

Temperature dependence and evolutionary adjustment of critical night length in insect photoperiodism

(evolution/circadian system)

COLIN S. PITTENDRIGH* AND TSUGUHIKO TAKAMURA

Hopkins Marine Station of Stanford University, Pacific Grove, CA 93950; and Entomology Department, Montana State University, Bozeman, MT 59715

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ABSTRACT The photoperiodic responses of *Drosophila auraria* are shown to involve its circadian system functioning as the “clock” that measures the duration of darkness at night. Attempts at further clarification of this finding were based on the widely held assumption that adaptive adjustment of critical night length is caused by change in the circadian system’s entrainment behavior. Three different experimental programs yielded data that are incompatible with this starting premise. Collectively, the observations suggest a new interpretation of the lability (phenotypic and genetic) of critical night length based on change in the level of response to all night-length measurements—not on the measurements themselves. This proposition is found especially relevant to the temperature dependence of photoperiodic responses and its role in controlling the onset and termination of the breeding season at different latitudes.

There are still a few insects, like *Megoura* (1), that continue to give no evidence of a circadian component in their utilization of day length (photoperiod) as a cue to season. But the great majority of the many organisms now tested—plant and animal—yield clear confirmation of Erwin Bünning’s (2) remarkable insight that the innate circadian organization of eukaryotes plays a significant role in the photoperiodic induction of seasonally appropriate metabolic pathways. Our continuing ignorance of its biochemical nature limits current understanding of this role to purely formal aspects. However, these include the important finding that the role of the circadian system is to measure the duration of darkness at night—it is the “clock” responsible for the time measurement at the heart of the photoperiodic phenomena.

The strength of this conclusion emerges from the entrainment behavior of circadian systems exposed to an exotic, 24-hr light/dark cycle in which two brief (e.g., 1 hr) light pulses separate two long dark periods of unequal length (e.g., 10 and 12 hr). By manipulating the initial conditions (3), one can establish either of two stable, steady-state phase relationships between the light cycle and the circadian system: in one of these, the system’s “subjective night” (4) falls in the shorter dark period, and in the other, it falls in the longer “night.” The two entrained steady states elicit different photoperiodic responses; the crucial issue is, evidently, the duration of darkness into which the system’s “subjective night” is compressed (5–7). Although the prevailing usage is to designate the photoperiod (duration of light) that elicits the 50% response as the critical day length (CDL), we think it more to the point to speak of critical night length (CNL) and do so throughout this paper.

At the outset it was hoped that the experiments we summarize in this preliminary report of work on *Drosophila auraria* would shed further light on this relationship between circadian entrainment and photoperiodic induction. We were attracted to *D. auraria* as a model system by the pioneering observations of Iwao and colleagues (8) on the photoperiodism of this species in Hokkaido. Our experimental program

was based on two major and widely held assumptions. The first is that systematic adaptive change in CNL (as along a latitudinal cline) will be paralleled by—indeed, derived from—systematic change in the system’s entrainment behavior. Therefore, it should be profitable to identify more precisely what aspect of entrainment systematically covaries with change in CNL. In this, of course, we are following the lead of Lankinen and colleagues at Oulu (9).

The second assumption—also shared, for example, at Oulu—is a justification for using the eclosion pacemaker as a marker of all other components in the multioscillator circadian system. As Engelmann and Mack (10) first reported, the pacemakers driving the eclosion and locomotory rhythms of *Drosophila pseudoobscura* are markedly different in both their periods (τ) and responsiveness to light. But in evolving the *Early* and *Late* strains of *D. pseudoobscura*, we found (11) that when the period of one pacemaker (eclosion) was changed by selection, that pressure also changed the other and in the same direction. Thus, even if the measurement of night length were effected by entrainment of a separate oscillator, its systematic variation should be sufficiently paralleled by that driving the eclosion rhythm to warrant using the latter, which is more easily assayed, as “marker.”

Preliminary work established that the photoperiodism of *D. auraria* does involve, as we expected, a circadian component that functions as the “clock” measuring night length. In three later series of experiments, our goal was to define more precisely what feature of circadian entrainment changed as CNL changed. The outcome of all three series was wholly unexpected: we found no evidence of any systematic covariation in circadian and photoperiodic parameters; on the contrary, it became clear that major change in CNL could be realized by selection without detectable impact on the circadian rhythm we studied. Our current conclusion is that the adaptive adjustment of CNL in nature is effected by change in the level of response to all night-length measurements, not by change in the sensory (circadian) system that executes the measurements.

