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Circadian Function in the Photoperiodic Induction of Gonadotropin Secretion in the White-crowned Sparrow, *Zonotrichia leucophrys gambelii*

(birds/reproduction)

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ABSTRACT **Photosensitive White-crowned Sparrows (*Zonotrichia leucophrys gambelii*) previously maintained on short day lengths (8-hr light; 16-hr darkness) were transferred to complete darkness. At various intervals thereafter (2–100 hr) they were exposed to a single 8-hr light period. To determine if the light period had been inductive, plasma luteinizing-hormone concentration was measured by radioimmunoassay in blood samples taken before and after the light period. Inductive light periods recurred at approximately daily intervals. The data provide further evidence for the involvement of a circadian rhythm in time measurement in the photoperiodic responses of birds.**

In the past decade much research has been directed towards understanding the mechanisms by which photoperiodic organisms measure the length of the day (or night) in the use of this information to time annual reproductive cycles. The major discussion has been whether this photoperiodic "clock" is based on an hourglass phenomenon, or on a circadian oscillator. The available evidence now argues for the involvement, in some manner, of a circadian rhythm in some species of plants, insects, birds, and mammals (1–11). But it also seems clear that hourglass-type systems occur in at least some species of insects (12, 13) and that the two types of timing mechanisms may not be mutually exclusive. Under certain conditions a circadian oscillator can show hourglass-like behavior (14, 15) and, in a general sense, circadian functions may be involved through their impact on the physiological well-being of the organism (16).

For photoperiodic birds, the evidence that circadian functions are involved in time measurement is considered to be strong (17, 18). It stems primarily from so-called "resonance" experiments in the House Finch (8, 22), Japanese quail (9, 17), and the White-crowned Sparrow (19, 20). In addition, Menaker and Eskin (10), from a rather different experimental approach, have presented data from the House Sparrow that are difficult to interpret unless a circadian function is invoked. The many "interrupted-night" experiments (9, 18, 19, 23, 24) are viewed as consistent with the hypothesis of a circadian component but are not conclusive. The essence of the "resonance" experiments, first introduced by Nanda and K. C. Hamner (21), is the demonstration that photoperiodic induction does not depend on a light period of specific duration but rather on the time at which light is administered. This is achieved by exposing the organism to cycles of light (L) and dark (D) in which the light period is short (6 or 8 hr) and is combined with increasing periods of darkness, for example, 6L 6D, 6L 30D, 6L 54D, and 6L 66D. Gonadal growth does not occur in those cycles that are multiples of 24 hr but does occur in the cycles of 12, 36, and 60 hr. While this argues powerfully that secretion of gonadotropin does not occur unless light coincides with a particular phase of an underlying circadian rhythm in "photosensitivity," there are drawbacks in the design. In order to obtain a measurable change in the end point, whether it be gonadal growth in a vertebrate, diapause in an insect, or flowering in a plant, it is usually necessary to repeat the light-dark schedule through several or many cycles which can lead to complex entrainment problems. Moreover, it has thus far proved impossible to show rhythmicity in "resonance" schedules longer than 72 hr. Both of these problems could be overcome with the measurement of a response that is induced by a single long day. Such became available in birds with the development of a radioimmunoassay (25) for the measurement of the plasma level of luteinizing hormone (LH). The transfer of Japanese quail from short to long days causes an increase in plasma LH after one long day and prior to a measurable increase in gonadal weight (26, 28). These findings suggested another means of testing the original suggestion of Bünning (29) that endogenous rhythms were involved in the measurement of day length in photoperiodic responses. Birds could be transferred from short days (8L 16D), in which the level of plasma LH is low, into darkness and at different times thereafter could be given a single 8-hr light period. According to the hypothesis, light should induce an increase in LH secretion, and hence a higher plasma level, only during periodically recurring intervals. Similar experiments have been carried out with species of plants in which flowering can be induced by exposure to a single long or short day (30, 31). This paper describes results obtained with the White-crowned Sparrow; experiments with Japanese quail have yielded similar results.

MATERIALS AND METHODS

Mature photosensitive male White-crowned Sparrows (*Zonotrichia leucophrys gambelii*) were used. The birds, held on short days (8L 16D) for a month prior to the first experiment, had small testes (<3 mg) and a low level of plasma LH (about 0.5 ng/ml); they were housed in pairs in a Hartshorne soundproof chamber illuminated by a 6W fluorescent lamp. Locomotor activity was monitored in each cage. Birds exposed to long periods of darkness (>24 hr) were supplied with a 10% sucrose solution which they invariably preferred to plain

Abbreviations: LH, luteinizing hormone; #L #D, cycles of light (L) and darkness (D) in hr (#).

