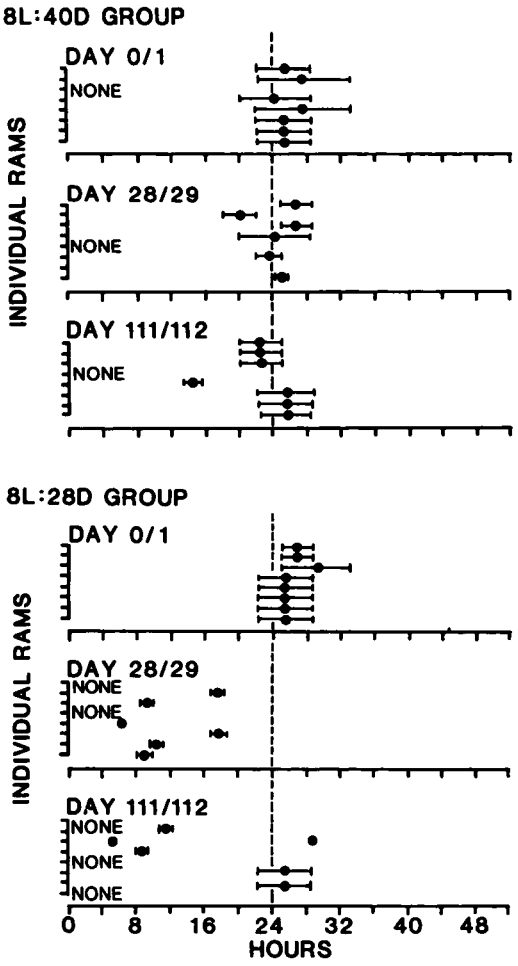

FIG. 8. Interval between significant peaks in the plasma concentration of melatonin (taken as the cycle length) based on blood samples from 8 adult Soay rams collected at hourly intervals for 2 days on 3 occasions during exposure to 16 weeks of 8L:40D *(upper panels)* or 8L:28D *(lower panels)*, following a period of 16 weeks of 16L:8D. *Day 0* is the last day of 16L:8D.

in the rhythmic patterns of hormone secretion. This allowed some correlations to be tested. There was a significant correlation between the rate of growth of the testes and the timing of the development of the sexual flush (Spearman rank correlation: $r=0.90$, $P<0.01$). There was a significant negative correlation between the timing of the increase in the size of the testes and the decrease in the 24-h mean plasma concentration of prolactin (Spearman rank correlation: $r=-0.70$, $P<0.05$), but no significant correlation with the changes in the 24-h mean concentrations of melatonin. The analysis of the hourly variations in the plasma levels of melatonin and prolactin revealed no obvious correlation between the rates of testicular growth and the rhythms in hormone secretion. An example of the melatonin profiles for the ram which showed the most rapid development of the testes and earliest sexual flush from the 8L:28D group is shown in Fig. 9 for comparison with similar data for the slowest ram in that group.

DISCUSSION

Experimental Design

The present study used artificial photoperiods to investigate the mechanism by which rams respond to changes in day length to time their seasonal reproductive cycle. Groups of Soay rams were initially exposed to long days (16L:8D) for 16 weeks to inhibit reproductive activity, and then their responses to photoperiods of 8L:16D, 8L:28D, 8L:40D and prolonged long days of 16L:8D were tested using the size of the testes and the appearance of the sexual skin flush as the reproductive indices.

With two groups of 8 rams available for the experiments, it was only possible to perform two of the lighting treatments concurrently. The 8L:28D and 8L:40D photoperiods were therefore tested initially, and then the remaining photoperiodic regimens were tested using the group of rams that had been exposed to the 8L:40D photoperiod; the other group of rams were not used since their reproductive cycles were less well synchronized after exposure to 8L:28D. The testicular response to a change from long days (16L:8D) to short days (8L:16D) in the rams from the 8L:40D treatment was very similar to that reported previously (Lincoln and Davidson, 1977), indicating that the exposure to the unusual photoperiod of 8L:40D had no long-term disruptive effect. While all four lighting treatments were not performed simultaneously, they are assumed to be comparable since they each involved an initial treatment period of long days (16L:8D) lasting 16 weeks during which the testes of the rams became uniformly regressed (Fig. 1).

