## Pineal Function: The Biological Clock in the Sparrow?

Abstract. *The pineal organ of the house sparrow,* Passer domesticus, *is essential for persistence of the circadian locomotor rhythm in constant conditions. Upon removal of the pineal body, activity becomes arrhythmic. However, pinealectomy does not abolish the rhythm of locomotor activity in birds exposed to light-dark cycles. Pinealectomized birds are entrained by light cycles in much the same manner as are normal birds. Our data demonstrate that the pineal organ is a crucial component of the endogenous time-measuring system of the sparrow.*

Under constant environmental conditions of light and temperature circadian rhythms persist for long periods. Most investigators interpret such persistence (without external cues) as reflecting the behavior of time-keeping machinery within the organism. Most surgical attempts to identify organs or tissues essential for circadian time-measurement have proved unsuccessful, and existing experimental support for the concept of endogenous control remains almost entirely formal. The effects of various surgical manipulations of the nervous system on the activity rhythms of cockroaches have been studied (*1*). Interpretation of much of this work is complicated by lack of criteria for distinguishing (i) between effects on the controlling system (that is, the biological "clock") and direct effects on the behavior assayed (locomotor activity) and (ii) between effects on the clock and effects on the process of photoreception. Richter (*2*), studying locomotor activity in wild rats, has destroyed the entrainment response to light by blinding the animals. However, the blind rat's circadian rhythm is retained, with a period different from that of the prevailing light-dark cycle. Removal of any of the endocrine glands of these blind rats, including the pineal gland, has no effect on the endogenous rhythmicity. Pinealectomized laboratory rats continue to exhibit a circadian rhythm of locomotor activity in constant dim light (*3*). Parallel investigations of the effects of removal of the pineal body on circadian rhythms in birds have not been reported. Previous studies of the avian pineal organ have attempted to correlate pineal function with reproductive regulation, and some slight effects have been noted (*4*).

The pineal organ of the house sparrow *Passer domesticus* is necessary for the expression of the free-running rhythm of locomotor activity in constant darkness (DD). Perching activity (*5*) in normal sparrows is invariably rhythmic in DD as exemplified by more than 200 lifelong records from our laboratory. Pinealectomy (*6*) abolishes this circadian rhythm. Fig. 1A is an example of the consistently obtained response of 32 sparrows in DD to pinealectomy (two exceptions are discussed below). An arrhythmic (*7*) pattern of activity develops 0 to 9 days after surgery in all pinealectomized birds. Such arrhythmic perching behavior persists in DD for as long as 9 months after surgery. The behavior of four pinealectomized birds in constant light (LL, about 500

