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Dormancy, Diapause, and the Role of the Circadian System in Insect Photoperiodism

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Abstract

Whole-animal experiments devised to investigate possible association between photoperiodic time measurement and the circadian system (Bünning's hypothesis) are compared with more recent molecular investigations of circadian clock genes. In *Sarcophaga argyrostoma* and some other species, experimental cycles of light and darkness revealed a photoperiodic oscillator, set to constant phase at dusk and measuring night length repeatedly during extended periods of darkness. In some species, however, extreme dampening revealed an unrepetitive (i.e., hourglass-like) response. Rhythms of clock gene transcript abundance may also show similar phase relationships to the light cycle, and gene silencing of important clock genes indicates that they play a crucial role in photoperiodism either alone or in concert. However, the multiplicity of peripheral oscillators in the insect circadian system indicates that more complex mechanisms might also be important.

INTRODUCTION

The traditional view of insect overwintering dormancy is that it is governed by periods of either quiescence or diapause. In quiescence, growth, development, and reproduction cease during periods of adverse conditions such as low temperature (e.g., due to cold torpor), and active metabolism resumes promptly once the adverse conditions terminate in the spring. It is therefore a direct response to adverse environmental conditions. In diapause, however, insects respond to environmental cues such as the autumnal changes in day length or night length (i.e., to photoperiod) that occur well in advance of approaching winter. It is also regulated by genetically programmed changes in the endocrine systems controlling development or reproduction, thereby constituting an alternative developmental pathway (12). Because photoperiodic changes are not in themselves adverse, such signals are regarded as token stimuli (31). There is also evidence for a low-temperature, non-photoperiodic regulation of diapause (36, 43). The selective advantages afforded by diapause—as opposed to quiescence—lie in the time available for the storage of metabolites, particularly lipids, before the onset of winter and in the synchrony engendered within the population of insects upon its reactivation in the spring. In some cases, there may also be an associated cold tolerance (21).

This review is an account of the development of an idea proposed in 1936 by the German plant physiologist Bünning (6) that photoperiodism was a function of the circadian system, an idea that became known as Bünning's hypothesis. In the 1950s and 1960s, slow progress was made, stimulated mainly by the work of Pittendrigh, an ardent supporter of Bünning's proposal, comparing the properties of photoperiodic time measurement with the emerging knowledge of circadian rhythms (53, 56, 58). Despite these advances, progress continued to be slow because the nature of circadian rhythmicity itself remained obscure. This situation began to change in 1971, when Konopka & Benzer (25), working with *Drosophila melanogaster*, isolated the first clock mutants. However, this model insect had rather poor photoperiodic responses, and further progress had to wait until modern molecular techniques became applicable to non-model species. Today, we are on the cusp of describing the photoperiodic responses of many insects in terms of the genes and proteins making up their seasonal clocks.

The first part of this review describes whole-animal experiments providing evidence for the circadian basis of photoperiodic timing, mainly using the flesh fly *Sarcophaga argyrostoma*. The second part reviews molecular evidence from a wider range of species to support this association, and in the last section, the complex multioscillator nature of the circadian and photoperiodic systems is discussed.

THE CIRCADIAN BASIS OF PHOTOPERIODIC TIME MEASUREMENT IN INSECTS

Photoperiodic Induction of Diapause

Figure 1 shows the photoperiodic response curves for two species of fly isolated at 55°N: *S. argyrostoma*, which has a pupal diapause (65), and *Calliphora vicina*, which diapauses as a fully fed larva (76). These curves show several characteristic features: (*a*) continuous nondiapause development during the long days (or short nights) of summer, separated from the diapause-inducing short days (or long nights) of autumn by an abrupt critical day length of approximately 14.5 h (or critical night length of approximately 9.5 h); (*b*) the effect of temperature on diapause incidence; and (*c*) the characteristic fall in diapause incidence under ultrashort photoperiods. **Figure 1*b*** also shows four ranges of photoperiod, two of which (*i* and *iv*) do not occur in the natural environment and one of which (range *ii*) only occurs during the winter, when the flies are already in diapause. Only range *iii* is of ecological importance; this is dominated by the critical point and demonstrates the essentially autumnal nature of the photoperiodic phenomenon.