Response to Different Photoperiods

The results obtained by exposing rams to long days (16L:8D) for more than 16 weeks are of particular importance in interpreting the effects of the other three treatments. When the animals were placed under long days, the testes

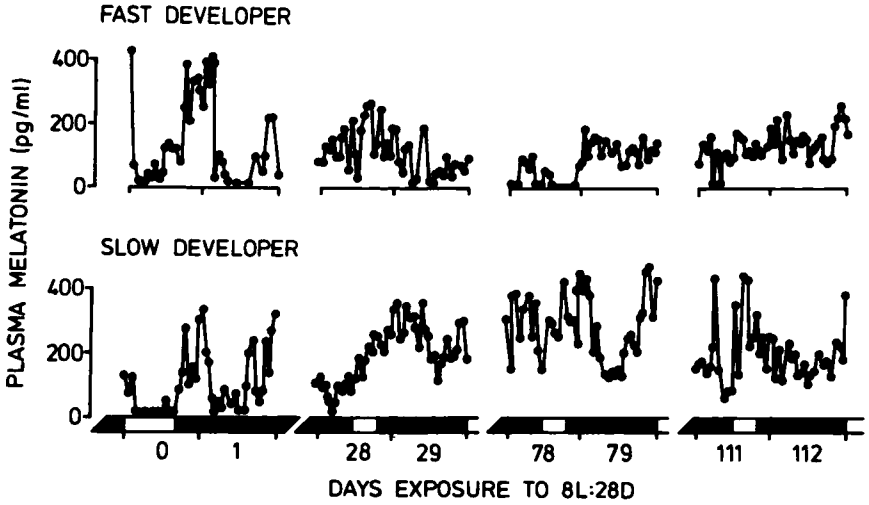

FIG. 9. Changes in the concentration of melatonin in the plasma of two adult Soay rams based on blood samples collected at hourly intervals for 2 days on 4 occasions during exposure to 16 weeks of 8L:28D, following a period of 16 weeks of 16L:8D. *Day 0* is the last day of 16L:8D. The animals were selected to represent a fast developer *(upper panel)* and a slow developer *(lower panel)* based on the rate of growth of the testes and the timing of the reappearance of the sexual skin flush. The timing of the periods of light *(open bar)* and darkness *(closed bar)* are shown at the bottom.

became fully regressed after some 12 weeks, by which time the sexual skin flush had disappeared. However, after 16-20 weeks under long days, the testes began to redevelop spontaneously and the skin flush reappeared. This result indicated that while exposure to long days acted to inhibit reproductive function, this effect was lost after some 16 weeks. Spontaneous reactivation of the testes under long days has been described previously in rams (Lincoln and Davidson, 1977; Lincoln, 1978; Howles et al., 1980), and is presumably an example of the development of photoperiodic refractoriness which occurs in other seasonal breeding mammals such as the golden, Djungarian and Chinese hamster in response to prolonged exposure to an otherwise inhibiting photoperiod (Turek and Campbell, 1979; Reiter, 1980; Hoffmann, 1981a; Bartke and Parkening, 1981).

In the present study, the three other lighting

TABLE 3. Summary of the rhythmical changes in the concentrations of melatonin, prolactin, sodium and potassium in blood plasma collected from adult Soay rams on three occasions during exposure to the 8L:40D or 8L:28D regimen. The values represent the interval between two significant peaks in the plasma profiles of an individual animal using the initial value of the peak as the reference point (see text); median values ± range for each group are shown.

