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Circadian Systems, VI. Photoperiodic Time Measurement in *Pectinophora gossypiella**

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Abstract. Diapause (100% incidence) occurs in the moth *Pectinophora gossypiella* when it is exposed to 24-hour light/dark cycles involving 12 hours of red light (600 nm); only 2% occurs when the photoperiod is extended to 14 hours, again with 600-nm light. This wavelength fails to synchronize all the known circadian oscillations of the moth. These observations appear, therefore, to constitute positive evidence that the photoperiodic time measurement is not mediated by a circadian oscillation. However, it remains possible, even plausible, that the photoperiodic clock is a separate circadian oscillator coupled to light by a red-absorbing pigment. That possibility is testable. The nature of the clock—oscillator or not—remains open.

For more than 10 yr this laboratory has been interested in the proposition made initially in 1936 by Erwin Bünning that what we now call circadian rhythmicity is causally involved in the photoperiodic phenomena; that the "clock" which measures the seasonally varying duration of darkness is the same circadian clock system involved in time-compensated sun orientation and in daily periodicities more generally. Papers by Pittendrigh and Minis in 1964[1] and Pittendrigh in 1966[2] attempted: (1) to formulate Bünning's proposition in an explicit way amenable to test in terms of newer knowledge of circadian rhythmicity; and (2) to show that the then-available evidence attested strongly to the validity of Bünning's proposition for several species.

In their 1964 paper Pittendrigh and Minis noted explicitly that Bünning's hypothesis implicitly demands two quite distinct functions of the daily cycle of light and darkness: (1) the light pulse in each daily cycle entrains the innate circadian oscillation and thereby establishes a determinate phase relationship (ψ) between the light cycle and every phase point in the oscillation, and (2) the light does, or does not, effect photoperiodic induction depending on whether or not some specific phase point (ϕ_i) in the oscillation coincides in time with light. They noted that since the phase relationship (ψ_{OL}) of the innate oscillation to the light cycle changes with change in photoperiod (that is, with day length or, its reciprocal, night length), ϕ_i will be illuminated under certain photoperiodic regimes and not under others. In developing these analytically distinct roles of the light cycle (entrainment vs. induction), they noted that the two functions could well be mediated by distinct photoreceptors and that different action spectra for entrainment and induction were therefore a possibility to be reckoned with.

They emphasized, too, for reasons laid out in their 1964 paper, that in developing an evaluation of Bünning's idea, analyses of photoperiodic induction and of entrainment of circadian rhythmicity should be pursued concurrently in some suitable single species: for what is at stake is the proposition that photoperiodic induction is a function of some limited class of entrained steady states of the circadian system. There are significant differences among species in their entrainment responses to light cycles. This laboratory has performed such parallel studies in the moth *Pectinophora gossypiella* in which the avoidance of diapause, an arrest of normal development in the fourth larval instar, is effected by a long photoperiod; and in which we have found and assayed three distinct circadian rhythms. The time at which the caterpillar hatches from the egg is controlled by a circadian oscillation; so, too, is the time at which the adult emerges from the pupal case. The egg-laying activity of adult females also displays circadian rhythmicity. The very different phase relationships of these three rhythms to the external light cycle and to their light-sensitive driving oscillations is discussed by Pittendrigh and Minis;[3,4] these authors also note that while all three driving oscillations are similar in the form and phase of their phase-response curves, the oscillations are nevertheless each distinguishable by either their free-running period (τ) or their sensitivity to red light. They warn that a multicellular circadian system comprises a population of driving oscillations, and that some current investigations in the photoperiodic time measurement rest on assumptions about such circadian systems (including the mode of their coupling to light) which are far from well founded in fact. As we shall see below, the uncertainty attending one of these assumptions becomes crucial in evaluating a surprising discovery: *Pectinophora gossypiella* effectively measures night length in spite of the fact that all three currently assayed oscillations have their phases distributed at random with respect to a cycle consisting of monochromatic (600 nm) light and darkness.

Materials and Methods. Details of the rearing methods and procedures for induction experiments have been previously described.[5,6] Descriptions of the assay of the rhythm of egg hatch,[6] of the rhythm of adult oviposition,[5] and of the rhythm of eclosion[3] have also been published.

For the experiments reported here, the larvae were reared on a modification of the wheat germ diet developed by Adkisson *et al.*[7] The curve for development as a function of photoperiod at 26°C under our laboratory procedures is essentially the same as that obtained when a cottonseed meal diet is used—there is a slight difference in degree of response, but the critical features of the response curve are identical.