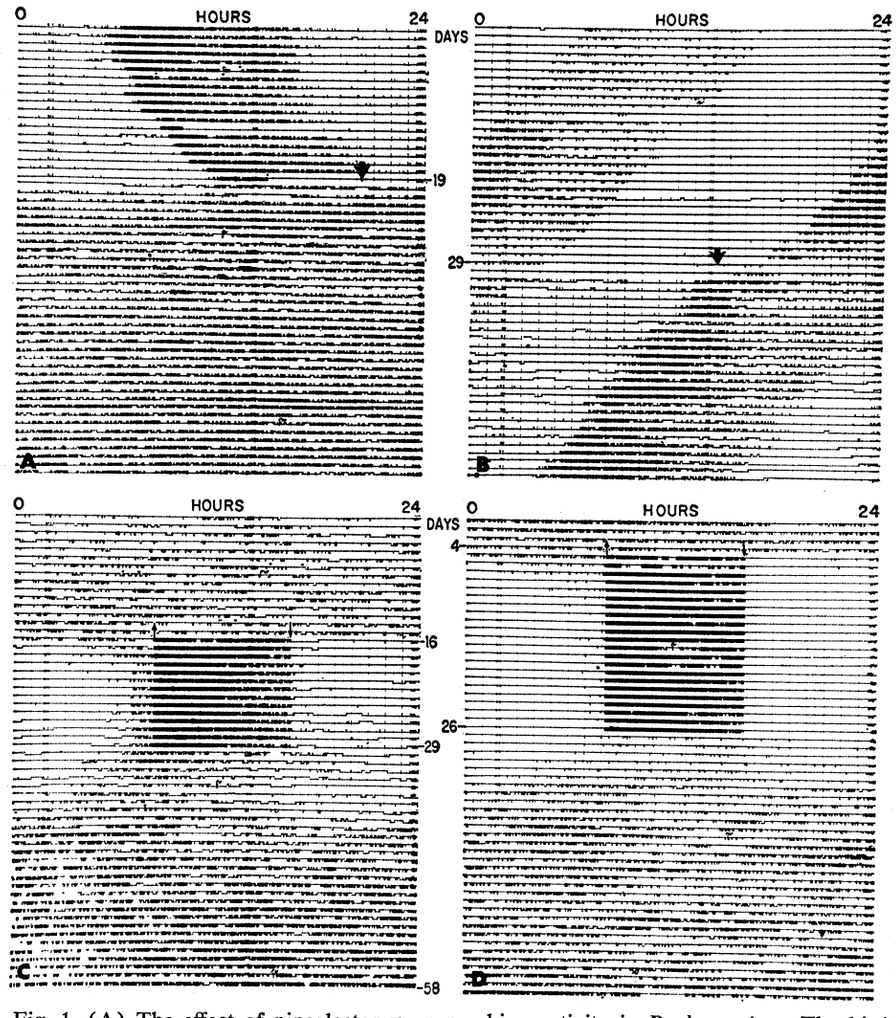


Fig. 1. (A) The effect of pinealectomy on perching activity in *P. domesticus.* The bird is in DD and constant temperature (about 23°C) throughout the record. On day 19 the pineal organ was removed as indicated by the large arrow. Within 2 days after the operation no circadian rhythm is discernible. (B) The effect of a sham pinealectomy on the rhythmic activity of a bird in DD. The operation was performed on day 29 (large arrow). The free-running period before the operation was about 23 hours 38 minutes; subsequently it was about 23 hours 40 minutes. Also note the phase shift of approximately 2½ hours resulting from the surgical procedure. C and D show entrainment patterns of two pinealectomized sparrows. The beginning and end of the daily light period are marked with arrows. The dense black bars during the light fraction indicate intense perching activity. In C, days 1 to 15 demonstrate arrhythmic activity in DD. On days 16 to 29, the bird received 8 hours of light followed by 16 hours of darkness per 24-hour period and from day 39 to 59, the bird was once again in DD. A positive phase angle of about 1 hour is evident after the 3rd day of the light cycle. After the light cycle was discontinued, about 8 days were required for this bird to reestablish an arrhythmic pattern. In D, days 1 to 4 show arrhythmic activity in DD, on days 5 to 26 the bird is on LD *8* : 16; and on days 27 to 59 the bird was in DD. Notice the "decay" of rhythmicity on days 27 to 33, illustrating the transition to arrhythmicity more clearly than does C. The pattern of this decay, with activity onsets occurring earlier and activity terminating later each day is characteristic of pinealectomized birds released in DD from LD entrainment.