That interpretation immediately resolves the (apparent) conflict between the well-known temperature compensation of circadian pacemakers and the proposition that they execute the (temperature dependent) measurement of night length: it is, we think, not the measurement of night length but the insect’s response to it that temperature impacts so strongly. The details of temperature dependence emerge as a major feature controlling the onset and duration of the insect’s breeding season; and the adaptive adjustment of CNL to latitude is seen as change in the temperature dependence of the photoperiodic response. These ecological aspects are so central

Abbreviations: CNL, critical night length; PPRC, photoperiodic response curves.

*To whom reprint requests should be addressed at: 8434 Wagon Boss Road, Bozeman, MT 59715.

to our changed interpretation of CNL evolution that we develop them first.

MATERIALS AND METHODS

Most of the work was concentrated on four of the nine latitudinal races of *D. auraria* generously given us by members of the Biology Department of Tokyo Metropolitan University. These four came from Hokkaido (42.9°N), Akita (39.8°N), Kawasaki (35.8°N), and Miyake (34.2°N). Stocks were maintained on a standard corn meal/molasses medium in cabinets at 17°C provided with a 14:10 light/dark cycle that assured escape from diapause. Photoperiodic responses were assayed in flies reared in smaller temperature-controlled cabinets where the experimental lighting regimes were controlled by electronic timers. Two photoperiodic responses were assayed routinely after 10 days of imaginal (posteclosion) life: (i) what percentage of the females lacked mature ovaries (reproductive diapause), and (ii) what percentage had a black cuticle (short-day response) as opposed to yellow (long-day response). The reproductive diapause of females (failure to develop ovaries) can be fully determined by the photoperiod after the pharate insect has eclosed: flies reared in a 10:14 light/dark cycle would all diapause were that regime continued in posteclosion life, but all will develop ovaries in 10 days if transferred to a 14:10 light/dark cycle after emergence.

The *D. auraria* eclosion rhythm was monitored with the same infrared sensors found useful (10) in earlier work on *D. pseudoobscura*.

RESULTS

Adjustment of CNL to Temperature and Latitude: Control of the Breeding Season. Photoperiodic response curves (PPRCs) were measured for all four latitudinal races of *D. auraria* at three different temperatures, viz: 15°, 17°, and 20°C. Fig. 1 gives the PPRCs for one race (Akita, 39.8°N) at three temperatures and for four different latitudinal races (≈34° to ≈43°N) at 15°C. *D. auraria* has the same perplexing nonmonotonic PPRC characteristic of other long-day insects. In responding to change of either temperature or latitude, critical night length (a 50% response) is adjusted, formally, in precisely the same way—not by shifting the PPRC along its

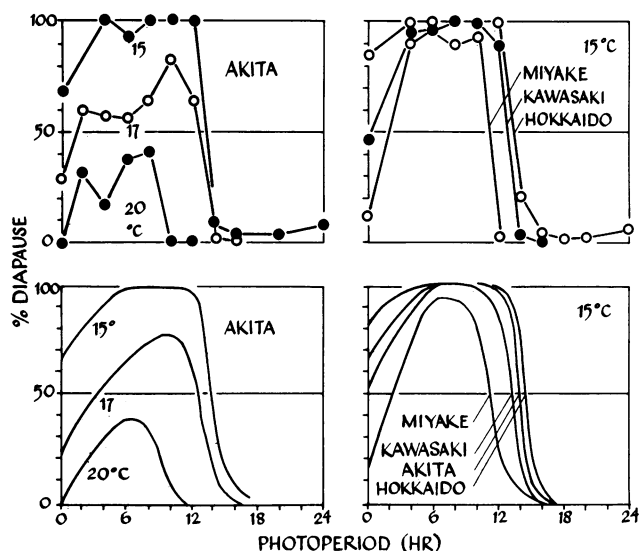


FIG. 1. (Upper) PPRC for induction of diapause in *D. auraria*. (Lower) Curves illustrating the degree of smoothing (of curve in Upper) used in Fig. 2.

photoperiodic axis but by elevating or depressing the entire curve on its ordinate (level of response).