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water. For each experiment, 16 such chambers were used (32 birds). The birds tolerated the long periods of darkness well; only four died in the series of 11 experiments. Periodic laparotomies indicated little or no testicular growth.

Blood samples (50–100 μl) were collected in capillary tubes from either a leg or wing vein. The tubes were sealed, centrifuged, and stored at −20°. All samples from each experiment were assayed together.

Plasma LH was measured by radioimmunoassay (25). The assay was developed against chicken LH (25), but it also cross-reacts with plasma and pituitary extracts from a number of other avian species (26, 28, 32). To measure the LH activity in the small volumes of plasma withdrawn repetitively from White-crowned Sparrows (body weight 25 g), it was necessary to modify the assay method slightly: 10- or 20-μl samples of plasma (or standard) were used together with 20 μl of the anti-chicken LH. Labeled chicken LH (fraction AE1, ref. 32: 3000 cpm per tube) was added in 20 μl as was the precipitating antiserum. The standard was a highly purified chicken LH fraction (AE1). Normally, it was possible to obtain at least duplicate estimates of the LH activity in each plasma sample.

In the common design used for the 11 experiments the birds were maintained on a basic regime of 8L 16D and then on the first day of an experiment blood samples were taken between hr 2 and 4 after real dawn to provide pre-experimental values for the plasma LH. On that day the lights went off as usual after hr 8. Thereafter, the birds remained in darkness until they received the "test" period of 8 hr of light. Between 7 and 16 hr after the end of this light period, blood samples were again taken. The birds were then returned to their original daily schedule of 8L 16D for 10–14 days before the next experiment. By the end of this period the LH concentration was again minimal. As far as possible, treatments were so arranged that birds receiving a "noninductive" schedule in one experiment were given an "inductive" schedule in the following experiment. The onsets of the test light periods were varied from 2 to 100 hr after the beginning of darkness (i.e., beginning 10–108 hr after the last real dawn). From six to 18 birds were subjected to each particular light treatment.

Since samples were analyzed for individual birds before and after treatment, the data were analyzed by paired "t"-tests.

RESULTS AND DISCUSSION

This test of the Bünning hypothesis required that White-crowned Sparrows show an increase in plasma LH after a single long day as had been found with Japanese quail (26, 27). This was demonstrated in three experiments with photosensitive males that had been maintained on 8L 16D for a month and then photostimulated by long days (20L 4D). In 24 birds the mean LH concentration in blood taken just after the onset of the first long day was minimal: 0.656 ± 0.087 (SEM) ng/ml. After the first long day the level in these same birds had risen to 2.016 ± 0.221 ng/ml, a very highly significant increase. For the test it was necessary to expose the birds to a light period that, in a normal 24-hr cycle, is noninductive. An 8-hr light period was selected since this consistently increased plasma LH when given in the schedule 8L 4D 8L 4D. The plasma level at the beginning of this schedule was 0.651 ± 0.077 ng/ml (16); 24 hr later it had increased to 1.433 ± 0.127 ng/ml, a highly significant increase ($P <$ 0.001). When shorter light periods of 2 or 4 hr were used, not all birds responded or the sampling schedule failed to detect responses. The increase in plasma LH caused by exposure to the uninterrupted long day of 20 hr was significantly greater ($P < 0.02$) than the response to the 8 hr of light placed 12–20 hr after dawn. This suggests that the birds are responsive to light from 8 to 12 hr after subjective dawn, an observation consistent with previous experiments on this species (34).

