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RELATIONSHIPS BETWEEN PHOTOPERIODISM AND CIRCADIAN RHYTHMS OF ACTIVITY IN THE HOUSE FINCH

BY W. M. HAMNER* AND J. T. ENRIGHT†
Department of Zoology, University of California, Los Angeles, California

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INTRODUCTION

The problem

The research described in this article addresses itself to a question posed by Bünning (1936): is there, within an organism, a single physiological time-measuring system which is responsible for all manifestations of daily rhythmicity?

The background

Under field conditions, a vast majority of the behavioural and physiological properties of an organism show rhythmic variation with a daily repetition. Although some of these rhythms may be passive systems, driven by the external environment, an increasing body of evidence indicates that, instead, most of the daily rhythms appear to be endogenous, and are merely synchronized by the environment. Under constant laboratory conditions, the periods of the rhythms usually deviate consistently from the exact 24 hr. value seen in the field. Such rhythms are now commonly designated as 'circadian' (Halberg, Halberg, Barnum & Bittner, 1959), and an extensive literature has developed describing their general properties. The endogenous origin of daily rhythms has been appreciated for many years; the experimental analysis of rhythms in plant-leaf movements has a particularly long history (Bünning, 1960).

Many organisms, in addition to daily rhythmic patterns, also exhibit seasonal rhythms. In 1920 Garner & Allard discovered that daylength was involved in the control of seasonal floral morphogenesis, a discovery which stimulated extensive experimental work in photoperiodism and eventually led to the conclusion that daylength was directly responsible for the timing of the seasonal responses of many plants, insects and birds. Impressed by the fact that light cycles can synchronize plant leaf movements, and that daylength controls flowering, Bünning proposed in 1936 the hypothesis that 'the physiological basis of...photoperiodism lies with the endogenous daily rhythms...'. Further, he suggested that an essential step in the study of plant photoperiodism was an analysis of leaf movement rhythms, because these are an easily measured expression of the many other internal changes that are equally 'regulated by the endogenous daily rhythm'. Implicit in this hypothesis are two major tenets: (1) that a daily endogenous rhythm times the photoperiodic response; and (2) that there is *one* 'master-clock', and that an observer can therefore 'read the hands' of that clock by examining any endogenous daily rhythm of the organism in question.

* Present Address: Department of Zoology, University of California, Davis, California.
† Present Address: Department of Oceanography, University of California, La Jolla, California.

Although neither aspect of the Bünning hypothesis received much experimental support for the next 20 years, recent studies with plants have documented conclusively the first of these tenets: that an endogenous diurnal rhythm is involved in the timing of the photoperiodic responses of many plants (see review by K. C. Hamner & Takimoto, 1964). More recently a similar set of experimental techniques has demonstrated that the photoperiodic response of at least one animal, the house finch, *Carpodacus mexicanus*, depends upon an endogenous circadian sensitivity to light (W. M. Hamner, 1963, 1964).

The second aspect of Bünning's hypothesis (the 'master-clock' concept) remains largely unsubstantiated, although the utility of the proposition would be extensive if it could be validated. The nature of the photoperiodic system is such that one cannot directly observe the rhythm, but only infer, from an otherwise improbable distribution of subsequent data, that a rhythmic mechanism must have been responsible. Furthermore, such experiments are costly, both in numbers of experimental organisms and in the duration of the experiments. If Bünning's 'master-clock' suggestion is correct, it should be possible to study photoperiodic rhythms more economically and easily by observing another, more direct manifestation of that clock.

Clearly an animal in a natural environment behaves as a temporal unit; the rhythms of locomotor activity, of feeding, of body temperature, etc., show fixed phase relationships with each other, and in many instances it is probable that these fixed phase relationships represent a rigid coupling of functions within the organism. In fact, the literature on endogenous rhythms is replete with references to *the* biological clock, conveying (by implication rather than by experimental evidence) that an organism possesses a single, discrete 'master timer' which serves to drive all those rhythmic functions which have daily periods.

It is also conceivable, however, that there are several—or many—physiological systems in higher metazoa which can show quasi-autonomous oscillations. If these hypothetical multiple oscillatory systems were to be completely independent, it would seem necessary to postulate that under natural conditions the normally fixed phase relationships between rhythms are affected by separate environmental synchronization of each of the several systems. Hence, this alternative hypothesis seems more complex than the single-clock hypothesis, but evidence bearing on the question is scanty.

Suggestive evidence *against* a single biological clock can be found in experiments with human subjects under unusual environmental regimes (Aschoff, 1965; Lobban, 1965), in which certain physiological functions apparently may become uncoupled from each other. These lines of evidence, however, deal with phenomena of no clear ecological relevance, and such instances may not represent a reasonable test of the Bünning hypothesis, which was, in fact, advanced for photoperiodism.

A more appropriate kind of evidence was obtained by Hoffmann (1960), who examined the ecologically relevant question of whether there is close coupling between circadian rhythms in sleep-wakefulness, and in time-compensated celestial orientation. He concluded, on the basis of experiments with starlings, that orientation and locomotion are probably both functions of a single time-measuring system. Extensive replication would make this interpretation more compelling, but the results, for the two animals studied, do indeed present a remarkable correlation.

Three experimental approaches relating to photoperiodism have also been published, but none of them seems to us to provide a conclusive answer. Bünsow (1960) has demonstrated certain interesting correlations between rhythms in petal movement and photoperiodic responses under several light–dark cycles. Menaker (1965) has found that certain of his observations on testicular regression in the house sparrow could be most easily interpreted as indicating that locomotor activity provides an index for the phase of a photoperiodic rhythm which he thought might be responsible for testicular regression. In contrast, Pittendrigh & Minis (1964), working with a photoperiodic insect, were not able to demonstrate a consistent and significant relationship between the circadian rhythm of egg-laying and a hypothetical circadian photoperiodic rhythm. Nevertheless, the logical elaboration of the Bünning hypothesis which Pittendrigh has emphasized was important for the design and interpretation of certain experiments which we have undertaken.

In this article we describe a group of thirteen experiments which have been performed in order to determine to what extent the circadian locomotor activity rhythm of the house finch is coupled with the circadian rhythm which underlies testicular maturation. The observations reported include data on testicular responses of 105 birds and a total of 2836 daily activity recordings (1300 m. of recording).

Materials and methods

Male house finches were captured, handled, and maintained as previously described (Hamner, 1966). The experimental animals were provided with enough food and water for approximately 2 weeks, and supplies were then renewed during the main light period. The birds were in individual hardware-cloth cages approximately 40 × 20 × 20 cm. fitted with one perch which was centrally located and attached externally to two microswitches wired in parallel to a 20-channel Esterline-Angus event recorder. Thirteen separate experiments were conducted. Details of type of experiment, number of birds, duration of experiment, degree of isolation during experiment, and average light intensities are presented in Table 1.

Table 1

Expt. no.	Type of expt.	No. of surviving birds	Expt. began	Duration (days)	Individual isolation	Average light intensity (lux)*	
						Bright	Dim
1	LD† 6:42, light gradient	6	8. xi. 63	23	–	300	.
2	LD 6:30	15	8. xii. 63	34	–	260	0·05
3	LD 6:42	10	8. v. 64	24	–	800	0·006
4	LD 6:54	10	8. v. 64	25	–	800	0·006
5	LD 6:66	10	8. v. 64	24	–	800	0·006
6	LD 6:16	10	3. vii. 64	28	–	800	0·006
7	LD 6:20	10	3. vii. 64	28	–	800	0·006
8	LD 3:19	6	14. vii. 65	34	–	718	0·01
9	LD 3:23	6	14. vii. 65	34	–	1780	0·01
10	Free-run, Light early	5	18. xii. 64	35	+	10	0·002
11	Free-run, Light late	6	18. xii. 64	35	+	11	0·007
12	Free-run, Light early	5	3. ii. 65	29	+	45	0·015
13	Free-run, Light late	6	3. ii. 65	29	+	37	0·014

* Light intensities were measured either with a Weston model 756 photometer or a Photovolt Model 520 M photometer, with the sensor at perch level, fully exposed to the light source.

† LD 6:42 means a 48 hr. light cycle, with 6 hr. of bright light, 42 hr. of darkness (or dim light).

The birds used in these studies were not laparotomized before experimentation. However, all the birds used received at least 8 weeks of LD 6:18 (a 24 hr. light cycle with 6 hr. light, 18 hr. darkness) before experimentation; this pre-treatment is adequate to assure that the birds have small gonads, and that they are not refactory to appropriate photostimulation (Hamner, unpublished). Furthermore, pre-experimental laparotomies of laboratory-held animals with comparable prehistories convince us that testicular responses obtained in the present studies cannot be accounted for as the result of using birds with previously enlarged testes. In all cases the testes sampled from similar non-experimental animals were undeveloped, i.e. did not exceed 2·0 mg./testis. At the termination of the experiment the weight of the left testis of each bird was recorded. Thereafter the birds were released.

In Expt. 1 partial visual isolation of the birds was involved (see below). In Expts. 2–9 the birds were in adjacent registration cages, neither visually nor acoustically isolated from those receiving similar treatment. In Expts. 10–13 the birds were isolated individually in registration cages placed in ventilated light-tight boxes (inoperative refrigerators). Auditory isolation in these experiments was not complete, but we have no evidence that vocal communication between the birds, each within a separate refrigerator, affected the results.