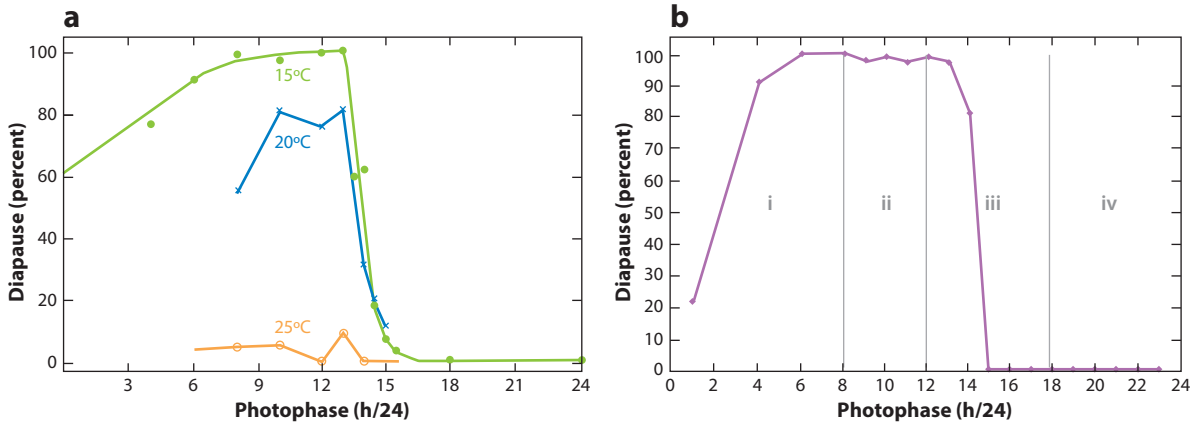

Figure 1

Photoperiodic response curves for two insect species. (*a*) Pupal diapause induction in the flesh fly *Sarcophaga argyrostoma* isolated at 55°N, showing critical day length of approximately 14.5 h (or critical night length of approximately 9.5 h), the fall of diapause incidence under ultrashort day lengths, and the reduction in diapause with increased temperature (65). Closed circles indicate 15°C, crosses indicate 20°C, and open circles indicate 25°C. (*b*) Larval diapause induction in the blow fly *Calliphora vicina* at 55°N induced by maternal exposure to different photoperiods at 19°C and larval rearing at 12°C in constant darkness (schematic) (77). Photoperiods in regions *i* and *iv* do not occur in the natural environment, and those in *ii* only occur during the winter, when flies are already in diapause. Only region *iii* is of ecological significance: This region is dominated by critical day length (or critical night length) directing development down the alternate diapause or nondiapause pathways.

Bünning's Hypothesis and Coincidence Models

In 1936, Bünning suggested that photoperiodic time measurement was a function of circadian rhythmicity, then known to regulate persistent daily oscillations—in the case of his work, the up-and-down movements of the leaves of bean seedlings (6). He proposed that the daily cycle comprised a 12-h light-requiring half cycle (the photophil) and a 12-h dark-requiring half cycle (the scotophil). Short-day (long-night) effects then resulted when light was restricted to the photophil, but long-day (short-night) effects ensued when light extended into the scotophil (**Figure 2**).

More recent versions of Bünning's general hypothesis include external and internal coincidence (54, 70, 80) (**Figure 2**). The former resembles Bünning's original model in that it comprises a single oscillator and two roles for light: entrainment by the light cycle and photoinduction by light coinciding with a light-sensitive phase in the night. Internal coincidence, in contrast, comprises two (or more) oscillators, one (or some) phase-set by dawn and one (or some) phase-set by dusk, and a single role for light: that of entrainment.

Experimental Evidence for the Circadian Basis of Photoperiodism

Numerous experiments attest to the association between the circadian system and photoperiodic timing, the most commonly used being the Nanda-Hamner (NH) protocol (45). In this experimental design, a fixed photophase is coupled, in different experimental subsets, with a range of dark periods to give overall cycle lengths up to 72 h or more. In so-called positive NH responses, alternate peaks and troughs of diapause incidence occur at roughly 24-h intervals as cycle length is extended, the interpeak interval reflecting the period of the underlying circadian oscillation(s) (**Figure 3**). Essentially negative NH responses lacking these peaks and troughs were

