Induction of diapause in *Drosophila melanogaster*: Photoperiodic regulation and the impact of arrhythmic clock mutations on time measurement

By: David S. Saunders, Vincent C. Henrich, and Lawrence L Gilbert

Abstract:

The fruit fly *Drosophila melanogaster* displays an ovarian diapause that is regulated by photoperiod. Newly eclosed female flies (Canton-S wild type) exposed to short days (<14 hr of light per day) at 12°C (or 10°C) enter a fairly shallow reproductive diapause. Females exposed to long days (16 hr of light per day) at the same low temperature undergo ovarian maturation. The short day induced diapause continues for 6-7 weeks under a 10:14 light/dark cycle at 12°C but is terminated rapidly after a transfer to higher temperature (18 or 25°C) or to long days (18:6 light/dark cycle). Females from three strains homozygous for alleles of the period (*per*) locus, reportedly arrhythmic for behavioral circadian rhythms, and females that possessed two overlapping deletions of *per* were also capable of discriminating between long and short days, although, when compared with the wild-type flies, the critical day length was shifted to shorter values by ≈2 hr. It is concluded that the period locus is not causally involved in photoperiod time measurement.

Abbreviations: PPRC, photoperiodic response curve; DD, constant darkness; LL, constant light.

Article:

The majority of insects inhabiting the temperate zones develop and reproduce during the summer months but become dormant as winter approaches. Although ecological distinctions are becoming blurred, this dormancy may be a direct response to unfavorable conditions (i.e., cold torpor or quiescence), or it involves programmed responses to seasonal changes in day length (or photoperiod), which results in interruptions or alterations in endocrine regulation (i.e., diapause). Photoperiodic regulation of diapause has been documented for a large number of species representing '13 insect orders (1).

A central problem in the study of photoperiodism is the nature of the "clock" used by organisms to discriminate long days from short days (or short nights from long nights) as the seasons change. The mechanism of this time measurement remains obscure, but a favored model suggests that night length is measured by a clock based on the system of circadian oscillations, which also regulate overt daily rhythms (2-4). In the 50 years since its formulation, this proposition has become known as "Bunning's hypothesis."

In view of the importance of fruit flies (*Drosophilidae*) to genetics, photoperiodic regulation of overwintering diapause in this family has attracted considerable attention. Of the 18 species of Drosophila for which data are currently available, 16 overwinter in a reproductive or ovarian diapause in which short days (or long nights) elicit a block to vitellogenesis, 1 (*D. deflexa*) is reported to overwinter as a larva (5) and another (*D. alpina*) overwinters as a pupa (6). Of those diapausing as adults, most attention has been paid to *D. phalerata* (7, 8), *D. littoralis* (10, 11), and *D. auraria* (12, 13).

D. melanogaster occupies an almost unique position in biological research because of its amenability for molecular and genetic analysis. Therefore, the nature of its overwintering strategy, whether diapause or quiescence, is also of considerable interest. To our knowledge, no published observations on this aspect of its biology have appeared, although it would be surprising if some attention had not been given to the problem, especially since the circadian system is known to underlie photoperiodic time meaurement [at least in *D. auraria* (13)], and the clock or period (*per*) locus is being subjected to intensive molecular and genetic analysis (14-17).

This paper describes experiments with *D. melanogaster* in which newly eclosed adults were exposed to a range of photoperiods at temperatures somewhat lower than those normally used in routine laboratory maintenance and experimentation in an attempt to mimic the shortening days and falling temperatures encountered in the natural environment. The data reveal that *D. melanogaster* possesses a short day induced ovarian diapause (block to vitellogenesis), but that neither the reportedly arrhythmic mutant alleles of the *per* locus (per^{01}, per^{02}, and per^{03}) nor a "double deletion" of the *per* gene prevents the ability of female flies to discriminate between long and short days.

