Long-term rhythms of testicular volume and plasma prolactin concentrations in rams reared for 3 years in constant photoperiod

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Summary. Two groups of 6 rams were kept under constant photoperiod consisting of short days (8 h light (L): 16 h dark (D); Group S) and long days (16 h L: 8 h D; Group L) from 4 to 38 months of age. Five other rams were reared under a photoperiod representative of that occurring naturally (Group N). Testis size and plasma prolactin concentrations were obtained weekly. These data were subjected to time series analysis. The results indicated that there were persistent periodic excursions in both parameters measured. In Group N, the average cycle length for both testis volume and plasma prolactin was about 1 year and the peaks in plasma prolactin preceded those in testis volume by about 18 weeks. Rams from Group L also showed rhythmical changes in these parameters with periodicities of around 35 weeks and it is suggested that these cyclic changes may constitute true endogenous circannual rhythms; again the prolactin peaks preceded those of testis volume by about 18 weeks. Overall, rams from Group S had excursions of testis growth of a similar magnitude to those of Group L but the changes were less regular than those of Group L. Plasma prolactin was significantly lower in Group S than in Group L and there was little evidence for rhythmicity. It is proposed on the basis of the temporal relationship between peaks of prolactin and testis volume in Groups N and L, that prolactin may play a role in the timing of the reproductive cycle in the ram.

Introduction

The testes of rams maintained under constant photoperiod exhibit spontaneous cycles of development and regression. Plasma prolactin concentrations in such rams also show rhythmic changes, apparently unrelated to other environmental influences such as temperature, and this led to the suggestion that testicular growth and prolactin concentration rhythms in the ram may be endogenous, merely being entrained by the light cycle and not driven by it (Lincoln & Davidson, 1977; Howles, Webster & Haynes, 1980). This is in accord with evidence from males of a variety of species, that certain rhythmic changes in physiological characteristics normally considered to be under seasonal control continue in constant laboratory conditions. For example, rhythms in sexual activity and plasma testosterone concentrations in male rhesus monkeys (Michael & Keverne, 1971; Robinson, Scheffler, Eisele & Goy, 1975; Michael & Bonsall, 1977; Wickings & Nieschlag, 1980), testicular growth in starlings and ducks (Schwab, 1971; Assenmacher, 1974), plasma testosterone and testis weight in laboratory rats (Kinson & Liu, 1973; Mock, Kamel, Wright & Frankel, 1975; Mock & Frankel, 1978a, b) and various

physiological rhythms in chipmunks and ground squirrels (Heller & Poulson, 1970; Pengelley & Asmundson, 1974). The ram experiments mentioned above (and a number of the other studies also) were, however, of relatively short duration and did not fulfil a major criterion laid down by Farner & Follett (1966) in regard to endogenous periodicities; namely that "two consecutive accurately timed cycles under constant conditions would constitute the minimum evidence for the existence of such periodicities; more would be desirable". In consequence, the experiment described previously (Howles *et al.*, 1980), in which rams were maintained in constant photoperiod for 18 months, was continued for a total of 3 years to establish whether the rhythms in testicular growth and plasma prolactin concentrations persist and thus provide more evidence for endogenous rhythmicity.

Materials and Methods

General management of animals and routine data collection

These have already been described in detail by Howles *et al.* (1980). Briefly, two groups of 6 rams were kept under constant photoperiod consisting of short days (8 h light : 16 h dark; Group S) and long days (16 h L : 8 h D; Group L) from 4 to 38 months of age. Five other rams were reared under a photoperiod representative of that occurring naturally (Group N). Temperature was not controlled in the experimental rooms and was highest between June and August (mean range 9–19°C) and lowest (mean range 0.4–7°C) between December and March. Two rams died (N4 in Week 78 and L2 in Week 109) due to illness unrelated to the experimental conditions. The length and width of the testes was measured weekly (Tuesdays between 09:00 and 10:30 h) from Weeks 19 to 99 for Group N, Weeks 25 to 149 for Group S and Weeks 25 to 152 for Group L and blood samples were collected by jugular venepuncture immediately before each testis measurement. Samples collected between Weeks 19 and 90 for Group N, Weeks 10 and 149 for Group S and Weeks 10 and 151 for Group L were assayed for prolactin.