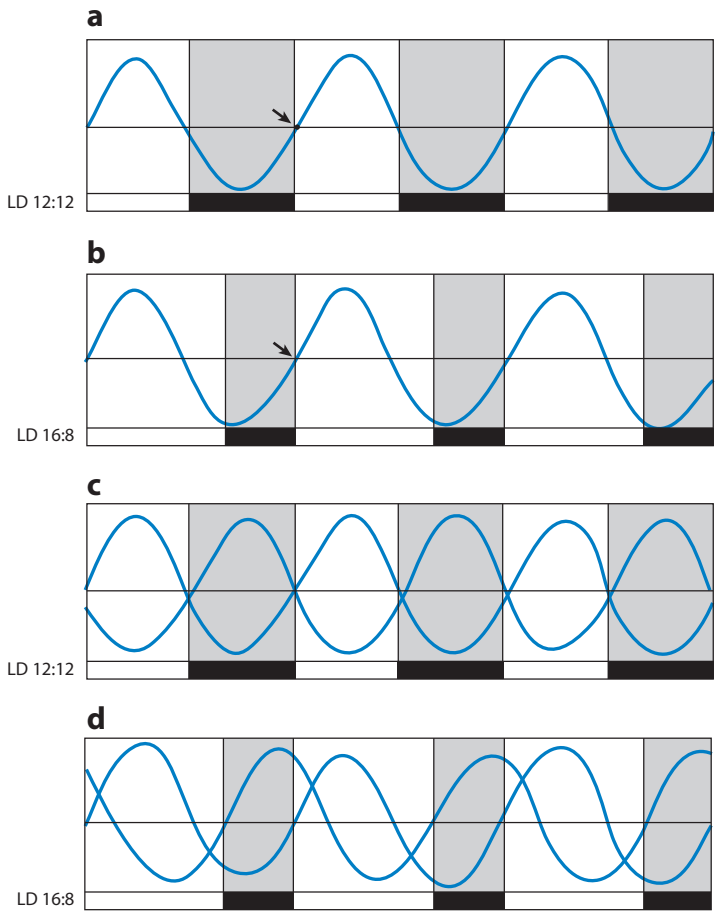

Figure 2

Models for the insect photoperiodic clock. The top two panels illustrate Bünning's original model [referred to as external coincidence (54)] under (*a*) a short-day (long-night) photoperiod in fall and (*b*) a long-day (short-night) photoperiod in summer. In both regimes, the photoperiodic oscillator is phase-set (entrained) by the onset of light (at dawn) (*small arrows*) and passes through a 12-h light-requiring (or photophil) half cycle (above the mid-line) and a 12-h dark-requiring (or scotophil) half cycle (below the mid-line). Under an autumnal long night (*a*) light (L = 12 h) and darkness (D = 12 h) form a light–dark cycle (LD 12:12 h) in which light is restricted to the photophil, whereas under a short-night summer photoperiod (LD 16:8 h) light extends into the scotophil. Light, therefore, has two roles in external coincidence: entrainment of the oscillator and coincidence (or not) between light and a photoinducible phase, leading to nondiapause development. Schematic created after Bünning (6, 7). (*c*,*d*) An alternative model, internal coincidence, comprising two oscillators, one phase set by light-on (at dawn) and the other by light-off (at dusk). Under a long-night regime (*c*), the two oscillators are held in antiphase, whereas under short summer nights (*d*), mutual (internal) phase relationships change to cause an overlap between the two, leading to nondiapause development. Unlike external coincidence, light has only one role in this model: that of entrainment. Schematic modified from Pittendrigh (54).

initially considered to indicate noncircadian or hourglass-like photoperiodic timers (32, 33) but are now thought to indicate the involvement of heavily dampened circadian oscillations (8, 36). Experimental evidence for such oscillator dampening has been found in *S. argyrostoma* (85) and in two species of *Drosophila* living at high latitudes (4, 24, 93).

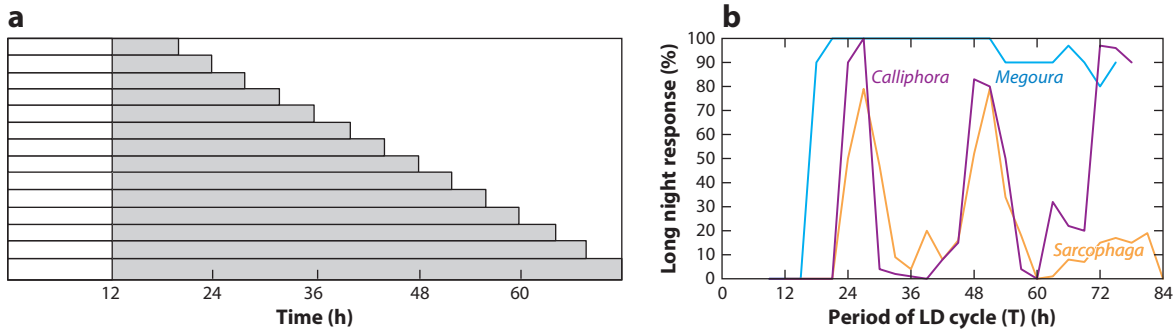

Figure 3

The Nanda-Hamner experimental protocol designed to reveal the oscillatory nature of the insect photoperiodic clock. (*a*) Insects are exposed to light–dark (LD) cycles containing a constant photophase (12 h, in this case) coupled, in different experimental subsets, to a dark period of increasing length to give overall cycle lengths up to 72 h or more. This light cycle is repeated throughout the insect's photoperiodically sensitive period of development. (*b*) Responses to Nanda-Hamner experiments in three species: In *Sarcophaga argyrostoma* and *Calliphora vicina*, peaks of high diapause incidence occur at cycle lengths of 24, 48, and 72 h (close to multiples of the endogenous circadian period), indicating involvement of the circadian system in photoperiodic time measurement. In the aphid *Megoura viciae*, however, such peaks and troughs are lacking, initially suggesting a noncircadian (hourglass-like) clock. *Sarcophaga* data are from Saunders (66). *Calliphora* data are from Saunders (77). *Megoura* data are from Lees (33).

The Photoperiodic Clock Measures Night Length Rather Than Day Length

Independent variation of the light and dark phases throughout the photoperiodic sensitive period has shown that, in most species, the period of darkness (the scotophase) is more important than that of light (the photophase) (78, 79). In *S. argyrostoma*, for example, the incidence of pupal diapause was found to be very low in cycles containing a short 8-h night [e.g., Light:Dark (LD) 12:8 and LD 16:8] but approached 100% in cycles containing a long 12-h night (e.g., LD 12:12 and LD 16:12), regardless of whether the accompanying light component was short or long (66). A possible exception to this is provided by the linden bug *Pyrrhocoris apterus*, which appears to measure day length rather than night (75). However, resolution of this apparent difference may be as follows: *P. apterus* is a diurnal species, often seen to be active in bright sunlight. It is possible, therefore, that what is bright light to *Sarcophaga* is merely dim light to the linden bug. Light-sensitive species may therefore perceive an experimental light source as bright, whereas relatively light-insensitive species may see the same light source as dim. Consequently, if brighter light pulses were used in experiments with *P. apterus*, then the timing system might be shown to measure night rather than day, as in the fly. Perhaps, in such studies, subjective light intensity should be considered rather than absolute intensity. This aspect has yet to be tested, but its importance is further demonstrated in the next section.