Fig. 2 again uses Akita to explore the ecological implications of temperature dependence. It gives smoothed PPRCs (Fig. 2 Top Left) for the natural range of photoperiods at 15°, 17°, and 20°C. Transects 1–7 are made across the three PPRCs at the particular photoperiods that (as 30 day means) characterize each month at Akita: transect 1 is for December; 2, for January and November; 3, for February and October, etc. The response to a given day length at each of the three temperatures measured is then used (Fig. 2 Middle Left) to estimate the impact of a wider temperature range on the response to that particular photoperiod. Clearly, the curves based on only three points are, at best, approximations, especially for the lower temperatures; but this shortcoming has little bearing on our current use of them. Combined with information on the day-length and temperature characteristics (as means) of each month at Akita, the curves yield an estimate of what fraction of the female population will be in diapause throughout the year. This approach predicts the Akita breeding season will begin (diapause down to 50%) in mid-May and end (diapause up to 50%) in late September (Fig. 2 Bottom Left).

One adaptive aspect of these effects has long been recognized: both the onset and termination of the breeding season will be appropriately and independently adjusted in response to unseasonable temperatures. Other aspects have received less notice. First, were the photoperiodic control of diapause strictly temperature-independent [as in the now perplexing case of *Pieris brassicae* (12)], the breeding season would be unnecessarily truncated as a consequence of the phase-difference between the annual cycles of temperature and day length. Fig. 3 shows that if a 13.4-hr photoperiod were

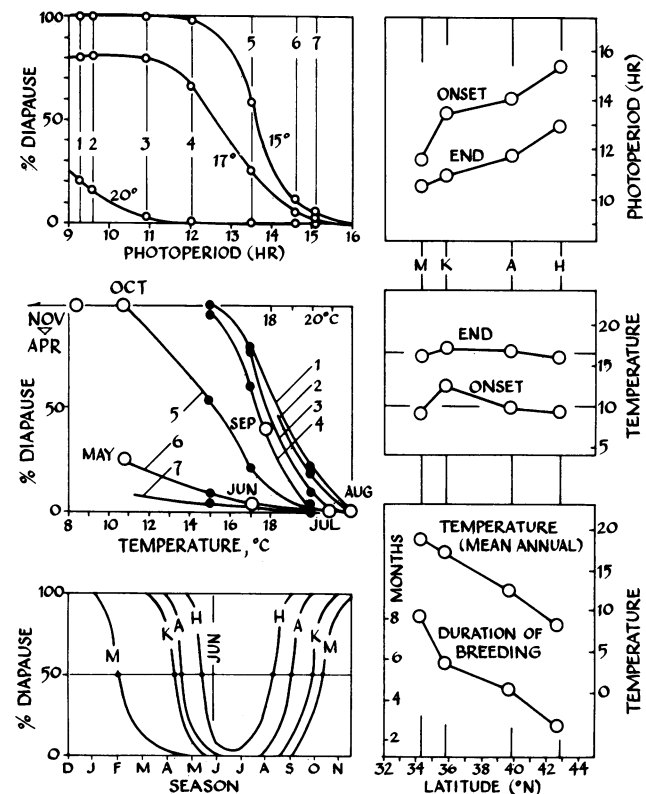


FIG. 2. Interaction of temperature and photoperiod in controlling the seasonal onset and end of *D. auraria* breeding activity at four latitudes. M, Miyake; K, Kawasaki; A, Akita; H, Hokkaido. (Middle Left) Small solid points from transects of Top Left; large open points give the combination of temperature and photoperiod at each month in Akita. See the text.

selected to initiate Akita's breeding as soon as the temperature was adequate (say 10°C) in the spring, an unqualified use of the same signal would terminate the season soon afterwards in spite of the temperatures being not only warm but still rising (A in Fig. 3 Left). However, the autumnal approach to CNL (50% diapause) begins at higher (summer) temperatures—thus, shortening the effective photoperiod and postponing the season's "end"—than those (winter temperatures) preceding the "onset of breeding" (50% ovaries), which accordingly awaits a longer photoperiod (B in Fig. 3 Left; also Fig. 2 Top Right).

