foundress number, the square of foundress number, and calculated levels of inbreeding for each species to be significantly correlated to brood sex ratio in the expected pattern ($P < 0.001$). For foundress numbers greater than one, the theoretical curves lie within the 99 percent confidence intervals in all species. Differences among crops within species, total number of pollinator-parasitizing wasps, and total number of pollinator wasps reared per brood are not significantly correlated with the sex ratio in the sampled broods. Thus, sex-dependent mortality through parasitism and sex ratio skewing in response to resource availability do not account for the observed patterns.

21. M. J. Wade, *J. Theor. Biol.* **112**, 109 (1985).

22. I thank D. M. Windsor who made this work possible, S. J. Wright who was instrumental in the development of the model, M. D. Rausher for statistical help, R. B. Foster for *Ficus* identification, J. T. Wiekes for *T. costaricensis* identification, L. F. Reid, D. E. Wheeler, M. J. Ryan, D. M. Feener, S. W. Skinner, S. E. Via, E. G. Leigh, Jr., A. R. Kiester, R. Lande, M. J. Wade, D. W. Schemske, T. P. Young, R. K. Colwell, D. S. Webster, H. F. Howe, and W. D. Hamilton for useful suggestions or help in preparing the manuscript. Special thanks to M. Rabinowitz. Support was provided by the Smithsonian Tropical Research Institute through its Environmental Sciences Program and by the University of Iowa through its Teaching and Research Fellowship Program. Equation 2 was derived independently by both J. H. Werren and M. K. Uyenoyama (personal communications).

14 February 1984; accepted 7 March 1985

Multiple Circadian Oscillators Regulate the Timing of Behavioral and Endocrine Rhythms in Female Golden Hamsters

Abstract. *A single daily "surge" in pituitary luteinizing hormone release was observed in ovariectomized–estrogen-treated hamsters expressing an intact circadian rhythm of locomotor activity. In contrast, two luteinizing hormone surges occurred within a single 24-hour period in hamsters whose activity rhythm had dissociated or "split" into two distinct components. These observations indicate that both behavioral and endocrine circadian rhythms are regulated by the same multioscillator system, which seems to be composed of at least two distinct circadian oscillators.*

Circadian rhythms are endogenously generated biological oscillations with periods of approximately 24 hours (*1*). These rhythms have been observed in biochemical, physiological, and behavioral events in both unicellular and multicellular organisms. That the circadian system in mammals is composed of multiple circadian oscillators (*2*) is supported by the finding that during prolonged exposure to constant light the rhythm of locomotor activity in rodents can dissociate or "split" into two distinct circadian components (*3*). During the early stages of splitting, the two activity components often show different free-running periods for several cycles before becoming stably synchronized to each other, with the two activity onsets occurring about 12 hours apart. The simplest explanation for this phenomenon is that the rhythm of activity is regulated by two mutually coupled oscillators, or sets of oscillators, each of which regulates one activity component (*3, 4*).

Although splitting of circadian rhythms into two components has been observed in many vertebrate species, all clear cases of splitting have been of either the rhythm of locomotor activity or behavioral rhythms (such as feeding and drinking) that depend on the activity-rest state of the animal for their expression (*3, 5*). It is not clear if splitting is a general property of vertebrate circadian rhythms or if it is related only to the activity-rest cycle of the animal. To determine whether nonbehavioral rhythms can also dissociate into two circadian components, we monitored the timing of the "surge" of luteinizing hormone (LH) in ovariectomized hamsters treated with estrogen (OVEX-E) and having either an intact or a split rhythm of locomotor activity. A surge in pituitary LH release occurs daily in ovariectomized hamsters after treatment with estrogen. In contrast, a single preovulatory LH surge occurs once every 4 days in hamsters with ovaries (*6*).