The Circadian System Is Set to a Constant Phase at Light-Off

Working with the adult emergence (eclosion) rhythm of *Drosophila pseudoobscura*, Pittendrigh (53) showed that eclosion became arrhythmic under extended periods of light, but rhythmicity became re-apparent after transfer to darkness. These data suggested that the rhythm was damped out in extended periods of light but restarted after the constant light-to-darkness transition. At least for practical purposes, extrapolation back to this point indicated that the oscillation restarted at a phase equivalent to the beginning of the subjective night (at a phase called Circadian Time, CT 12), where it was in a position to commence night length measurement. A similar phenomenon has

been shown for the rhythm of adult activity in the blow fly *C. vicina*, in which locomotor activity became arrhythmic in bright light (approximately 48 lux) but resumed rhythmic activity after transfer to darkness, also at a phase equivalent to CT 12 (20, 82). In contrast, during constant light of lower intensity (approximately 2 lux), flies remained rhythmic—although with different individual and lengthened periods—and phases after transfer to darkness were not synchronized to CT 12. The intensity of the light is therefore crucial in this phenomenon.

In *S. argyrostoma*, both the photoperiodic oscillator [shown by the peaks of high diapause in NH experiments (**Figure 3**)] and the rhythm of adult eclosion are reset to a phase close to CT 12 after an extended period of light (68). This suggests that, under these conditions, night length measurement begins at dusk, and that the overt rhythm of eclosion may be taken as an independent measure of the underlying but otherwise invisible photoperiodic oscillator in further analyses of the timing phenomenon.

The Photoinducible Phase and Its Location in the Night

Search for the light-sensitive (or photoinducible) phase predicted by Bünning's hypothesis and its derivation, the external coincidence model, began with night interruption experiments in which the dark period of a diapause-inductive long-night cycle was systematically interrupted by short light pulses (1, 2). Unexpectedly, this procedure produced two such points, rather than one, the first (point A) occurring early and the second (point B) late in the night (**Figure 4**). Resolution of this apparent conundrum was achieved by Pittendrigh & Minis (59). He pointed out that light falling at point A, acting as a new dusk, would cause a delay in subsequent phases of the oscillation (phase delay), while that falling at point B, acting as a new dawn, would cause advances (52). Since

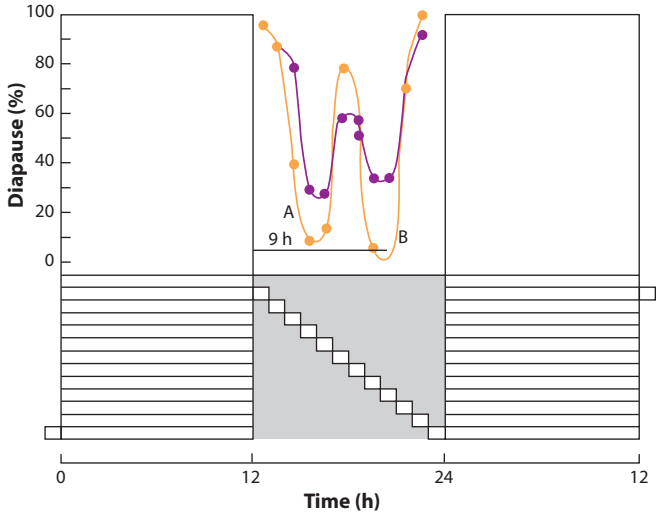

Figure 4

Night-interruption experiments in *Sarcophaga argyrostoma*. Larvae were exposed to diapause-inducing light–dark cycles of LD 12:12 with the 12 h night systematically interrupted by a 1 h supplementary light pulse. In two replicate experiments, the incidence of pupal diapause was high (as in the uninterrupted controls) except for pulses falling early in the night (at point A) or late in the night (at point B). Point B occurs 9 to 9.5 h after the onset of darkness and marks the position of the photoinducible phase. Figure created with data from Saunders (67).

the photoperiodic oscillation was phase-set to a constant phase (CT 12) at the transition from light to darkness (see above), the photoinducible phase—dubbed φ_i—must be at point B and not point A. Phase delays caused by light early in the night would lead to nondiapause development by delaying φ_i into the following photophase, whereas light falling late in the night would cause such effects by coinciding directly with φ_i, leading once again to nondiapause development (**Figure 4**). In the case of *Sarcophaga*, φ_i would lie at the end of the critical night length, i.e., at CT 12 + 9 to 9.5 h, or at approximately CT 21.5 h.

Use of the Eclosion Rhythm of *S. argyrostoma* as an Overt Indicator of Phase of the Photoperiodic Oscillator

With an overt behavioral or physiological rhythm, phase changes may be observed directly, but this is not the case with a covert photoperiodic oscillator. For this reason, Bünning suggested that an overt rhythm—in the case of his work, the daily up-and-down movements of bean leaves—could be used as an independent measure of phase for the photoperiodic oscillator (7). This analytical approach has been used to investigate the photoperiodic regulation of pupal diapause in the flesh fly using the rhythm of adult emergence as an indicator of the photoperiodic system. To do this, a family of responses (94) to single light pulses ranging from 1 to 20 h was established and used to track the phase of φ_i (at CT 21.5) in a variety of simple and more complex light regimes in a computer program designed to determine entrainment of the covert photoperiodic oscillator through the development of the fly from the beginning of the photoperiodic sensitive period (in first instar larvae) to its termination (as mature larvae entering the soil to form puparia, after approximately 19 days at 18°C) (69, 72).

In a wide variety of experiments involving both simple and more complex light cycles—including simulated natural photoperiods, night interruption experiments, regimes formed from two short 1 h pulses per cycle (symmetrical skeleton photoperiods) and the NH protocol—computations indicating that φ_i fell in the dark led to a high incidence of pupal diapause, whereas those indicating that φ_i was illuminated resulted in nondiapause development to the adult fly (67, 69).

