Carbon Dioxide Output as an Index of Circadian Timing in 
*Leanna* Photoperiodism

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**ABSTRACT**

Previous work on flowering suggested that photoperiodism in *Lemna perpusilla* 6746 involves an endogenous circadian "clock," but direct evidence requires study of an overt rhythm in the same plant. The CO2 output rate of axenic cultures supplied with sucrose has been studied in a system using infrared analysis and monitoring four sets of cultures at once. Alternations of 14 to 21 hours of dim red light with darkness in 24-hour cycles can entrain the CO2 output. In darkness following either continuous dim red light or entrainment to a 12(12) light:dark schedule, the rate oscillates through two maxima and two minima, with a circadian periodicity, before apparently damping. In continuous red light, the rate is linear. The skeleton photoperiodic schedule 14(5,10)14(18), with its two portions highly unequal, rapidly entrains the CO2 output in a phase relationship which is the same irrespective of which dark period is given first. The schedules 14(13)14(10.5) and its inverse, however, with two portions more nearly equal in length, differ markedly from each other with respect to manner of entrainment, as they do in their effects on flowering. These and other results strongly support the concept that a circadian clock is an important component of photoperiodism, and they provide a new experimental system in which to study its action.

The view that photoperiodism is a function of the "biological clock" is becoming widely accepted (6–8, 19). Nevertheless, there are few experimental systems in which relationships between a photoperiodic response and some overt, continuous rhythm—as an indicator of the state of the clock—can be established with ease and precision. Thus the penetrating studies of phytochrome photoreception in higher plant photoperiodism (2, 3) have not been fully matched by investigations of photoperiodic timing, which, with a few notable exceptions, have relied largely on demonstrating oscillations in the photoperiodic response itself. One persistent obstacle has been the frequent unavailability of a reliable overt rhythmic process in precisely those plants or conditions where it might be most useful. As an ironic result, the most definitive evidence relating an overt rhythm to photoperiodic responsiveness was obtained on a *Coleus* in which the role of phytochrome is not understood (9, 10). An additional difficulty with higher plants is that long experiments with brief light exposures, as in "skeleton" photoperiodic schedules, are difficult without an energy source other than photosynthesis.

The photoperiodic control of flowering in the duckweed *Lemna perpusilla* 6746 has been extensively studied with respect to light quality, chemical effects, and other factors (14, 17, 18); in experiments under nonphotosynthetic conditions made possible by axenic culture on media with sucrose, the effects of certain skeleton photoperiodic schedules suggest the activity of an endogenous circadian rhythm (12, 15). Until now, however, a continuous measure of the state of the clock was lacking. The experiments to be reported show that the rate of CO2 output serves well for this purpose, thus further complementing the already abundant advantages of the *Lemna* system and strongly confirming the view that an endogenous clock is part of the mechanism of photoperiodism.

**MATERIALS AND METHODS**

**Stock Cultures.** *L. perpusilla* 6746 was maintained in 125-ml Erlenmeyer flasks on 50 ml of 0.5 × strength Hutner's medium plus 1% sucrose, 600 mg/liter tryptophane, and 100 mg/liter yeast extract, under about 200 ft-c (2152 lux) of continuous cool white fluorescent light at 25 to 27°C. New cultures were started weekly with one three-frond colony (14).

**Flowering Experiments.** These proceeded as described earlier (12–14) except that cultures were grown on 30 ml of 1.1 × strength Hutner's medium plus 1% sucrose in 25–× 150-mm culture tubes, and that the red light used was about one-fifth the "standard" intensity (13) and gave a total energy of 8 to 15 μW·cm⁻² between 550 and 800 nm.

**Assay of CO2 Output Rate.** A schematic summary of the system used is given in Figure 1. Each culture is started by pouring a single 11- to 16-day-old stock culture, with medium, into a cotton-stoppered 250-ml narrow mouth reagent bottle containing 150 ml of 1.1 × Hutner's medium plus 1% sucrose. Generally, two cultures are placed in each vessel system. The differences between the CO2 levels maintained in vessels with plants and those maintained in precisely similar vessels lacking only plants, but with culture bottles and medium, constitute the basic data. Such values are obtained once every 30 min for each of four sets of plants, with two vessels used as blanks. During the course of the experiments, the ambient (blank) level of CO2 ranged between about 310 and 370 ppm depending on weather conditions and time of day. The temperature was 28 to 30°C in all experiments.