Four-watt "cool white" fluorescent lamps in water jackets were used as a light source and were set in a wooden housing on the ceiling of each rearing cabinet. An aperture 1 in. in diameter in the base of the housing, 5 cm from the lamp, was fitted with a plastic filter holder into which interference filters could be placed. Monochromatic light was generated by Oriel Optics 1-in. diameter narrow-band interference filters (band width 10 nm at 50% peak transmission). Light intensities were measured with a Photovolt photometer (model 514) whose sensitivity to the given wavelengths had been calculated.

The lamps were controlled by Intermatic timers whose operations were continuously monitored by an Esterline Angus operations recorder.

The identical cabinet and optics were used for rhythm assay and induction experiments under a given wavelength.

In the induction experiments the insects were reared during their entire development,

including the egg stage, in the test light regime (white light, monochromatic light, or constant dark). A technique has been developed to obviate the need for transfer of newly hatched larvae to the food vials, so that light is never needed, operationally, in the rearing procedures.

The insects were maintained in the particular light/dark regimes under study for 40 days, at which time they were taken into white light and scored for diapause or completed development.

Results and Discussion. Bruce and Minis[8] recently obtained an action spectrum for initiation by light of the circadian oscillation in the eggs of *Pectinophora gossypiella*. That spectrum has a broad maximum in the blue and a sharp drop-off to zero between 490 and 510 nm. As such it is qualitatively very similar to action spectra obtained by Sargent and Briggs[9] and Frank and Zimmerman[10] for circadian systems in *Neurospora* and *Drosophila* respectively. Knowledge of the spectral sensitivity of the circadian system prompted us to seek an action spectrum for photoperiodic induction. We began with the simple experiments summarized in Table 1. When *Pectinophora gossypiella* is reared in constant

TABLE 1. *The results of exposing populations of Pectinophora to 24-hr cycles consisting of 14 hr of monochromatic light, 10 dark, or 12 hr of monochromatic light, 12 dark, at 25.5 ± 0.5°C.*

Wavelength of 14-hr photoperiod (±5 nm)	Intensity (ergs/cm²/sec)	N	Development (%)
White control	40	53	100
480	1.3	52	98
560	1.4	48	98
600	1.4	49	100
640	0.59	46	95
680	0.14	46	83
Wavelength of 12-hr photoperiod (±5 nm)			
White control	40	49	2
370	0.13	27	7
380	0.06	25	4
400	0.56	29	3
480	1.3	50	2
540	6.6	57	0
620	0.57	55	2
Constant dark (DD)	...	142	73
Constant light (LL)	40	...	15

darkness, about 73% of individuals escape diapause and complete development. In constant light only about 15% complete development. The dependence of the incidence of diapause on photoperiod is summarized in Figure 1, which is based on data published by Adkisson *et al.*[11] and supplemented by unpublished observations of Minis and Pittendrigh. In a 24-hr light/dark cycle involving a 14-hr photoperiod, 100% of the insects complete development; when the photoperiod is 12 hr, only 2% complete development. It is important to note that both of these values are significantly different from the 73% completion of development which characterizes populations in constant darkness.

Table 1 summarizes the effects of *14*:10 and *12*:12 light/dark cycles by using monochromatic light of various wavelengths. In every case the insects involved

derive from eggs which had been harvested in complete darkness and transferred immediately to the light cycle and wavelength indicated in the table. The general result is strikingly clear. Light/dark cycles involving photoperiods of 12 hr suppress development for all wavelengths tested up to 620 nm and they suppress it as effectively as cycles involving white light itself. Similarly, *14*:10 light/dark cycles are equally effective in inducing complete development for all wavelengths up to 640 nm and probably to 680 nm. It is recalled that in constant darkness the incidence of completed development is only 73%.

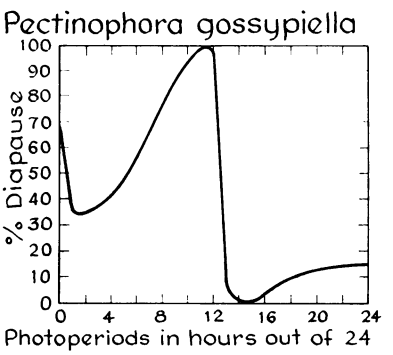


FIG. 1. The photoperiodic response curve for *Pectinophora grossypiella*. Based on combined data from Adkisson[11] and unpublished work in this laboratory.