Experiment 1

Rationale and methods

It has been reported for other species that supplementary light of very low intensities (*c.* 1 lux), administered during the dark phase of a non-stimulatory light–dark cycle, does not induce testicular maturation (Farner, 1959). Once testicular growth has been induced, however, rates of growth are somewhat dependent on intensity, within the limited range from about 1 to 100 lux; above about 100 lux growth rate seems again to be independent of intensity. Consequently, the intensity of light during the main light period of a light–dark cycle, as long as it exceeds a minimum value, is thought to be of little photoperiodic concern. However, in order to measure the activity of birds during the normally dark phase of a cycle (a requirement for our subsequent experimentation), a dim background light was needed because house finches, like most non-migratory passerines, will not usually leave a perch when in absolute darkness. The planned experimental program required, therefore, that we find a dim background light intensity which would not in itself induce gametogenesis and yet would allow the bird to move about during the 'dark' phase of a lighting cycle. Presumably, an intensity approximating moonlight would satisfy these requirements. Six birds were placed on an LD 6:42 regimen, a cycle shown previously to be photoperiodically non-inductive (Hamner, 1964), and each bird was exposed additionally to one of six continuous, dim, background light intensities.

The birds were separated by suspended layers of cheesecloth which hung between the cages, and which progressively decreased the continuous light intensity, along a gradient, initiated by a 10 W. bulb at one end of a 5 m. room. The bright light was provided by four overhead fluorescent tubes (2·4 m. long) which illuminated all cages. Should testicular growth occur under this treatment, it would indicate that the supplemental background illumination was above the threshold for gametogenetic induction, and would be an unacceptable intensity for further experiments. The LD 6:42

cycle was chosen because it would permit spontaneous locomotor activity during the dim light on alternate days.

Results and discussion

Plate 1 contains the locomotor activity patterns of the six birds, and Table 2 lists the background light intensities and the testicular weights attained. It can be seen that bird no. 1, which was exposed to the brightest of the supplementary lights, was almost continuously active with no sustained intervals of rest; this bird was the only one of the six which showed testicular maturation. The dim background lighting presented

Table 2. *Light gradient*

Bird no.	Background intensity (lux)	Left testis wt. (mg.)
1	50	11·6
2	1·5	1·0
3	0·34	0·8
4	0·20	0·8
5	0·056	0·4
6	0·031	0·5

to the other five birds did not induce testicular maturation, but did, none the less, permit locomotor activity during the 'dark' phase of the lighting cycles, activity which was broken by distinct intervals of rest. Note the marked differences in both intensity of locomotor activity and timing relationships between activity and the main-light treatments, as the background intensity decreased. The background light intensity experienced by bird no. 6 was selected as an appropriate guide-line value for use in further experiments. This light intensity seemed clearly to be below the photoperiodic threshold, and also resulted in clear-cut synchronization of the activity rhythm, with most intense activity confined to the main light stimulus and with no major phase drift during treatment. The intensity was, nevertheless, sufficient to permit appreciable spontaneous activity in the absence of additional stimulation.

Experiments 2, 3, 4, 5

Rationale

One of the lines of evidence used to demonstrate that a circadian rhythm controls the photoperiodic responses of the house finch was that light-cycles of LD 6:18, 6:42, and 6:66 (i.e. 1-, 2- and 3-day cycles) did not stimulate testicular development, whereas light cycles of LD 6:6, 6:30, and 6:54 were strongly stimulatory (Hamner, 1963). This peculiar dichotomous distribution of data is difficult to interpret unless one invokes the hypothesis that a circadian rhythm times this photoperiodic response, a hypothesis which has subsequently been confirmed by interrupted-night experiments (Hamner, 1964). If there is, as postulated in the introduction, a close linkage between the circadian rhythm controlling gonadal development and the circadian rhythm leading to locomotor activity, one should expect to find a similar dichotomous pattern of activity responses to these two sets of photoperiodically inductive and non-inductive lighting cycles. Experiments 2–5 were conducted to ascertain whether such a specific correlation exists between inductive LD regimens of 6:30 and 6:54, and the non-inductive regimens of 6:42 and 6:66.

Results and discussion

Table 3 lists the testis weights of the birds on each light regimen in the same order as their respective activity records appear in Pls. 2, 3, 4 and 5.

The testicular responses observed during these experiments correspond, in general, with those reported for three previous experiments (Hamner, 1963, 1964). The testes of the birds on the LD 6:30 cycle developed rapidly, as did the testes of the birds on the 6:54 cycle; the testes of the birds on the LD 6:42 and 6:66 cycles were significantly smaller ($P < 0.01$, Kruskal–Wallis analysis of variance by ranks). Therefore, consistent with previous observations, we conclude that light-cycles approximating a natural short-day, or whole multiples thereof (6:42 and 6:66), are sufficient to entrain the photoperiodic sensitivity rhythm without initiating testicular enlargement; in contrast, the lighting schedules of the LD 6:30 and 6:54 cycles resulted in rapid testicular development.

Table 3. *Weight of left testis (mg.)*

Bird and channel no.	Cycle duration (hr.)			
	36	48	60	72
1	49·1	1·7	79·8	36·5
2	33·0	64·0	25·0	42·8
3	39·0	2·2	0·5	11·1
4	32·6	3 8	27·5	9·7
5	32·6	3·6	38·2	2·2
6	42·7	3·8	53·1	4·2
7	*	7·3	33·2	2·1
8	41·4	2·7	75·9	1·0
9	*	2·4	29·8	3·6
10	*	4·1	35·6	5·3

In the 36 hour experiment, there was no bird registered on channel 5; the asterisks on channels 7, 9 and 10 indicate that the records of the testicular weights for these birds have been lost. Additional testicular weights from this experiment for birds from channels 11, 12, 14, 16, 18 and 19 are: 30·0: 8·8; 45·3; 33·9; 33·5 and 26·6 mg. respectively.

The testes of some of the birds on the 48 hr. and 72 hr. cycles, however, developed beyond the size which had been observed in previous similar experiments (Hamner, 1963, 1964, 1966). None of the 26 birds in these prior experiments with LD 6:42 treatment had enlarged testes, and only 2 birds in previous experiments on LD 6:66 showed testicular growth. In contrast, 6 of the 10 birds in the present 48 hr. cycle treatment, and 7 of the 10 on the 72 hr. cycle showed significant (though usually slight) testicular enlargement. We conclude that the dim background light was probably responsible for the testicular growth of these birds, since this is the only known and conceivably significant difference between these experiments and those previously conducted.

The patterns of locomotor activity shown by the birds which received 48 hr. and 72 hr. light cycles (Pls. 3, 5) are relatively uncomplicated. During the pre-treatment on LD 6:18 about half the birds showed some 'phase-lead', i.e. the onsets of locomotor activity regularly preceded the onset of the 6 hr. light treatment by an amount which varied from one bird to another, ranging from a few minutes up to $2\frac{1}{2}$ hr. During the long dark portions of the actual treatment cycles, the birds all continued to show regular waking–sleeping rhythms with intervals of perch-hopping activity

occurring about every 24 hr. At those times when locomotor activity coincided with the 6 hr. treatment, activity was intense, with frequency of perch deflexions similar to that seen during the pre-treatment regimen. Activity, when not reinforced by the bright light, was usually weaker and more sporadic, but the overall pattern is clearly that of an endogenous daily rhythm, subject to reinforcement by bright lighting.

Some of the birds showed slight changes in phase relationship to the lighting during the 24 days of treatment, but in no case are the differences between initial and final phase relationships more than 2 hr., and in most cases far less. Thus, the apparent overall average period of the activity rhythms was in all cases not more than 5 min. different from 24 hr. The simplest interpretation of such results, then, is that a 6 hr. light treatment, presented every other cycle, or every third cycle in the activity rhythms, was sufficient to synchronize the rhythmicity; that although slight adjustment of the phase relationships took place during treatment (as was also the case during pre-treatment), light cycles with periods of 48 hr. and 72 hr. produced entrainment of the endogenous rhythms to a 24 hr. period.

The patterns of locomotor activity on the 36 hr. and 60 hr. light cycles (Pls. 2, 4) are more complex, and can probably be best understood by an initial consideration of the 60 hr. cycle. As can be seen in Pl. 4, the pattern of locomotor activity was repeated at intervals of 120 hr., not 60 hr. For the first 60 hr. of the cycle, the activity pattern reproduced that seen on the 72 hr. cycle, with intense activity during the first 6 hr. lighting, and two subsequent intervals of weaker activity, beginning about 24 hr. and 48 hr. after the last onset of the lights.

The second 6 hr. light treatment within the 120 hr. activity pattern occurred about 12 hr. out of phase with preceding activity, but, nevertheless, induced intense activity for the full duration of the lights. The timing of the subsequent spontaneous intervals of activity merits special attention. Had the lighting during the normal sleeping interval had no effect on the endogenous rhythmicity, the first subsequent burst of activity should have begun some 24 hr. after the preceding spontaneous onsets of activity, i.e. some 4–6 hr. after the end of the lighting; had the light treatment initiated a new 24 hr. rhythmicity, these onsets would have been expected to occur some 24 hr. after the beginning of the light treatment. In fact, the results indicate that neither of these expectations was accurately fulfilled. Instead, the supplementary lighting some 12 hr. after onset of spontaneous activity delayed the onset of subsequent, spontaneous activity by some 2–3 hr. The next spontaneous onset then occurred some 24 hr. later; and the repeating pattern thereafter began anew, with intense locomotor activity, reinforced by the 6 hr. of light.