The output rate was measured under schedules of darkness combined with dim red light. The latter was provided for each vessel by a 4-w cool white fluorescent tube behind a 3-mm thickness of Rohm and Haas 2444 red Plexiglas. As measured with an Agricultural Specialties Company (Beltsville, Maryland) model...
3052 spectroradiometer, this source at plant level gave about 3.5 
\( \mu \text{W} \cdot \text{cm}^{-2} \) total energy between 800 nm and the low wave length
cutoff about 550 nm, with an intensity of 0.072 \( \mu \text{W} \cdot \text{cm}^{-2} \cdot \text{nm}^{-1} \)
in the peak range of 648 to 660 nm and of 0.014 \( \mu \text{W} \cdot \text{cm}^{-2} \cdot \text{nm}^{-1} \) at
730 nm. Without the red filter, the intensity was approximately
80 ft-c (861 lux) as measured with a General Electric MR-100 light meter. Because of the complex geometry involved, the actual energies experienced by the plants varied several fold from the values measured.

In spite of the lack of direct contact between the plants and the
moving air stream, diffusion through the cotton stopper appar-
ently allows the system to respond quite rapidly; Figure 2
shows how quickly the effects of shaking or nonshaking are
recorded. The shaking frequency used was selected arbitrarily
and is probably not optimal, but much more rapid shaking soon
causes obvious damage to the plants. For this reason, and since
interpretations of the data sought depend only on relative maxima
and minima and not on absolute values, little attention has been
paid to the latter. The general range is suggested by the following
information on the experiment started July 7 (Fig. 9). Blotted
fresh weights of five cultures like those used to start the experi-

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**FIG. 1.** Schematic summary of *Lemna* CO\(_2\)-monitoring system.

**FIG. 2.** Response time of *Lemna* CO\(_2\)-monitoring system as shown
by response to shaking. CO\(_2\) output in arbitrary units.
Fig. 3. CO₂ output rate (3-hr-hourly, arbitrary units) of cultures on various light/dark schedules. Time as number of hours after the start of the second light period. All cultures, from continuous light, were given the succession: dark period 1, light period 1, dark period 2, light period 2, etc. 8(16) and 1(23) data from experiment started 5/13/69; 16(8) from experiment started 6/17/69.

ment were 0.96, 1.20, 1.25, 1.45, and 1.86 g. At flow rates of about 0.8 liter/min, two of these cultures per vessel gave a CO₂ difference of 40 to 80 ppm. However, the relationship between total frond number (or fresh weight) and the CO₂ value is not a simple one. During the experiments involving frequent red light, the cultures grow rapidly, probably doubling every 2 to 4 days, but CO₂ output remains much more level. A reasonable explanation (though with no direct evidence) is that, even with gentle shaking, the major part of the activity recorded is that of the fronds in the top layers of the cultures, with those below contributing a decreasing proportion. Whatever its cause, the relatively level CO₂ output under these conditions immensely simplifies both data collection and the location of peaks and troughs; it also, however, strongly emphasizes the purely relative nature of the data and should be borne in mind when considering possible mechanisms.

Many earlier experiments (examples are in Figs. 4 and 6) were done with an apparatus differing from that described in several respects, notably in its dependence on random leakage rather than a diffusion stone to retrieve the difference between inflow rate and sampling rate, and in the method of drying the sample stream. The results differed from those with the later equipment chiefly in showing a (spurious) strong underlying uptrend due to a pressure buildup, but conclusions with respect to the timing of maxima and minima did not differ.

