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27. Part of the research described in this report was carried out by the Jet Propulsion Laboratory, California Institute of Technology, under contract with the National Aeronautics and Space Administration (NASA). Y.L.Y. was supported by NASA grant NAGW-413 to the California Institute of Technology. We acknowledge several valuable conversations with M. Allen, W. DeMore, C. B. Farmer, J. Margitan, M. Molina, J. Rodriguez, G. Toon, and J. Sander. We thank D. Hofmann, R. DeZafra, R. Schindler, C. B. Farmer, C. Howard, D. McKenna, and D. Hartmann for preprints.

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Circadian System Controlling Release of Sperm in the Insect Testes

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Release of mature sperm from the testis into seminal ducts of the gypsy moth exhibits a circadian rhythm. The rhythm of sperm release was shown to persist in vitro, in isolated complexes of testis and seminal ducts cultured in light-dark cycles or in constant darkness. The phase of the rhythm was also reset in vitro by exposure to shifted light-dark cycles. Therefore, the testis–seminal ducts complex from the gypsy moth is photosensitive and contains a circadian pacemaker, which controls the rhythm of sperm movement. This finding extends the range of structures in multicellular organisms that are known to contain circadian oscillators and provides a new model system in which circadian mechanisms may be studied.

STUDIES OF THE CIRCADIAN SYSTEMS in multicellular animals have focused on identification of the structures and mechanisms involved in generating rhythmicity. Several pacemakers, that is centers capable of self-sustaining oscillations that control various behavioral and biochemical rhythms, have been localized in anatomically discrete sites by means of tissue transplants or by the monitoring of the rhythm in the organs cultured in vitro. Circadian oscillators have been reported primarily in parts of the nervous system. Examples include optic lobes of insects (*1*), eyes of mollusks (*2*), pineals of lizards (*3*) and birds (*4*), and suprachiasmatic nuclei of mammals (*5*). In addition, the insect prothoracic glands have been implicated as the site of a circadian clock (*6*) and insect cuticle has exhibited circadian-like growth in vitro (*7*). More experimental data are needed to determine whether circadian oscillators of multicellular animals are confined to nervous and endocrine tissues or whether they also exist in other tissue types. We present here direct evidence that the testis–seminal ducts complex of the gypsy moth, *Lymantria dispar*, contains a light-sensitive circadian pacemaker, which controls rhythmic release of sperm from the testis.

In Lepidoptera, as in most other insects, sperm cells develop in clones, and mature spermatozoa are released from the testis in the form of enlongated sperm bundles. The intriguing observation that the sperm release occurred in a daily rhythmic pattern was first made for the flour moth, *Ephestia kuehniella* (*8*). In the gypsy moth the release of sperm from the testes is also rhythmic and starts several days before adult eclosion when spermatogenesis is completed (*9*). The rhythm of sperm movement can be entrained by different photoperiods in intact animals, as well as in isolated abdomens in both flour moth (*10*) and gypsy moth (*9*). The rhythm has a circadian nature since it persists in constant darkness with the temperature-compensated period of approximately 24 hours (*11*). In male gypsy moths kept in light-dark cycles consisting of 16

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hours of light and 8 hours of dark (16L:8D), sperm bundles were released from the testis into the upper vas deferens (UVD) during an interval of approximately 4 hours shortly before lights-off (Fig. 1). Sperm remained in the UVD throughout the dark period and then 2 to 4 hours after lights-on was transferred all at once into the next segment of the vas deferens, the seminal vesicle (SV). During the light period, the sperm was gradually relocated into the duplex where batches of sperm released in subsequent cycles accumulated until mating.