In all cases the energy flux involved is well above our *estimate* (0.02 ergs/cm²/sec) of the threshold for effectiveness in photoperiodic induction. In any case a more rigorous validation of that statement proves to be unnecessary for the essential point of this paper: *light cycles involving known energy fluxes at wavelengths greater than 520 nm are fully effective in photoperiodic induction* (the data in Table 1) *but are as certainly ineffective in entraining the known circadian rhythmicities of Pectinophora gossypiella.*[12]

As discussed in the beginning of this paper, it is not, in itself, surprising that wavelengths greater than 420 nm are photoperiodically inductive in *P. gossypiella* even though they fail to initiate a circadian rhythm, as Bruce and Minis have shown. What *is* surprising about the results of Table 1 is that they were obtained from populations which were never exposed—*at any stage in their life history*—to any light cycle that could have entrained, and hence synchronized, the circadian oscillation in the individual insects involved. Thus they provide *prima facie* evidence that populations of insects can discriminate completely (100% vs. 2%) between 14 and 12 hr of light (10 and 12 hr of darkness) no matter what the phase relationship of the light (or dark) to their circadian oscillation. If the discrimination between 14 and 12 hr of light is indeed effected independently of the phase relationship of the light to the oscillation, the coincidence model of Pittendrigh and Minis must be rejected for this species, and so for that matter—*for this species*—must every other specific formulation of Bünning's general proposition that the time measurement of photoperiodism is effected by a circadian system. At face value Table 1 is not only a failure to support Bünning's hypothesis, it is positive evidence that for *Pectinophora gossypiella* the Bünning mechansim is not what is involved.

This entire line of argument rests, however, on two crucial assumptions: (1) that light above 510 nm is as ineffective when seen repeatedly in entrainment as it is when seen as a single initiating pulse (which was the Bruce-Minis assay); and (2) that *all* the circadian driving oscillations in the total *Pectinophora* system are coupled to the light by the same pigment. Clearly these two assumptions need scrutiny.

(1) The experiments summarized by Figures 2, 3, and 4 were designed to ascertain whether or not red light (600 nm) which is known to be ineffective as a rhythm initiator is nevertheless effective as an entraining agent when seen repeatedly. The experiments compare the entraining effectiveness of 480 nm and 600 nm: and they involve all three of the known assayable circadian rhythms in *Pectinophora gossypiella*. As expected, 480 nm determines the phase of the circadian rhythm in all three cases: the rhythm of egg hatch, the rhythm of pupal eclosion, and the rhythm of oviposition by adult females. Cycles of red light (600 nm) are, however, completely ineffective entraining agents, as we had originally deduced on the basis of the Bruce-Minis analysis of single pulses. The facts are especially clear in the pupal and adult rhythms. In two regimes of *LD* 14:10, using 600 nm, the pupal rhythm free-runs with a period clearly shorter than 24 hr. *LD* 14:10 (480 nm) entrains as effectively as *LD* 14:10 (white). In the adult, when the phase of a previous 14:10 *white light*/dark cycle is shifted by 6 hr, with 480-nm monochromatic light, the phase shift of the rhythm is prompt and complete. However, when the light used for the phase-shifted light cycle is 600-nm monochromatic, there is no phase shift in the rhythm which simply free-runs. It should be noted that there is some evidence that red light is not completely without effect on the rhythms we have studied in *Pectinophora gossypiella*. The "free-run" in the red/dark cycles of Figure 3 has a period perceptably shorter than that characteristic of the free-run in constant darkness. The same is true of the egg-hatch rhythm which free-runs in red light after transfer from white, that is, the period of the free-run is shorter in constant red light than in constant darkness (Bruce and Minis, unpublished observations). But the essential fact remains unaffected by this qualification:

