CIRCADIAN CLOCK MUTANTS OF *DROSOPHILA MELANOGASTER*

Thesis by

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ABSTRACT

Three mutants of Drosophila melanogaster have been isolated in which the free-running period of the circadian eclosion rhythm and the adult locomotor activity rhythm is affected. One mutant is arrhythmic, another has a short period of 19 hours, and the third has a long period of 28 hours. The mutants retain their phenotypes over the temperature range 18° to 25° C. All three mutants map near the tip of the X chromosome (distal to the centromere). By deficiency mapping, the short-period mutation has been localized to the 3B1-2 region. Complementation tests show that all three mutations affect the same functional gene.

Analysis of activity rhythms of individual mosaic flies indicates that the site of action of the short-period mutation is probably located in the head of the fly. A few activity patterns of split-head and mixed-head mosaics appear to possess both mutant and heterozygous components, suggesting that the fly head may contain two complete clocks capable of maintaining their periodicities independently.

The short-period mutation affects both the duration of the light-insensitive part of the oscillation and the degree to which the clock can be reset during the light-sensitive part of the oscillation.

Both the short-period and long-period mutant eclosion rhythms can be entrained to a period of 24 hours by a 12:12 light-dark

cycle having a light intensity at least two orders of magnitude greater than that required to entrain the normal rhythm. The arrhythmic mutant does not entrain under these conditions. In the presence of a temperature cycle, however, the arrhythmic mutant does entrain, but its rhythm damps out when the temperature cycle is removed.

Evidence is presented that Pittendrigh's two-oscillator model for the clock in D. pseudoobscura applies to D. melanogaster as well. The three clock mutations primarily affect the light-sensitive driving oscillator. The arrhythmic mutation appears to have eliminated the driving oscillator while leaving the temperature-sensitive driven oscillator relatively intact.

CONTENTS

CHAPTER 1.

Introduction to Genetics of Circadian Rhythms

Rhythmic phenomena are frequently encountered in biological processes (1,2). They may be found on all levels of organization, from the subcellular (e.g., variations in enzyme activity (3)) to the organismic (e.g., locomotor activity (4)). The periods of known rhythms range over several orders of magnitude, from less than a second (e.g., the repetitive firing of certain neurons (5)) to a year (e.g., migration (6)).

Some rhythms appear to require an external driving cycle for their manifestation (7); when the organism is removed from the external influence, the rhythm disappears. Many rhythms, on the other hand, persist apparently undiminished for at least several days after the organism is placed in an essentially constant environment (1). Such rhythms seem to arise from a source endogenous to the organism, although it is difficult to eliminate the possibility that the organism is responding to some barely perceptible external cycle. The endogenous nature of certain rhythms is further supported by the observation that their periods, when running in a constant environment, do not exactly equal those of known environmental cycles, but rather deviate by several minutes to an hour or more (1,8). In addition, the periods of the rhythms of individual organisms under constant conditions often differ significantly from each other (9). This is opposed to the situation wherein the same organisms are under entrainment by an external cycle, in which case their periods coincide

(9). The most notable of endogenous rhythms are the circadian rhythms which have a free-running period of approximately, but not exactly, one day (10). Circadian rhythms typically have an additional property, that of temperature compensation; the period of the rhythm is relatively independent of temperature (11). However, like most biological systems, circadian rhythms are sensitive to certain external stimuli. They may be reset or driven by pulses of, for example, light or temperature (11,12).

There is very little known concerning the basic biochemical mechanism of the oscillation underlying circadian rhythmicity—the circadian clock. A wide variety of inhibitory substances, as well as metabolic cofactors and hormones, have been tested and found to have little effect on the phase and period of circadian rhythms, although many drastically affect the metabolism of the organism used (1,13). Heavy water changes the period of the clock under constant environmental conditions, and inhibitors of RNA and protein synthesis also affect it (14-21). However, these experiments have not yielded any new insights into the clock mechanism, beyond indicating the involvement of these molecules. Recent experiments in which pulses of potassium ions have been used to reset a circadian rhythm of neural spike activity implicate the cell membrane as an input and output element of that circadian system (22). The sole model for a circadian clock that has been set down in fairly specific molecular terms is the Chronon model (23). According to this model, the 24-hour periodicity of the clock is derived from repeated transcription of RNA from postulated polycistronic units of DNA called

chronons. No critical test of this model has yet been devised, and in view of current limitations in knowledge and available techniques relevant to the study of nucleic acid biochemistry in higher organisms, a proper test of the model may not occur for some time. Many mathematical models have been constructed which describe various aspects of the behavior of circadian oscillators but do not suggest any specific biochemical or cellular system which might be responsible for the oscillation (24-27).

For the analysis of a circadian clock, a genetic approach may be incisive. By obtaining mutants having their clocks altered in different ways, one might be able to draw conclusions concerning the organization of the clock system. Alternatively, if clock mutations were found that also had other demonstrable effects on the organism, some clue to the nature of the chemical pathway underlying the oscillation might be obtained. Identification of the altered gene product associated with a clock mutation could provide an access route into the mechanism of the clock. In spite of these possibilities, there have been relatively few genetic studies related to circadian systems.

Most of the studies which have been reported have dealt with differences among strains or individuals whose genetic backgrounds had not been strictly controlled; as a result, the inheritance patterns of the genes involved proved difficult to determine. Bunning, for example, crossed two strains of Phaeseolus whose leaf movement rhythms had periods of 23 and 26 hours (28). The F_1 exhibited an intermediate period (25 hours), but it could not be decided whether the segregation

was Mendelian. Danilevsky likewise obtained an intermediate value for the day length required for diapause induction in a cross involving strains of a butterfly with two different values (29). In this case, the intermediate value persisted in the F_2 and in backcrosses; again no clear segregation was determined. Neumann performed a similar experiment with the insect Clunio marinus, crossing two strains with differing times of emergence. He also obtained intermediate values for the emergence times of the F_1 and F_2 generations and in backcrosses (30). Rensing studied the oxygen consumption rhythm of various strains of Drosophila bearing visible and lethal mutations, noting the time of appearance of the maximum when the flies were grown in a light-dark cycle (31). No one gene appeared to drastically affect the rhythm. There did, however, seem to be a sex difference in the time of peak oxygen consumption; this suggested that the X:autosome ratio was important in determining which of the two peaks would be expressed. Rensing proposed that one peak position might be controlled by the X chromosome and one by the autosomes. The data presented, however, were quite variable, and it was never fully established that the peaks observed and correlations drawn were statistically significant.