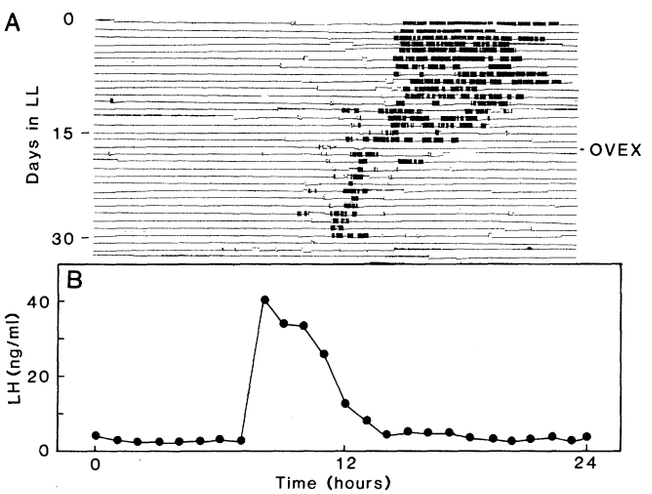
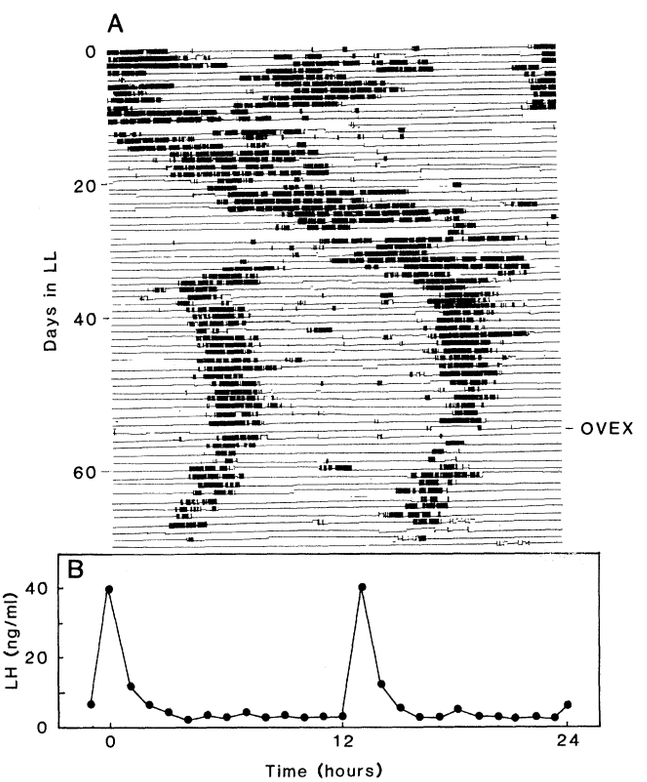


Fig. 1 (left). Continuous record of wheel running activity (A) and serum LH concentrations (B) of a representative OVEX-E hamster whose activity rhythm remained intact during exposure to constant light. The LH profiles are plotted so that each hourly sample corresponds to the time of sampling on the last day of each chart. Peak LH concentration was seen 1 to 4 hours before the onset of activity in all six animals with intact activity rhythms. Fig. 2 (right). Continuous record of wheel running activity (A) and serum LH concentrations (B) of a representative OVEX-E hamster whose activity rhythm had split into two components during exposure to constant light. Peak LH concentration occurred 0 to 4 hours before the onset of an activity bout in all ten animals with split activity rhythms.



Eighteen female golden hamsters (*7*), individually housed in cages equipped with running wheels (*8*), were transferred from a light-dark cycle (LD 16:8) to constant light (LL) (*9*). After 3 to 9 weeks, the ovaries of all animals were bilaterally removed (*10*). Two weeks after ovariectomy, each animal had a 4-mm-long Silastic capsule filled with estradiol benzoate implanted subcutaneously, and an indwelling cannula was inserted into the left atrium of the heart (*11*). Blood samples (0.4 ml per sample) were drawn hourly from each animal (*12*) over a 24-hour period beginning 48 hours after capsule implantation (*13*). Serum from each sample was later analyzed by radioimmunoassay for LH content (*14*).

At the time of blood sampling, six females had intact activity rhythms and ten had split rhythms. The activity patterns of the remaining two animals were disorganized and could not be characterized as either split or intact; therefore, data from these two animals have not been included in this report. All six females with an intact rhythm showed a single sustained increase in serum LH 1 to 4 hours before the onset of activity (Fig. 1). During the surge (*15*), the serum concentrations of LH were increased 3- to 14-fold above baseline, and the increase was sustained for 4 to 6 hours. In contrast, each female with a split activity rhythm showed two sustained increases in serum LH during the 24-hour sampling period; each LH surge occurred at any time up to 4 hours before the onset of one of the activity bouts (Fig. 2), and the LH remained elevated for 3 to 6 hours. The mean LH released per surge (*15*) was significantly less [$t(14) = 2.56$, $P < 0.05$] in animals with split activity rhythms than in animals with intact rhythms (Table 1). There was no significant difference between the two groups in the mean time interval between the peak of the LH surge and the onset of the next activity bout.