Prolactin assay

This was carried out as described by Howles *et al.* (1980). Group N samples were measured in random order in one assay. For Groups S and L, samples (in random order from 3 Group-S and 3 Group-L rams per assay) up to Week 75 were measured in 2 assays and samples from Week 75 in a further two. Sensitivity, expressed as the value of twice the s.d. from the binding obtained with zero concentration of prolactin was 250 pg/tube. The intra-assay coefficient of variation (CV) of duplicate pairs was 10.7% ($n = 50$). The inter-assay CV (from a standard plasma sample included in each assay) was 6.3% ($n = 5$).

Time series analysis

The testis volume and prolactin concentration measurements were examined for periodicity by autocorrelation and spectral analysis techniques (Jenkins & Watts, 1969). Data from individual rams were considered in period lengths of k intervals, the first $N-k$ values being paired with the last $N-k$ values and correlated according to the following formula in which N is the total number of data points, $\bar{x}$ is the mean of all data values, x is an individual datum value and i varies from 1 to N .

$$A_k = \frac{\sum_{i=1}^{N-k} (x_i - \bar{x})(x_{i+k} - \bar{x})}{\sum_{i=1}^N (x_i - \bar{x})^2}$$

A_k is the autocorrelation for period length k .

Large positive correlations at a particular period length when autocorrelations are plotted against their associated period lengths (a correlogram) indicate cyclicity with that particular

period. Features which are obscure in the correlogram can often be clarified by the production of a spectrogram. This requires transformation of the autocorrelations to give a spectral density estimate for a series of chosen frequencies. One of the simplest transformations of the autocorrelation function is the Fourier transform which is smoothed to remove spurious peaks. The formula used for computation of the smoothed spectral density function is:

$$\bar{R}_{xx}(l) = 2 \left[1 + 2 \sum_{k=1}^{L-1} \left(A_k W_{(k)} \frac{\cos \pi l k}{F} \right) \right]$$

Calculated at unit intervals of l between $0, 1, \dots, F$ and at a frequency $l \times \frac{1}{2F}$

where L is the truncation point that defines the bandwidth or degree of smoothing as follows:

$W_{(k)}$ the Bartlett smoothing window $= 1 - \frac{k}{L}$ when $0 \leq k \leq L$ and $= 0$ when $k > L$

and F is a constant chosen to define the frequency interval, $\frac{1}{2F}$.

The choice of which smoothing window to use makes little difference to the final spectrogram. However as the choice of truncation point does affect which peaks are considered spurious, the transformation was calculated at a number of bandwidths and scanned for consistent peaks. Such peaks were tested for significance by the method of Rahe, Owens, Fleeger, Newton & Harms (1980). This test confirmed that these peaks were representative of a significant periodicity in the data.

Examination of the raw data in Groups N and L suggested that the prolactin peak preceded that of testis volume by some 16–20 weeks. In order to determine the lag period (k) more accurately, the first $N-k$ values for the prolactin data were correlated against the last $N-k$ values for the testis volume for individual animals. A range of lags were used and the one resulting in the highest significant correlation coefficient was taken to represent the phase difference between the two parameters.