In an attempt to locate the mechanism controlling the rhythm of sperm release, testis with the attached UVD and SV were removed from pharate adults of gypsy moths and placed in culture (*12*). The rhythm of sperm release from the testis to the UVD and its transfer from the UVD to the SV in intact control animals kept in a 16L:8D photoperiod is shown in Fig. 2A. In isolated testis-UVD-SV complexes cultured under the same photoperiod, the release of sperm also proceeded rhythmically for the duration of the experiment (three cycles), although in vitro the mean number of bundles released decreased with subsequent cycles (*13*). Timing of both the release of sperm from the testis to the UVD and the transfer of sperm from the UVD to the SV was similar in vitro and in intact control animals (Fig. 2B). To determine whether the rhythm of sperm release was endogenous and circadian, we cultured testis-UVD-SV complexes in constant darkness. The periodicity of sperm movement persisted, but release of sperm became less synchronous as might be expected when the rhythm is free-running during constant conditions leading to progressive asynchrony between different testes (Fig. 2C). These experiments demonstrated that the testis-UVD-SV complex of gypsy moths contains a circadian pacemaker, which controls the rhythm of sperm movement.

To determine whether the phase of sperm release rhythm could be reset in vitro, we placed testis-UVD-SV complexes in culture (*12*) and then exposed them to a 6-hour phase advance or a 6-hour phase delay of the original 16L:8D photoperiod. By the second cycle in the new photoregime, the time of sperm release had either advanced or delayed to resume the normal position relative to the light-dark cycle (Fig. 3). Moreover, when testes exposed to the phase-advanced photoperiod were subsequently placed in constant darkness, the reset phase of the rhythm persisted, which showed the stability of the entrainment. These results provided clear evidence that testis or vas deferens of gypsy moth, or both, contains a photoreceptor that can respond directly to

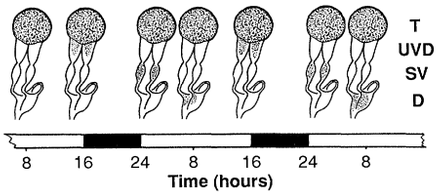

Fig. 1. Schematic representation of the rhythm of sperm movement in the gypsy moth males. Batches of sperm bundles (indicated by dotted areas) are released from the testis (T) into the upper vas deferens (UVD) shortly before the dark period. After lights-on, sperm is rapidly transferred into seminal vesicles (SV) and then, gradually, into the duplex (D). Black bars on the time scale indicate the dark phase.

environmental light conditions by prompt entrainment (*14*). The in vitro entrainment of the circadian rhythm of sperm release was completed after only one exposure of the testis to the shifted light-dark cycle, as occurred in intact animals (*9*). In a related study of circadian rhythm of the optic nerve impulses in the mollusk *Aplysia*, entrainment to the shifted photoperiod took one cycle in vivo but four to five cycles in the isolated eyes in vitro (*15*). A possible explanation for these differences could be that in *Aplysia* factors from other parts of the body could affect the rate of entrainment in the eye, whereas in gypsy moth entrainment may not involve organs other than the testis.

It is not known which cells within the testis–seminal duct complex might be involved in the circadian mechanism. Since other known circadian oscillators are located in the neural tissue (*1–5*), several testis-UVD preparations were examined with light and electron microscopes for the presence of innervation. We did not observe any nerve fibers, local ganglia, or neurosecretory cells in this part of the reproductive system. Similarly, no innervation of the testis-UVD complex was found in another moth known to have a rhythmic release of sperm in vivo (*16*). In leaving the testis, sperm bundles must pass in between cells of the basilar membrane, which separates testicular follicles from the lumen of the UVD in Lepidoptera (*17*). These cells are likely to play a role in the circadian mechanism controlling the release of sperm.

The role of rhythmic sperm movement may be to synchronize complex morphological changes that lead to final maturation of sperm in posttesticular organs as described in other Lepidoptera (*18*). The rhythm of sperm release is essential for normal reproduction in the gypsy moth, since in constant light, where this rhythm is disrupted, the amount of sperm leaving the testis is reduced and dispersal of spermatozoa from sperm bundles is impaired. Sperm of males exposed to constant light do not acquire

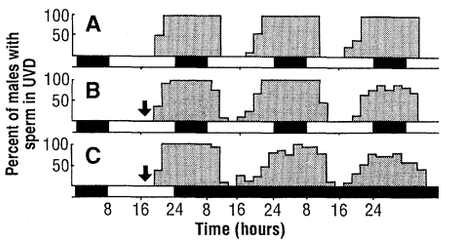

Fig. 2. The rhythm of sperm movement in intact gypsy moth males kept in a 16L:8D photoperiod (**A**) or in isolated testis-UVD-SV complexes placed in vitro at the time indicated by the arrow and kept in a 16L:8D photoperiod (**B**) or in constant darkness (**C**). Black bars on the time scale indicate dark phase. To determine the proportion of UVDs containing sperm, we dissected 8 to 12 preparations every 2 hours.