Pittendrigh has described an experiment wherein Drosophila were selected for early and late eclosion for many generations; this selection yielded two stable lines, one of which consistently emerged early and one late (32). This result showed that at least one parameter of the rhythm, its phase, could be altered in an organism by selection, presumably by changing its genetic constitution. By

using the response to short light pulses as an assay of the behavior of the clock, however, Pittendrigh showed that the basic oscillator remained the same in both strains; some other aspect of the circadian system was affected by the selection.

Strain differences have also been observed in rhythms of Paramecium. Karakashian, for example, has found that, in the case of three strains of Paramecium aurelia which exhibited mating rhythms in light-dark cycles, two rhythms persisted in constant darkness and one did not (33). Likewise, Barnett described two strains of Paramecium multimicronucleatum which differed in the expression of a rhythm of mating-type reversal (one appeared to be acyclic); both strains, however, exhibited a rhythm in cell division (34). This result leaves open the possibility that the coupling of the clock to the mating-type reversal rhythm was affected rather than the clock itself.

The most interesting data concerning the genetics of rhythms come from the fungi. Here mutants have been isolated which exhibit a rhythm of growth or conidiation, whereas the wild-type strains ordinarily do not. (It has recently been found, however, that many if not all wild-type strains can be made to express a rhythm by changing their growth conditions.) The rhythm observed may or may not be truly circadian. In Ascobolus and Neurospora, for example, mutants have been isolated which differ from wild-type by a single gene and which exhibit periodic growth (35,36). The growth rhythm is, however, very sensitive to temperature and not entrainable by light or temperature cycles (35,37,38). In the Neurospora mutant

"clock," the period varies from 22 to 72 hours depending on the growth medium (38,39). It therefore appears as though the growth rhythm of these mutants should not be considered truly circadian, since they lack several properties which "true" circadian rhythms normally possess.

In the case of the Neurospora mutants "timex" and "patch," the observed rhythm, that of conidiation, is indeed circadian; the period is relatively insensitive to temperature, the rhythm persists in constant darkness, and it can be entrained by light and phase-shifted by a single light pulse (40-43). Changes in the growth medium affect the degree of expression of the rhythm in "timex" but not the period (40). The "patch" phenotype was first observed in a proline-requiring strain and appears to partially suppress the proline requirement (42). "Patch" strains grow to excess ("escape") on medium containing sorbose (42); they do not grow in colonies as do wild-type strains grown on this medium. The "patch" mutation segregates as a single gene (43). The "timex" mutant, on the other hand, differs from wild-type by two genes, one of which appears to be the structural gene for invertase ("timex" lacks invertase activity) (41). The mechanism by which the rhythm is produced has not yet been worked out, although the strain has been used to demonstrate, in conjunction with an albino mutant, that the photoreceptor for light effects on the rhythm is probably not a carotenoid (41).

It is interesting to note that, although "clock" is very different from "timex" or "patch," the three mutants are all rhythmic, and all seem to be linked to sugar metabolism. "Timex," as mentioned

above, lacks invertase activity (41). "Patch" escapes on sorbose (42). The "clock" strain used by Bianchi did not grow on minimal medium with sorbose, but grew and banded on minimal medium supplemented with sorbose and sucrose (37). Berliner and Neurath found that growth of a "clock" strain was arrhythmic unless sugars were added to the medium (44). Finally, a wild-type strain investigated by Sussman et al. produced a "patch" phenotype when grown on minimal medium to which sorbose and sucrose had been added (36). These observations strongly suggest that at least the coupling of a clock to growth or conidiation, if not the clock itself, is in some way connected with sugar metabolism.

Recently it has been possible to measure both the non-circadian growth rhythm and the circadian conidiation rhythm in the same strain of Neurospora (45). It may therefore be feasible to determine whether there are any elements common to the mechanisms underlying the two rhythms by isolating mutants affecting both rhythms. So far, only mutants which change the period of the growth rhythm but leave the conidiation rhythm unaffected have been obtained (45). Feldman and Waser have also reported the isolation of mutants in which the circadian conidiation rhythm is affected (46); the usefulness of the mutants has so far been limited by the sterility of crosses involving the mutants and other strains.

As an organism in which to study the genetics of a circadian rhythm, Drosophila offers several advantages. The rhythm of eclosion of the adult fly from the pupal case has been studied in great detail in Drosophila pseudoobscura, especially by Pittendrigh and his

co-workers (47). The effects of light and temperature on the circadian oscillator have been thoroughly examined, and a model has been proposed to explain the mechanism by which these agents entrain the oscillator (26,47-54). The oscillator has been shown to be coupled to the eclosion event but not to other events which occur at specific times during the development of the organism (55); in addition, the oscillator itself has been shown to be independent of the developmental stage of the insect (56). There is strong evidence for the existence of a circadian rhythm of locomotor activity in adult Drosophila (57-59), and studies have been made of light-dark fluctuations in egg-laying, pupation, puff patterns, oxygen consumption, and morphology of neuroendocrine cells (60-62). Much is known about the genetics of Drosophila, the species melanogaster having been studied for over half a century (63). In addition, Drosophila is becoming widely used as an organism for biochemical (64), developmental (65), and behavioral investigations (66,67), primarily because of the extensive knowledge of its genetics.

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CHAPTER 2.

Clock Mutants of *Drosophila melanogaster*

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Clock Mutants of *Drosophila melanogaster*

(eclosion/circadian/rhythms/X chromosome)

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ABSTRACT Three mutants have been isolated in which the normal 24-hour rhythm is drastically changed. One mutant is arrhythmic; another has a period of 19 hr; a third has a period of 28 hr. Both the eclosion rhythm of a population and the locomotor activity of individual flies are affected. All these mutations appear to involve the same functional gene on the X chromosome.