Materials and Methods
Animals. Wild-type (Canton-S) and mutant flies were raised at 25°C under 12 hr of light and 12 hr of darkness (LD 12:12) on a standard agar-cornmeal medium. The mutant fly strains were homozygous for three alleles of the period (*per*) locus, designated per^{01}, per^{02}, and per^{03}. These mutants apparently lack overt circadian eclosion and adult locomotory rhythms (14, 17). Flies from these strains were verified independently as arrhythmic by standard laboratory tests (J. M. Ringo and H. B. Dowse, personal communication). Furthermore, the *per* mutant strains used for this study were derived from the same Canton-S strain (14) that has been maintained on a light/dark schedule for at least 20 years. To evaluate the possible effects of the per locus on the photo- periodic induction of diapause, a balanced stock [Df(y64j4)/ Df(y^{2}TEM202/w$^+$Y)] that carries two overlapping deletions of the *per* locus were used. The females of this stock carry one of each deletion, which entirely removes the *per* locus of each chromosome but does not cause death, as either deletion does when homozygous; this genotype will be referred to as the *per* double deletion (18).

Environmental Conditions. Within a few hours of emergence, flies were transferred to fresh culture bottles or vials and placed at 10 or 12°C in a variety of photoperiods [LD 3:21 to continuous light (LL)]. Temperatures were maintained in refrigerated incubators and light cycles were provided by 15-W fluorescent tubes regulated by commercial timers. Conditions of continuous darkness (DD) were obtained by wrapping the culture vessels in aluminum foil. Each group of newly emerged flies was divided into two subgroups, one exposed to an expected long day (e.g., LD 14:10, 16:8, or 18:6) and the other exposed to an expected short day (e.g., LD 8:16, 10:14, or 12:12). Therefore, comparisons between siblings at long or short days could be made in most cases.

Quantification of Diapause. At intervals, flies were etherized, the females were dissected in saline, and ovarian development was assessed (19). Briefly, stages 1-7 cover the previtellogenic development of the egg follicle, stage 8 shows a slight deposit of yolk in the oocyte, stages 9-11 are the main vitellogenic stages (with one-third, one-half, and three- quarters of the oocyte occupied by yolk, respectively), while stages 12-14 constitute the stages of chorion formation and final maturity of the ovarian egg. In the present experiments, all vitellogenic oocytes (stage 8 and over) were regarded as developing (i.e., "nondiapausing"), whereas all previtellogenic stages (stages 1-7) were regarded as nondeveloping (i.e., "diapausing").

Each ovary of *D. melanogaster* contains an average of 16 polytrophic ovarioles (20), but these are characterized by a lack of interovariole synchrony. For this reason, the state of ovarian development was assessed according to the most advanced follicle present. Since some flies contained only one or two chorionated eggs, and these were scored as nondiapausers, the scoring method provided a conservative criterion for diapause. In each group of flies dissected, the percentage of diapausing flies was calculated as the proportion of the total with entirely previtellogenic ovaries.

RESULTS

Ovarian Development at 12°C in Long or Short Days. Groups of wild-type (Canton-S) flies were placed at 12°C in either a long day (LD 18:6) or a short day (LD 10:14) photoperiod and were sampled at intervals to establish a time course for ovarian development (Fig. 1). Newly emerged females had entirely previtellogenic ovaries, but by day 8, flies in LD 18:6 showed a considerably higher incidence (≈80%) of yolky ovaries than those in LD 10:14 (≈40%). Four weeks after eclosion, 20% of the flies in the short day photoperiod had yolky follicles, whereas those in the long day photoperiod were almost entirely vitellogenic and producing a steady trickle of eggs. The short day flies with previtellogenic ovaries were considered to be in ovarian diapause.

At ≈6 weeks after eclosion, the ovaries of the short day group underwent a spontaneous recovery of ovarian maturation that was completed by the 8th week. During this period of recovery, the ovaries became filled with vitellogenic follicles in all stages of yolk deposition. Under the experimental conditions (12°C, LD 10:14), therefore, ovarian diapause was maintained for 6-7 weeks.
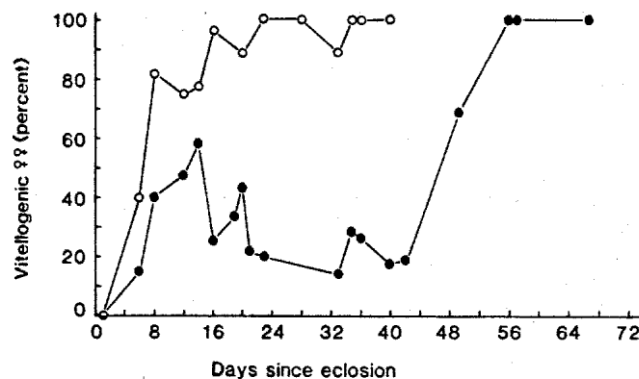