Notation for Light(Dark) Treatments. All numbers refer to hours, and parentheses indicate darkness. Schedules with a sum of 24 hr are understood to be repeated each day. A “skeleton” schedule (12, 16) refers to total darkness interrupted by brief periods of light—here, \( \frac{3}{4} \) hr. Thus \( \frac{3}{4}(7)\frac{1}{4}(16) \) means \( \frac{3}{4} \) hr light followed by \( 7 \frac{1}{4} \) hr darkness followed by \( \frac{3}{4} \) hr light followed by \( 16 \frac{1}{4} \) hr darkness, repeated, and can be regarded as the “skeleton” of 8(16). All treatments begin from continuous (cool white or dim red) light, and the skeleton photoperiods, in particular, are to be read in the order given. Thus a treatment designated \( \frac{3}{4}(13)\frac{3}{4}(10) \) (continuous light followed by a 13-hr dark period, then \( \frac{3}{4} \) hr of light, then \( 10 \frac{3}{4} \) hr of darkness, then \( \frac{3}{4} \) hr of light, then 13 hr of darkness, and so forth. A treatment designated \( \frac{3}{4}(10)\frac{3}{4}(13) \) has the same succession of elements except that the first dark period experienced is \( 10 \frac{3}{4} \) hr long. Obviously, following continuous light, it is immaterial whether a schedule is designated \( \frac{3}{4}(7)\frac{1}{4}(16)\frac{3}{4} \) or \( \frac{3}{4}(7)\frac{3}{4}(16) \), except for a 15 min difference in the start of darkness.

RESULTS

Light(Dark) Schedules with 24-hr Periodicity. If, after an initial 6- to 24-hr equilibration under continuous dim red light, the cultures are started on light(dark) schedules with a 24-hr periodicity, the CO₂ output rate entrains to that periodicity. Portions of two such experiments are shown in Figure 3. These data and those of numerous similar experiments indicate, first, that the dim red light used probably has only the slightest direct photosynthetic effect on the CO₂ output rate, for the effects of turning it on or off depend almost entirely on the cycle time at which it is done. Note, for example, the great perturbation at the end of the 16-hr light period as compared with the small effect at the end of the 8-hr light period. Secondly, in such simple 24-hr cycles the maximum CO₂ output rate depends on the start of the light period for its timing, not the start of the dark period. In Fig. 3, the maximum is at approximately (depending primarily on how one handles the perturbation at the close of the 16-hr light period) 15 to 17, 16 to 17, or 16 to 17 hr after the start of 16-, 8-, or 1-hr light period, respectively. Similar conclusions appear to hold for schedules from \( \frac{3}{4}(23) \) to \( 21(3) \), with a maximum falling between 14 and 20 hr after the start of the light period. However, some light period lengths lead to greater immediate “light off” perturbations than others, and these may complicate the picture. In addition, recent experiments suggest that, again with certain light period lengths, true steady state entrainment...
Lemna respect the data succession: units. It does these data is of continuous oscillation light cated.

FIG. 5. Above: CO₂ output rates (1/4-hourly, arbitrary units) of three sets of cultures. All cultures from continuous light were exposed to the succession: 12 hr dark, 12 hr light, 12 hr dark, 12 hr light, 12 hr dark (D3). 12 hr light (L3). Below: The three records above summed with respect to time from the start of L3.

may not be reached without at least four or five cycles, so that the data in Figure 3 may not be truly representative.

Circadian Rhythmicity in Darkness. Flowering experiments with skeleton photoperiods (12, 15) suggested that transfer from continuous light to darkness initiates a circadian oscillation in Lemna cultures. Data such as those in Figure 4 indicate that such an oscillation is also detectable in the rate of CO₂ output, though it does not persist very long. In the experiment shown, the hourly rates of CO₂ output for three sets of cultures are plotted in arbitrary units. (As noted earlier, the spurious general uptrend in these data is a technical problem, but it does not affect the conclusions.) Initially, all three sets were held in dim red light, and the one (channel 3) that remained so throughout the experiment shows a simple linear change of CO₂ output rate with time. The light was turned off on the other two channels at the times indicated by the arrows, following which each rate oscillated for a first maximum, a first minimum, a second maximum, and a second minimum, with timing dependent on the transition to darkness. If the values of the two darkened cultures are summed as functions of the time in darkness, the first maximum is found at hour 9 and the second at hours 30 to 36 (mean, 33), while the first and second minima are at hours 16 to 18 (mean, 17) and hour 40, respectively. Thus the periodicity is clearly circadian. The scatter in the data and the rapid damping prevent more precise evaluation of the period, but the general conclusion has been confirmed in a number of experiments.

The possibility that cultures previously entrained to 24-hr cycles might show a more persistent oscillation in darkness was tested in several experiments. Typical results are shown in Figure 5, for three sets of cultures given three (12)12 (dark) light cycles following continuous light. The upper panels show the data for the individual sets in constant darkness, which in the lower panel are summed as functions of time after the third (and last) start of light. Here again, clear first and second maxima and minima, with circadian timing, are evident, but with no greater persistence than starting from continuous light (Fig. 4).