The close relation between the timing of the surge and the bouts of activity in both the intact and the split state supports the hypothesis that the circadian rhythms of LH release and locomotor activity are regulated by the same circadian oscillators (*16*). Alternatively, the rhythms of LH release and locomotor activity could perhaps be regulated by separate but tightly coupled circadian oscillators, although at present there is little evidence to support this hypothesis. The finding that both oscillators underlying the activity rhythm also contribute to the release of pituitary LH extends our earlier observation that the onset of lordosis (a behavioral marker for the LH surge) could be linked to either activity component of the split activity rhythm (*17*). However, because lordosis behavior lasts for about 18 hours, whereas the two activity components occur no more than 12 to 13 hours apart in the split state, we were unable to detect more than a single lordosis onset in any 24-hour period.

Table 1. Incidence of LH surges, mean (± standard error of the mean) interval between peak LH concentration and onset of activity, and mean (± standard error of the mean) LH concentrations released per surge for animals with either an intact ($n = 6$) or a split ($n = 10$) rhythm of locomotor activity.

Activity rhythm	Incidence (%)		LH surge to activity (hours)	Mean LH per surge (ng/ml)
	One surge	Two surges		
Intact	100	0	2.2 ± 0.5	77.9 ± 17.9
Split	0	100	2.1 ± 0.2	40.2 ± 6.2

A number of possible explanations exist for the single LH surge in OVEX-E hamsters with an intact activity rhythm, even though two circadian oscillators can stimulate pituitary LH release. In the nonsplit state, two separate oscillators may not be present; rather, a single oscillator may be able to divide into two suboscillators under certain environmental conditions, such as prolonged exposure to constant light. Alternatively, if both oscillators were functional in the nonsplit state, they might signal for the release of LH at about the same time, which would result in only a single surge of LH. The latter possibility is supported by our finding that the total LH released per surge in the animals with an intact activity rhythm is more than twice that released by the animals with a split activity rhythm. This interpretation should be viewed with caution, however, as the females with intact rhythms were bled after only 5 weeks in LL, whereas the females with split rhythms were bled after 9 to 11 weeks in LL (*10*). Prolonged exposure to continuous light may diminish the LH surge in OVEX-E hamsters.

The use of the OVEX-E animal as a model has enabled us to show that the neural signal regulating the LH surge can be expressed twice in 1 day. However, it is not clear whether two LH surges would also be seen in hamsters with ovaries and a split rhythm of locomotor activity. The decrease in serum estrogen or the rise in serum progesterone after the initial LH surge might inhibit the expression of a second neural signal for the release of LH in hamsters with ovaries and a split rhythm of activity.

Thus, two circadian oscillators seem to contribute to the timing of pituitary LH release in female hamsters, and these oscillators are apparently the same ones that regulate the rhythm of locomotor activity. Both behavioral and endocrine circadian rhythms seem to be regulated by a multioscillator system, and many circadian rhythms may have the capacity to split into two distinct components.

JENNIFER M. SWANN*
FRED W. TUREK
Department of Neurobiology and Physiology, Northwestern University, Evanston, Illinois 60201