Confirmation of the role of the photoinducible phase in the photoperiodic induction of pupal diapause in *S. argyrostoma*, and thus of the validity of the external coincidence model, was then provided by two further experiments. In the first, originally devised for the aphid *Megoura viciae* (33), larvae of *S. argyrostoma* were exposed to night-interruption experiments with the interrupting pulse either (*a*) falling early in the night (at point A) and then being followed by a dark period ranging from 7 to 13 h or (*b*) falling later in the night (at point B) and then being followed by a final 12 h of darkness, a scotophase longer than the critical value. The first regime showed that the nondiapause-inducing effect of light falling at point A could be reversed by a final dark period greater than the critical night length, whereas a similar light pulse falling on point B could not. The photoinducible phase was therefore at B and not A. Goto & Numata (16) later showed that the spectral sensitivity of the early night interruption was greatest at short wavelengths (470 to 583 nm; ultraviolet to green), whereas for a light pulse late in the night, the action spectrum was much broader (395 nm to 660 nm; into the red). This difference suggested that two distinct photoreceptors were involved (16), an observation consistent with earlier results for *M. viciae* (33). For example, entrainment at both points A and B might involve the blue-light-sensitive CRYPTOCHROME, whereas sensitivity to longer wavelengths at B might involve opsin-based photoreceptors (see below).

In a later experiment, larvae of *S. argyrostoma* were exposed throughout their sensitive period to regimes containing a single 1 h pulse of light per cycle in cycles ranging from 21.5 to 29.5 h

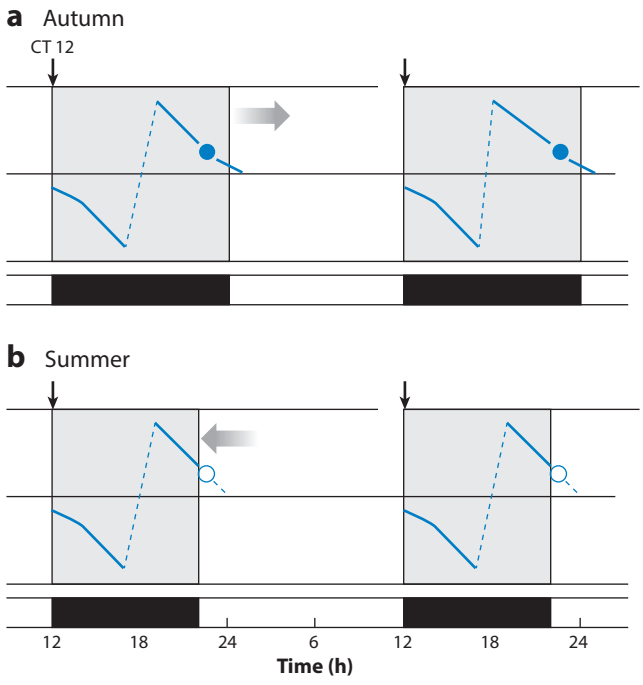

Figure 5

The external coincidence model as applied to *Sarcophaga argyrostoma*, showing the model under (*a*) short and (*b*) long days. The photoperiodic oscillator is phase-set to dusk at Circadian Time (CT) 12 at the light-to-dark transition (*small vertical arrows*), and the photoinducible phase (φ_i) occurs 9–9.5 h (the critical night length) later. Under autumnal short days or long nights (*a*), φ_i falls in the dark, and diapause supervenes; under the long days or short nights of summer (*b*), it is illuminated by dawn light, resulting in nondiapause development. Large horizontal arrows show the movement of dawn in relation to the photoperiodic oscillator. Closed and open circles show the phases of φ_i in autumn and summer, respectively.

covering the primary range of entrainment of the photoperiodic oscillator to 1 h pulses of light. In such experiments (59), entrainment theory predicts that, when the period of the light cycle is greater than the endogenous period of the oscillator (about 24 h), the light pulse must occur in the early part of the night, where it causes the phase delay necessary to convert the latter to the light cycle. Conversely, when the period of the light cycle is less than the period of the oscillator, the light pulse must occur late in the night to cause the necessary phase advance, again to correct the latter to the former. Simply altering the period of the light cycle therefore allowed different parts of the night to be selectively illuminated. The results of this experiment showed that only when the 1 h light pulse illuminated the photoinducible phase (late in the night at CT 21.5) was nondiapause development recorded. In all other regimes, this phase fell in the dark, and diapause supervened (71, 73). These data suggest a more up-to-date version of external coincidence (**Figure 5**) that is more appropriate for *S. argyrostoma*.

Ultrashort Photophases, Light Intensity, and Transient Number

Illumination or non-illumination of the photoinducible phase accounts for the switch in development at the critical night length but cannot explain the fall in diapause incidence in very short photoperiods (**Figure 1**). Even though these ultrashort photoperiods play no role in the natural

environment, explanation of their action is necessary and provides additional evidence for the involvement of the circadian system in photoperiodic timing.

Working with *D. melanogaster*, Winfree (94) showed that shorter (and/or dimmer) light pulses induce small phase shifts, whereas longer (and/or brighter) pulses cause larger phase shifts. Consequently, since the rate at which an oscillation reaches entrainment to a light cycle may be less rapid with shorter (or dimmer) light pulses, the oscillation passes through more intermediate or transient cycles before final or steady-state entrainment is achieved.