Skeleton Photoperiods: Comparison of 1/4(13)1/4(10) with 1/4(10)1/4(13). When plants are transferred from continuous light to 6 or 7 days of a skeleton photoperiodic regime consisting of alternating 101/4- and 13-hr dark periods separated by 1/4-hr light pulses, flowering depends on which dark period is given first (12). The course of CO₂ output was therefore followed under comparable conditions. A typical result for the first 3 days is shown in Figure 6, in which continuous light ended at 1400 on the first day shown, and the 1/4-hr light breaks are indicated by vertical lines. For the first 24-hr period, the patterns are essentially alike. In the second, however, the maximum under 1/4(10)1/4(13) comes early in the 13-hr dark period, while under 1/4(13)1/4(10) (lower panel) it is late in the 13-hr period with some perturbation into the start of the next 101/4. By the third 24-hr period the difference is no longer subtle. The 1/4(13)1/4(10) pattern has established a 24-hr rhythmicity with its peak surrounding the transition between (13) and (10), while the other is almost arhythmic, with no marked maxima or minima. Thus, two skeleton schedules with markedly different effects on flowering differ also in their action on CO₂ output rate; at least for the first few cycles.

While the effects of 1/4(10)1/4(13) and its inverse differ greatly in short flowering experiments, the difference is reduced with longer treatments. In a typical experiment, flowering percentage values for 1/4(13)1/4(10) were 53, 53, 58, and 66 on days 6, 7, 8, and 9, respectively, while the corresponding values for 1/4(10)1/4(13) treatment in the same experiment were 1, 13, 11, and 39. Hence, it seemed worth examining the CO₂ output in longer experiments also, to see whether the difference visible in the first few days disappears. Data from a 6-day experiment appear in Figure 7. The results for the first 3 days resemble those in Figure 6, in spite of the different method and presentation used. In particular, on day III, 1/4(10)1/4(13) again shows essentially no maxima or minima, while 1/4(13)1/4(10) peaks just at or before the 13 101/4 transition. By day VI, however, a 24-hr periodicity is evident also in 1/4(10)1/4(13). Experiments long enough to determine the stable pattern, if any, finally attained under 1/4(10)1/4(13) treatment have not been done, but it is obvious that the arhythmic

Fig. 6. CO₂ output rate of two sets of cultures; curves drawn between points represent hourly rates in arbitrary units. Vertical lines represent 1/4-hr light breaks; numbers in panels indicate lengths of dark period in hours.
condition of days III and IV, the most obvious difference between the two schedules, does not persist indefinitely.

**Skeleton Photoperiods with Highly Unequal Portions.** Earlier results with *Lemna* flowering (11) as well as with *Drosophila* (16) indicate that if the two portions of a skeleton schedule differ sufficiently in length—e.g., $\frac{1}{4}(5\text{hr})\frac{3}{4}(18)$ instead of $\frac{1}{4}(10\text{hr})\frac{3}{4}(13)$—then the response does not depend significantly on which dark period is given first. Confirming this, a flowering experiment comparing four treatments, $\frac{1}{4}(10\text{hr})\frac{3}{4}(13)$, $\frac{3}{4}(13)$, $\frac{1}{4}(10\text{hr})\frac{3}{4}(4)$, $\frac{1}{4}(5\text{hr})\frac{3}{4}(18)$, and $\frac{1}{4}(10\text{hr})\frac{3}{4}(5)$, gave flowering percentage values of 2, 37, 61, and 82 on day 7, and 7, 53, 71, and 78 on day 8. A 6-day experiment on CO₂ output comparing the same schedules is shown in Figure 8. In the top two panels are the now familiar differences between $\frac{1}{4}(10\text{hr})\frac{3}{4}(13)$ and its inverse, with the former essentially flat for days III and IV and the latter rapidly entraining the output rate to a 24-hr periodicity. The two lower panels, on the contrary, indicate that both of the schedules with high unequal portions entrain the output rapidly to a 24-hr periodicity regardless of whether the long or the short dark period is presented first. Within two full cycles in each case, the same phase relationship between schedule and CO₂ output seems to have been established, with the maximum rate roughly 9 to 11 hr after the start of the long dark period—that is, 14 to 16 hr after the first light break defining the short dark period.