Rhythmic variations in behavior are displayed by many organisms, ranging from single cells to man (1). When the rhythm persists under constant conditions, and has a period of around one day, depending little on temperature, the rhythm is called circadian (2). Many experiments have attempted to probe the mechanism (3), but the nature of the underlying oscillation remains unknown (4). Perturbations by inhibitors of RNA or protein synthesis suggest that such molecules are involved (5–8). Biochemical systems that oscillate with much shorter periods have been demonstrated both *in vivo* and *in vitro* (9, 10), but their relation to circadian rhythms is not clear.

An approach that has been successful in unravelling mechanisms in some systems is the use of genetic alterations. Since the expression of a rhythm requires an integrated system, mutation of the genes responsible for development and function of the system could lead to abnormal rhythms. Various aspects of circadian rhythms have indeed been shown to be sensitive to genetic makeup (11–18). For genetic dissection of circadian rhythms in an organism having a nervous system, *Drosophila* offers certain advantages. Much is already known about the rhythm of eclosion (emergence of the adult fly from the pupa), and genetic methodology is readily available. This paper describes the first result of such an analysis.

MATERIALS AND METHODS

Isolation of mutants

D. melanogaster of the C-S (Canton-Special) wild strain was maintained on cornmeal medium. Mutagenesis by ethyl methane sulfonate was according to Lewis and Bacher (19), the treated males being mated to virgin attached-X females, so that each F_1 progeny male carried a treated X chromosome received from his father. Each male was mated individually to attached-X females, producing a stock of males bearing identical X chromosomes, plus normal-rhythm, attached-X females. The stocks were reared at constant temperature under LD 12:12 (12 hr of 50 foot-candles or more of white fluorescent light, 12 hr of darkness each day).

To detect X-linked rhythm mutants, the stocks were examined for ones in which males emerged abnormally. The normal females in each bottle served as an internal control, at least twice as many emerging during the light as during the dark period. In a few bottles, males emerged in approximately equal numbers during day and night. Each mutant candidate was examined in more detail by raising pupae in LD 12:12, then monitoring the adult eclosion rhythm in constant darkness. From a total of about 2000 F_1 males, three rhythm mutants were obtained.

Determination of eclosion and locomotor activity rhythms

Eclosion rhythms, free-running in constant darkness, were determined with automatic "bang boxes" (20), generously loaned by Dr. Colin Pittendrigh. Several hundred pupae, raised in LD 12:12, were transferred to the apparatus at the end of a light cycle. The apparatus was thereafter maintained in constant darkness. Fractions were collected every hour, yielding an eclosion profile.

Locomotor activity of individual adult flies was measured by monitoring their movement with infrared light, which does not affect the *Drosophila* clock (21). The devices, designed and built by Dr. Yoshiki Hotta, used a small incandescent lamp, a Wratten No. 87C filter transmitting only wavelengths greater than 800 μm, and a chamber (3 mm thick $\times$ 4 mm wide $\times$ 45 mm high) containing the fly, some food, and a cotton plug. Two silicon solar cells were arranged so that one received light transmitted through the upper third of the chamber, the other the lower third; they were wired so that the output voltage was zero when equal light fell on both cells. As the fly moved into or out of the area monitored by either solar cell, the resulting imbalance was converted to an all-or-none response registered on an event recorder. The sharpest rhythms were obtained with young flies (within one week of eclosion) previously exposed to at least three cycles of LD 12:12.

Genetic mapping of rhythm mutants

The rhythm mutants had normal morphology, but their abnormal eclosion patterns could be used as markers in recombination experiments. Males bearing an X-linked rhythm mutation were crossed to females homozygous for the visible markers *yellow-2*, *scute*, *vermilion*, *forked*, and a wild-type allele of *yellow* located near the centromere. These markers had no effect upon rhythm. F_1 males, receiving their X chromosomes from their mothers, were all $y^2\ sc\ v\ f\ \cdot\ y^+$, and the females were all heterozygous, having the rhythm mutation on one X chromosome and $y^2\ sc\ v\ f\ \cdot\ y^+$ on the other. These males and females were mated to each other. F_2 males, receiving X chromosomes that had an opportunity to undergo recombination in their mothers, included various recombinants for the rhythm mutation and the morphological markers.

F_2 progeny were raised in LD 12:12 at 25°C, collected in the pupal stage, and transferred to "bang boxes," as above. The morphological phenotype of each male fly was scored by microscopic examination, and the eclosion profile was plotted for each parental and recombinant class.

Complementation tests on rhythm mutants

Flies heterozygous (in the *trans* arrangement) for two rhythm mutations were constructed as follows. Males bearing one of the mutations were crossed to females carrying the balancer X chromosome FM 7, which contains multiple inversions to suppress crossingover between the two X chromosomes, as well as the dominant marker *Bar* for identification (22). Virgin progeny females (mutant/FM 7) were crossed to males bearing the second rhythm mutation, and the double heterozygotes (identified by lack of the *Bar* marker) were selected. These were tested individually in the locomotor-activity meter. The same procedure was used for constructing flies heterozygous for rhythm mutations and various X-chromosome deletions.

RESULTS

Eclosion rhythms of normal and mutant strains

Fig. 1*A* shows the normal circadian rhythm of eclosion of adults. The data shown are for attached-X females (carrying the genetic markers *yellow* and *forked*), which were routinely used as internal controls in experiments involving mutants (see *Methods*). Their rhythm was indistinguishable from that of the C-S males from which the rhythm mutants were isolated. These eclosion peaks are somewhat broader than those reported for *D. pseudoobscura* (23). In *pseudoobscura*, the period of the eclosion rhythm has usually been determined with reference to the median point of each successive eclosion peak. For *melanogaster*, a more sharply definable point is the time at which the peak rises to half its maximum value. The average period for normal flies (Fig. 1*A*) is about 24 hr.