The critical question for the interpretation of this complex pattern of activity is whether the activity, which was induced by the second light treatment in the midst of what otherwise would have been an interval of sleep, represents an additional, full cycle of the biological oscillation, or only an exogenously evoked response which then produced a 2–3 hr. lengthening of that cycle in the rhythm. In other words, should the observations be interpreted as a sequence with approximate periods of 24, 24, 12, 15, 24 and 21 hr., or as a sequence with periods of 24, 24, 27, 24 and 21 hr.?

We prefer the latter interpretation, since it is consistent with the 'response curve' derived from other sorts of experiments with the house finch (Enright, 1965), and since it avoids the difficulties inherent in accounting for a full cycle of the biological

oscillation within 14–15 hr. (periods which do not generally occur spontaneously in circadian systems and periods for which it is commonly reported that entrainment is impossible; however, see Kavanau, 1962). This preferred interpretation implies, then, that the biological rhythms underwent 5 cycles for each 2 of the light cycles; that the light treatments, which occurred in the middle of the 'subjective night' (as measured by spontaneous activity onset) produced 2–3 hr. phase delays in the rhythm and that the alternate light treatments, which preceded the 'expected' activity onset by 2–3 hr. produced a saturation phase advance which essentially 'restarted' the rhythm at the onset of the lights.

Since no major changes in phase relationship occurred during the 24-day treatment, it is concluded that the 60 hr. light cycle effectively entrained the biological rhythm, a result accomplished by alternating phase delays and phase advances.

A completely analogous interpretation is proposed for the results of the experiment in which the birds experienced a 36 hr. light cycle. The pattern in the activity cycle was repeated at intervals of 72 hr., twice the period of the light cycle. The light treatments during 'subjective night' did not initiate new rhythms, but instead phase-delayed the subsequent activity onsets by 2–3 hr. The bright lights then came on some 22 hr. later and 'surprised' the birds (note lack of phase lead in the left portions of both Pls. 2 and 4), producing the phase advance necessary to reinitiate the pattern. It appears, thus, that both the 36 hr. and the 60 hr. light cycles entrained the rhythm to a period which, on the average, was exactly 24 hr. This was accomplished in both cases by alternately inducing phase delays and phase advances.

An unexpected further similarity of the 36 hr. and 60 hr. cycles is that in both cases the birds showed activity patterns twice the length of the lighting regimen (a 120 hr. pattern for the 60 hr. cycle, and a 72 hr. pattern for the 36 hr. cycle), patterns which were related to the phase of the *pre-treatment* lighting. These two experiments illustrate dramatically how persistent the effects of prior history can be on subsequent activity performance.

This extended discussion of the activity patterns observed in the experiments using 36 hr. and 60 hr. cycles is of considerable importance to the general theory of circadian rhythms, since certain physical and mathematical models (Wever, 1962) suggest that entrainment to light-cycles which are not integral multiples of a circadian period (3/2 and 5/2 in these cases) is not to be expected (see also Enright, 1965). For present purposes, however, the significant issue is that the patterns of locomotor activity which were observed under two photoperiodically non-inductive light cycles (three, if one includes the LD 6:18 cycle of pre-treatment) were basically similar to each other, and differed markedly from the activity patterns which were observed under photoperiodically inductive light cycles. These experiments, then, indicate an excellent correlation between photoperiodic responses and patterns of locomotor activity under certain ahemeral light cycles; the data, to this point, are consistent with the Bünning hypothesis, that these distinct temporal manifestations may be different reflections of an identical internal timing mechanism.

A note of caution can, however, be injected at this point: there was considerable variability between birds in the details of locomotor activity pattern shown under the treatment light cycles, but no significant correlation was found between these differences in locomotor activity and the variations in testicular response. For example,

bird 9 on the 72 hr. cycle, which showed the most intense activity during the dark portion of the cycle, and the greatest phase-lead (anticipation of the 6 hr. light treatment), had testes smaller than birds 1, 2 and 3, all of which were far less active. This lack of correspondence within a group, as opposed to statistically significant inter-group differences, is a difficulty we have experienced throughout in data interpretation.

22 hr. and 26 hr. cycle experiments (experiments 6, 7, 8, 9)

Rationale

Consistent within the theoretical framework of 'phase–response curves', and in accord with empirical observations on activity records of house finches from other experiments (Enright, 1965), is the following expectation: the activity of birds placed on an LD cycle of 22 hr., with a short main light period, should be completely synchronized to the light phase of the cycle, with all of the activity occurring during the light and with no phase-lead; locomotor activity of birds on an LD cycle of 26 hr., with a comparably short main light period, should be entrained to the cycle, but should 'anticipate dawn' by as much as 10 hr. In the 22 hr. cycle, birds should always experience light at approximately the same time that it had been 'expected', during the morning of their subjective day as measured from activity onset; in the 26 hr. cycle birds should repeatedly receive light late in their subjective day. If one can, indeed, 'read the hands of the master clock' by examining the activity records of the birds, and if the photoperiodic oscillation is driven by that same timing mechanism, then one would further expect that the testes of the birds on the 22 hr. cycle (light early) would remain undeveloped because these birds should never experience light during the second, stimulatory phase of the photoperiodic rhythm. On the other hand, the testes of the birds on a 26 hr. cycle (light late) should rapidly enlarge, the light falling coincident with the sensitive phase of the photoperiodic rhythm.

Recent conceptual developments in circadian rhythm theory (such as free-run, phase-shift, response-curves, entrainment, etc., see Pittendrigh & Minis, 1964), concepts not available to Bünning in 1936, suggest to us that the above interpretation, based on 'subjective circadian time', embodies both the essence of the original Bünning hypothesis and the body of more recent experimental contributions, without doing violence to either.

Results and discussion

Pls. 6 and 7 present samples of the locomotor activity patterns observed during the first experiments involving 22 hr. and 26 hr. light cycles; Table 4 presents the final testicular weights and data on phase-lead. As anticipated in the experimental design, the birds which experienced 22 hr. light cycles confined nearly all their locomotor activity (more than 99 %) to the time when the main light stimuli were on. However, the birds which experienced the 26 hr. cycle showed marked phase lead, i.e. anticipation by several hours of the onset of the lighting. The 26 hr. cycle induced long subjective days, as measured by duration of locomotor activity, with light treatment falling late in the subjective day, while the 22 hr. cycle led to a 6 hr. activity interval coincident with the lighting. Although none of the birds showed strong testicular maturation, clear growth did occur in many cases; the median testicular weight of the birds from the 26 hr. cycle was, furthermore, significantly greater than that of the

birds from the 22 hr. cycle ($0{\cdot}01 < P < 0{\cdot}05$, Mann–Whitney U test). The short duration of the experiment, coupled with the fact that several days passed before the birds on the 26 hr. cycle attained large phase-lead, may well be responsible for the fact that in those cases in which testicular growth occurred, full maturation was not attained.

Table 4

22 hr. treatment		26 hr. treatment			
				Phase-lead (hr:min)	
Channel no.	Testicular weight (mg.)	Channel no.	Testicular weight (mg.)	Cycle 10	Cycle 15
1	0·6	9	7·4	9:25	10:30
2	1·7	2	6·8	9:05	7:25
3	1·0	7	6·5	7:00	7:05
4	1·5	1	5·5	7:40	7:10
5	1·3	4	2·5	5:40	4:30
6	5·0	10	2·1	5:15	1:35
7	1·5	3	2·0	8:50	8:00
8	1·6	8	2·0	5:50	6:05
9	2·5	5	1·8	4:55	4:20
10	5·0	6	1·4	6:10	5:00

As Table 4 indicates, there was also positive correlation within the 26 hr. experiment, between phase-lead and the final testicular size attained. Note that the data for the 26 hr. group are tabulated in order of decreasing testicular weight, and that phase-lead shows a similar trend. This correlation was generally statistically significant (Kendall's $\tau = 0{\cdot}53$, $P < 0{\cdot}05$, for example, in cycle 10). In other words, there is a significant trend, within the 26 hr. experiment, for longer subjective days to be associated with greater testicular growth. It would be easy to regard these experiments as strong evidence in favour of the Bünning hypothesis, but such a conclusion ignores two anomalies: (1) within the 26 hr. experiment, the bird which consistently had the second or third longest subjective days (channel 3) had one of the smaller testes measured in this experiment; and (2) even more disconcerting is the appreciable testicular growth by two birds (channels 6 and 10) on the 22 hr. cycle. While accessory hypotheses might be invoked to explain the first discrepancy (e.g. large inter-individual differences in 'critical day-length'), we are at a loss to account for the clear testicular growth of the birds which responded during the 22 hr. cycle treatment.