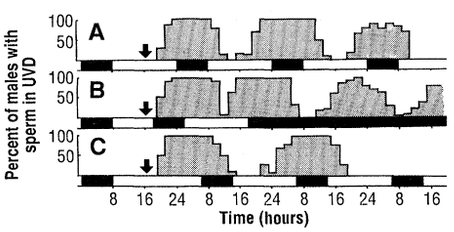

Fig. 3. The rhythm of sperm movement in testis-UVD-SV complexes isolated in vitro at the time indicated by the arrow. Preparations were subsequently kept in the original 16L:8D photoperiod (**A**), or subjected to a 6-hour phase advance followed by constant darkness (**B**) or to a 6-hour phase delay (**C**). Black bars on the time scale indicate dark phase. To determine the proportion of UVDs containing sperm, we dissected 8 to 12 preparations every 2 hours.

fertilizing capacity so that females mated to these males fail to oviposit and behave like virgins (*19*).

The testis-UVD-SV complex is a unique circadian system in which photoreceptor, pacemaker, and the regulated process are all contained in a highly specialized, sex-specific organ. The search for features common for pacemakers located in the testis and in the nervous system may help us to understand the underlying mechanisms of the circadian clock.

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12. Testis-UVD-SV complexes were dissected with aseptic technique and placed individually in 0.4 ml of Graces insect medium (Gibco) containing 10% fetal bovine serum (Gibco) and 1% antibiotic-antimycotic solution (Sigma) in four-well Nuclon Multidishes. Cultures were kept at 25°C in airtight incubator chambers (Billups-Rothenberg) filled with oxygen and humidified. At designated times 8 to 12 preparations were dissected to determine the presence of sperm bundles in the UVD and SV.
13. Mean number of sperm bundles released during one cycle in intact pharate adult males was 55.2 (SE = 21, n = 8). In cultured testis-UVD-SV complexes the mean number of sperm bundles released was 39.1 (SE = 9, n = 8) during the first cycle in vitro, 16.6 (SE = 10, n = 10) during the second cycle, and 4.3 (SE = 3, n = 8) during the third cycle.
14. Photoperiod rather than temperature is the main entraining factor for the rhythm of sperm release. When pupae were exposed to a 16L:8D cycle and to a cycle of 16 hours at 28°C and 8 hours at 23°C so that the light and temperature cycles were 6 hours out of phase with each other, the timing of sperm release was in phase with the photoperiod, not the temperature cycle.
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A Chitin-Binding Lectin from Stinging Nettle Rhizomes with Antifungal Properties

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Rhizomes of stinging nettle contain a small-sized lectin that exhibits binding specificity toward chitin. This lectin inhibits growth of several phytopathogenic and saprophytic chitin-containing fungi in vitro. The antifungal action of the nettle lectin differs from the action of chitinases, which are a ubiquitous class of antifungal plant proteins. Moreover, the nettle lectin acts synergistically with chitinase in inhibiting fungal growth. The nettle lectin may be a promising candidate for possible applications in the genetic engineering of disease-resistant crops.

IN SPITE OF THE VAST RESEARCH ON plant lectins, their physiological role remains a matter of controversy (*1, 2*). As early as 1975, an attractive proposal was forwarded suggesting that wheat germ agglutinin (WGA), a chitin-binding lectin from wheat embryos, plays a role in the defense of seedlings against fungal attack (*3*). This notion was based on the observation that WGA inhibits spore germination and hyphal growth of *Trichoderma viride*. Several investigators have proposed arguments supporting such a protective role for WGA (*4*) and other chitin-binding plant lectins (*5, 6*). More recently, however, it was shown that contaminating chitinases, not the lectins themselves, are responsible for the observed in vitro inhibition of fungal growth (*7*). To reopen the debate, we report that a chitinase-free chitin-binding lectin from stinging nettle rhizomes is a potent inhibitor of fungal growth.