Figs. 1*B*, 1*C*, and 1*D* show the rhythms for males of three mutant types, each isolated by one-step mutation from the normal C-S strain. One mutant is essentially arrhythmic; another has a short period of about 19 hr; the third has a long period of about 28 hr. These profiles are reproducible in repeated runs for each strain and the properties of the mutants have been hereditarily transmitted over many generations.

Effect of temperature on the eclosion rhythms

Between 18°C and 25°C, the period of the eclosion rhythm of normal *D. melanogaster* remains constant to about 1 hr (the interval used in collecting fractions). The same is true for the short- and long-period mutants. The arrhythmic mutant remains arrhythmic in this temperature range.

Locomotor activity rhythm in individual flies

Eclosion occurs only once in a fly's lifetime; to study the clock that controls eclosion, one must observe an entire population. This raises a question for the apparently arrhythmic mutant: Is the absence of an eclosion rhythm due to lack of expression of the clock or simply desynchronization of the various individual flies? To answer this, it is necessary to assay some ongoing phenomenon in a single fly. We chose to measure locomotor activity, using the photoelectric device described in *Methods*. Earlier studies have demonstrated the existence of a rhythm of locomotor activity in *Drosophila* (24–26).

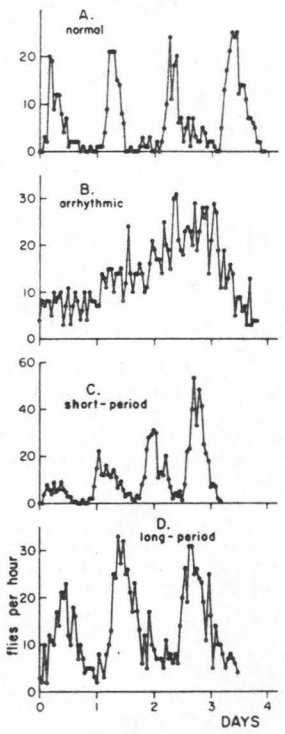

FIG. 1. Eclosion rhythms, in constant darkness, for populations of rhythmically normal and mutant flies, previously exposed to LD 12:12. T = 20°C.

Fig. 2*A* shows the activity, as registered on an event recorder, for a rhythmically-normal female (*yellow*, *forked*, attached-X). The fly was raised in LD 12:12, then placed in the monitoring device at the end of a light cycle. In these records, the offset of activity was typically more abrupt than the onset, so that the free-running period could be best determined by measurement of the average drift in time of offset per day. The rhythm shown in Fig. 2*A*, therefore, has a period of about 25 hr. For 8 females studied, the average period was 24.5 ± 0.4 hr. Fig. 2*B* shows the activity of a female homozygous for the arrhythmic mutation. The activity appears, by comparison, random in time. Thus, this mutation has indeed abolished the locomotor rhythm in individual flies. Four females studied gave similar results, with no evident periodicity.

Fig. 2*C* shows the activity for a homozygous short-period female. To better illustrate the short period, these records are displayed using a modulus of 19 hr. The locomotor activity rhythm for 5 short-period females was 19.5 ± 0.4 hr. Fig. 2*D* is for a homozygous long-period female, presented *modula* 28 hr. The average period for 4 females was 28.6 ± 0.5 hr.

Males of each mutant strain were also monitored (7 arrhythmic, 6 short-period, 4 long-period). The results were similar to those for females, giving average periods of 19.2 ±

Fig. 2. Locomotor activity rhythms, monitored in infrared light, for individual rhythmically normal or mutant flies previously exposed to LD 12:12. Activity registered by event recorder. Records read from *left* to *right*, each new line representing the start of a successive interval. For visual continuity, each successive interval is also replotted to the right of the immediately preceding interval. The traces for normal and arrhythmic are plotted *modulo* 24 hr; for the short-period mutant *modulo* 19 hr is used; the long-period mutant is plotted *modulo* 28 hr. T = 25°C.

0.5 hr for the short-period mutant and 28.5 ± 0.5 hr for the long-period mutant, while no arrhythmic male showed any evident periodicity. Eight normal C-S males showed an average period of 23.8 ± 0.5 hr. Thus, in every case, the rhythm of ongoing locomotor activity in the adult corresponds to the rhythm of eclosion for the population.

Genetic mapping of rhythm mutants

To locate the mutant genes on the X chromosome, recombination was measured with respect to morphological markers with known position. Eclosion profiles were determined for various recombinant types and compared to the normal parental type. Fig 3 illustrates the method for the short-period mutant. The result is that recombinants lacking the portion of the marked chromosome carrying the genes for *yellow-2* and *scute* (and, hence, having obtained this portion from the rhythm-mutant chromosome) display the mutant period. The reciprocal recombinants (not shown) have a normal period. Thus, this rhythm mutation would appear to be located toward the left end of the X chromosome (the centromere being at the right end). The same procedure was also followed for the arrhythmic mutant; it also mapped to the same portion of the chromosome. The mapping was repeated for both mutants using the X-linked visible markers *white*, *singed*, and *miniature;* the results confirmed the assignment of both mutations to the left end of the X chromosome.

As a further check on all three rhythm mutants, recombinant males were recovered from crosses using the markers *white*, *singed*, *miniature*, or *yellow*, *white*, *split*. Each male was mated to virgin attached-X females to produce a stock of identical males, and the eclosion profile or locomotor rhythm of the stock was determined. The results in all 27 cases tested (9 for each rhythm mutant) were consistent with location of all three mutations to the left of *white*.