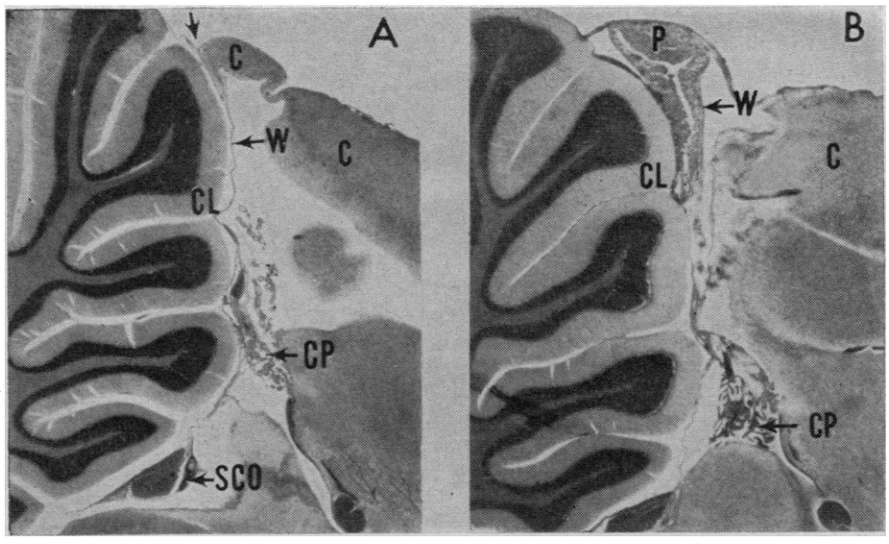


Fig. 2. Sagittal sections through the brains of (A) a pinealectomized sparrow, and (B) a sham-operated sparrow, at equal magnifications. The unlabeled arrow in A designates the site from which the pineal body was removed. *C*, cerebrum; *CL*, cerebellum; *P*, pineal organ; *CP*, choroid plexus of the third ventricle; *W*, posterior "wall" of the cerebrum; and *SCO*, subcommisural organ (in B the section is lightly lateral to the midsagittal subcommissural organ).

lux) is indistinguishable from their behavior in DD and from the behavior of normal birds in LL (*8*). In all of 14 sparrows subjected to sham pinealectomy (*6*), rhythmicity persisted (see Fig. 1B); all demonstrated circadian free-running periods in DD after surgery.

The circadian locomotor rhythms of normal sparrows entrain to (are synchronized by) 24-hour light-dark cycles (*9*). Birds on short photoperiods assume a positive phase angle to the light, beginning their activity prior to the onset of the light. On longer photoperiods the onset of activity usually coincides with that of the light. In contrast to its effect on free-running rhythms, pinealectomy does not abolish the entrainment response to light circles. Data on three pinealectomized birds entrained to LD *16*:8, three entrained to LD *12*:12, four entrained to LD *8*:16 (Fig. 1, C and D), and six entrained to LD *3*:21 reveal no obvious differences from the entrainment patterns of normal birds.

To be sure pinealectomy was complete and to assess brain damage to surrounding areas, we made histological (*10*) studies of the whole brain when sufficient activity data from each bird had been obtained (Fig. 2). Although some necrosis was occasionally evident in the cerebellum and in the cerebrum of some of the brains examined, the brain damage sustained by the pinealectomized animals was no greater than that suffered by the sham-operated birds. Furthermore, several of the pinealectomized birds showed no evidence of brain damage. Thus far, the brains of 16 pinealectomized birds have been examined (other birds referred to are still in experiments). Twelve had no detectable pineal tissue whereas four had traces of pineal parenchymal tissue.

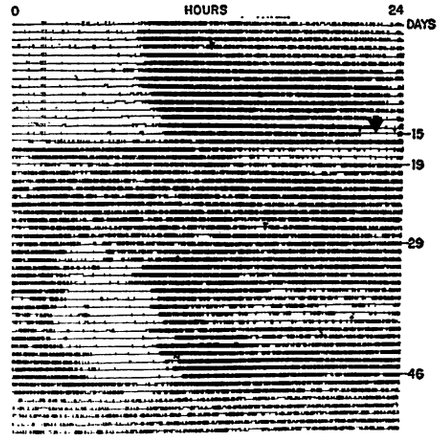


Fig. 3. Record of a bird in DD whose pineal organ was incompletely removed, as revealed by subsequent histology. Most of the pineal was removed on day 15 (arrow). Indications of remaining rhythmicity (and a very large phase shift) are evident on days 16 to 18. The bird became arrhythmic from days 19 through 29, developed a circadian rhythm from day 29 to 46, and returned to arrhythmic activity pattern after day 46.