		Interval between peaks (h)		
Hormone	Treatment (n)	Days 0-3	Days 28-29	Days 111-117
Melatonin	8L:40D (8)	24(20-30)	23(21-32)	25(18-32)
	8L:28D (8)	25(18-30)	21(12-52)	39(12-52)
Prolactin	8L:40D (8)	24(19-31)	24(18-25)	.....
	8L:28D (8)	25(12-32)	31(17-52)	29(10-52)
Sodium	8L:40D (4)	25(20-26)	25(23-27)	25(25-26)
	8L:28D (4)	24(19-26)	24(24-26)	23(22-23)
Potassium	8L:40D (4)	22(18-22)	25(23-27)	24(22-24)
	8L:28D (4)	23(22-27)	26(20-27)	26(23-27)

treatments involved changing the photoperiod after 16 weeks exposure to long days (16L:8D); this was at the time when spontaneous redevelopment of the testes was about to begin. Using the results obtained from the rams exposed to the prolonged period of long days for comparison, it is evident that such a switch to either 8L:16D or 8L:40D resulted in an increase in the rate of redevelopment of the testes and an earlier reappearance of the sexual flush. This stimulatory effect was not observed, however, in the animals exposed to 8L:28D after the same pretreatment period of long days (Fig. 1). Under the 8L:28D conditions, the pattern of testicular recrudescence was similar to that occurring in the rams exposed to 16L:8D for more than 16 weeks.

At the end of 16 weeks under 8L:28D, the photoperiod was changed to 8L:16D (a short day). At this stage the rams had relatively large testes, and sexual skin flush had redeveloped although there was considerable variability within the group. After the switch to 8L:16D the size of the testes was maintained, and there was an intensification of the sexual flush in all animals after 5-8 weeks (Fig. 2). This synchronized effect, occurring at the same time as the sexual flush develops after a change from 16L:8D to 8L:16D (Fig. 1), indicates that the rams responded to 8L:16D as a stimulatory photoperiod after prior exposure to 8L:28D.

The combined results show that there is not a simple relationship between the change in photoperiod and reproduction. In particular, the 8L:16D, 8L:28D and 8L:40D regimens all involved a reduction in the absolute amount of light per day compared to 16L:8D, but only the 8L:16D and 8L:40D regimens resulted in a stimulated response. Indeed, the change from 8L:28D to 8L:16D involved an increase in the absolute number of hours of light per day and yet resulted in a stimulated response. The results are therefore inconsistent with a simple hourglass model for photoperiodic time measurement and require further explanation. Our data are similar to those described by Stetson et al. (1975) working with the golden hamster. They showed that photoperiods of 6L:18D and 6L:42D were interpreted as "short" days while photoperiods of 6L:30D and 6L:54D were interpreted as "long" days; in contrast to the sheep, a long day response in the hamster is indicated by stimulation of testicular activity. The conclusion from the studies on the hamster was that the photoperiodic response involved an interaction between the photoperiodic cycle and an endogenously generated circadian rhythm in sensitivity to light.

Role of Melatonin

There is good experimental evidence from work with seasonal breeding mammals including the sheep, that the secretion of melatonin by the pineal gland plays a central role in the mechanism by which changes in the photoperiod affect reproduction (Reiter, 1980; Cardinali, 1981; Lincoln and Almeida, 1981). Results from studies on sheep have shown that the circulating concentration of melatonin changes markedly from day to night and that the timing of the daily rhythm is dictated by the prevailing photoperiod (Rollag et al., 1978; Kennaway et al., 1981; Arendt and Symons, 1981; Lincoln et al., 1982). Modification of the pattern of secretion of melatonin in rams by pinealectomy or superior cervical ganglionectomy leads to disruption of the photoperiodic control of reproduction (Barrell and Lapwood, 1979a,b; Lincoln, 1979; Arendt et al., 1981; Lincoln et al., 1982) and recently it has been shown that continual administration of melatonin also blocks the photoperiodic response (Lincoln and Almeida, 1981). These results provide good evidence that the pattern of melatonin secretion plays a causal role in the regulation of reproduction in sheep, as originally proposed by Rollag et al. (1978).