References and Notes

1. J. Aschoff, *Handbook of Behavioral Neurobiology No. 4: Biological Rhythms* (Plenum, New York, 1981).
2. F. W. Turek, *Annu. Rev. Physiol.* **47**, 49 (1985).
3. C. S. Pittendrigh, in *The Neurosciences: Third Study Program*, F. O. Schmitt and F. G. Woden, Eds. (MIT Press, Cambridge, Mass., 1974), pp. 437–458; ——— and S. Daan, *J. Comp. Physiol.* **106**, 333 (1976); F. W. Turek, D. J. Earnest, J. Swann, in *Vertebrate Circadian Systems*, J. Aschoff, S. Daan, G. Groos, Eds. (Springer-Verlag, Berlin, 1982), pp. 203–214.
4. S. Daan and C. Berde, *J. Theor. Biol.* **70**, 297 (1978).
5. Z. Boulos and M. Terman, *J. Comp. Physiol.* **134**, 75 (1979).
6. M. H. Stetson, M. Watson-Whitmyre, K. S. Matt, *Biol. Reprod.* **19**, 40 (1978).
7. Adult *Mesocricetus auratus* [Lak:LVG(SYR)] were obtained from the Lakeview Hamster Colony, Newfield, N.J., at 9 weeks of age.
8. Each revolution of the wheel was recorded on a continuous event recorder (Esterline Angus).
9. Cages were arranged on a metal rack so that the light intensity at the top of the cage ranged from 300 lux at the top of the rack to 80 lux at the bottom.
10. After exposure to LL for about 50 days, the activity rhythm of as many as 80 percent of the hamsters in any particular study in our laboratory show a split rhythm of activity. Therefore, to obtain enough animals with intact rhythms, it was necessary to bleed six animals early during the exposure to LL, before the spontaneous development of the split condition. The animals with split rhythms ($n = 10$) were ovariectomized 7 to 9 weeks after exposure to LL to allow for the full development of the split condition.
11. Cannulas, constructed of polyethylene tubing (inner diameter, 0.58 mm; outer diameter, 0.965 mm) with a bubble 2.7 cm from one end, were inserted while the animals were under pentobarbital anesthesia into the right atrium of the heart, bubble-end first, through the jugular vein. The cannula was anchored to the vein with a suture tied behind the bubble and threaded under and out through the skin below the back of the animal's head.
12. During the bleeding session, cannulas were connected to additional polyethylene tubing, which extended outside the cage. Each cannula was flushed with heparinized (10 USP/ml) saline between the taking of samples. After every second bleeding, each animal received an equal volume (1 ml) of donor blood prepared from pooled animals' red blood cells that had been resuspended in 5 percent human protein plasma fraction (Plasmanate, Cutter, Berkeley, Calif.) and 5 percent phosphate dextrose solution [J. G.

Gibson, S. B. Rees, T. J. McManus, W. A. Scheitlin, *Am. J. Clin. Pathol.* **26**, 8537 (1957)].
13. The LH surge in OVEX-E hamsters was at maximum amplitude 48 hours after estradiol capsules were implanted (J. M. Swann and F. W. Turek, unpublished results).
14. G. D. Niswender, A. R. Midgely, Jr., S. E. Monroe, L. E. Reichart, Jr., *Proc. Soc. Exp. Biol. Med.* **128**, 807 (1968); C. A. Blake, R. L. Norman, C. H. Sawyer, *Biol. Reprod.* **8**, 299 (1973). Minimum and maximum detectable LH concentrations corresponded to 2.0 and 40 ng of NIH-LH-S16 per milliliter.
15. An LH surge is defined as two or more consecutive points exceeding the 95 percent confidence limit above baseline. Mean LH (nanograms per milliliter) released per surge was calculated as an average of the total area under the curve of each LH surge for all the animals in each group.
16. M. H. Stetson and P. J. Anderson, *Am. J. Physiol.* **238**, R23 (1980); M. L. Moline *et al.*, *Horm. Behav.* **15**, 451 (1981).
17. J. Swann and F. W. Turek, *Am. J. Physiol.* **243**, R112 (1982).
18. Supported by NIH grant HD-09885 and by a grant from the Whitehall Foundation. J.M.S. is the recipient of a predoctoral fellowship in NIH training grant HD-07068, and F.W.T. is the recipient of research career development award K04 HD-00249. We thank G. D. Niswender for providing antiserum (No. 15). We dedicate this report to Colin S. Pittendrigh on the occasion of his 65th birthday.
* Present address: Reproductive Endocrinology Program, 300 North Ingalls, University of Michigan, Ann Arbor 48109.

16 August 1984; accepted 3 January 1985

Diurnal Rhythms of *N*-Acetylserotonin and Serotonin in Cerebrospinal Fluid of Monkeys

We reported a diurnal rhythm for serotonin in monkey cerebrospinal fluid (CSF) (*1*) that was suppressed by exposure to continuous light and by the β-adrenergic blocking drug, propranolol (*2*). For the serotonin assay, samples were treated with acetic anhydride prior to extraction, converting serotonin into *N*,*O*-diacetylserotonin (*1*, *3*). Any endogenous *N*-acetylserotonin (NAS) present would be converted to the same compound and would therefore subsequently be indistinguishable from serotonin.

To distinguish between the two compounds we have now modified the first stage of the derivatization process, substituting propionic anhydride for acetic anhydride as the acetylating agent (*3*). Serotonin now yields *N*,*O*-dipropionylserotonin, while NAS yields *N*-acetyl-*O*-propionylserotonin (Fig. 1).

The spirocyclic electron-capturing compounds formed by subsequent treatment with pentafluoropropionic anhydride may be distinguished on the basis of their gas-liquid chromatography (GLC) retention times as well as on the basis of the masses of their major ions. Furthermore, for serotonin only, the asymmetrical methyl group (R_2) results in the formation of two isomers that are partly separated by the capillary column, resulting in a characteristic GLC trace which further increases the certainty of identification of this compound.