FIG. 1. Time course for ovarian maturation of *D. melanogaster* (Canton-S) at 12°C under long days (○, LD 18:6) or short days (●, LD 10:14). After a month, all long day flies were vitellogenic, whereas 80% of short day flies are in ovarian diapause. Diapause in the short day group is terminated spontaneously after 6–7 weeks.

Reversal of Ovarian Diapause by High Temperature or Long Days. Canton-S flies maintained at 12°C under a short day regime (LD 10:14) and showing, ≈40% vitellogenesis—i.e., 60% ovarian diapause, by day 20, were transferred to either of two higher temperatures (18 and 25°C) at short day length (LD 12:12) or to a long day regime (LD 18:6) at the same temperature (12°C). Fig. 2 shows that ovarian maturation resumed rapidly in these flies, most promptly at 25°C LD 12:12, and least promptly at 12°C LD 18:6. Control flies that remained at 12°C and LD 10:14 continued to exhibit 20% vitellogenesis—i.e., 80% diapause. These results demonstrate that diapause may be broken by a transfer to a long day length or to a higher temperature, the latter overriding the diapause-maintaining effects of continuing short days.

Photoperiodic Response Curves (PPRCs) for *D. melanogaster* (Canton-S). Groups of Canton-S flies were incubated in a range of photoperiods from LD 3:21 to LD 20:4 at either of two temperatures (10 or 12°C). Additional groups were maintained in the aperiodic regimes of DD and LL. An average of seven groups was established at each photoperiod, a total of 1826 female flies (203 per photoperiod) were examined at 10°C, a total of 2991 (272 per photoperiod) were examined at 12°C. The incidence of diapause was determined for each group after 14 days (12°C) or 21 days (10°C); the PPRCs are shown in Fig. 3.

At any one combination of photoperiod and temperature, the variability between the samples studied was rather large. The reason for this variability is currently unknown, and for the purpose of the present paper the PPRCs are based on the proportions of the total number of flies at each condition that remained previtellogenic. Accordingly, the data presented in Fig. 3 indicate that the overall incidence of diapause at 10°C was high (75-95%) in all photoperiods up to LD 12:12 and then fell to ≈38% at LD 16:8, but it increased again to a high value (96%) at LD 20:4. At 12°C, the incidence of diapause was lower in all regimes, but particularly so in DD (16%)

and in the long day photoperiods from LD 14:10 to LD 20:4 (23– 14%). The lowest incidence of diapause was at LD 16:8 (8%).

The shape of the PPRC at 12°C is typical for a long day species of insect in that nondiapause development (ovarian maturation) occurs in long days but is blocked in short days (1). The critical day length separating long days from short days is not well marked in *D. melanogaster*, but it is evident at ≈14 hr of light per day. The decline in diapause incidence in DD is commonly observed in long day species (1); the increase in diapause in very long days and in LL is less frequently observed and gives the PPRC of *D. melanogaster* a characteristic "wave-shaped" profile.
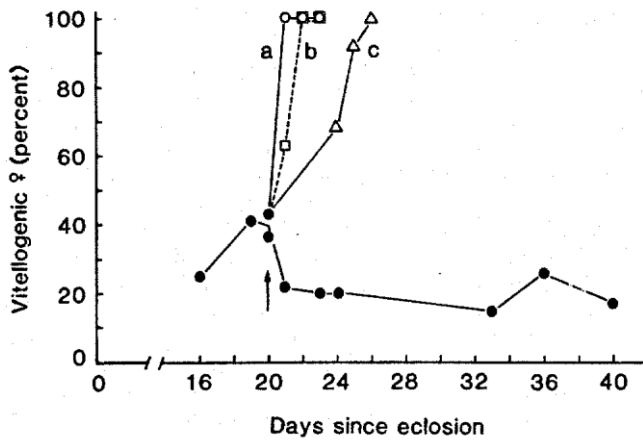