Some years ago a monomeric lectin was isolated (by affinity chromatography on chitin) from rhizomes of stinging nettle (*Urtica dioica L.*) (*8*). With a molecular size of 8.5 kD, *Urtica dioica* agglutinin (UDA) is the smallest plant lectin known. UDA is unusually heat and acid resistant, has high contents of cysteine and tryptophane (*8*), exhibits specificity toward *N*-acetyl-D-glucosamine oligomers (*9*), and shows a striking amino acid sequence homology with WGA (*10*).

Experiments indicated that UDA preparations strongly inhibited fungal growth in vitro. To make sure that our UDA preparation was free of contaminating chitinase, the lectin fraction obtained by affinity chromatography on chitin was further purified by ion-exchange chromatography, gel filtration, and affinity chromatography on ovomucoid Sepharose (*11*). Throughout all these purification steps antifungal activity eluted with hemagglutination activity. The final UDA preparation exhibited no detectable chitinase activity when up to 100-μg amounts were tested by the ^{3}H-labeled chitin degradation assay (*12*), which has a detection limit of 20 ng purified chitinase (*13*). Moreover, chitosanase, β-*N*-acetylglucosaminidase, and β-1,3 glucanase activities were negative when 100-μg amounts were employed in the appropriate assays (*14–16*).

To quantify the fungistatic effect of UDA, an assay was used based on the measurement of hyphal growth of germinated spores. Dose-response curves were determined for seven different fungi that contain chitin in their cell walls (*Botrytis cinerea, Collectotrichum lindemuthianum, Phoma betae, Phycomyces blakesleeanus, Septoria nodorum, Trichoderma hamatum*, and *Trichoderma viride*) and one chitin-negative fungus (*Phytophthora erythroseptica*) (*17*) (Fig. 1). Concentrations required for 50% growth inhibition (IC_{50}) of the chitin-containing fungi varied from 20 to 125 μg/ml, whereas the chitin-negative *Phytophthora erythroseptica* was insensitive to

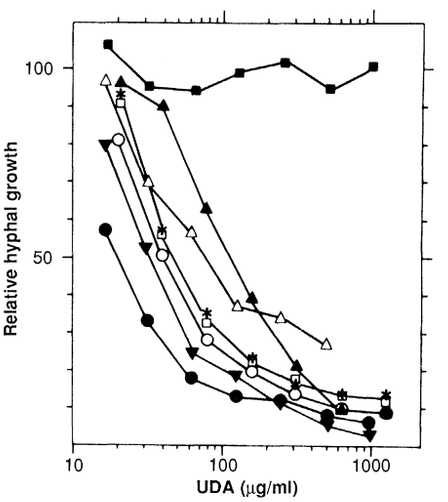

Fig. 1. Antifungal activity of UDA. Hyphal growth of germlings was measured at varying concentrations of UDA (*11*) with the following test organisms (*23*): *B. cinerea* (○), *C. lindemuthianum* (●), *Phoma betae* (△), *Phycomyces blakesleeanus* (▲), *Phytophthora erythroseptica* (■), *S. nodorum* (▼), *T. hamatum* (□), *T. viride* (*). Relative hyphal growth is expressed as percentage of the hyphal growth of control cultures; data are means of three independent experiments. Ratios of standard error to mean were 17% or less. Hyphal growth inhibition assay was performed as follows: 100-μl amounts of spore suspensions (2×10^4 spores/ml) in potato dextrose broth (Difco) were incubated in flat bottom multiwell plates (Nunc) at 22°C until the hyphae of the germlings had an average length of 40 μm. Then, test solutions (50 μl) were added to the germlings, and the plates reincubated at 22°C until the control germlings (50 μl of water added) attained an average length of 500 μm. Average length of 50 individual hyphae was determined from photomicrographs made with an inverted microscope.

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