Complementation tests on rhythm mutants

The recombination experiments indicated similar positions on the X chromosome for the 3 rhythm mutations, raising the question whether these mutations represent changes in the same functional gene (cistron). This can be tested by constructing females bearing a different rhythm mutation on

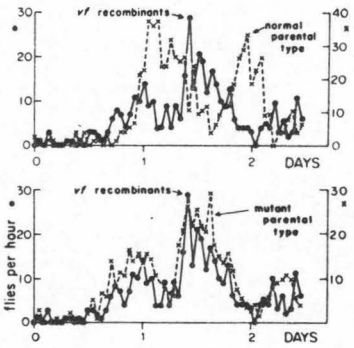

Fig. 3. Genetic recombination of the short-period gene with marker genes on the X chromosome. The eclosion profile is shown for one recombinant type emerging from the cross, compared to the normal and mutant parental types.

each of the two X chromosomes, and observing the resultant rhythms. This has been done for all combinations of the 3 rhythm mutant genes with each other and with the normal gene, measuring the activity rhythm on individual flies. Table 1 gives the results. Note the cases of heterozygotes with a mutant gene on one X chromosome and a normal gene on the other. For both the arrhythmic and the long-period mutants, the result is a rhythm with period close to normal. Thus, these mutant genes may be regarded as recessive to the normal one. In the case of the short-period mutant, however, the period of the heterozygote is intermediate between short and normal. This gene can, therefore, influence the rhythm even in the presence of a normal gene; it is only partially recessive. When the short-period mutant gene is opposed to the arrhythmic one, the rhythm displays a short period. Similarly, the arrhythmic gene is overshadowed by the long-period one. When the short-period and long-period mutants are tested together the result is a period close to

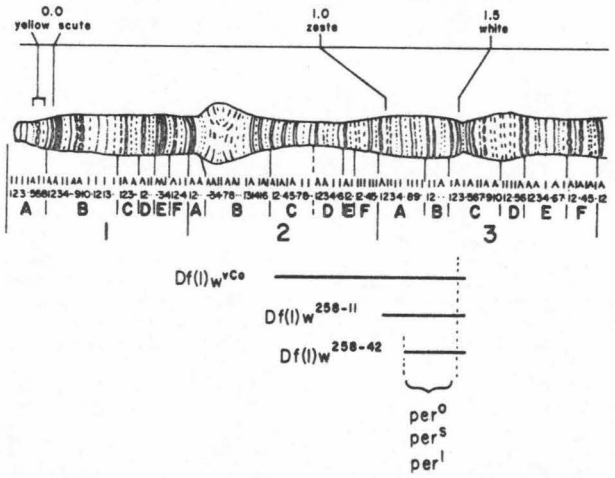

Fig. 4. Bridges' map (28) of the X chromosome of *Drosophila melanogaster*, showing about one-sixth of the chromosome at the end distal to the centromere. Each deficiency mutant lacks bands over the range indicated (9). *per°* = arrhythmic mutant, *per*^{s} = short-period mutant, *per*^{l} = long-period mutant.

normal. This particular result does not provide a distinction between an additive effect or complementation of the two mutant genes to produce a normal period.

Complementation tests were also performed, using various deficiencies of the X chromosome, as illustrated in Fig. 4. All 3 rhythm mutations were tested in females heterozygous for the largest deficiency, w^{vCo}. The mutant phenotype was fully expressed in each case. Thus, this deficiency lacks the normal gene. The short-period mutant was similarly tested against the 2 shorter deletions shown. Again, this mutation was fully expressed; the heterozygotes had a short period. This result, combined with the fact that recombination experiments place the rhythm gene to the left of *white*, locates the gene within bands 3A6 to 3C2 (Fig. 4).

In sum, the results are consistent with the hypothesis that the arrhythmic mutant gene is simply inactive. Neither the short-period gene nor the long-period gene complements with the arrhythmic one to produce a normal rhythm. If all 3 mutations are, in fact, point mutations, they would appear to affect the same functional gene. However, it is not excluded that the arrhythmic mutation could be a deletion that overlaps the other two. Such a deletion could not be very large, since, unlike most known deficiencies, the arrhythmic mutant is fully viable and fertile, as are also the short- and long-period mutants.

DISCUSSION

As in many other aspects of behavior, alteration of a single gene can drastically change the properties of circadian rhythms. The different phenotypes of the three mutants described might be explained if, for instance, the long- and short-period mutants contain missense mutations producing alterations in quality or quantity of a gene product involved in the clock mechanism, while the arrhythmic mutant lacks the substance altogether. If this is so, the arrhythmic mutant may serve to identify the missing substance by comparison with normal flies.

TABLE 1. *Free-running period of locomotor activity*

Genotype				
First X chromosome	Second X chromosome	N	Period ± SD	Phenotype
normal (C-S)	normal (FM 7)	4	24.4 ± 0.5	normal
arrhythmic	arrhythmic	4	arrhythmic	arrhythmic
short-period	short-period	5	19.5 ± 0.4	short-period
long-period	long-period	4	28.6 ± 0.5	long-period
arrhythmic	normal (FM 7)	8	25.2 ± 0.4	~ normal
short-period	normal (FM 7)	5	21.9 ± 0.4	intermediate
long-period	normal (FM 7)	5	25.5 ± 0.5	~ normal
short-period	arrhythmic	6	19.5 ± 0.4	short-period
long-period	arrhythmic	5	30.6 ± 1.3	long-period
short-period	long-period	6	22.9 ± 0.4	~ normal

The fact that the *period* of the rhythm under constant conditions is altered implies that the mutations are affecting the basic oscillator. All three mutations affect both the pupal eclosion rhythm and the adult activity rhythm, as if a single clock system controls both. Since the rhythm that determines eclosion can be initiated and reset during the larval and pupal stages (27), it would appear that this clock system persists through metamorphosis. At the very least, both systems have some gene product in common. By measurement of the effect of the mutations on other rhythms (e.g., egg-laying, mating), it should be possible to determine whether rhythms having different phases are all coupled to the same basic oscillator.

The long- and short-period mutants retain a property of normal rhythms in that their periods change very little with

temperature. Thus, whatever mechanism is responsible for temperature compensation of the rhythms is still operative in these mutants.

It is striking that the first three rhythm mutants isolated, having very different phenotypes, all affect the same functional gene, since one might expect that many genes play a role. Isolation of additional mutants on the X chromosome, as well as the autosomes, may turn up other relevant genes.

The anatomical site of action of a rhythm mutation can be investigated by using genetic mosaics, i.e., flies composed of mutant and nonmutant parts, and determining which parts control the rhythm of the composite fly. Preliminary results indicate that the rhythm corresponds to the genotype of the head.