In the present study it was possible to assess the role of melatonin by measuring the hourly changes in the levels of melatonin in the peripheral blood of rams exposed to either 8L:28D or 8L:40D following a period under 16L:8D. These two lighting treatments resulted in different patterns of testicular development. Therefore if the secretion of melatonin was involved in the photoperiodic effect, differences in the patterns of secretion of melatonin would be expected in the animals from the two treatments. The results confirmed this prediction. In the rams in which the photoperiod was changed from 16L:8D to 8L:40D, the daily rhythm in the plasma levels of melatonin was evident on all occasions when blood samples were collected during the 16 weeks of the treatment. The rhythm had a period close to 24 h and was well synchronized between all animals in the group. During exposure to 8L:40D, the daily peak in the levels of melatonin occurred earlier in the day relative to the onset of the light period, compared to that under the

previous lighting regimen of 16L:8D. The change in the pattern of melatonin secretion is very similar to that observed when rams were transferred from 16L:8D to 8L:16D (Lincoln et al., 1982).

In the rams in which the photoperiod was changed from 16L:8D to 8L:28D, the daily rhythm in the levels of melatonin became modified in a way quite different from that observed under 8L:40D. Under the 8L:28D photoperiod there was a loss of the 24-h periodicity in the levels of melatonin. The peaks of secretion occurred at irregular intervals with no obvious relationship to the time of day or occurrence of the 8-h period of light. Furthermore, there was little consistency in the patterns for the individual animals in the group.

The differences in the plasma profiles of melatonin following exposure to the two artificial lighting regimens can be correlated with the differences in the testicular response. Thus, the maintenance of a 24-h rhythm in the plasma levels of melatonin, with a change in its phase so that peak values occurred earlier relative to the onset of the light period as observed following the change from 16L:8D to 8L:40D, was correlated with photoperiodic stimulation of testicular activity. The same modification in the pattern of melatonin following the change from 16L:8D to 8L:16D (Lincoln et al., 1982) was also correlated with stimulation of testicular activity (e.g., Fig. 1). In contrast, the arhythmical pattern in the plasma levels of melatonin as occurred during the 8L:28D regimen was correlated with a lack of stimulation of testicular development.

Circadian Rhythms

The way in which the rhythms in the plasma levels of melatonin were affected by the 8L:28D and 8L:40D photoperiods can be interpreted by assuming that the daily rhythm in melatonin is generated endogenously, and unless affected by the prevailing light cycle, has a period close to 24 h; i.e., it is a circadian rhythm. Any photoperiodic cycle repeating itself every 24 h (e.g., 16L:8D or 8L:16D) or a multiple of this (e.g., 8L:40D) would be effective in entraining the rhythm since the cues from the light cycle would come at a consistent time relative to the endogenous circadian rhythm. The effect would be the expression of a rhythm in melatonin with a period close to 24 h even if the photoperiodic cycle had a period of 48 h (e.g., 8L:40D). If light acts as the time cue, then the duration of the light period would affect the phase of the rhythm. These predictions are consistent with the results from the rams exposed to 16L:8D, 8L:40D and 8L:16D (Figs. 3, 6 and 7; Lincoln et al., 1982).

Under a photoperiodic cycle with a period grossly different from 24 h or a multiple thereof (e.g., 8L:28D with a cycle of 36 h), the circadian rhythm controlling melatonin will have to adjust to receiving cues from the light cycle coming at different times. The response under these conditions would be unpredictable. In the present study, the exposure of rams to the 8L:28D photoperiod resulted in a breakdown of 24-h rhythmicity in the plasma levels of melatonin and there was much variation in the patterns shown by the individual animals. There was some evidence of a rhythm in melatonin with a period close to 18 h on Days 28 and 29 of the treatment (Fig. 8), suggesting that the 8L:28D cycle causes two cycles to be produced in the space of 36 h for a time. A consistent relationship between the melatonin profiles and the photoperiod was not obvious, however, and there were occasions when peaks in melatonin occurred during the 8-h light period, a feature seldom observed under ordinary 24-h light cycles.