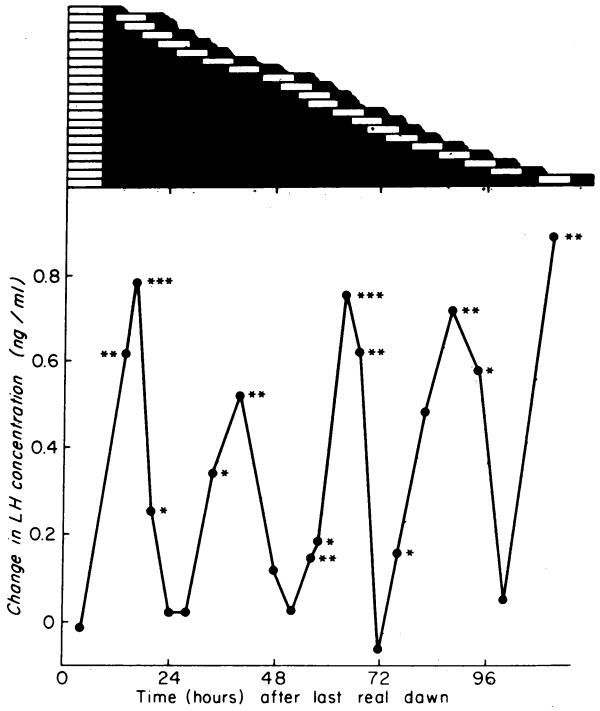

FIG. 1. The effect of an 8-hr photoperiod given at various intervals after entry to darkness on plasma LH concentration in White-crowned Sparrows. The *white* and *black* bars at the top illustrate the various treatments. Birds were previously maintained on 8L 16D and a pre-experimental blood sample was taken for all birds early in the last 8-hr light period. The post-experimental sample was taken 7–16 hr after the end of the test photoperiod. The *ordinate* shows the change in plasma LH concentration between these two samples that resulted from a particular treatment. The time after the last real dawn is shown on the *abscissa*. Full details are given in Table 1.

The responses to the 8-hr test period are shown in Table 1 and Fig. 1. Inductive periods clearly occur periodically at approximately daily intervals. The most marked contrast in the inductive effect of the light period is seen when the groups receiving light from 12 to 20 hr after subjective dawn are compared with those receiving it from 0 to 8 hr after subjective dawn. All of the former groups (Table 1, treatments beginning 12, 36, 60, 84, and 108 hr after last dawn) responded with a highly significant increase in the plasma LH. This increase was similar in the five groups, arguing that even after 100 hr of darkness the birds were as sensitive to light as on the first day. In contrast, little or no increase in LH occurred when the light period fell during the first 8 hr after subjective dawn (Table 1, treatments beginning 0, 24, 48, 72, and 96 hr after last dawn), although in the 8L 64D 8L treatment some (5 out of 18) showed a response. This was sufficient to cause a significant increase ($P < 0.05$) in the mean for this treatment. The rise was much lower, however, than

TABLE 1. *The effect on plasma LH levels in White-crowned Sparrows of an 8-hr light period given at different times after entry to darkness*

Treatment	Number of birds	Pre-experimental LH concentration (ng/ml)	Post-experimental LH concentration (ng/ml)	Change in LH concentration (ng/ml)	P*
8L 16D only	16	0.231 ± 0.024	0.216 ± 0.027	−0.015 ± 0.031	0.5
8L 2D 8L	6	0.537 ± 0.112	1.148 ± 0.115	+0.612 ± 0.104	0.01
8L 4D 8L	16	0.651 ± 0.077	1.433 ± 0.127	+0.783 ± 0.071	0.001
8L 8D 8L	8	0.248 ± 0.051	0.484 ± 0.086	+0.249 ± 0.082	0.05
8L 12D 8L	5	0.358 ± 0.036	0.380 ± 0.061	+0.022 ± 0.082	0.5
8L 16D 8L	8	0.355 ± 0.081	0.375 ± 0.054	+0.020 ± 0.033	0.5
8L 22D 8L	4	0.393 ± 0.111	0.733 ± 0.073	+0.340 ± 0.065	0.05
8L 28D 8L	18	0.434 ± 0.057	0.950 ± 0.104	+0.516 ± 0.113	0.001
8L 36D 8L	6	0.375 ± 0.085	0.488 ± 0.045	+0.113 ± 0.091	0.2
8L 40D 8L	17	0.579 ± 0.070	0.599 ± 0.087	+0.020 ± 0.051	0.5
8L 44D 8L	6	0.277 ± 0.048	0.417 ± 0.042	+0.140 ± 0.018	0.001
8L 46D 8L	6	0.362 ± 0.064	0.542 ± 0.084	+0.180 ± 0.053	0.02
8L 52D 8L	11	0.542 ± 0.140	1.206 ± 0.221	+0.755 ± 0.155	0.001
8L 56D 8L	8	0.698 ± 0.077	1.306 ± 0.159	+0.609 ± 0.118	0.01
8L 60D 8L	7	0.966 ± 0.137	0.869 ± 0.118	−0.097 ± 0.132	0.5
8L 64D 8L	18	0.574 ± 0.069	0.756 ± 0.102	+0.182 ± 0.077	0.05
8L 70D 8L	8	0.945 ± 0.106	1.403 ± 0.278	+0.458 ± 0.260	0.05
8L 76D 8L	10	0.568 ± 0.091	1.280 ± 0.165	+0.712 ± 0.199	0.01
8L 82D 8L	7	0.640 ± 0.092	1.204 ± 0.234	+0.564 ± 0.217	0.05
8L 88D 8L	5	0.794 ± 0.078	0.842 ± 0.161	+0.048 ± 0.164	0.5
8L 100D 8L	8	1.164 ± 0.260	2.048 ± 0.454	+0.884 ± 0.221	0.01