The second series of 22 hr. and 26 hr. experiments was undertaken using only a 3 hr. light stimulus, with the expectation that the birds on the 26 hr. cycle would show even greater phase-lead than with a 6 hr. stimulus, and would, thereby, receive light later in the subjective day. As indicated in Pl. 8, which shows samples of the activity patterns measured on the 26 hr. cycle, this expectation was largely fulfilled. On the 22 hr. light cycle with 3 hr. stimulus (not shown), nearly all locomotor activity was confined to the times of bright lighting, as was the case when a 6 hr. stimulus was given every 22 hr. (Pl. 6). Final testicular weights are summarized in Table 5.

On the 26 hr. cycle only one bird showed major testicular growth, and again this was a striking correlation, since this was the bird which consistently showed the greatest phase-lead. Treatment was, however, continued longer in this experiment than in the first 26 hr. experiment, and we are therefore unable within the framework of the Bünning hypothesis to account for the observation that only one bird in six showed

strong response and that several birds which repeatedly received the light stimulus 10 hr. or longer after activity onset showed no appreciable testicular growth. As Pl. 8 indicates, the bird which showed major testicular growth (no. 6) had an activity pattern characterized by very intense perch-hopping at the time of spontaneous activity onset, but in terms of the timing of the stimulus within the subjective day, it is difficult to accept that the differences between that bird and at least three others which did not respond is great enough to account for the differences in growth. The long-day control data (Table 5) indicate that these birds were certainly not refractory to light stimuli.

Table 5

22 hr. treatment		26 hr. treatment		LD 18:6 controls:
Channel no.	Testicular weight (mg.)	Channel no.	Testicular weight (mg.)	testicular weight (mg.)
1	1·4	1	3·2	43·4
2	1·7	2	2·0	52 4
3	1·3	3	2·0	100·6
4	2·3	4	2·5	39·2
5	2·1	5	1·4	59·1
6	0·9	6	44·1	

Furthermore, had the experiment been conducted as a typical night-interruption treatment, with a 3 hr. stimulus given 10 hr. after an activity onset induced by a non-stimulatory light-cycle (e.g. 6 L, 4 D, 3 L, 11 D), strong testicular growth would have been expected in all cases (Hamner, unpublished). These experiments suggest that timing within a subjective day, which begins with a spontaneous activity onset, is not, for photoperiodic responsiveness, completely comparable with a subjective day initiated by a light stimulus. In brief, then, the Bünning hypothesis, in its simplest form, seems inadequate to account for the details of observations from the 22 hr. and 26 hr. experiments, *in spite of* statistically significant differences and correlations in the group responses.

Free run experiments (*experiments* 10, 11, 12, 13)

Rationale and methods

These experiments were designed to evaluate an alternative interpretation of our previous results. To this point, it has been assumed that only the second light stimulus of the 36 hr. and 60 hr. LD cycles (Expts. 2, 4) could have been an effective photoperiodic stimulus; that the light stimulus which produced phase-advance, occurring some 21–22 hr. after a spontaneous activity onset (Pls. 2, 4) set the phase of the activity rhythm, but was non-stimulatory for the gonads. Although this first light stimulus induced patterns of locomotor activity which seem comparable with those of non-stimulatory cycles, it is conceivable that this light (or a portion of it) also served to stimulate growth by falling very late (hours 21–24) in the preceding subjective day, rather than representing the initiation, from hour zero, of a new subjective day. This alternative interpretation might also account for the two cases (Table 4) of testicular growth under the 22 hr. cycle.

The 'free run' experiments were based on measuring subjective time in the absence of a predetermined forcing light-cycle (see Hoffman, 1960). Two groups of birds were allowed to 'free-run' for 4 days in constant dim light. Then, in one group, a 6 hr.

Table 6. *Free-run experiments* 10 *and* 11

Overall average period (hr.:min.)	Separate free-runs: Median period (hr.:min.)	Separate free-runs: Range − 2	Duration of activity; and change in duration (hr. approx.)	Intensity of activity; and changes	Phase shifts (− = advance; + = delay): Median (hr.:min.)	Phase shifts (− = advance; + = delay): Range ÷ 2	Light: Background (lux × 10^{-3})
24:15	24:40	0:55	8 (0)	Weak (0)	+0:55	1:35	0·7
23:35	23:40	0:25	17 (−6)	Weak (0)	0:00	0:35	0·6
24:30	24:40	1:10	11 (0)	Weak (0)	−0:10	1:10	2·8
24:25	24:45	1:10	11 (−5)	Strong (−)	−0:30	1:05	2·6
23:55	24:25	0:50	8 (0)	Weak (0)	−1:15	1:15	1·4
25:35	.†	.	16 (0)	Weak (0)	+3 to +6 hr.		1·1
25:10	24:55	0:30	6 (+2)	Weak (0)	+4:30	2:00	3·6
25:30	24:25	1:15	8 (+2)	Weak (0)	+3:00	3:15	3·3
24:20	24:10	0:35	12 (−3)	Weak (+)	+1:55	0:40	12·5
25:05	24:10	0:30	7 (+5)	Weak (0)	+3:20	1:50	13·0
24:20	23:40	0:35	13 (+7)	Moderate (+)	+1:45	0:55	9·5

or: 6 hr. light stimulus given on two successive days (24 hr. interval), once during treatment.
y weak activity, individual estimates of period and phase shift cannot be made reliably.
ors: one light stimulus of 11 hr. duration instead of 6 hr.; one inter-stimulus interval of 3 days rather than 4 days.
rall average period' were derived from the time difference between initial and final activity onsets, and the number of interv
thus also include any phase-shifts induced by light stimuli. Period values from 'separate free-runs' were derived from t
onsets between light stimuli; the median, ± range/2 gives an approximation of the full observed distribution of values. Roug
of activity during the first week of treatment were compared with similar estimates for the last week of treatment; '17 (−6)
lasted about 17 hr. per cycle during the first week and about 11 hr. during the last week. Intensity of activity was evaluate
last weeks of treatment; o, + and − mean respectively no change, increase and decrease in the intensity of locomotor activity
Phase shifts were measured by established methods (DeCoursey, 1961) on the first day following each stimulus; 'transients' (a
one cycle) were, as DeCoursey found for nocturnal rodents (DeCoursey, 1961, 1964), common for phase advances but not re
intensities were measured at the centre bottom of the cages with the photometer cell pointed vertically upwards, toward the lig

Table 7. *Free-run experiments* 12 *and* 13

(See Table 6 for explanation of column headings)

Overall average period (hr.: min.)	Separate free-runs: Median period (hr:min.)	Separate free-runs: Range ÷ 2	Duration of activity; and change in duration (hr. approx.)	Intensity of activity; and changes	Phase shifts (− = advance; + = delay): Median (hr.:min.)	Phase shifts (− = advance; + = delay): Range ÷ 2	Light: Background (lux × 10^{-3})
22:45	23:10	0:20	6 (0)	Weak (0)	−0:25	0:45	16
23:40	23:45	0:50	6 (0)	Weak (+)	0:00	0:55	18
23:55	24:10	0:30	6 (+2)	Weak (0)	−0:35	0:35	13
23:25	23:30	0:15	5 (+3)	Weak (+)	−0.35	0:20	14
23:25	23:15	0:25	12 (0)	Weak (+)	+1:00	1:00	12
24:00	23:55	0:35	9 (+4)	Moderate (+)	+1:00	0:30	12
23:55	23:25	0·30	13 (+4)	Strong (0)	+1:50	1:05	15
23:30	23:00	1:00	16 (0)	Strong (0)	+0:45	0:40	15
24:15	23:35	0:30	8 (+8)	Moderate (+)	+2:00	1:05	15
24:20	23:50	0:15	8 (+7)	Weak (+)	+2:00	0:15	11
23:10	22:40	1:30	12 (0)	Moderate (0)	+1 30	0:45	18

light stimulus was given early in the 'subjective day', as measured by spontaneous activity onsets, and a similar stimulus, for the second group, was given late in the subjective day. Light-early stimuli were consistently administered to begin within 90 min. after spontaneous activity onset; light-late stimuli began 12 hr. after spontaneous activity onset. According to the Bünning hypothesis, the light-early treatment should not cause testicular growth, and the light-late treatment should strongly stimulate testicular growth.

Light-early and light-late experiments were performed concurrently, using birds with identical pretreatments. Twelve birds were placed in the twelve separate treatment chambers, each with its own lighting system; assignment to 'light-early' or 'light-late' treatment was made arbitrarily before activity recording was begun. Two birds were rejected in the first free-run experiments during the first week of treatment because the animals showed little or no locomotor activity; these were replaced with birds from the pretreatment stock (LD 6:18). In each of the sets of free-run experiments one bird on the 'light-early' schedule was rejected from the experiment after 2–3 weeks of treatment. These later rejections were made on the *a priori* basis that treatment could not be continued because the overt rhythms had deteriorated to a state of continuous activity, with no clear rest time recognizable.

Results and discussion

Experimental results for the remaining eleven birds in each series are summarized in Tables 6 and 7. The testicular-weight data indicate that the 'light-late' treatment was, in general, far more stimulatory than the 'light-early' treatment. The group median testicular weights are significantly different ($0{\cdot}01 < P < 0{\cdot}05$; and $P < 0{\cdot}01$, Mann–Whitney U test), but in both Tables anomalies in testicular growth are evident, as will be further discussed below.