Results

Testis volume and plasma prolactin data for individual animals in Groups N, L and S are shown in Text-figs 1 and 2. Over the study period there was no significant difference between mean testis volumes of Group S ($358 \pm 22 \text{ cm}^3$) and Group L ($370 \pm 16 \text{ cm}^3$). The overall mean level of plasma prolactin was significantly lower ($P < 0.001$; ANOVA) in Group S ($21 \pm 3 \text{ ng/ml}$) than Group L ($45 \pm 4 \text{ ng/ml}$). Significant periodicities in the data as revealed by time series analysis are shown for each animal in Table 1. For Group N, cycle length was around 60 weeks for testis volume and 55 weeks for plasma prolactin concentrations. Correlation of these data for Group N indicated that peaks in plasma prolactin preceded those in testis volume by about 19 weeks (Table 2). Group L rams also underwent rhythmic changes in testis volume and plasma prolactin. With one exception, testis volume showed a periodicity of about 37 weeks. For prolactin, there was evidence to suggest that there may be two rhythms; with one periodicity of about 35 weeks and another of around 10 weeks. Correlation of these data again revealed a phase difference of about 17 weeks between a plasma prolactin peak and peak of testis volume. The range of periodicities in testis volume within rams for Group S (25 to 76 weeks) was much greater than that for Group L. In only one ram, S3, was there evidence for a rhythm in prolactin secretion. There were no significant correlations indicative of a particular phase difference between prolactin secretion peaks and testis volume for Group S.

Text-fig. 1. Testis volume (expressed as the volume of a cylinder incorporating both length and width measurements) for individual rams in (a) Group N, (b) Group L and (c) Group S.

Discussion

The results indicate that cyclicity in testis volume and plasma prolactin concentrations under constant photoperiodic conditions as suggested in the previous shorter study (Howles *et al.*, 1980) indeed persist over a long period. Treatment of data from Group N by time-series analysis revealed the usefulness of this technique for establishing the periodicity of rhythms in that a significant rhythm of around 52 weeks was found in both testis volume and plasma prolactin concentrations. That the rhythms were not exactly 52 weeks was probably due to the large confidence limits of the power spectra since there were limited numbers of data points and these analyses require large sample numbers (Williamson, 1975). There is no doubt that weekly sampling limited the analysis and, in retrospect, more frequent sampling would have been desirable. Notwithstanding this, it was considered that such an analysis offered the best means of establishing the nature of any periodicities existing in plasma prolactin concentrations and testis volume in the groups under constant photoperiod. In Group L, three consecutive, regular cycles

A question raised in some studies in which rhythms are found under constant photoperiodic conditions is whether such rhythms are innate or require some form of imprinting. For instance, observed rhythms in Sika deer under constant photoperiod were reinforced by age and to some extent depended upon whether the animal had previously experienced normal changes in photoperiod (Goss, Dinsmore, Grimes & Rosen, 1974). Also, in white-tailed deer, the presence

Table 1. Significant ($P < 0.05$)* cycles in testis volume and plasma prolactin concentrations for individual animals in Groups N, L and S with the number of data points used in the analysis (n)

Ram	Length (weeks) of significant major cycles			
	Testis volume	n	Plasma prolactin	n
Group N				
1	58	81	52	72
2	58	81	55	72
3	66	81	58	72
4	—†	59	—†	59
6	62	81	55	72
Group L				
1	38	128	35 (11)‡	142
2	38	83	None (16)	102
3	35	128	34 (10)	142
4	37	128	34 (10)	142
5	62	128	37	72
6	40	128	None (10)	142
Group S				
1	71	125	None	140
2	35	125	None	140
3	25	125	None (11)	140
4	26	125	None	140
5	37	125	None	140
6	76	125	None	140

* The limited number of data points precluded testing at a higher level of significance.

† Insufficient data points.

‡ Minor rhythms that were detectable.

Table 2. Phase differences between peaks of plasma prolactin concentrations and testis volume for individual animals (1–6) in Groups N, L and S

Phase differences ($P < 0.05$) in weeks					
Group N		Group L		Group S	
1	19	1	19	1	NS
2	23	2	18	2	NS
3	NS	3	18	3	NS
4	17	4	14	4	NS
		5	15	5	NS
6	16	6	18	6	NS

NS, not significant.

of the pineal gland was necessary for the organization of the first antler, sexual and feeding cycles of young deer, but removal of the pineal gland from mature animals caused only slight modification of established cycles (Brown, Cowan & Kavanaugh, 1978). In the current study 'remembered' pre-experience was unlikely to be a factor responsible for the rhythms since the animals received only 4 months of natural increasing photoperiod between birth and transfer to constant conditions.