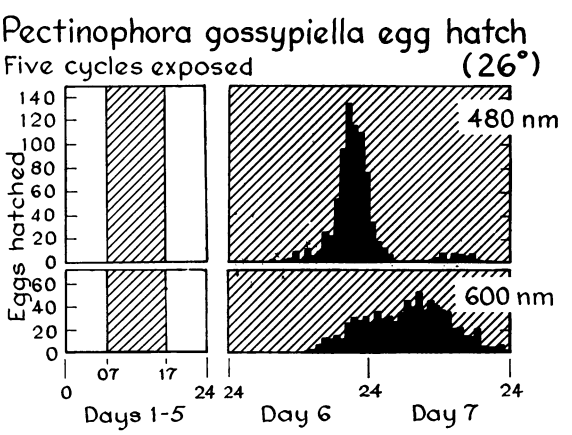
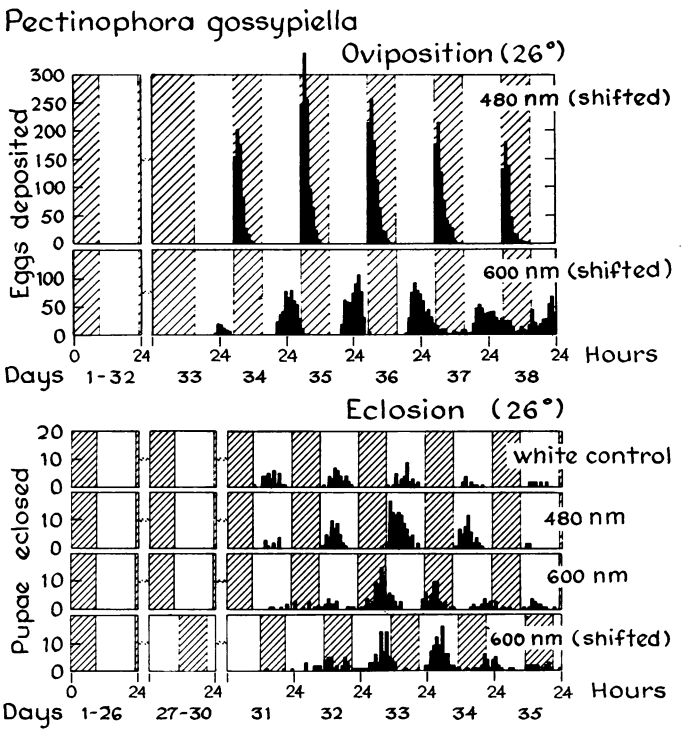


Fig. 2. The distribution of egg hatching (*Pectinophora gossypiella*) in darkness after 5 days of light/dark cycles involving light of 480 and 600 nm. After exposure to cycles of 600-nm light, the distribution is aperiodic. After exposure to cycles of 480-nm light, the distribution is bimodal reflecting the presence, and synchrony, in the population of gating oscillations in the individual insects. Most develop in time to hatch in the first "gate" of the free-run in darkness; a few slower developers are forced to await the second gate. See Pittendrigh and Minis[3] for a discussion of egg-hatch gating.



(*Below*) Fig. 3. All populations were raised in *LD 14*:10 (white light) for the 26 days. A control group continues to develop and eventually ecloses in white light (*top panel*). Another group (*second panel*) is shifted on the 27th day into blue light (480 nm) which continues to entrain the rhythm effectively. The third and fourth groups were transferred on the 27th day into cycles involving red light (600 nm). The phase of the light cycle is unchanged in the third group but shifted by 12 hr in the fourth. Both groups free-run with identical phase: the residual rhythmicity of the population (synchrony of individuals) derives from the prior white light, and is free-running in the red light.

(*Above*) Fig. 4. Moths were raised in *LD 14*:10 (white light) for 32 days and then transferred, as adults, into *14*:10 light/dark cycles involving either 480 or 600 nm. In both cases the phase of the monochromatic light cycle was shifted by 6 hr relative to the previous white light cycle. The population in 480 nm is immediately entrained by that light: oviposition occurs as usual in the dark. The population in 600 nm is unentrained by the new light regime: the residual rhythmicity of the population (synchrony of individuals) derives from the prior white light, and is free-running in the red light.

Figures 2, 3, and 4 leave no doubt that the red end of the spectrum fails to entrain the known circadian rhythmicities of *Pectinophora gossypiella*. The first of our two assumptions is valid.

(2) The second assumption—that all driving oscillations in *Pectinophora gossypiella* are coupled to light by the same pigment—is less easy to evaluate. Its significance, on the other hand, is clear: we are faced with the possibility that there is a distinct circadian oscillator in the system which (*a*) effects the photoperiodic time measurement, (*b*) has no control over the phase of hatching, eclosion, or oviposition, and (*c*) is coupled to the light cycle by a distinct pigment that absorbs in the red as well as in the blue.

This prospect, somewhat unattractive to us esthetically, is by no means implausible in view of the known diversity of drivers in a multicellular system and the increasing evidence that they (like the induction mechanism itself) are *directly* coupled to light. To test this second assumption we need experimental designs that do not involve the use of any of the other circadian oscillations in the system. These are provided by what Pittendrigh and Minis[4] call "bistability" and (following the terminology of Brinkmann) "resonance" experiments. Strong versions of both such experiments using 600-nm light have yet to be performed, and in the meantime it remains an open question whether or not the measurement of photoperiod in *Pectinophora gossypiella* is mediated by a circadian oscillator.

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[12] Adkisson, P. L., in *Proceedings of the Conference on Electromagnetic Radiation in Agriculture* (New York: Illuminating Engineering Society and the American Society of Agricultural Engineers, 1965), p. 30. Adkisson has published preliminary results for *Pectinophora* showing that orange (610 nm) was partially effective in preventing diapause when used as a supplement to a 12-hr white photoperiod, but red (640 nm and above) was without effect.