There was appreciable correlation in the first experiments (Table 6, light-late) between background light intensity and testicular response; and the intensity of the bright stimulus lights was in several cases less than 10 lux, and hence conceivably below the threshold for photoperiodic responses. Therefore, light intensities were adjusted before the second series of experiments. These background intensities were, in general, increased, with the intention of inducing more intense spontaneous activity; the intensities of the treatment stimuli were also slightly increased.

A brighter background intensity resulted, in general, in activity rhythms which had shorter periods, as predicted by Aschoff's rule (Hoffmann, 1965). The median free-run period for the seven birds which experienced background intensities from 0·6 to $3{\cdot}6 \times 10^{-3}$ lux ranged from 23 hr. 40 min. to 24 hr. 55 min. with a group median of 24 hr. 40 min. (Table 6); for the fourteen birds at background intensities between 9·5 and 18×10^{-3} lux, median free-run periods ranged from 22 hr. 40 min. to 24 hr. 10 min., with a median of 23 hr. 38 min.—approximately 1 hr. shorter (Tables 6, 7).

The Tables indicate that light-late treatment resulted in consistent phase delay of the activity rhythms, with some suggestion that the delay was greater in the first experimental series than in the second; light-early treatment tended to produce phase advance, but of far smaller magnitude than the delays induced by light-late. These results are qualitatively consistent with a response curve, derived by other means, for the house finch (Enright, 1965).

A thorough examination of Tables 6 and 7 will reveal a number of correlations between properties of the activity rhythms and testicular responses, but none of these seems more consistent than the general experimental prediction, that light-late is a more stimulatory treatment than light-early. Nevertheless, we are not satisfied that the outcome should be interpreted as evidence in favour of the Bünning hypothesis. The prediction was that *no* testicular growth should occur when the light stimuli fell early in the subjective day, and any testis weight greater than 2·0 mg. must be interpreted as growth. We are thus unable to account for the testicular maturation of 4 birds (1 in the first experimental series, 3 in the second) within the framework of our working hypothesis.

Pls. 9 and 10 contain four of the activity recordings obtained in these experiments, records from a light-early and a light-late treatment from each experimental series. A comparison of the light-early figures illustrates the interpretational difficulties described above: one bird showed no testicular growth, the other appreciable growth, and the activity rhythms are extremely similar. Neither bird experienced a 'subjective day', as measured by locomotor activity, of longer than 8 hr.

A large body of background information (Hamner, 1963, 1966, and unpublished) indicates that if male house finches are maintained on an LD 6:18 light cycle, the testes will remain immature, *without exception*. Those four birds which showed anomalous testicular growth in the free-run experiments received intense lighting for 6 hr., once every 4 days, at a time, relative the activity cycle, which is closely comparable with the timing under an LD 6:18 treatment. Two possible interpretations of these anomalies occur to us: either the testicular responses are the result of the background lighting; or the anomalous responses indicate that the timing of locomotor activity is an inadequate criterion by which to determine the phase of the circadian rhythm of sensitivity to light as a photoperiodic stimulus. We prefer to reject the dim-lighting argument because the intensities involved were at least one order of magnitude dimmer than full moonlight, and presumably far below the photoperiod threshold.

GENERAL DISCUSSION

The experiments described herein fall into three categories: methodological, correlational and predictive.

Methodological. Expt. 1 led to a choice of light intensities which, at the time, seemed adequate for recording locomotor activity, but not sufficient to induce gonadal growth. In retrospect, insufficient replication may have been misleading. The 48 hr. experiment, no. 3, similar to Expt. 1, can be interpreted as suggesting that dim light intensities of the order of only 0·005 lux, which is far below the apparent photoperiodic threshold of 1 lux indicated by experiment 1, allowed the strong testicular development of the testes of 1 of the birds, and testicular sizes above minimal in 9 out of 10 birds. This result is in marked contrast to the total lack of testicular development above 2 mg. from four previous separate experiments involving 26 birds on LD 6:42 cycles without additional background illumination. This contrast raises serious doubts about the validity of the concept that there is a fixed threshold intensity for photoperiodic responses. Furthermore, this finding presents a complication in the interpretation of our subsequent experiments. If, as seems to be the case, a background illumination

of only 0·005 lux can permit testicular development on a lighting cycle which is otherwise non-stimulatory, the other cases of anomalous testicular responses which we have observed *may* have been induced similarly.

Correlational experiments. The locomotor activity responses observed on the 36, 48, 60 and 72 hr. cycles were strongly correlated with the types of testicular responses which had been expected, although certain discrepancies appeared in the testicular weights which actually resulted from these light cycles (see preceding paragraph). On the whole, the results permit the conclusion that those ahemeral light cycles which reproducibly induce strong testicular growth are correlated with patterns of locomotion which are, in turn, distinctly different from those which appear under non-stimulatory ahemeral cycles.

This observed *correlation* can be interpreted by a modification of the Bünning hypothesis, substituting 'subjective dawn' for the light-on stimulus as a phase reference point. This rephrasing of the hypothesis led us to design two sorts of predictive experiments.

Predictive. The eight experiments designed to permit prediction of the photoperiodic response from the activity rhythms of individual birds were successful from a statistical point of view. However, the adequacy of the predictions for groups of birds has been marred repeatedly by individual variations which we believe should not be ignored.

Whereas the *lack* of response of three or four birds on the first 26 hr. cycle is not particularly impressive (negative evidence), the fact that two of the birds on the 22 hr. cycle did show testicular enlargement is inescapably positive evidence that at least these two birds were stimulated by a light cycle which involved a 6 hr. stimulus which occurred apparently at the beginning of the 'subjective day'. Similarly, 1 of the 6 birds in the first free-run experiment, and 3 out of the 5 in the second, showed some testicular enlargement, contrary to expectations for birds that received light early in the subjective day. Although these responses were by no means maximal, there is every reason to expect that, had treatment been more prolonged, full testicular maturation would have taken place under treatment regimens which involved presentation of the 6 hr. stimulus immediately after activity onset.

These results are, then, incompatible with the rigorous formulation of the Bünning hypothesis, unless one invokes stimulation by the background lighting as an explanation for the many anomalous responses. Since, however, the intensity used for background lighting was always less than that of full moonlight by more than an order of magnitude, this form of 'special pleading', in an attempt to rescue the initial hypothesis, makes little sense in terms of testicular responses under field conditions.

We have herein attempted to examine the 'master-clock' hypothesis by formulation of strong predictions, and by testing these predictions with large numbers of animals. We conclude that the circadian rhythms of locomotor activity and testicular responsiveness, while perhaps coupled, and closely coupled, in the field, can become phase-shifted relative to one another in the laboratory and may represent different systems entirely.

SUMMARY

1. The Bünning hypothesis proposes that many rhythmic physiological processes, including photoperiodic responsiveness, are all based upon a single, endogenous circadian time-measuring system ('die physiologische *Uhr*'). We have attempted to test this hypothesis by examining correlations between the circadian waking–sleeping rhythm of the house finch (*Carpodacus mexicanus*), and the circadian rhythm of sensitivity to light, which underlies the photoperiodic testicular responses of this species.

2. Experimental techniques included (1) comparisons of locomotor activity patterns induced by specific non-daily light cycles which stimulate gametogenesis (LD 6:30 and 6:54) with those induced by other cycles which are non-inductive (LD 6:18, 6:42 and 6:66); (2) comparisons of gametogenesis resulting from light cycles which produce large phase-lead in the activity rhythms and thereby result in photic stimulation late in the 'subjective day' (LD 6:20 and 3:23) with results from similar cycles which cause no phase lead (LD 6:16 and 3:19); and (3) comparisons of gametogenesis under free-running (unsynchronized) conditions in which a 6 hr. stimulus was intermittently administered early in the 'subjective day', with other treatments in which the same stimulus was administered late in the 'subjective day'.

3. In all experimental series, when only group responses are considered, there were clear and strong correlations between testicular growth and the patterns observed in locomotor activity. The nature of the large intra-group variability, however, convinces us that the Bünning hypothesis, as here interpreted, is inadequate to account for all the results. Either the two circadian rhythms may be independent, similar systems; or, if there is a single 'master clock', the two manifestations of this timing system are apparently not phase-locked under artificial laboratory conditions. It is not clear to us how these two alternatives are experimentally distinguishable.

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EXPLANATION OF PLATES

PLATE 1

Exp. 1: activity records of six male house finches, subjected to a 48 hr. light cycle (LD 6:42), with varying intensity of continuous background lighting. Successive 48 hr. portions of record from each bird are mounted beneath each other. Part 1 is the record from the bird which received the brightest background illumination, part 6 from the bird with dimmest background. The timing of the 6 hr. main-light treatment is indicated by vertical lines. Resulting testicular weights contained in Table 2. See text for further experimental details.

PLATE 2

Exp. 2: activity record of fifteen male house finches subjected to a 36 hr. light cycle. First 72 hr. band represents the activity of all birds during the first 72 hr. of treatment; subsequent 72 hr. sequences of treatment follow in order. The 6 hr. main-light treatment is indicated by blocks of intense activity. The first block of activity in the upper left coincided with an extension of the times of lighting during the pretreatment (LD 6:18). Two treatment errors of about 30 min. duration each are indicated by irregularities in the central blocks of activity.