This work was supported by a grant from the National Science Foundation to S.B. and a National Science Foundation predoctoral fellowship to R.K. We thank Dr. Colin Pittendrigh for enlightening discussions and for the loan of several bang boxes, Dr. Yoshiki Hotta for design and construction of the activity detectors, and Barbara Stewart for help with the mapping experiments.

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CHAPTER 3.

Additional Data

DEFICIENCY MAPPING OF THE SHORT-PERIOD MUTANT: ADDITIONAL DATA

In order to further localize the short-period mutation on the X chromosome, several X chromosome deficiencies, in addition to those listed in Konopka and Benzer (1), were tested for complementation. Flies were constructed as described in Konopka and Benzer (1). The results are given in Table 1. For five of the deficiencies examined, including the three listed in Konopka and Benzer (1), the period of the short-period/deficiency heterozygote is very close to that of short-period females; these deficiencies therefore overlap the mutation. In the case of the other two deficiencies, the period of the heterozygote is similar to that of short-period/white or short-period/FM7 females; these deficiencies do not overlap the mutation. (Short-period/white females were included as short-period/normal rhythm controls since all deficiencies tested were either mutant or deficient for white.) These experiments localize the short-period mutation to the 3B1-2 region.

TABLE 1.

COMPLEMENTATION TESTS OF THE SHORT-PERIOD MUTATION

WITH X-CHROMOSOME DEFICIENCIES

Short-period homozygotes and short-period/normal heterozygotes

Genotype	Number tested	Period of activity rhythm	Phenotype
short-period/short-period	5	19.5 ± 0.4 hrs.	short-period
short-period/normal (FM7)	5	21.9 ± 0.4 hrs.	intermediate
short-period/normal (w)	13	21.3 ± 0.4 hrs.	intermediate

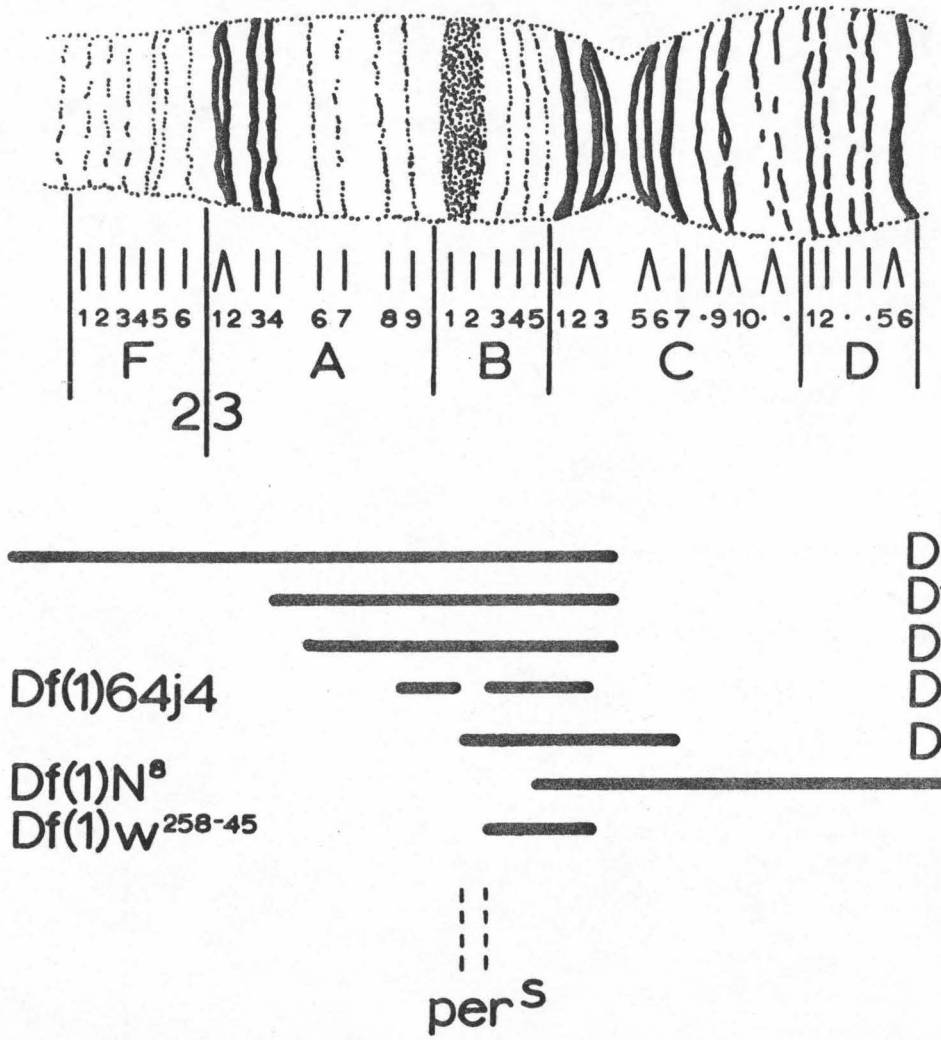
Deficiencies which uncover the mutation

Deficiency	Break points	Number tested	Period of activity rhythm	Phenotype
w^{vCo}	2B17-C1;3C4-5	8	19.7 ± 0.4 hrs.	short-period
w^{258-11}	3A3-4;3C3-5	5	19.8 ± 0.3 hrs.	short-period
w^{258-42}	3A4-6;3C3-5	6	19.9 ± 0.3 hrs.	short-period
64j4	3A8-9;3B1-2	5	19.9 ± 0.3 hrs.	short-period
62d18	3B1-2;3C6-7	4	19.9 ± 0.3 hrs.	short-period

Deficiencies which do not uncover the mutation

N^{8}	3B4-C1;3D6-E1	13	21.6 ± 0.4 hrs.	intermediate
w^{258-45}	3B2-3;3C2-3	7	21.4 ± 0.4 hrs.	intermediate