FIG. 2. Reactivation of diapausing ovaries of *D. melanogaster* (Canton-S) by transfer to 25°C, short days (LD 12:12) (a, ○); 18°C short days (LD 12:12) (b, □), or 12°C long days (LD 18:6) (c, △). ●, Control flies continuing in diapause-maintaining conditions (12°C, LD 10:14). Arrow shows the point of transfer from 12°C LD 10:14 to the new conditions.
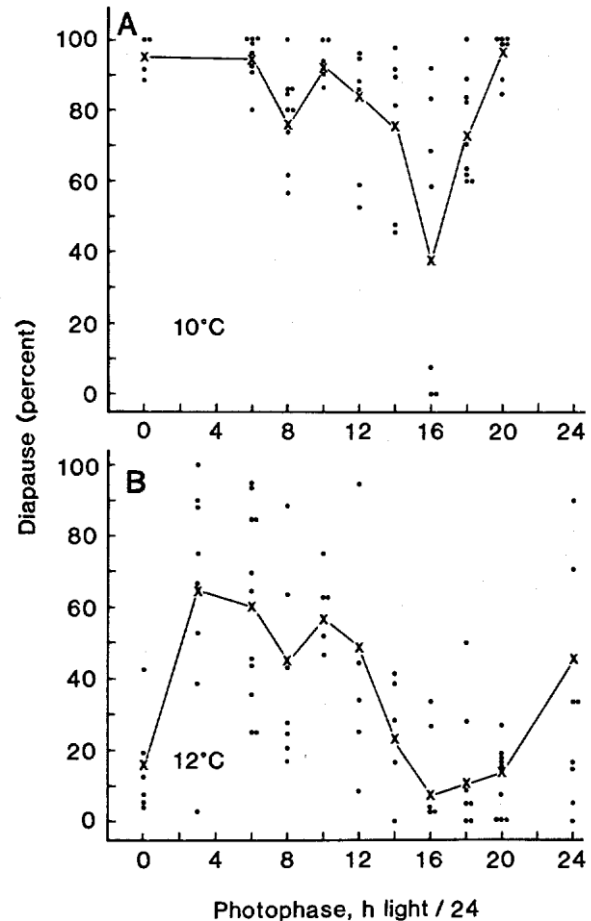
FIG. 3. Photoperiodic response curves for *D. melanogaster* (Canton-S) at 10°C (*A*) and 12°C (*B*). Points show proportions of each group that remained previtellogenic (diapause) after 21 days (10°C) or 14 days (12°C); X, overall incidence of diapause at each photoperiod.

Ovarian Development of Behaviorally Arrhythmic Flies (*per*⁰ and Double Deletions of *per*). An average of 91 arrhythmic mutant flies were exposed at 12°C to a range of photo- periods from LD 3:21 to LL. Female flies from all three *per*⁰ strains, as well as the *per* double deletion stock, were able to discriminate between a short day and a long day, although the apparent critical day length was shifted to a shorter value than in the Canton-S control group (Fig. 4). In all four cases, photoperiods of LD 12:12 or longer induced a low incidence of ovarian diapause—i.e., a high incidence of vitellogenesis—whereas LD 10:14 or shorter elicited 60-80% diapause. The critical day length for all four arrhythmic strains was between 10 and 12 hr of light per day. As in the Canton-S control group, diapause incidence in the mutant strains fell in ultrashort photoperiods and increased again in very long photoperiods (LD 18:6) to provide a wave-shaped PPRC. The mutant flies thus appeared to maintain a functional photoperiod clock regulating diapause/vitellogenesis, although genetic changes at the *per* locus clearly affected time measurement.