PLATE 3

Exp. 3: activity records for group of ten birds during 48 hr. light cycle (LD 6:42), together with activity recorded during 20 days of pretreatment (LD 6:18). Each 48 hr. strip of recording includes the activity of all ten birds. Times of 6 hr. main-light treatment are shown by blocks of intense activity.

PLATE 4

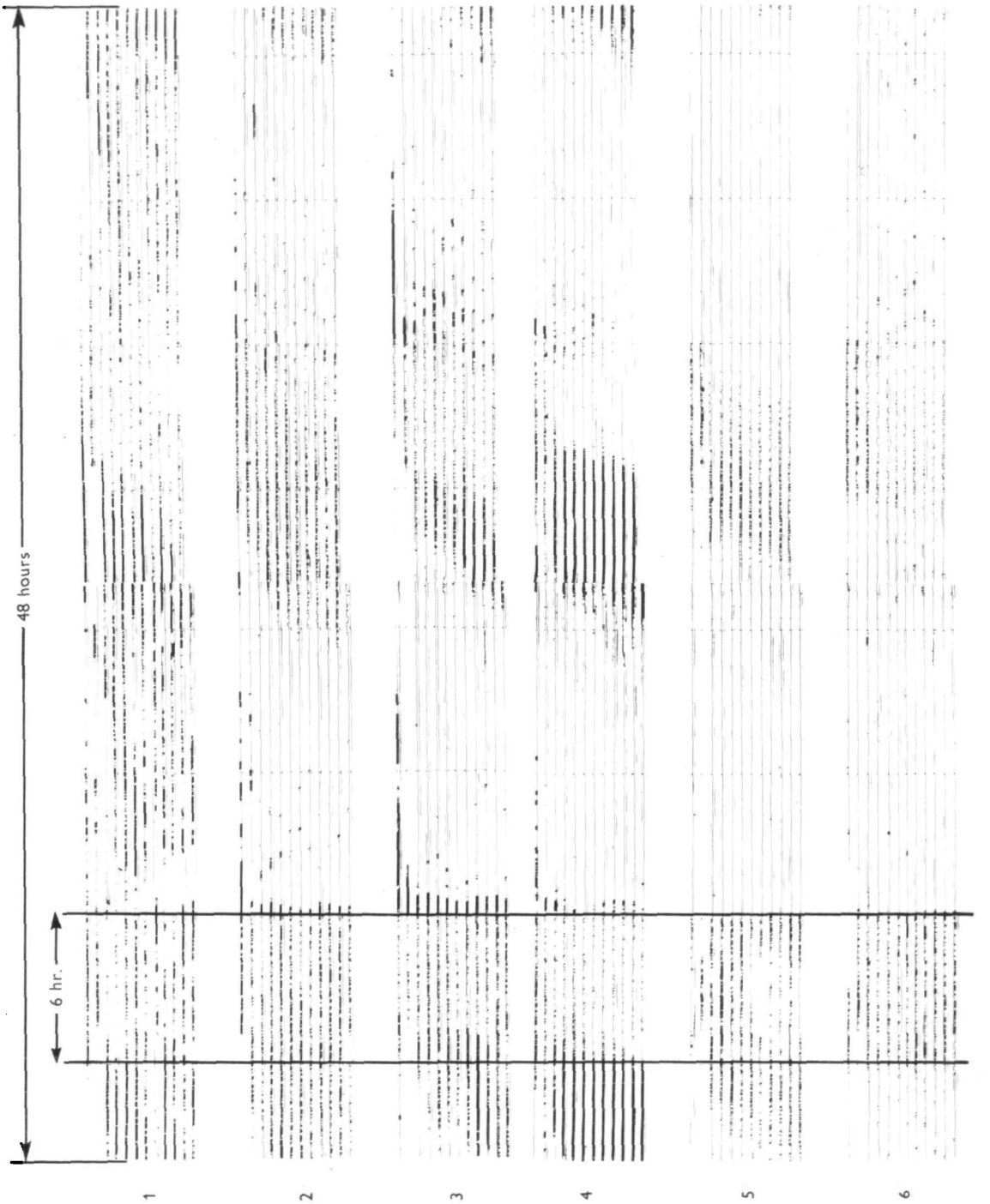
Expt. 4: LD 6:54. Recordings are mounted in sequential 120 hr. strips, together with 20-day pretreatment. The vertical lines drawn at 24 hr. intervals serve to indicate the phase-delay of the activity rhythms following alternate light stimuli.

PLATE 5

Expt. 5: LD 6:66. Recordings mounted in sequential 72 hr. strips, together with 18 days of pretreatment.

PLATE 6

Expt. 6: LD 6:16. Selected 22 hr. portions of activity recording, each for all ten birds. 'P' denotes last day of pretreatment recording; numbers to the left of other 22 hr. strips denote the cycle number, i.e. '5' denotes activity recorded during the fifth cycle of treatment.



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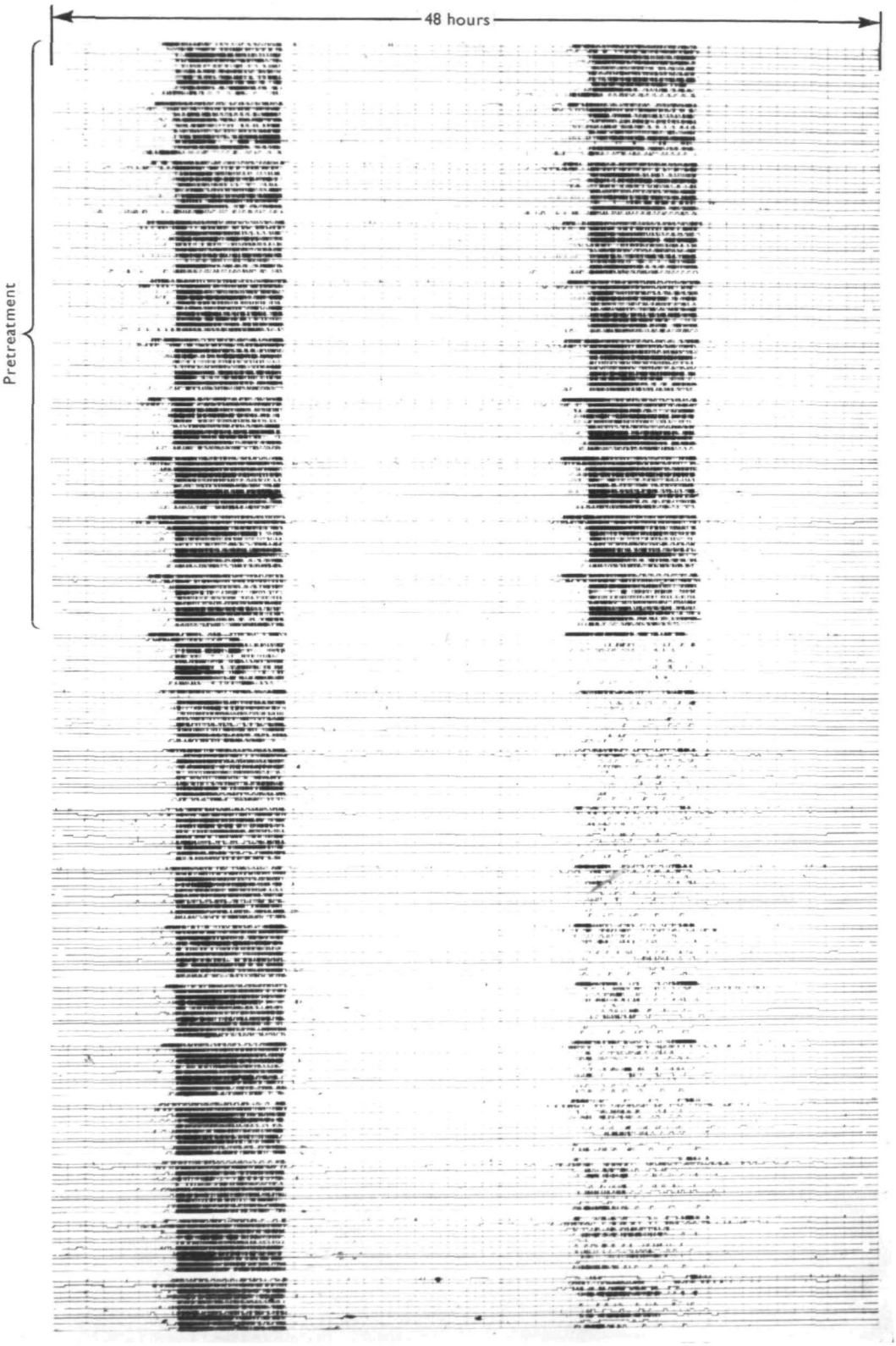