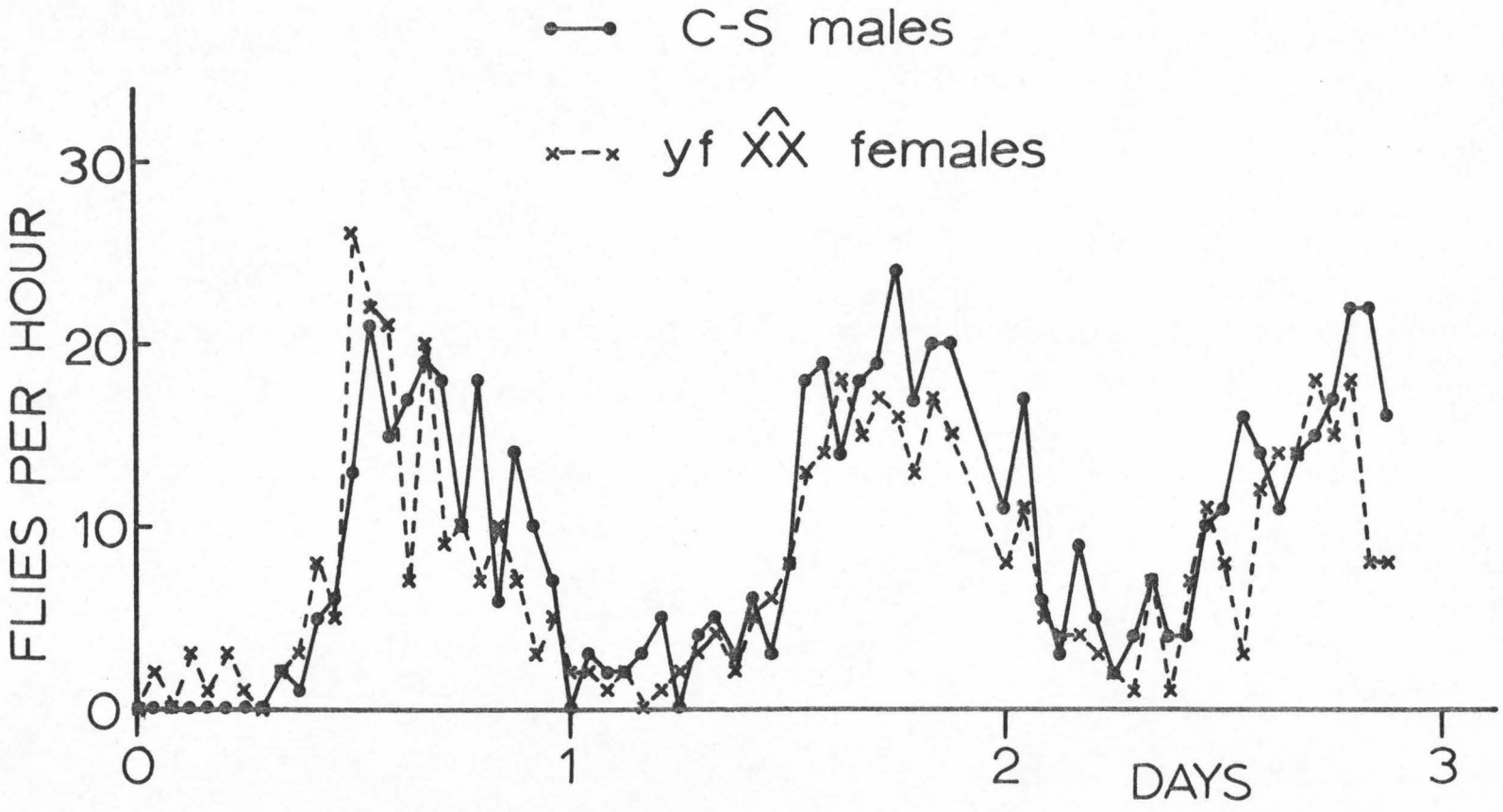
Figure 1. A map of the X chromosome bands missing in the deficiencies used to localize the short-period mutation. The missing bands are indicated by the black lines. Only the region 2F to 3D is shown. The stock containing the 64j4 deficiency also contained the w^{258-45} deficiency, as shown.


1 2 3 4 5 6
1 2 3 4
6 7
8 9
1 2 3 4 5
1 2 3
5 6 7 · 9 10 · ·
1 2 · · 5 6
F
A
B
C
D
2 3
Df(1)w^vCo
Df(1)w^258-11
Df(1)w^258-42
Df(1)w^258-45
Df(1)62d18
Df(1)64j4
Df(1)N^8
Df(1)w^258-45
per^s

THE SIMILARITY OF THE ECLOSION RHYTHMS OF RHYTHMICALLY NORMAL MALE AND FEMALE FLIES

Eclosion rhythms of mutant strains have routinely been determined using a stock in which males carried the mutant X chromosome, while the females carried two X chromosomes attached together and were rhythmically normal. The purpose of using such a stock is to provide an internal control, namely, the rhythmically normal females, to insure that the experiment had been properly executed. The appropriateness of using attached-X females as controls rests upon the similarity of their eclosion rhythm to that of the wild-type (Canton-S) males from which the mutants had been isolated (see Konopka and Benzer (1)). Figure 2 shows the eclosion rhythm of Canton-S males and attached-X females determined simultaneously in constant darkness. It is apparent that no major differences exist between the rhythm of males and the rhythm of females.

Figure 2. The eclosion rhythms of Canton-S males and yellow, forked, attached-X females, determined in constant darkness at 24° C. The pupae had been previously raised in LD 12:12. They were then transferred to constant darkness at the end of a twelve hour light period. The start of the plot corresponds to the beginning of the constant dark period.