DISCUSSION

The Photoperiodic Regulation of Diapause in *D. melanogaster*. The present results demonstrate that *D. melanogaster* has evolved a photoperiodically regulated ovarian diapause similar to that of other species of the genus. Evidence that we are dealing with a true photoperiodic response includes the following: (*i*) the sharp decrease in diapause incidence as a function of increasing day length, with the transition occurring at a day length that is ecologically appropriate for a strain of flies originally collected at a latitude of 43°N (Canton, OH); (*ii*) the fall in diapause in DD that is observed in the majority of PPRCs (1); and (*iii*) diapause is terminated by the transfer from short to long days. That the diapause state may also be terminated by the application of juvenile hormone (D.S.S., D. S. Richard, S. W. Applebaum, M. Ma, and L.I.G., unpublished data) also points to its physiological basis as an interruption in the brain–corpus allatum axis, with a temporary decrease in juvenile hormone titer resulting in a block to the uptake of yolk polypeptides from the hemolymph. There is also evidence, however, that the ovarian diapause of *D. melanogaster* is rather "weak" or "shallow" since it only seems to occur at rather low ambient temperatures and is easily broken by transfer to a higher temperature and short days. The low temperature requirement for its expression presumably explains why it has escaped attention in the past.

Several attempts to classify photoperiod diapauses have proved to be of limited value because they clearly attempt to force categories on a continuum (21-23). Muller (21) recognized three types of diapause: parapause, an obligate cessation of development occurring annually in a univoltine species; eudiapause, a facultative arrest induced by photoperiod but terminated after a period of chilling; and oligopause, a facultative diapause both induced and terminated by photoperiod. More useful, perhaps, is a model introduced specifically for *Drosophila* spp. by Lumme (6), which recognizes a latitudinal dine in response to seasonal cues. For a hypothetical example, populations in southerly latitudes may not show a true diapause but overwinter in a cold torpor quiescence. Further north, with an increase in latitude, a photo- periodic diapause may occur, with the critical day length lengthening and the number of summer generations falling. Southerly populations might show "oligopause" responses, and "eudiapause" might be characteristic of insects at mid- latitudes, with "parapause" appearing further north.

D. melanogaster is of tropical origin (24) and is thought to have moved northward with humans as a synanthrope; Lumme (6), for example, records it as a "commensal" in Finland. It has been implicitly assumed that such commensal species are devoid of a photoperiodic diapause (see ref. 25 for *D. melanogaster*) even though insects living in a peridomestic situation must be subject to low temperature and poor food supply during the northern winter, and an appropriate overwintering strategy would provide a distinct selective advantage. It is easy to see how the responses observed in the present paper could provide that advantage: autumnal flies exposed to shortening day lengths at a cool temperature (the average October temperature for central Ohio is close to 12°C) would suspend ovarian maturation, probably redirect metabolites to the fat body, possibly undergo behavioral changes leading to overwintering site selection, and then become completely dormant as winter temperatures fall still further. Reactivation would occur, regardless of the photoperiod, at the earliest favorable spring temperature. With this overwintering strategy, *D. melanogaster* could be regarded as an example of an insect with a weak oligopause.