In *S. argyrostoma*, light pulses of approximately 6 h or more produce large phase shifts, whereas pulses of 1 to 4 or 5 h produce smaller or lower-amplitude responses (69). As the photoperiodic oscillator undergoes entrainment to shorter pulses, lower diapause incidence might result from the greater number of transient cycles occurring before steady-state entrainment is achieved, whereas increased light intensity might lead to more rapid entrainment and a higher incidence of diapause. Experiments using short light pulses of increased intensity provided evidence in favor of this conclusion (74).

The fall in diapause incidence under these ultrashort photoperiods may thus be attributed to three variables: (*a*) the rate of achievement of steady-state entrainment via transient cycles during a time-limited sensitive period; (*b*) subjective light intensity (sensitivity of photoreceptors); and (*c*) low-temperature induction of diapause—separate from photoperiodism—most clearly observed, of course, in constant darkness (43, 83).

Interim Summary

The whole-animal experiments described above were largely conceived and conducted in a premolecular era, when the phenomena of circadian rhythmicity and photoperiodism were known almost entirely from their behavioral and ecological perspectives. These experiments cannot prove a strictly causal association between the circadian system and the photoperiodic clock, but they provide many parallels suggesting such a connection. More concrete evidence can be provided by examining the role of known circadian clock genes in the photoperiodic phenomenon. This approach is described in the next section.

THE ROLE OF CIRCADIAN CLOCK GENES IN PHOTOPERIODIC INDUCTION

The circadian clock in *D. melanogaster* is now known to be regulated by a nested system of autoregulatory feedback loops based on the transcription and translation of several clock genes (17, 18, 87). These include, inter alia, the negative regulators *period* (*per*) and *timeless* (*tim*); the positive regulators *Clock* (*Clk*) and *cycle* (*cyc*); and *cryptochrome1* (*cry1*), which encodes the blue-light photoreceptor CRYPTOCHROME that mediates entrainment. Comparative studies, however, have revealed significant differences among the insects. In the monarch butterfly *Danaus plexippus*, other Lepidoptera, Heteroptera, and Hymenoptera, mammalian-type *cry2* is also expressed, which, unlike *Drosophila*-type *cry1*, acts as a transcriptional repressor; this form of the clock is regarded as ancestral (38, 96). In the derived *Drosophila* clock, *cry2* has been lost, whereas in the wasp *Nasonia vitripennis*, both *cry1* and *tim1* have been lost (63, 86). If these differences also occur in the photoperiodic mechanism, then substantial differences in the way clock genes operate in photoperiodism might be expected.

Assessment of the role of circadian clock genes in the photoperiodic induction of diapause—and thus in the validity of Bünning's hypothesis—has been performed in six insect orders (Orthoptera, Homoptera, Heteroptera, Lepidoptera, Diptera, and Hymenoptera). Most of these

studies have involved two main procedures: (*a*) determination of the time course of clock gene mRNA transcripts in long and short night cycles and (*b*) gene silencing (RNAi) techniques to knock down genes to ascertain their possible role in the phenomenon. The first of these techniques is of more limited utility, as it might merely investigate aspects of circadian rhythmicity per se rather than photoperiodic regulation of diapause; the second, although more instructive, has problems of interpretation that require resolution (see below). Arriving at a consensus is therefore difficult, not only because of differences between species, but also because different investigators have studied different genes, making comparisons difficult.

However, rhythmical expression patterns of the negative regulators *per*, *tim*, and *cry2* have been investigated in seven species (identified here as genera): *Chymomyza* (51), *Culex* (39), *Sarcophaga* (15, 28), *Nasonia* (5, 43), *Acyrthosiphon* (3, 9), *Pyrrhocoris* (19, 29) and *Sesamia* (26, 27, 30). In *Sarcophaga crassipalpis*, *Culex pipiens*, and *Sesamia nonagrioides*, *per* and *tim* mRNA levels peaked in the nights of both long- and short-night cycles and appeared to take their time cue from the L-to-D transition, thereby indicating night-length measurement; these rhythms also persisted in DD but with some dampening. These results are therefore in accordance with the external coincidence model as outlined in the previous section and thus further suggest that *per* and *tim*, or the PER/TIM heterodimer that subsequently enters the nucleus, might be important components of the photoperiodic oscillation. In contrast, rhythmic expression of *per* and *tim* in *Acyrthosiphon pisum*, and of *per* and *cry2* in *N. vitripennis*, seem to take their principal time cue from dawn, suggesting day-length measurement. However, under a photophase of higher light intensity, these last two examples might also be shown to be due to species measuring night length. In the aphid *A. pisum*, this interpretation might resolve the problem found by Lees (34, 35), who observed night-length rather than day-length measurement in whole-animal experiments with *A. pisum*. Although negative regulators were often rhythmically expressed in LD—as was the positive regulator *cyc* in *Cx. pipiens* and *Se. nonagrioides*—*Clock* (and *cry1*) did not oscillate in the mosquito.

Studies using gene knockdown techniques (RNAi) have been more informative. For example, knockdown of *tim* in *Chymomyza costata* induced a phenotype resembling the *non-photoperiodic diapause* (*npd*) variant (51); knockdown of *per* in *N. vitripennis* (43) and in the cricket *Modicogryllus siamensis* (64) suggested that *per* was essential for the photoperiodic response; and RNAi directed against *per*, *tim*, and *cry2* in *Cx. pipiens* reared under long nights redirected development along the nondiapause pathway (39).