Two of the latter four birds produced activity records in DD which showed intermittent periods of rhythmicity lasting 2 to 4 weeks preceded and followed by arrhythmic activity. These two are the only instances of rhythmicity in sparrows on which pinealectomy was attempted (Fig. 3).

Our data show that the pineal body of the sparrow is required for maintenance of its locomotor activity rhythm in constant conditions but is not necessary for the entrainment response to light cycles. We tentatively conclude that:

1) The pineal organ does not merely exert direct control over the expression of locomotor activity. Were this the case, pinealectomy would render the birds either continuously active or continuously inactive. In fact, pinealectomized sparrows are rhythmic in an LD regime, and it is clear that, under these conditions, the activity is not simply forced by the light cycles because pinealectomized birds are continuously active in DD but inactive during much or all of the dark portion of an LD cycle, and pinealectomized birds maintain positive phase angles to short photoperiods (that is, they begin their activity some hours before the onset of the light).

2) The pineal organ cannot represent the entire mechanism underlying circadian timing of locomotor activity. When a light cycle entraining a pinealectomized bird is discontinued, the pattern of activity "decays" rhythmically into arrhythmic activity. This pattern of gradually attained arrhythmicity occurs in the first 1 to 4 weeks of DD after entrainment. It occurs each time a pinealectomized bird is entrained to a light cycle and subsequently subjected to DD. The attainment of arrhythmicity requires a greater number of cycles in pinealectomized birds released into DD from LD entrainment than in birds pinealectomized while free running in DD (compare Fig. 1A with Fig. 1, C and D). In addition, the maintenance of positive phase angles to light cycles by pinealectomized birds indicates that some portion of the timing system remains.

3) Pinealectomy might produce in birds subjected to it the perceptual illusion that they were in constant light when in fact they were being held in constant darkness. This interpretation would account for the observed decay of the locomotor rhythm following

pinealectomy as a second order effect, no different, except in the manner by which it is achieved, from the effect of LL itself. This possibility cannot be excluded on the basis of our data, but it appears unlikely in view of the fact that pinealectomized birds can be entrained by light cycles.

4) The pineal could be a self-sustained oscillator, normally driving a damped oscillator which in turn directly controls locomotor activity. In this case, the pattern of "decay" into arrhythmicity after entrainment would reflect the damping of the remaining oscillator. On the basis of this interpretation, the entrainment response of pinealectomized birds would result from direct driving of the damped oscillator by the light cycle.

5) The pineal could be a coupling device within a multi-oscillator clock system. Two or more oscillating components of the timing machinery might be coupled, and fixed phase relationships might be maintained among them by some action of the intact pineal organ. Arrhythmicity in DD could result from their uncoupling by removal of the pineal organ and their subsequent drifting out of phase with each other. Entrainment in the absence of the pineal organ would indicate that the light cycle alone is sufficient to maintain fixed phase relationships among the component oscillators.