Model for the Photoperiodic Response

The results of these experiments are consistent with a circadian model for photoperiodic time measurement, with melatonin acting as one of the principal hormones in the relay of photoperiodic effects upon reproduction. Melatonin is believed to influence the release of luteinizing hormone releasing hormone, dopamine and other factors from the hypothalamus which regulate the secretion of luteinizing hormone, follicle-stimulating hormone and prolactin from the anterior pituitary, and thus influence the gonads (Reiter, 1980; Lincoln and Short, 1980; Cardinali, 1981). When a comparison is made between the melatonin profiles of rams exposed to 8L:40D and 8L:28D, it is evident that differences exist in the timing of the peaks of secretion but not in the overall 24-h mean concentrations which increased in both groups, or in the duration of the daily bouts of secretion (Figs. 6 and 7; Table 2). Similarly, a comparison of melatonin profiles under 16L:8D and 8L:16D show a difference in the timing of the onset of the period of melatonin secretion rather than differences in the 24-h mean concentration or the duration of the

bouts of secretion (Lincoln et al., 1982). These results emphasize the probable importance of the *timing* of the rhythm in melatonin release in producing a short-day or long-day reproductive response.

To explain why the same concentration of melatonin produced at different times of day has totally different effects, it is necessary to assume that there is a rhythm in sensitivity to melatonin. This feature has not been studied in the ram, but there is good experimental evidence from work with other seasonal breeders including the golden hamster (Tamarkin et al., 1976), Djungarian hamster (Hoffmann, 1981b) and ferret (Herbert, 1981; Carter et al., 1982) that injections of melatonin given at different times of day produce different effects on the activity of the gonads. This applies to pineal intact animals as well as to pinealectomized animals in which the complication of the endogenous secretion of melatonin has been removed. If such a rhythm in responsiveness to melatonin occurs in the ram, it is logical that the photoperiodic response depends on the way the light cycle entrains the rhythm in the release of melatonin in relation to the rhythm in responsiveness to melatonin (an example of internal coincidence; Pittendrigh and Minis, 1964).

In order to investigate this internal coincidence model it is necessary to be able to measure physiological parameters which reflect the changing activity of the hypothalamus related to the time of day. With this aim, we measured the hourly changes in the plasma levels of prolactin and electrolytes in the rams exposed to the 8L:40D and 8L:28D photoperiods; these parameters are indirectly regulated by the hypothalamus. The results proved difficult to interpret, however, since the overall plasma concentration of prolactin decreased to a low level during the experiment in both groups (albeit faster under 8L:40D) preventing the detection of any possible rhythm in prolactin levels related to time of day. Even at the beginning, when the prolactin levels were high, the daily rhythms were not very distinct (Fig. 4). In the case of the measurement of electrolytes, the daily rhythms were clearly evident and consistent between animals but they were similar in the rams exposed to both 8L:40D and 8L:28D, and did not change during the experiment (Fig. 5). At the present time it is not possible to use these results to develop the model for photoperiodic time measurement in sheep and this must await more detailed studies on the control of circadian rhythms in the activity of the hypothalamus and the consequent effects.

Conclusion

The results of this study indicate that the mechanism by which photoperiod affects testicular activity in the ram involves the generation of circadian rhythms in hormone secretion and the way different photoperiods affect the expression of these rhythms. The control of melatonin secretion is considered of central importance since it appears to relay the influence of photoperiod by acting at a specific time of day to affect the hypothalamic-pituitary axis and reproduction.

ACKNOWLEDGMENTS

We are grateful to Norah Anderson and Rhona Cunningham for their help in these experiments, and to Pamela Warner for her statistical expertise. The time series analysis was kindly performed by Mr. J. Craigon and Dr. C. M. Howles. The prolactin and melatonin antisera were kindly provided by Drs. A. S. McNeilly and G. D. Niswender, respectively, and ovine prolactin was provided by the NIAMDD, U.S.A. O.F.X. Almeida was supported by a Medical Research Council (U.K.) Studentship.

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