However, Bradshaw and his group (13) suggested that studies to determine the role of circadian rhythmicity by knockdown of single genes present complications in interpretation. They observed that the circadian clock comprised an interacting group of genes and proteins in feedback loops that together formed a functional unit, or module. The photoperiodic mechanism, whatever that might be, was also a module. If the circadian clock were to act as a photoperiodic timer, then it would have to do so in its entirety, i.e., by modular pleiotropy, whereas if a single gene was implicated, then this could be via simple gene pleiotropy, implying that it might not be a component of the photoperiodic clock itself but a more downstream factor. This distinction between modular and gene pleiotropy has influenced many of the later investigations into the veracity of Bünning's hypothesis.

In the northern house mosquito, *Cx. pipiens*, clock gene involvement was investigated by knockdown (RNAi) of *per*, *tim*, and *cry2* in long-night (diapause-inducing) cycles, together with the circadian-related gene *pigment-dispersing-factor* (*pdf*) (39). RNAi directed against the negative regulators *per*, *tim*, and *cry2* caused females reared under long nights to avert diapause. In contrast, knockdown of *pdf* caused short-night females to accumulate the greater lipid stores, resembling a diapause-like state. It was concluded that circadian clock genes were causally involved in the induction and maintenance of diapause in this mosquito.

Work with the bean bug *Riptortus pedestris* by Numata and colleagues (22, 50) has provided some of the most persuasive evidence to date that circadian clock genes, acting as a module, provide the molecular machinery for photoperiodic time measurement. Ikeno et al. (22) used gene silencing (RNAi) techniques against the negative regulator *per* and the positive regulator *cyc* in conjunction with daily growth layers in the endocuticle as an indicator of circadian rhythmicity (as hands of the clock). These endocuticular layers, first described by Neville (47, 48) in several hemimetabola, consist of daily pairs of light and dark cuticles that free-run in constant conditions with a circadian oscillation that is both temperature compensated and entrainable by environmental (temperature) cycles. As such, they act as a reliable indicator of the endogenous circadian system.

Results showed that RNAi directed against *per* led to nondiapause ovarian development even under otherwise diapause inductive LD12:12 and to the deposition of a single dark layer of cuticle instead of the normal banding pattern. In contrast, RNAi directed against *cyc* suppressed ovarian development (i.e., induced a diapause-like state) even under LD 16:8 and led to the deposition of a single non-laminate layer of light cuticle. These results suggested that *per* and *cyc* were crucial for both the photoperiodic and circadian phenotypes.

Later studies examined the effects of gene silencing of circadian clock genes on both diapause regulation and lipid accumulation in the fat body (50). As before, RNAi directed against *per* led to ovarian development instead of diapause under both long and short photoperiods, whereas RNAi against *Clk* led to diapause-like results, again under both photoperiodic conditions. Silencing *per* also resulted in the suppression of lipid accumulation in the fat body, whereas silencing of *Clk* led to lipid accumulation. These results suggested that the circadian clock as a module played a pivotal role in both diapause regulation and lipid accumulation, despite their different output pathways.

Results of the whole-animal experiments discussed above, together with those from emerging molecular investigations, strengthen the intuitive suggestion made over eight decades ago by Bünning (6) that photoperiodic time measurement is a function of the circadian system. This association is now almost universally accepted in taxa from fungi to mammals (46). Among the insects, evidence appears to be particularly strong for an external coincidence type of clock in *Sarcophaga*, and perhaps in other Diptera. Most if not all insect photoperiodic clocks appear to comprise dampening circadian oscillators, with a pronounced trend toward heavily dampened hourglass-like responses in more northerly populations and in aphids.

THE MULTIOSCILLATOR NATURE OF THE PHOTOPERIODIC CLOCK

Circadian rhythmicity is fundamentally a cellular phenomenon. A wide variety of cells and tissues are therefore potential circadian (and photoperiodic) clocks. Behavioral and developmental phenomena such as activity (sleep–wake) cycles, molting, eclosion, and reproduction are probably all central processes controlled by clock(s) in the brain–retrocerebral complex. As early as 1971, Truman (88) identified two types of circadian clock. Type 1, exemplified by those clocks regulating eclosion, egg hatch, and molting, had endocrine outputs and tended to damp out in extended periods of light and to become arrhythmic in LL. Type 2 clocks, in contrast, controlled rhythms neurally, free-ran in both constant darkness and constant light, and regulated rhythms of locomotor activity (as well as sun compass orientation and the time sense or Zeitgedächtnis of honey bees). Photoperiodism, with its endocrine regulation, was placed in Type 1.

In *S. argyrostoma*, systematic sampling of hemolymph throughout larval development revealed peaks of ecdysteroids corresponding to the larval–larval molts and puparium formation, which were identical in both short-night and long-night insects. After puparium formation, however, short-night (nondiapausing) insects showed a large peak of ecdysteroids during pharate adult development, whereas in long-night (diapause-destined) insects, a very low titer of ecdysteroids

followed as they entered diapause (62). Ring glands (RGs) were then maintained in vitro to determine whether they could respond to stimulation by prothoracicotropic hormone (PTTH) from pupal brains. Results showed that, although diapausing brains contained as much PTTH as nondiapausing brains, RGs from diapausing pupae became unresponsive to PTTH approximately 4 days after pupariation (61). This showed that pupal diapause in *S. argyrostoma* was regulated by a two-tier brain–RG system.