It is clear that the season's end could be postponed still further by additional adjustment of the photoperiodic response's temperature dependence. The status quo in which preparation for winter (diapause up to 50%) is initiated at temperatures well above the lowest exploitable (e.g., the springtime onset) is surely determined by selection as being teleonomically conservative. The same is likely true of the response to the terminating (autumnal) photoperiod being much more temperature dependent than the response to the initiating photoperiod in the spring. And temperature plays a larger role in the terminating "decision" as one goes north (Fig. 4).

The same procedures used on Akita yield estimates of the breeding season for the other three latitudinal races analyzed (Fig. 2 Bottom Left). Dependence of the responses on temperature has been adjusted by selection to initiate and end the breeding season at very similar temperatures (compare ≈10°C and 16.5°C) at all latitudes (Fig. 2 Middle Right); the strategy of selection has evidently been to conserve the temperature range within which *D. auraria* breeds. One consequence is the close dependence of breeding season duration on the mean annual temperature (Fig. 2 Bottom Right). The only phenological observations to compare with the Fig. 2 estimates are those of Iwao *et al.* (8), who document the brevity of the Hokkaido season.

The Circadian System Measures Night Length. Elsewhere we have reported preliminary experiments using the Nanda-Hamer protocol on the Hokkaido strain (at 17°C) that showed circadian periodicity is indeed involved in the photoperiodism of *D. auraria* (13). These were followed by "bistability" experiments (Fig. 5) in which the circadian system was entrained by an exotic light/dark cycle that included two long dark periods (one of 10 hr and the other of 12 hr) separated by 1-hr light pulses. In entraining to this cycle, the *Drosophila* pacemaker can assume either of two stable phase relationships (ψ_{14} or ψ_{12}) to this light cycle—hence the "bistability" jargon introduced in refs. 5 and 3. These differ only in the duration of the dark period (10 or 12 hr) that embraces the oscillator's "subjective night." The

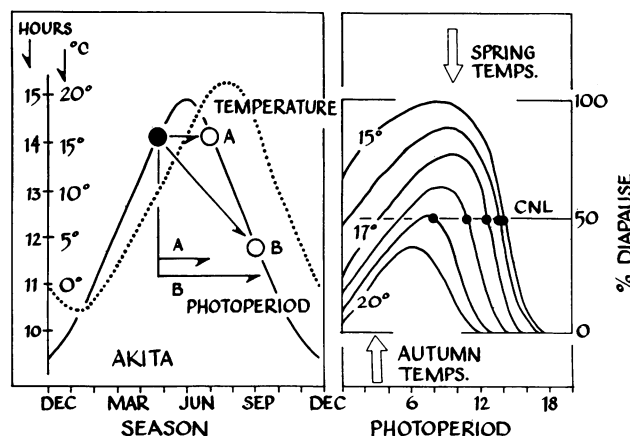


FIG. 3. The temperature dependence of photoperiodic responses prolongs the breeding season. See the text.

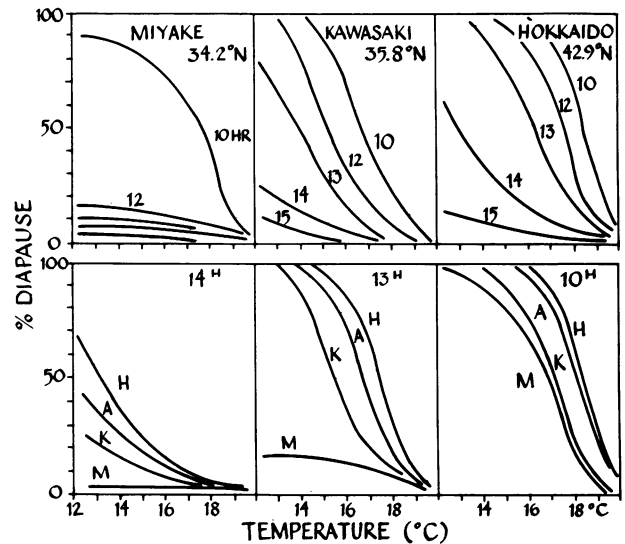


FIG. 4. The temperature dependence of responses to photoperiods ranging from 10 to 15 hr at three different latitudes. See the text. M, K, A, and H are as in Fig. 2.