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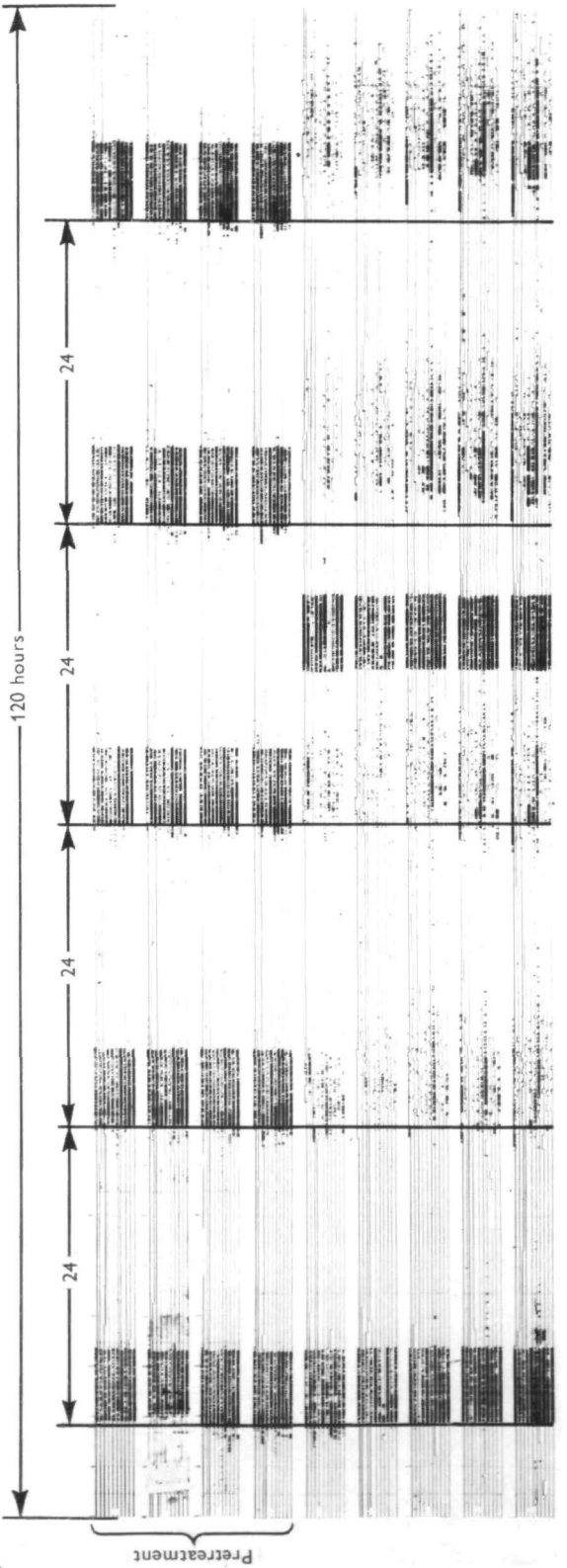

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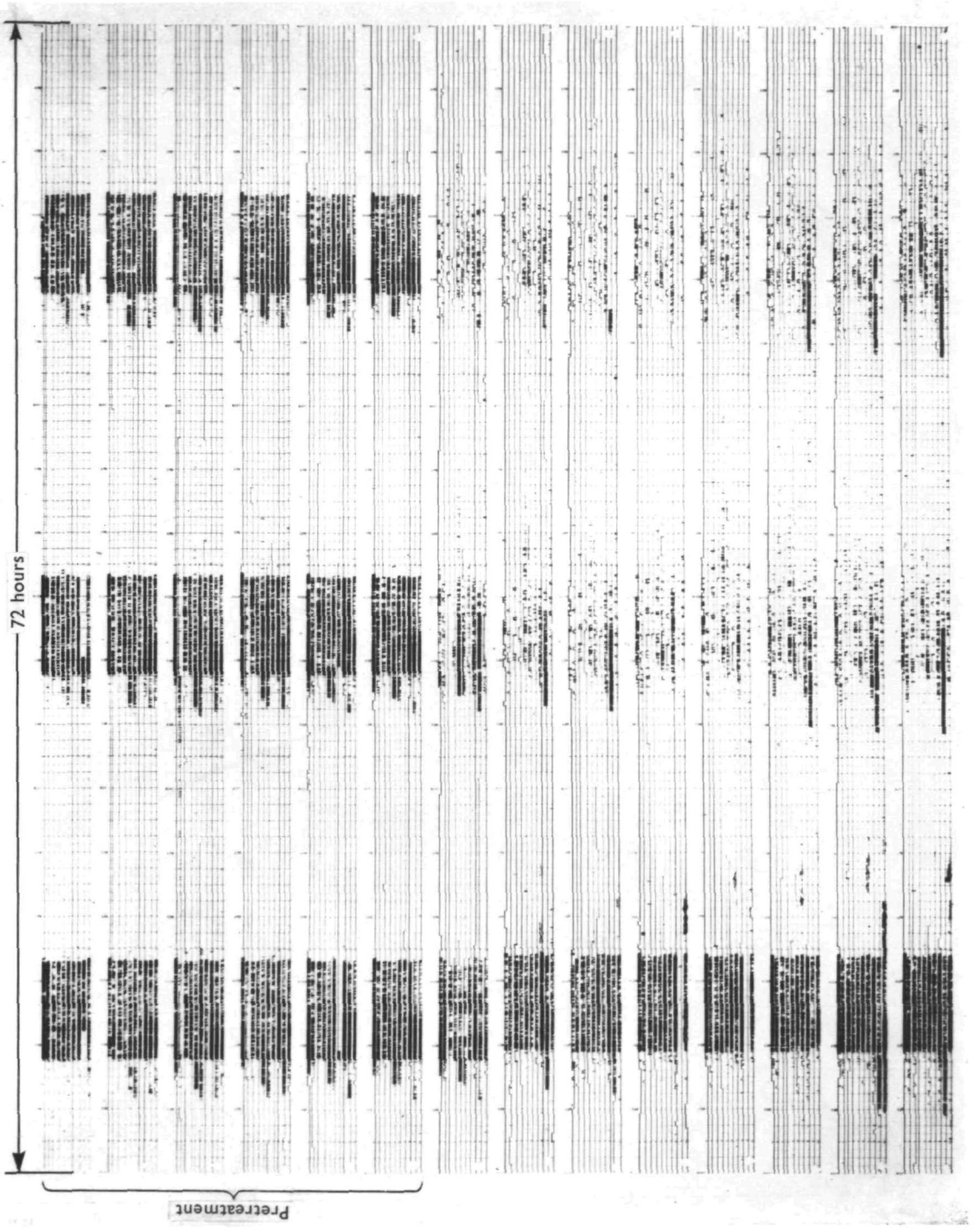