This two-tier arrangement resembled the classical scheme proposed by Pittendrigh for the regulation of eclosion rhythmicity in *D. pseudoobscura*. Pittendrigh's original model, dating from the 1950s (56–58, 81), theorized that the physiological mechanism immediately underlying eclosion was governed by one oscillator (the slave) that was distinct from another, light-sensitive oscillator (the pacemaker). The pacemaker was thought to be directly entrained by the light cycle, self-sustained, and temperature compensated. The slave, in contrast, was a driven element, coupled to the pacemaker but not directly controlled by light. This model further theorized that the pacemaker was immediately reset by light, but that the slave required several non-steady-state or transient cycles before it attained synchrony with the pacemaker. Early papers by Pittendrigh and his colleagues reviewed the experimental evidence for this pacemaker–slave regulatory scheme.

Examples of similar two-tier regulation of insect developmental rhythms have been described in the silk moth *Samia cynthia ricini* (40, 41) and the bloodsucking bug *Rhodnius prolixus* (89, 90); these involved a clock in the brain producing a rhythm of PTTH and clock(s) in the (paired) prothoracic glands (PGs) producing circadian pulses of ecdysteroids, although it should be stressed that, unlike in Pittendrigh's model, both brain and PGs were light sensitive in these examples.

Vaz Nunes et al. (91, 92) suggested a similar, theoretical scheme to account for both circadian rhythmicity and the photoperiodic induction of diapause in flies and other insects. This control systems model was an extended version of the damped circadian oscillator model (36, 83, 84) consisting of two feedback oscillators: a self-sustained (or slightly damping) pacemaker and a strongly damping slave coupled to the pacemaker, both of which were entrained by the light cycle. This model accounted for many features of circadian rhythmicity, including transients in continuous darkness after a light pulse, temperature compensation, and—most importantly for the photoperiodic clock—changes in the phase relationship occurring between pacemaker and slave when the system was entrained by light–dark cycles comprising different photophases.

In the photoperiodic version of this model (92), both pacemaker and slave oscillators were entrainable by light and temperature cycles. Anatomical location of these components was not specified, but the pacemaker could be in the brain (perhaps in neurons adjacent to the PTTH cells), and the slave could be in the RG. It was proposed that night-length measurement was performed by the slave according to the principles of external coincidence, although it is equally likely that discrimination of long from short nights could be performed in the brain. Such a two-tier clock is relevant to the induction of pupal or larval diapause in *S. argyrostoma* and *C. vicina*, which involves the PTTH–ecdysteroid pathway. A similar pacemaker–slave clock could be involved in reproductive diapause induction along the brain–corpus allatum pathway, or in changing phase relationships between pacemaker and slave in a form of internal coincidence. There are clearly diverse ways that a clock of this type may measure day or night length.

Peripheral circadian clocks have been found in a wide range of tissues and organs outside the central nervous system, including sense organs (eyes, antennae), Malpighian tubules, gut, gonads, epidermal cells (secreting daily growth layers), fat bodies, and endocrine glands (14, 23, 42, 60). Many of these peripheral oscillators are independent of a central brain clock and are directly entrained by light. Some of them, however, also receive entrainment from the brain (44). In addition, microarray analysis has shown that a substantial portion of the *Drosophila* transcriptome is rhythmic (37), confirming that the insect circadian system has a complex, multioscillator construction.

The external coincidence model proposed for *S. argyrostoma* (which probably holds true for other species) is both adequate and simple. However, Albert Einstein was reputed to observe that everything should be as simple as possible, but not simpler. Perhaps the complexity of the insect circadian system means that the all-pervading photoperiodic phenomenon is equally complex and may contain, or consist of, internal coincidence characteristics. If the multiplicity of peripheral clocks now known to occur in insects consists of linked parts of output pathways from the brain, or oscillations independent of the brain but linked together, then these clocks could interact in complex internal coincidence devices like that envisaged by Pittendrigh (55; see also 81); these ideas need further consideration.

SUMMARY

This comparative approach to the problem of photoperiodic diapause has revealed huge diversity in detail, often with little apparent relation to phylogeny. On the one hand, this suggests that the phenomenon has evolved on multiple occasions as insects extended their distributions into areas with adverse winter conditions. On the other hand, however, since dormancy and diapause have also evolved in the tropics (10, 11, 49, 95), these traits may be considerably more ancient, predisposing subsequent extensions into more northerly latitudes. Evolution of the season within has provided many solutions. Some cases, such as that of *Drosophila* spp., where the interval between induction and the resulting diapause is short, or those examples where photoperiodic sensitivity extends through diapause itself, offer the best opportunity of explaining the phenomenon of photoperiodic regulation. Other systems—perhaps the majority—in which differentiation of long from short photoperiods occurs in advance of the resulting diapause create additional problems. In extreme cases in which the sensitive period occurs many instars before diapause supervenes, or even in an earlier generation, photoperiodic regulation must involve a cascade of events such as the accumulation of photoperiodic information, its storage, and its onward transmission through intervening molts and metamorphic events or even through the ovary from one generation to the next without affecting normal development. Most insect photoperiodic systems appear to be based on dampening oscillators, with extreme dampening resembling hourglass-like clocks occurring in some species (aphids and populations of insects living at high latitudes). A variety of circadian-based photoperiodic clock mechanisms probably exists. External coincidence seems to be a valid model for *S. argyrostoma*, at both formal and molecular levels. A multioscillator basis for the insect photoperiodic clock seems likely, but the validity of the internal coincidence model remains to be established.

There can be little doubt, however, that Bünning's intuitive suggestion of 1936 is largely true, but that Bünning could not have appreciated its complexity at that time. It is a truism that when one asks a question in Science, one may receive a partial answer—but also many further questions. It is this complexity in the natural world that is its most fascinating aspect.

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