Dual internal standards $^2H_4$-serotonin and $^2H_4$-NAS were used, enabling the two compounds to be quantified independently. The collection and handling of the rhesus monkey CSF samples, the remainder of the assay, and the assay of melatonin were otherwise as previously described (*1*, *2*).

Serially collected samples of CSF from seven control animals and two that had had the pineal removed have been examined. Although serotonin was detected in five of the seven controls and in one of the pinealectomized animals at concentrations of 20 to 640 pg/ml, there was no evidence of a regular nighttime rise in concentrations as previously described. Elevated serotonin concentrations were observed during the day as well as at night, and the serotonin peaks were not coincident with the daily nighttime melatonin elevations. NAS was detected in six of the seven control animals at CSF concentrations ranging from 20 to 1128 pg/ml, and in four cases a nighttime rise in levels similar to that previously ascribed to serotonin was observed. In each case in which a rhythm of NAS was detectable, melatonin also showed a rhythm, indicating the close connection between these two compounds. There was no detectable nighttime NAS in the pinealectomized animals, although both showed small amounts of NAS (up to 160 pg/ml) in daytime samples. Likewise, no nighttime melatonin elevations were observed in the CSF of the pinealectomized animals.

In light of these results, we conclude that our previous reports of nighttime elevations of serotonin in CSF were erroneous and may be principally ascribed to NAS. NAS is the limiting precursor of melatonin, with its synthesis under β-adrenergic control (*4*); it is therefore not unexpected that rhythms of NAS would be suppressed by continuous light and by propranolol (*2*). The finding of elevated nighttime levels of NAS in CSF is not in itself uninteresting, since specific brain receptors exist for this compound (*5*); it may therefore have an independent physiologic function as a pineal hormone.

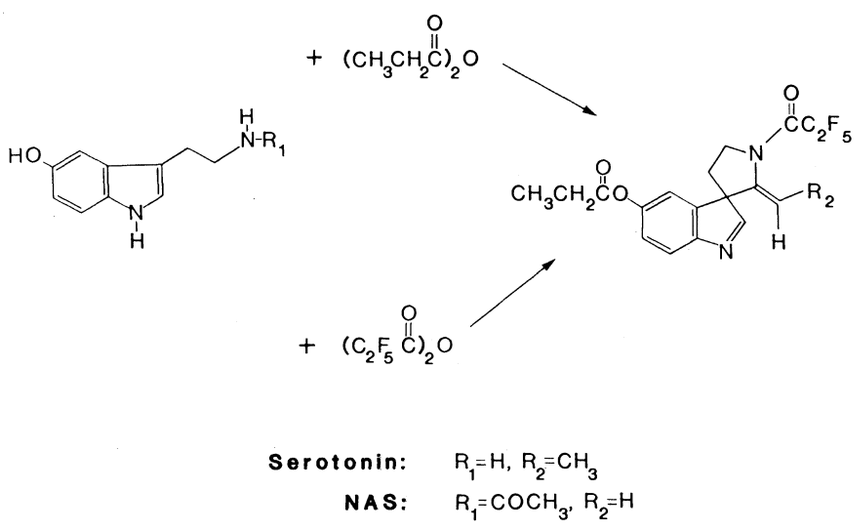


Fig. 1. Dual derivatization for serotonin and NAS.

PHILIP A. TAYLOR
MRC Reproductive Biology Unit, Centre for Reproductive Biology, Edinburgh EH3 9EW, Scotland
NANCY A. GARRICK
LAWRENCE TAMARKIN
DENNIS L. MURPHY
SANFORD P. MARKEY
National Institute of Mental Health, Bethesda, Maryland 20205

References and Notes

1. P. L. Taylor *et al.*, *Life Sci.* **31**, 1993 (1982).
2. N. A. Garrick *et al.*, *Science* **221**, 474 (1983).
3. S. P. Markey, R. W. Colburn, J. N. Johannessen, *Biomed. Mass Spectrom.* **8**, 301 (1981).
4. J. Axelrod, *Science* **184**, 1341 (1974).
5. L. P. Niles, G. M. Brown, R. K. Mishra, *Neuropharmacology* **22**, 1311 (1983).
6. We thank M. Mishkin for performing the pinealectomies and T. Miller and M. Linnoila for helpful discussions.

5 October 1984; accepted 10 December 1984