The Genetic Basis of the Photoperiodic Response. Strains derived from natural populations of *D. littoralis* display a range of critical day lengths for ovarian diapause that correlate with a latitudinal dine such that critical day lengths become longer at higher latitudes (9). Analysis of photoperiodic responses among inbred lines derived from eight natural strains reveals a segregation pattern indicative of several tightly linked loci or multiple alleles of a single autosomal locus. While these data do not rule out the possibility that other loci regulate the photoperiodic response in *D. littoralis*, a relatively small portion of its genome accounts for nearly all the variability in photoperiodic response observed among these strains.
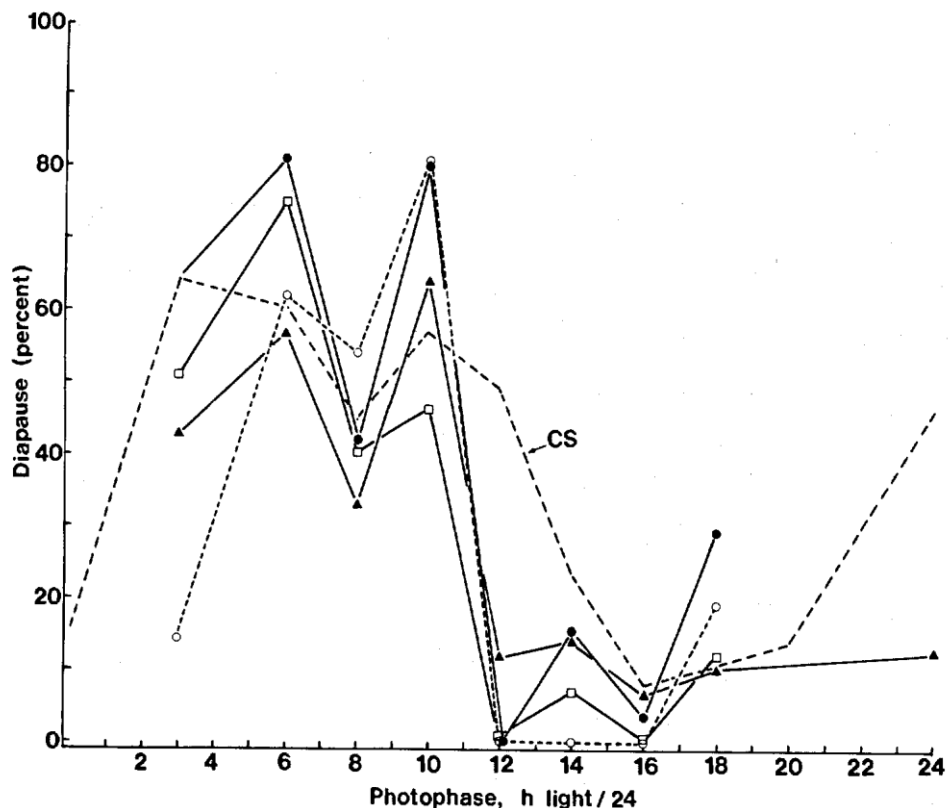
FIG. 4. Photoperiodic response curves for four arrhythmic circadian clock mutations of *D. melanogaster*: ▲, per^{01}; □, per^{02}; ●, per^{03}; ○, *per* double deletion (y64j4/TEM202/w⁺Y). The PPRC for Canton-S wild type (from Fig. 3) is shown for comparison (CS).

The importance of the demonstration of a photoperlodic response in *D. melanogaster* is, of course, that it opens up possibilities for the genetic and molecular "dissection" of both photoperiodism and the downstream pathways leading to vitellogenesis. The present investigation of the photoperiodic responses of the per mutations represents the first efforts in this direction. That all four "arrhythmic" mutant strains (per^{01}, per^{02}, per^{03}, and the double deletion of *per*) were capable of discriminating between a short day and a long day, albeit with an altered critical day length, suggests that the photoperiodic response remains largely intact in flies grossly altered with respect to their genotype associated with overt behavioral circadian rhythms.

With respect to Bunning's hypothesis (2, 3) that circadian rhythmicity is causally involved in photoperiodic time measurement, at least three interpretations of these data are possible. First, the circadian system is simply not involved and day length (or more probably night length) is being measured by a noncircadian or "hour-glass" type of clock in which the system is solely dependent on the daily light/dark cycle. Alternatively, the circadian system is causally involved in night length measurement, but the *per* locus is not—. i.e., one or more crucial circadian photoperiodic clock gene(s) lies elsewhere in the genome. Third, the possibility exists that the *per* locus is involved but that per^0 (and even the double deletion *of per)* are not totally arrhythmic. In this connection, these phenotypes show residual noncircadian rhythmicity in their adult locomotor activity patterns (26), and per^{01} flies are known to entrain to exotic light/dark cycles with characteristic phase relationships that are not consistent with the purely exogenous forcing of locomotory activity expected with totally arrhythmic flies (27). The residual rhythmicity and entrainability of per^0 flies might conceivably be sufficient in LD cycles to provide the entrained steady states suspected to act as the clock in modern versions of Bunning's hypothesis (3, 28). The present data do not distinguish between these possibilities in an unequivocal manner. However, since complete absence of the *per* locus DNA (the *per* double deletion) fails to render the photoperiodic clock inactive, we conclude that the second alternative—namely, that the *per* locus is not causally involved in time measurement and that the crucial genes lie elsewhere in the genome—is the most likely of the three. While the photoperiodic response observed in *D. melanogaster females* may involve the action of one or a few loci (as it might be in *D. littoralis),* the *per* locus is not among them, since even the absence of the locus does not eliminate a photoperiodic response. It appears, however, that mutational disruption of the *per* locus exerts a

modulatory influence on the diapause response. Further studies of the genetic and molecular basis of photoperiodism, therefore, will entail searching for genetic loci, possibly circadian in nature, at other sites.