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### References and Notes

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5. Activity recordings were obtained under conditions previously described; M. Menaker, in *Circadian Clocks*, J. Aschoff, Ed. (North-Holland, Amsterdam, 1965), p. 386. The birds were fed about every 14 days without interruption of constant darkness.
6. Pinealectomy was accomplished as follows: After the bird was anesthetized with Equithesin, the skin on the dorsal surface of the skull was slit and pulled laterally while the head was steadied by a stereotactic instrument. A piece of skull directly over the pineal body was drilled out with a dental burr and removed. The pineal organ was excised along with the meninges to which the dorsal part of the gland adheres. The removed skull piece was then replaced and sealed with Replica plastic filling material, and the skin sutured. Large venous sinuses which surround the pineal gland and receive blood from it, as well as from other parts of the central nervous system, were necessarily cut in removing the pineal body. Sham operated birds underwent an identical surgical procedure, including the cutting into these sinuses; however, the organ was not removed. During all surgical procedures the birds were exposed to light for 1 to 2 hours, for most of which they were under anesthesia.
7. "Arrhythmicity" is provisionally used in this context, as quantitative analysis of the activity data is not yet available. The day on which onset and offset become indistinguishable is arbitrarily designated as the beginning of arrhythmicity.
8. Although published documentation is rare, it is a common observation that most circadian rhythms are not expressed in constant bright light. As the basis of this response to constant light is not understood for normal birds, the meaning of the similar arrhythmic behavior produced by pinealectomy and that produced by LL remains obscure.
9. Light was obtained from a fluorescent bulb (Ken Rad, 4 watt, cool white F4T5/cw) providing 200 to 900 lux depending on the bird's position in the cage.
10. Birds were injected with Bouin's solution and decapitated. Skin and eyes were removed from the skull, and the dorsal part of the skull was drilled and carefully removed (including Replica cap) under a dissecting microscope. The brain with remaining skull was placed in Bouin's solution for 3 to 14 days, after which the skull was dissected from the hardened brain tissue which was then left in running tap water for 12 to 24 hours. Brains were then dehydrated in an ethanol series, cleared in xylene, and embedded in Paraplast. They were sagittally sectioned at 15 to 20 $\mu$ and stained in Mayer's hemalum and eosin.
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## Antiserum to Lymphocytes: Interactions with Chemical Immunosuppressants

Abstract. *An injection of rabbit antiserum to mouse lymphocytes causes temporary lymphopenia and prolonged survival of A-strain skin grafts on CBA mice. Cortisone or amethopterin without further antilymphocytic serum prolongs lymphopenia and immunosupression. When cortisone or amethopterin precedes the administration of the antiserum, the immunosuppressant action of the combination is less than that of the antiserum given alone. Whether the serum or the drug is given first does not affect the induction of lymphopenia by the serum. Thus, immunosuppressant action of antilymphocytic serum can be distinguished from its ability to induce lymphopenia. The results suggest that this serum may act as a mitogenic agent redirecting the proliferation of lymphocytes into immunologically incompetent pathways.*

The immunosuppressant effects of heterologous antiserums against lymphocytes in prolonging skin and kidney allograft survival have been well documented (*1–3*); however, the many theoretical and practical hazards associated with the prolonged administration of this material or of any heterologous serum product to organ transplantation need to be considered. In addition to the obvious dangers of serum sickness and anaphylaxis (*3*), the administration of antilymphocyte serum (ALS) has been associated with severe systemic reactions (*2*), marked local reaction at the site of injection, anemia, fever (*3*, *4*), generalized wasting syndromes (*5*), the development of antibodies to the injected proteins (*6*), and deposition of proteinaceous material along the basement membranes of renal glomeruli (*7*). Some of these reactions may be avoided by suitable purification of antigen (*8*), by fractionation of the serum, or by absorption (*3*, *4*). We have been interested in determining whether the immunosuppressant effect induced by ALS could be prolonged by other immunosuppressant agents (*9–11*), in order to try to shorten the period of ALS administration and to reduce the required dosage of other toxic, immunosuppressant materials. It is possible to maintain the immunosuppressant action induced by ALS with a combination of purine and pyrimidine antagonists in mice (*9*) or with azathioprine in dogs (*10*). Our experiments are designed to find out whether the additive or synergistic interaction of ALS with chemical immunosuppressant agents is dependent on their sequence of administration.

Rabbit antiserum to mouse lymphocytes (ALS) was prepared according to the technique of Gray *et al.* (*11*), with some modifications (*12*). Suspensions of washed A/Jax mouse lymph node cells (90 percent small lymphocytes) in Hanks's solution were mixed with equal volumes of complete Freund's adjuvant (Difco) and injected into each of the four footpads of an adult New Zealand white rabbit; the total dose was 400 $\times 10^6$ cells in 1.0 ml. Three weeks later, booster injections of the same cells ($100 \times 10^6$ cells in 0.5 ml) were given