steady state realized (ψ_{14} or ψ_{12}) is fully determined by the initial phase relationship of oscillator to light cycle, as summarized in the details of figure 11 in ref. 3. Using those earlier computations, we entrained females, freshly emerged from a 10:14 light/dark cycle to either ψ_{14} or ψ_{12} . The incidence of diapause in both groups was assayed after 10 days. In each of 12 separate experiments (at ≈17°C), there was more diapause in those females entrained to ψ_{12} (long night) than in their sibs entrained to ψ_{14} (short night). The power of these experiments derives from both steady states being realized in exactly the same physical environment (same cabinet, same light, same temperature) during their entrainment to ψ_{14} or ψ_{12} : they differed only in the phase of the 10:14 light/dark cycle experienced prior to eclosion and, hence, in their phase relationship to the subsequent common experimental regime. The overall response level varied in the 12 experiments because of strain differences, minor temperature differences, and the extreme sensitivity of the insects to night-length variation (PPRC steepness) in the 10- to 12-hr range; but in each experiment the incidence of diapause among flies on ψ_{14} (short night) was only ≈73% of that among flies on ψ_{12} (long night) (see Fig. 5). Night length is appraised by the extent to which light invades the circadian pacemakers' subjective night.

Change in the CNL Does Not Require a Change in the Circadian System. Our next goal was to identify changes in the circadian system that paralleled and presumably caused

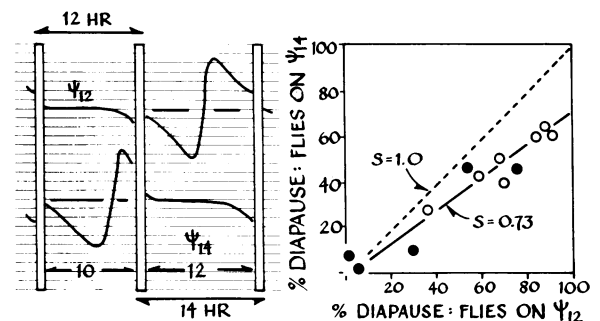


FIG. 5. Evidence from the "bistability" phenomena that the circadian system assesses night length (duration of darkness). (Left) The two steady-state phase relations of pacemaker to light cycle. (Right) ●, Miyake; ○, Hokkaido. See the text.

adaptive change in CNL. The first of three separate experimental programs sought latitudinal clines in the circadian system (eclosion rhythm) to account for the latitudinal cline in CNL we had found earlier (Fig. 2 *Top Right*). CNL for the onset of breeding increases steadily as one goes north—from 11.8 hr in Miyake (33.8°) to 15.2 hr in Hokkaido (42.9°N). We found several well-defined and related clines in the eclosion system, which will be documented more fully in a later paper. The period (τ) of the eclosion system becomes longer as one moves north, and the amplitude of the phase response curve declines steadily from south to north. The impact of these clines on the phase relationship (ψ_{EL}) of the eclosion rhythm (E) to the light cycle (L) is as expected when the system is entrained by brief photoperiods (e.g., 1:23): the northern peaks fall later in the day than those in the south (Fig. 6). The same is true when we assay ψ_{PL} —that is, the phase-relationship of the rhythm's pacemaker (P) to the light (L); ψ_{PL} , like ψ_{EL} , is more negative in the north (Fig. 6). But quite unexpected major complications were encountered when entrainment to longer (e.g., 14 hr) photoperiods was analyzed. There were equally clear latitudinal clines in both ψ_{PL} and ψ_{EL} , but both have the opposite sign to those found when the short photoperiod was used. Thus, the Hokkaido phase relationship lags that of Miyake on short photoperiods but leads it on long photoperiods. Since making these observations, we have found a theoretical explanation that involves the after effects of the photoperiod on ψ . However, irrespective of their explanation, these strain reversals of the phase relationship as a function of photoperiod raise an embarrassing challenge to our major premise that maintains that change in CNL is caused by change in the circadian system's entrained steady state. It appears that any model that explains the dependence of CNL on ψ for short photoperiods fails for long photoperiods because the sign of change in the level of photoperiodic response is the same for short and long photoperiods.