**Skeleton Photoperiods: Interrupting the Shorter Dark Period.** Earlier results on flowering indicate that if the shorter dark period of a skeleton schedule with relatively equal portions is interrupted by light, then its typical effects no longer appear (11). To see whether this might be reflected in the CO₂ data, the experiment shown in Figure 9 was performed. In the two upper panels the effects of $\frac{1}{4}(10\text{hr})\frac{3}{4}(13)$ are compared with and without an additional initial 8-hr dark period. In flowering experiments, the initial 8 hr of darkness renders the otherwise favorable schedule highly inhibitory. In Figure 9, it is evident that the initial 8 hr of darkness brings about a situation similar to that caused by $\frac{1}{4}(10\text{hr})\frac{3}{4}(13)$ in which the course of CO₂ output for days III and IV is rendered essentially flat in contrast to the marked periodicity otherwise brought about. In the two lower panels, however, the effect of an initial 8-hr dark period is tested on a schedule, $\frac{1}{4}(13)\frac{3}{4}(5\text{hr})\frac{1}{4}(4)$, which is simply $\frac{1}{4}(13)\frac{3}{4}(10\text{hr})$ with the 10\text{hr} dark period interrupted. Clearly, this interruption is sufficient to remove whatever ambiguity is present in the original schedule, for the initial 8-hr dark period has essentially no effect either on the rapid establishment of a marked 24-hr periodicity or on the phase relationship attained.

**DISCUSSION**

What Is Being Measured? Carbon dioxide output rates have been used to follow circadian periodicities in plants mainly by Wilkins (22) using detached *Bryophyllum* leaves, though a few other studies exist (6, 8). The work reported here is the first to attempt to correlate such data with the photoperiodic control of flowering. For this purpose it is probably satisfactory to regard CO₂ output rate as a meter on a "black box"—hopefully, in
fact, as "hands on the clock"—but it is worth remembering that the mechanism of the changes measured is unknown. (This is quite aside from the fortuitous quantitative situation, itself incompletely understood, already discussed in "Materials and Methods.") In the data presented, the entrainable variation in CO₂ output is generally between 10 and 20% of the total; this may represent any situation from a 0 to 100% variation in a reaction responsible for a small part of the total, to a small variation in a single process. The evidence in Bryophyllum points towards variation in CO₂ fixation by phosphoenolpyruvate carboxylase as the basis of the periodicity (22), but there is no evidence either for or against such a mechanism here. Alternatively, the Lema system might reflect changing respiratory rates (cf. Reference 5), and these in turn might reflect changing rates of frond production or other processes. The likelihood of rhythms in stomatal activity (6,8,19) is reduced here by the observation (Dr. H. Ziegler, personal communication, August 1969) that Lema stomates are nonfunctional. In the hands of Lema, further analysis of the system is obviously desirable; nevertheless, with all the limitations in mind, several significant conclusions can be drawn from the data now at hand.

**Rhythmicity in Continuous Darkness: Damping and Technical Problems.**

Experiments such as those summarized in Figures 4 and 5 show that in continuous darkness the CO₂ output rate oscillates through at least two maxima and minima, while remaining linear under continuous dim red light. The phase relationship of a given point on the oscillation to local time is determined either by the time of transition from continuous light to darkness or by the schedule previously used for entrainment. While the cycles do not persist long enough to be described as truly free-running (1), their period length seems clearly circadian. All these properties of the CO₂ output rate are those that would be predicted if it accurately reflected the kind of endogenous circadian rhythm inferred from the flowering experiments (12).

Damping in extended darkness unfortunately limits the types of experiments that can be done—excluding, for instance, rigorous phase-response tests—but it is not inconsistent with the original flowering data. Few higher plant systems, in fact, run for more than a small number of cycles under constant darkness, and L. perpusilla, in particular, is known to have a nonphotoperiodic light requirement for growth (12). However, there are at least two grounds for optimism on eventually measuring more cycles in darkness. One is the likelihood that the 28 to 30°C temperature used here is superoptimal (21), so that the number of cycles at, say 21°C might be much greater. The other is that the monitoring system itself can be improved to reduce random noise and external perturbations, both of which limit its use with cycles of small amplitude.