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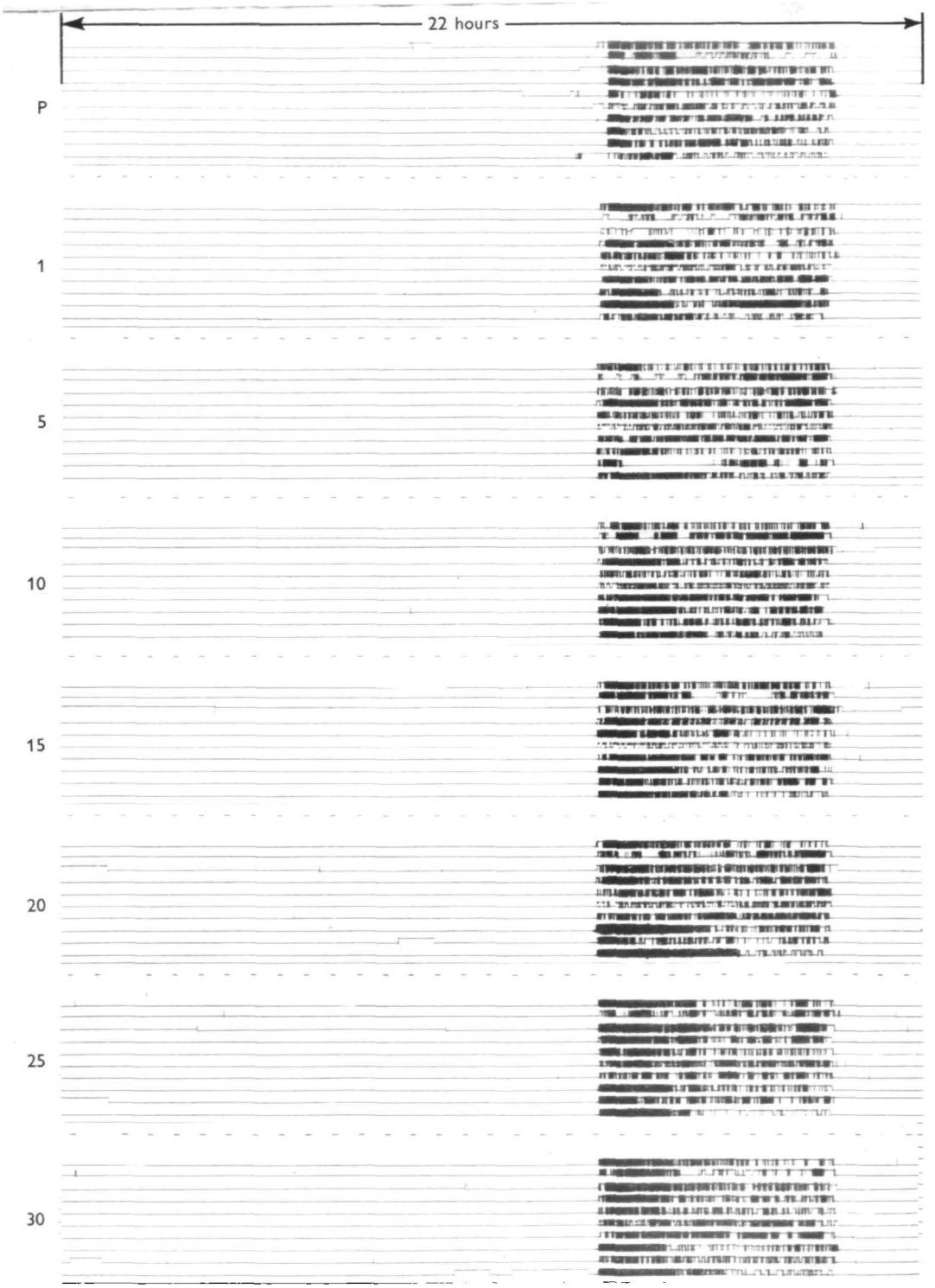
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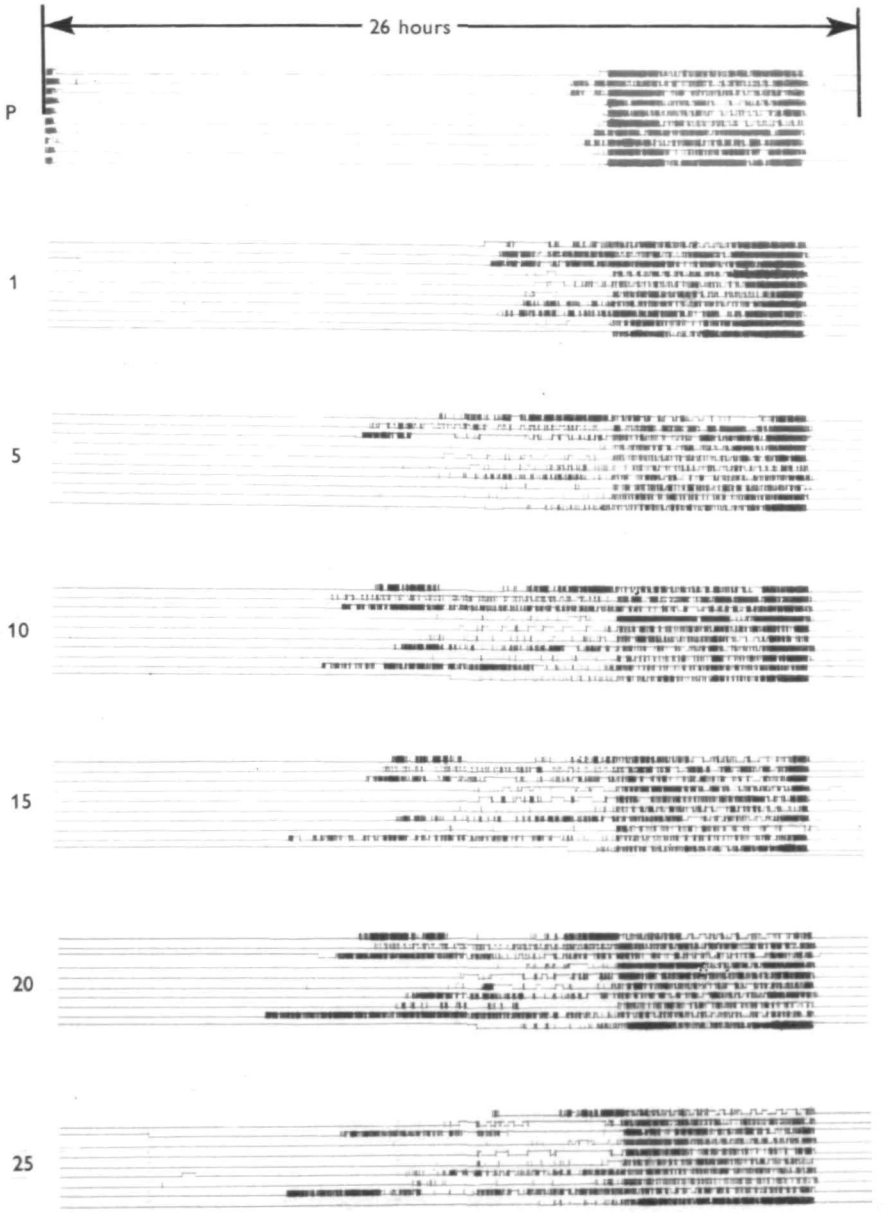
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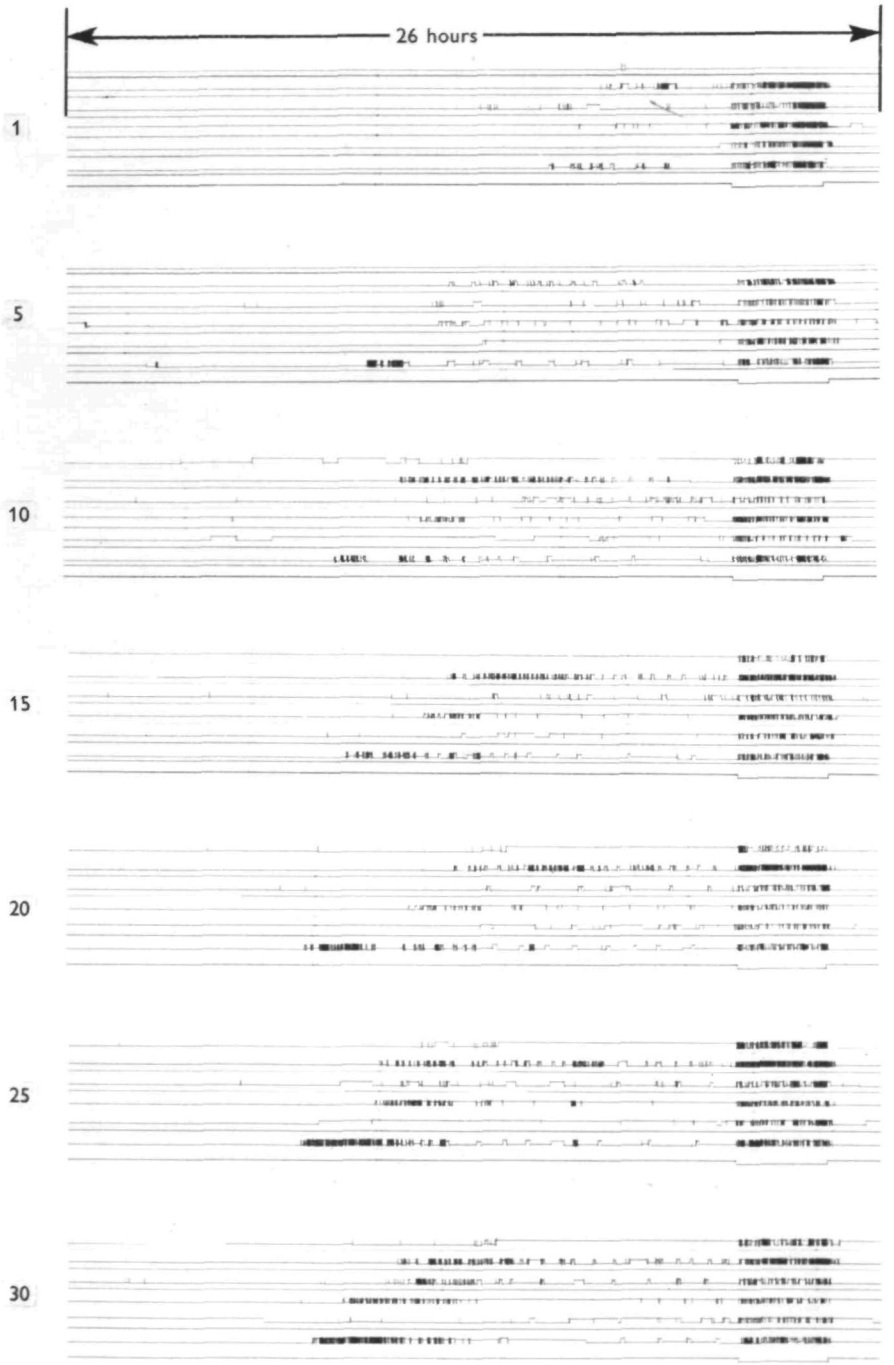
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24 hours
A
B


24 hours
A
B

PLATE 7

Expt. 7: LD 6:20. Selected 26 hr. portions of recording, each for all ten birds. Other symbols as in Plate 6.

PLATE 8

Expt. 9: LD 3:23. Recording for six birds. Other details as in Plate 6, except that light stimulus was of 3 hr. duration, indicated by blocks of intense activity.

PLATE 9

Complete activity recordings of two individual birds from light-early treatment. A, Expt. 10, third bird in Table 6; B, Expt. 12, third bird in Table 7; 24 hr. strips of recording have been mounted sequentially beneath each other. Heavy dots indicate times of activity onset; heavy horizontal bars are placed beneath activity record for duration of bright-light treatment, every fourth day.

PLATE 10

Complete activity recordings of two birds from light-late treatment; A, Expt. 11, ninth bird in Table 6; B, tenth bird in Table 7. See Plate 9 for symbols.