An intriguing feature of the current study is the differences found between Groups S and L, with the implication that the animal is not reading long or short photoperiods as merely constant,

but in some way distinguishes between the two. That prolactin levels are high in Group L compared to Group S is not surprising since there is evidence that a circadian photosensitive phase situated some 17 h after dawn exists for prolactin secretion in rams (Ravault *et al.*, 1976; Ravault & Ortavant, 1977). A similarly timed phase in the current experiments could lead to stimulation in one group but not the other. It is more difficult to account for the existence of rhythms of prolactin secretion in the constant long-day group. One possible explanation for this phenomenon was put forward by Gwinner (1973) with the hypothesis that the phase relationship between the circadian rhythm of photosensitivity and the light-dark cycle to which it is entrained is subject to circannual variations. He stated that this model could be used to account for certain data from the starling, namely that a circannual rhythm of testicular size was found in a 12L:12D cycle but not in 13L:11D or 11L:13D cycle (Schwab, 1971). These results could be explained by assuming that for birds kept on 13L:11D the photosensitive phase was always exposed to light, while for birds kept in 11L:13D it was continuously exposed to darkness despite the changing phase angle difference. A similar model could account for differences in prolactin secretion found between groups in the current experiments. However, Gwinner (1973) pointed out that there was evidence against this model since circannual rhythms existed in birds kept in constant light, i.e. a condition in which any circadian phase is permanently exposed to light. Such constant light data are not currently available for the ram and it is not possible, therefore, to come to any firm conclusions as to why prolactin shows cyclicity in Group L but not Group S rams of the present study, although the fact that they are different is of interest. Prolactin is an important component in the complex responsible for testis growth and testosterone production in some species and is considered to be a necessary stimulating factor in the seasonal breeding cycle of the Syrian hamster (Bartke, Hafeez, Bex & Dalterio, 1978). Although there is an inverse relationship between seasonal changes in prolactin and testis size in the ram (Lincoln, McNeilly & Cameron, 1978) it has been argued that the summer peak in prolactin is necessary to prime the testis for subsequent growth and testosterone production under the influence of LH and FSH (Buttle, 1974). However, Howles *et al.* (1980), reporting on the earlier part of this study, observed that testis development occurred in Group S in the absence of a priming peak of prolactin and suggested that prolactin had no more than a permissive role in testis growth in this species. Recent reports from other workers have also questioned whether prolactin plays a priming role in testicular function in the ram (Schanbacher & Ford, 1979; Barenton & Pelletier, 1980). Certain observations in the current study allow this argument to be extended in that prolactin may even inhibit testicular function; namely rhythms in prolactin secretion and testis size persist under the constant conditions of Group L, they are of similar durations of some 40 weeks and despite the fact that the duration is shorter than that found in the normal situation (Group N) the interval between the prolactin and testis peaks is the same, the prolactin peak preceding the testis peak by about 18 weeks. These consistent time relations suggest that prolactin may exert some controlling influence in the timing of the testicular cycle. There were, on the other hand, excursions of testicular growth in Group S of the same magnitude as those in Group L, but they lacked rhythmicity between animals. Prolactin secretion was significantly lower in Group S and there was little evidence for a rhythmic periodicity of secretion. It seems possible, therefore, that in Groups N and L testis growth and regression are timed by a rhythmic prolactin secretion, with high levels of prolactin being inhibitory and low levels allowing testicular growth. With low prolactin secretion and the effective absence of a prolactin rhythm as found in Group S, testicular cyclicity proceeds but without the 'prolactin clock' and so has an irregular frequency. The evidence for a causal relationship between prolactin and testicular development to date is only circumstantial. An equally plausible hypothesis would be that no causal relationship exists, merely that prolactin and testicular rhythms are controlled by the same 'clock'. Then it can be argued that disruption of such a 'clock' by, for instance, the photoperiodic conditions under which the Group S rams were kept could result in the observed situation, namely no prolactin cycles and irregular excursions in testicular size. Further experiments are needed to establish which, if either, of these hypotheses is correct.

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