Indeed, perturbation by some external factor or factors is evident in many experiments. For example, in the upper panels of Figure 5, while the major fluctuations are related primarily to the previous light (dark) schedule, there are also discontinuities in all three records at the same local times: 1400 hr on July 29 (800) and 1800 on July 30, and 1600 on July 31. Modifications such as close voltage regulation of the shaker may serve to eliminate such effects. In addition, the daily outdoor cycle of CO₂ level in the air—with much greater amplitude on some days than on others—reduces precision, and a system with constant CO₂ is being planned. Meanwhile, though the system used here is obviously adequate for work with periodicities either entrained or initiated by light-dark transitions, its openness to perturbation would disqualify it completely if the exogenous or endogenous basic timing mechanism, not merely of particular period, lengths and phase relationships, were the central question (4).

**Effects of Skeleton Schedules on CO₂ Output and on Flowering.**

The experiments with skeleton schedules are in effect tests of some of Pittendrigh's (16) formulations of Bunning's theory of photoperiodism. The latter, in its most general form, simply asserts that photoperiodic treatments act by interacting with an endogenous oscillation or circadian clock that functions as the basic timing mechanism (6,7). More specifically, Pittendrigh has proposed that skeleton schedules having different photoperiodic effects should entrain the endogenous oscillation in different phase relationships. Most specifically of all, a model developed from work with Drosophila was the basis of two precise predictions on the actions of skeleton photoperiods. First, schedules with two highly unequal portions should entrain the rhythm in the same phase relationship as full (nonskeleton) photoperiods in which the shorter fraction is the light period. That is, \(\text{\frac{3}{4}(6\%)} \text{\frac{1}{4}(17\%)}\) should act like \(7(17%)\), and this should be true irrespective of which dark period is given first—irrespective, in effect, of what phase of the endogenous oscillation (initiated by the transition to darkness) encounters the first light pulse. The second prediction is that for skeletons with relatively equal portions a "bistability phenomenon" would occur such that the final steady state phase relationship would depend entirely on which dark period was seen first—when in the endogenous oscillation the first light pulse would fall (16). What is striking is that all predictions, though from a Drosophila model, were fully consistent with data on Lema flowering obtained independently. A recent account of work elsewhere (15) confirming the flowering data still concludes that the original model is fully applicable. Use of the CO₂ results, however, provides a dimension previously lacking.

The least specific prediction—different entrainment by skeletons having different photoperiodic effects—seems fully borne out by the comparison between \(\text{\frac{3}{4}(13\%)} \text{\frac{1}{4}(10\%)}\) and \(\text{\frac{3}{4}(10\%)} \text{\frac{1}{4}(13\%)}\). The prediction that skeletons with highly unequal portions would effect the same steady state phase relationship irrespective of which dark period is given first is also borne out (Fig. 8, lower panels). Returning to \(\text{\frac{3}{4}(13\%)} \text{\frac{1}{4}(10\%)}\) and its inverse, the model also holds in the sense that the manner of entrainment depends on which dark period is seen first. However, there seems to be no bistability phenomenon of the kind predicted. Rather, the obvious difference between the two schedules is in the rapidity with which one entrains, and the other initially seems to suppress, rhythmicity in the CO₂ output. On the Drosophila model, the schedule \(\text{\frac{1}{4}(13\%)} \text{\frac{3}{4}(10\%)}\) would give a pattern similar to that caused by \(11(13)\), the corresponding full schedule that allows high flowering, while \(\text{\frac{3}{4}(10\%)} \text{\frac{1}{4}(13\%)}\) should give a pattern similar to that caused by the low flowering \(13\text{\frac{1}{4}}(10\%}\). This is certainly not observed within the first few days, so that to this extent the Drosophila model seems to fail. However, Figure 7, 8, and 9 suggest that in fact even \(\text{\frac{3}{4}(13\%)} \text{\frac{1}{4}(10\%)}\) fails to give truly steady state entrainment in 6 days, while preliminary experiments indicate that the full photoperiodic schedules \(11(13)\) and \(13\text{\frac{1}{4}}(10\%}\) are among those for which the steady state is also attained quite slowly (see "Results"). Hence longer experiments might well elucidate as the Drosophila model predicts.