A comparable challenge to our starting premises was encountered in the second experimental program, which established by laboratory selection differences in the *D. auraria* circadian system far greater than those between Miyake and Hokkaido. In the two selected lines (*Early* and *Late*), the period (τ) of the free-running rhythm differs by as much as 2.5 hr, and ψ_{EL} , on 1:23 light/dark cycle, differs by as much as 6 hr! (Fig. 7). In spite of this massive change in their circadian rhythmicity and its entrained steady state, the difference in the photoperiodic responses of the early and late *D. auraria* strains is trivially small and unrelated in kind to that associated with latitudinal change. The presence of some change, even small, is encouraging in one respect: it would be difficult to reconcile none at all with the clear evidence from

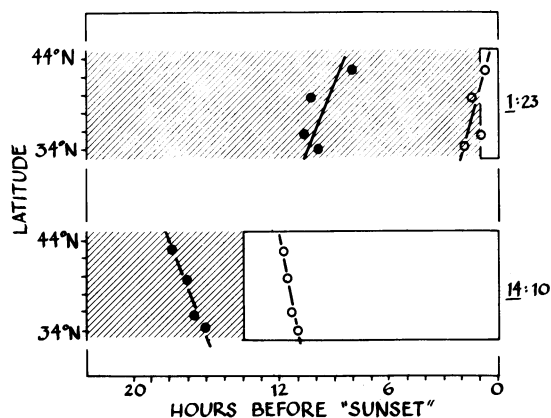


FIG. 6. Latitudinal clines in the phase relationship of the eclosion rhythm (○) and its pacemaker (●) to 24-hr light/dark cycles in which the photoperiod is either one (1:23) or 14 (14:10) hr.

“bistability” experiments that circadian entrainment is somehow involved in measuring night length. Nevertheless, the change in photoperiodic response we do find—a *small* shift of the PPRC along its photoperiod axis—provides no model of how CNL is adjusted by natural selection (that is, by elevation or depression of the response at all photoperiods).

It is the third experimental program (Fig. 7 *Bottom*) that clearly mimicked natural selection in adjusting CNL. In only five generations (on 12:12 light/dark cycle) of laboratory selection, we established two lines in which the incidence of black cuticle in one of them was increased to 96% (from 64% initially) and in the other was decreased to 21%. Moreover, when a complete PPRC was measured for both strains, the impact of selection (at 12:12 light/dark cycle) was seen at all photoperiods: the entire curve was elevated in the black⁺ line and depressed in the black⁻ line, mimicking the way natural selection changes CNL. And this extremely rapid change in CNL was effected without measurable impact on the circadian system's entrainment behavior, at least when assayed by the eclosion rhythm: ψ_{EL} is not detectably different in the black⁺ and black⁻ strains (Fig. 7).

DISCUSSION

Clearly none of the experimental programs summarized in the previous section provide support for our starting assumption