References:
1. Saunders, D. S. (1982) *Insect Clocks* (Pergamon, Oxford), 2nd Ed.
2. Bunning, E. (1936) *Ber. Dtsch. Bot. Ges.* **53,** 590-607. 3.
3. Pittendrigh, C. S. (1972) *Proc. Natl. Acad. Sci. USA* **69**, 27342737.
4. Lewis, R. D. & Saunders, D. S. (1987)1. *Theor. Biol.* **128**, 4759.
5. Basden, E. B. (1954) *Proc. R. Soc. London Ser.* A **29**, 114-118.
6. Lumme, J. (1978) in *Evolution of Insect Migration and Diapause,* ed. Dingle, H. (Springer, Berlin), pp. 145-170.
7. Geispitz, K. F. & Simonenko, N. P. (1970) *Entomol. Obozr.* **49**, 83-96.
8. Tyschenko, V. P., Goryshin, N. I. & Azaryan, A. G. (1972) *Zh. Obshch. Biol.* **33**, 21-31.
9. Lumme, J. & Oikarinen, A. (1977) *Hereditas* **86**, 129-142.
10. Lumme, J., Oikarinen, A., Lakovaara, S. & Alatalo, R, (19T4) *J. Insect Physiol.* **20**, 2023-2033.
11. Lankinen, P. (1986) *J. Comp. Physiol. A* **159**, 123-142.
12. Iwao, Y., Kimura, M. T., Minami, N. & Watabe, H. (1980) *Kontyu* **48**, 100-168.
13. Pittendrigh, C. S. & Takamura, T. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 7169-7173.
14. Konopka, R. & Benzer, S. (1971) *Proc. Natl. Acad. Sci. USA* **68**, 2112-2116.
15. Bargiello, T. A. & Young, M. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 2142-2146.
16. Zehring, W. A., Wheeler, D. A., Reddy, P., Konopka, R. J., Kyriacou, C. P., Rosbash, M. & Hall, J. C. (1984) *Cell* **39**, 369376.
17. Hall, J. C. & Rosbash, M. (1988)1. *Biol. Rhythms* **2**, 153-1T8.
18. Smith, R. F. & Konopka, R. J. (1981) *Mol. Gen. Genet.* **183**, 243-251.
19. King, R. C., Rubinson, A. C. & Smith, R. F. (1956) *Growth* **20**, 121-157.
20. King, R. C. (1979) *Ovarian development in Drosophila melanogaster* (Academic, New York).
21. Muller, H. J. (1970) *Nova Acta Leopold.* **35**, 7-27.
22. Mansingh, A. (1971) *Can. Entomol.* **103**, 983-1009.
23. Thiele, 11.-U. (19T3) *Can. Entomol.* **105**, 925-928.
24. Lemeunier, F., David, J. R., Tsacas, L. & Ashburner, M. (1986) in *The Genetics and Biology of Drosophila,* eds. Ash- burner, M., Carson, H. L. & Thompson, J. N., Jr. (Academic, New York), Vol. 3e, pp. 148-256.
25. Roberts, D. B. (1986) in *Drosophila, A Practical Approach,* ed. Roberts, D. B. (IRL, Oxford), Chap. 1.
26. Dowse, H. B., Hall, J. C. & Ringo, J. M. *(1987) Behay. Genet.* **17,** 19-35.
27. Helfrich, C. & Engelmann, W. (198T)*Z. Naturforsch. Teil C* **42***,* 1335-1338.
28. Pittendrigh, C. S., Elliott, J. & Takamura, T. (1984) in *Photo- periodic Regulation of Insect and Mulluscan Hormones,* eds. Porter, R. & Collins, J. M. (Pitman, London), pp. 26-41.