Two major questions remain unanswered in the comparison between \(\text{\frac{3}{4}(13\%)} \text{\frac{1}{4}(10\%)}\) and its inverse. First, explaining why one allows rapid flowering and the other does not will require locating the phase of the rhythm in which light is maximally inhibitory to flowering (cf. Reference 9). Attractive as the hypothesis might be, the difference in response cannot be attributed solely to rapid entrainment by one schedule and apparent temporary suppression by the other, though the latter may play some part in the low flowering. It is easy to find schedules—e. g., \(16(8)\)—that entrain rapidly but inhibit flowering completely. The second question concerns the meaning of the apparent temporary suppression itself. There are at least four possible mechanisms: (a) that the CO₂ output rate becomes uncoupled from an underlying clock, which itself carries on unaffected; (b) that the clock is deranged in some way, perhaps as in the "singular state" discovered in Drosophila (23); (c) that the clocks of individual fronds continue.
to run but that the population as a whole is desynchronized; or 
(d) that the apparently flat records result from a fortuitous suc-
cession of resettingsthatsimply do not happen to show sharply
maxima or minima, the only phase markers available. Given the
low resolving power of the method, the last seems perhaps most
likely. Nevertheless, there is a real possibility that the low in-
tensity light breaks used are insufficient, at certain phases, to
reset the clock unambiguously, and this could bring about condi-
tions b or c. In this case, high intensity skeleton photoperiodic
schedules would be expected to act very differently. Through such
experiments, as well as others, it should be possible to distinguish
among the mechanisms suggested.

Other Considerations. Overt circadian rhythms in a number of
other plant systems continue under light of various qualities and
intensities, while CO₂ output in *Lemna* cultures becomes
linear under continuous dim red light. This property, as already
noted, was predictable on the basis of flowering experiments, and
perhaps related to it is the ability of brief red light exposures to
entrain the rhythm. However, a point that must remain obscure
at present also illustrates the limitation of flowering experiments
alone in analyzing such properties. Before the present system
was available, *Lemna* flowering work suggested that single light
exposures were effective in resetting the phase of the rhythm only
if 4 to 6 hr long (14). Present evidence of entrainment even by 15-
min light periods seems inconsistent with such a conclusion, and
parallel experiments with the CO₂ system will be needed to resolve
the contradiction. It may, of course, be inappropriate to compare
single perturbation experiments with repeated cycle experiments,
particularly when the single perturbation was given at only one
particular phase of the rhythm.
The effects of light other than dim red on the *Lemna* CO₂
rhythm have yet to be tested; blue light, which permits flowering
even when continuously (13), will be of particular interest, if
complications due to photosynthesis can be avoided.

Takimoto and Hamner (20) have interpreted their data on
*Pharbitis* flowering as evidence for the existence of two circadian
components in the photoperiodic timing mechanism, a "light-
on" rhythm initiated by the darkness-to-light transition and a
"light-off" rhythm. In trying to relate the present experiments to
such a concept, one can note simply that, while a light-off signal
is clearly sufficient to initiate a circadian oscillation (Fig. 4), in
entrainment to 24-hr cycles it seems to be the light-on signal that
functions as the primary synchronizer (Fig. 3). Further work
with this system may make it possible to relate the Takimoto-
Hamner double rhythm proposal to models of the type proposed by
Pittendrigh (16), which at present seem to fit the data better
though imperfectly.

Conclusions. The CO₂ output rate of *L. perpusilla* 6746 under
combinations of dim red light and darkness appears to reflect the
activity of a circadian clock with properties expected from earlier
experiments on flowering (12). The effects of skeleton photo-
periodic schedules are consistent with the view that the action of
such schedules depends on the manner in which they entrain the
circadian oscillation, conforming in many, though not in all
respects to predictions from a model based on the *Drosophila*
eclosion rhythm (16). The results thus strongly support the con-
cept that a circadian clock is an important component of photo-
periodism (6, 7, 16), and they provide an experimental system in
which the actions of many factors on that component may be
examined separately from other effects.

Physiol. 10: 875–889) have recently published an account of
rhythmic changes in several aspects of respiratory metabolism in
*L. gibba*. Though not analyzed in relation to flowering responses,
these data should prove important in interpreting the results
presented here.

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