* Statistical comparisons were made between the pre- and post-experimental LH concentrations using a paired Student's "t"-test. The LH concentrations and the change in LH concentration are shown as Mean ± SEM.

that found with the 8L 76D 8L treatment ($P < 0.01$). Although all possible 8-hr light periods were not tested, Table 1 includes a number of treatments given at other times in the circadian cycle. Some of these gave intermediate responses that were significant but somewhat smaller than those seen with light pulses 12–20 hr after subjective dawn. For example, a light period falling 6–14 hr after subjective dawn (Table 1, treatments beginning 30, 54, and 78 hr after last dawn) caused a significantly smaller increase than the comparable light period begun 4 hr later. No increase occurred in the three groups in which the light period began 20, 44, and 68 hr after the last dawn. Here, the light period covered the last 4 hr of one "day" and the first 4 hr of the next.

The results clearly support the view that a circadian oscillator is somehow involved in photoperiodic time-measurement in the White-crowned Sparrow. The effectiveness of a short light period in inducing an increase in plasma LH recurs with a period of about 24 hr throughout at least five circadian cycles. This design provides a much stronger test of the Bünning hypothesis in birds than has been possible previously. This arises primarily because the re-entrainment problems due to the inductive pulse have been eliminated by using it once only. The results also go beyond the earlier "resonance" experiments (8, 9, 19, 35) where responses to three cycles only could be demonstrated. Although not too much reliance can be placed on the activity records, since each cage contained two birds, they did indicate a recurrence of perch-hopping in darkness with a periodicity of about 24 hr. In no case was it possible to determine a free-run period, but the results are consistent with the maintenance of circadian functions throughout the prolonged dark periods. A normal pattern of activity was rapidly re-established after return to 8L 16D. No attempt was made to determine the duration of the cyclic sensitivity to light in prolonged darkness. In a true free-run situation, the synchronization within a population on which this experimental design depends would soon be lost as the phase-angle differences become progressively greater. Obviously this did not occur after five cycles, although the few that responded to an otherwise "noninductive" light period (as in treatment 8L 64D 8L) may represent cases of a significant shift in phase. In the plant *Chenopodium rubrum*, cycles could be demonstrated for 7 days but by 10 days the clear rhythmicity had been lost (30).

From these experiments a definitive statement cannot be made as to the exact position or the length of the inducible phase within the circadian cycle. Although unlikely, it is possible that instantaneous phase shifts in the inducible phase could have occurred thus distorting somewhat the curve of sensitivity to light pulses. However, it seems probable that the phase lies between 8 and 20 hr after dawn. Light periods from 0 to 8 hr after subjective dawn were ineffective while those from 4 to 12 hr after subjective dawn elicited LH release but those from 20 to 24 hr (together with 4 hr of the next "day") did not. This accords with earlier night-interruption experiments with this species (18, 19).

Most studies on birds have been interpreted in terms of the Bünning-Pittendrigh external coincidence model of photoinduction (17, 18, 29). Our data also favor this model but do not preclude the possibility that the system operates by an internal coincidence arrangement (16, 36). However the circadian system is involved, it is, nevertheless, becoming clear that the concept that the hormones themselves are released daily on a rhythmic basis is an oversimplification. Although such may occur initially, thereafter rhythmicity in plasma LH concentration is much less apparent (27) and at all times the LH level remains elevated. This finding, together with the observation that LH secretion continues for many days after birds are returned to short days, suggests that induction

may initiate processes that continue for a long period of time. In the present experiments it was noted that following an inductive light period LH release continued at increased levels for up to 6 days.

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