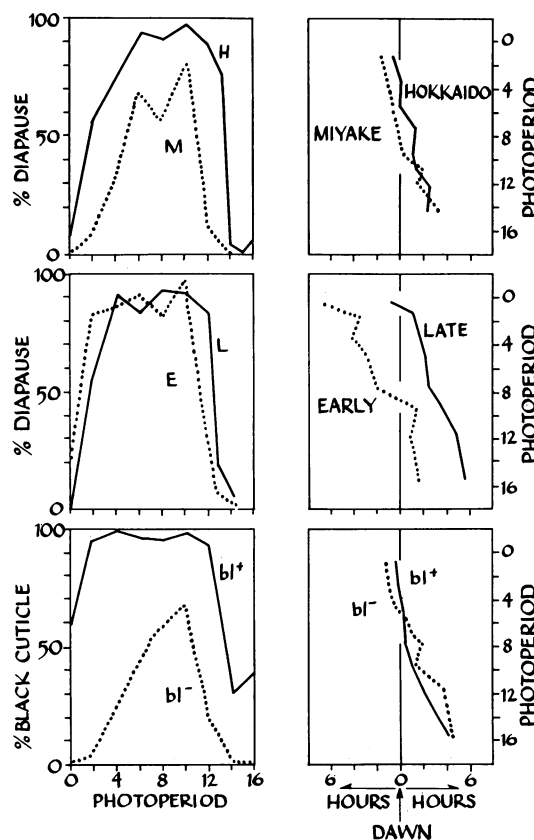


FIG. 7. Change in critical night length shows no systematic relation to change in the phase relations of eclosion rhythm to light cycle. See the text. (*Top*) Miyake (M) and Hokkaido (H) at 17°C. (*Top Left*) PPRCs for the induction of diapause. (*Top Right*) Phase relationship (ψ_{EL}) of the daily eclosion peak-to-dawn for photoperiods of 1 < 14 hr. (*Middle*) Two strains (*Early* and *Late*) selected on a 1:23 light/dark cycle at 17°C for a difference in the emergence time relative to dawn. (*Middle Right*) ψ_{EL} for photoperiods ranging from 1 to 14 hr. (*Middle Left*) PPRCs for induction of diapause. (*Bottom*) Two strains selected at 17°C for cuticle color—either black (bl⁺) or yellow (bl⁻). (*Bottom Left*) PPRCs (of females) for cuticle color. (*Bottom Right*) ψ_{EL} for photoperiods ranging from 1 to 14 hr.

that CNL adjustment is effected by change in the circadian clock's entrained steady state. And several of the observations, including the latitudinal clines and the cuticle color selections, strongly suggest that it is simply wrong. It is, of course, possible that the "troublesome" observations could as well be attributed to error in our second premise; thus, the eclosion pacemaker may not mirror change in a (putative) separate photoperiodic pacemaker as reliably as we have assumed, and further work may yet show that some change in a separate photoperiodic pacemaker does contribute to change in CNL without the eclosion pacemaker reflecting it. On the other hand, we think that such a contribution—if indeed it exists—will prove small and secondary to a different way of achieving the same end.

The interpretation of CNL lability we now prefer is based on the observation that, in responding to change of either temperature or latitude, the entire photoperiodic response curve is shifted (elevated or depressed) on its ordinate: adjustment of CNL is effected by changing the level of response to all photoperiods, not by changing the photoperiodic signals (circadian steady states) themselves.

The first clear merit of this interpretation is its resolution of the (apparent) conflict between a circadian pacemaker's well-known temperature compensation and CNL's equally well-known temperature dependence: the latter does not depend on change in pacemaker entrainment. There is also merit in the further implication that adjustment to temperature and latitude are not separate issues: at each new latitude invaded, natural selection evidently retunes the temperature dependence of the response mechanism to assure that the *D. auraria* breeding activity will exploit the same temperature range to which the rest of its physiology has already adapted at previous latitudes. And in adjusting temperature dependence to assure the onset of breeding at $\approx 10^\circ\text{C}$ and its end at $\approx 16^\circ\text{C}$, the CNLs necessarily become longer in the north.

There are other useful outcomes of this view of CNL lability. Since its adjustment entails the response to all day lengths, there is a lower temperature below which all females diapause and a higher temperature above which none do: photoperiodic control is only realized (and then temperature dependently) within a narrow window of temperatures. One consequence of adaptive evolution to new latitudes amounts to a shift of this window along the temperature scale. Its existence is, of course, responsible for the originally perplexing finding (14) that, although the Nanda-Hamner protocol yielded positive evidence of a circadian component in photoperiodic responses at some temperatures, it failed to do so at others, which we now see lie outside the window within which daylength makes any input. And the dependence of

this permissive window on latitude is beautifully documented by the important findings of Takeda and Skopik (15) on *Ostrinia nubilalis*. Three latitudinal races of the corn-borer differ in the temperature at which the Nanda-Hamner effect is clearest: the more southerly the strain, the lower the temperature for clear evidence of circadian periodicity in the diapause response.

Our principal concerns in this preliminary report of *D. auraria* photoperiodism have been: (i) the development of the functional significance of its temperature dependence and (ii) the likelihood that CNL adjustment is effected by change in the response to night length rather than in its measurement. Change in responsiveness alone, leaving the circadian system untouched, is not demanded by our data, but it is certainly compatible with all of the data, and that cannot be said of the usual assumption that bases CNL adjustment on circadian change. The only prospect of the latter making some contribution to CNL change is in showing that the second common assumption is wrong: that the eclosion system's behavior fails to mirror, adequately, the behavior of a separate "photoperiodic pacemaker."

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