C-S males
yf X̂X females
FLIES PER HOUR
30
20
10
0
0
1
2
3
DAYS

TEMPERATURE COMPENSATION OF NORMAL AND MUTANT RHYTHMS: ADDITIONAL DATA

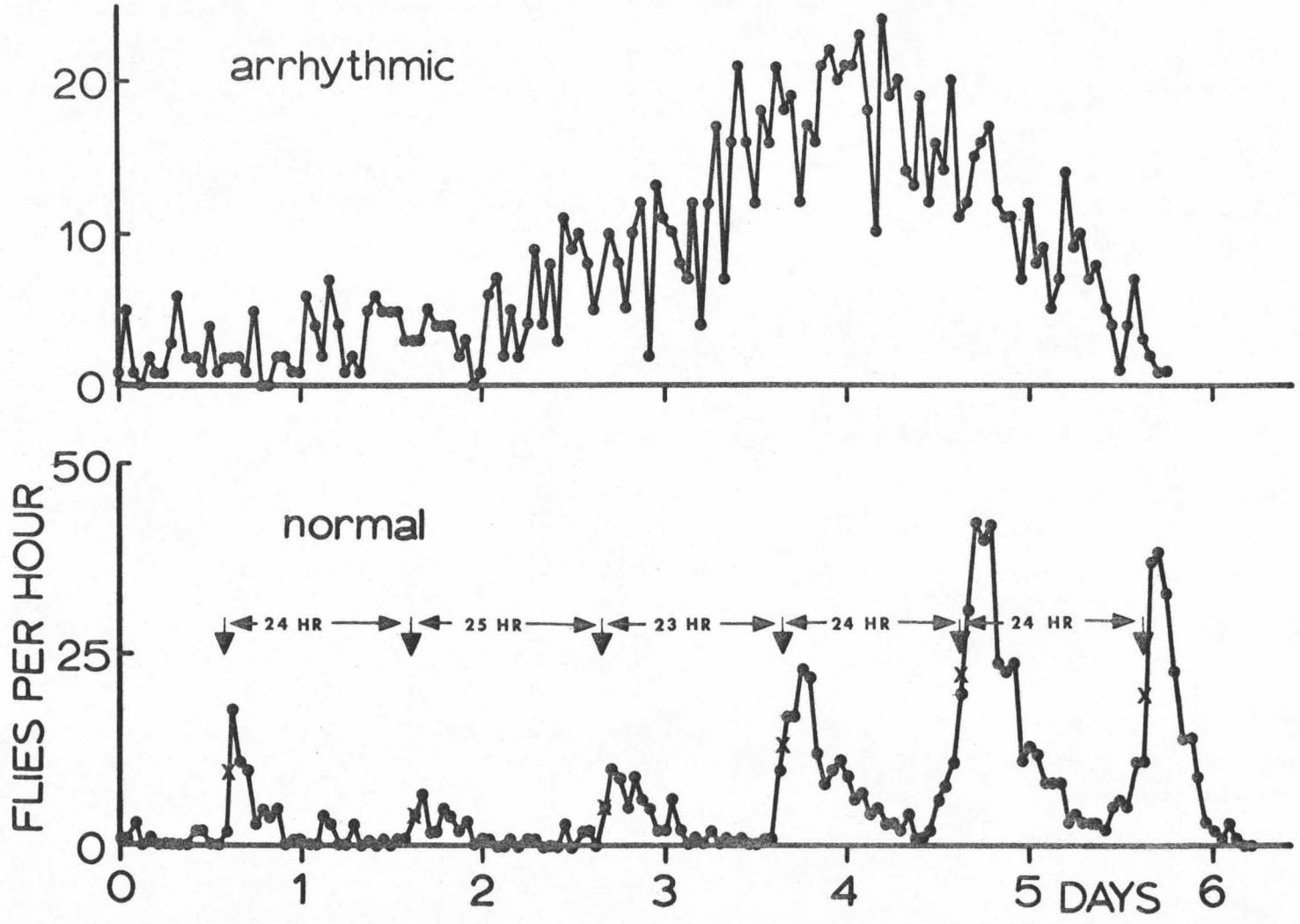
The period of a circadian rhythm in the steady state characteristically shows little variation with temperature. Since some circadian rhythms actually show a decrease in period with decreasing temperature (2), it is thought that the invariance of the period is due to a compensation mechanism rather than an insensitivity to temperature. To determine whether the mutant rhythms exhibit a similar phenomenon, the periods of normal and mutant rhythms were determined at 18° and 25° C. These limits were chosen because below 18°, the percentage of flies eclosing begins to fall sharply, and above 25°, eclosion occurs over too few cycles for accurate determination of the period.

Eclosion rhythms were determined according to the description in Konopka and Benzer (1). Figure 3 shows sample eclosion rhythms of rhythmically normal and mutant strains at 18° C. By measuring the interval between peaks (or between the half-rise times of successive peaks), it can be seen that the normal flies have a period of about 24 hours, while the short-period mutant has a period of about 20 hours and the long-period mutant about 28 hours. The arrhythmic mutant has no obvious rhythm at this temperature. Table 2 lists several determinations of the period of each rhythm; average periods were calculated over several cycles by measuring the time interval between the first and last peaks and dividing by the number of cycles. Figure 4 shows sample rhythms at 25° C. At this temperature, eclosion occurs over a span of about three days with the procedures used

routinely in these experiments. If three eclosion peaks are present, the first and last peaks may be skewed due to the small numbers of flies emerging at the beginning and end of the time interval over which eclosion occurs. For this reason, and because the fraction of time per day during which flies eclose is greater at 25° than at 18°, the period of each rhythm was determined as the time between the two eclosion minima. Table 3 gives these values for several determinations of each rhythm. The period of each rhythm at 18° is very close to the period at 25°; the arrhythmic mutant likewise remains arrhythmic over this temperature range. The change in the free-running period of the eclosion rhythm produced by the mutations does not appear to be due to a major alteration in the temperature compensation mechanism associated with the rhythm.

Figure 3. The eclosion rhythms of rhythmically normal and mutant strains at 18° C. in constant darkness. The rhythmically normal flies are yellow, forked, attached-X females. The short-period and arrhythmic mutant data are for males. The long-period mutant data are for males and females. The pupae were previously raised in LD 12:12 at 18°, then transferred to constant darkness at the end of a light period.

The midpoint of the rising phase of each peak is indicated by an X. The time span between successive midpoints is also shown.


arrhythmic
20
10
0
normal
50
25
0
FLIES PER HOUR
24 HR
25 HR
23 HR
24 HR
24 HR
0
1
2
3
4
5 DAYS
6

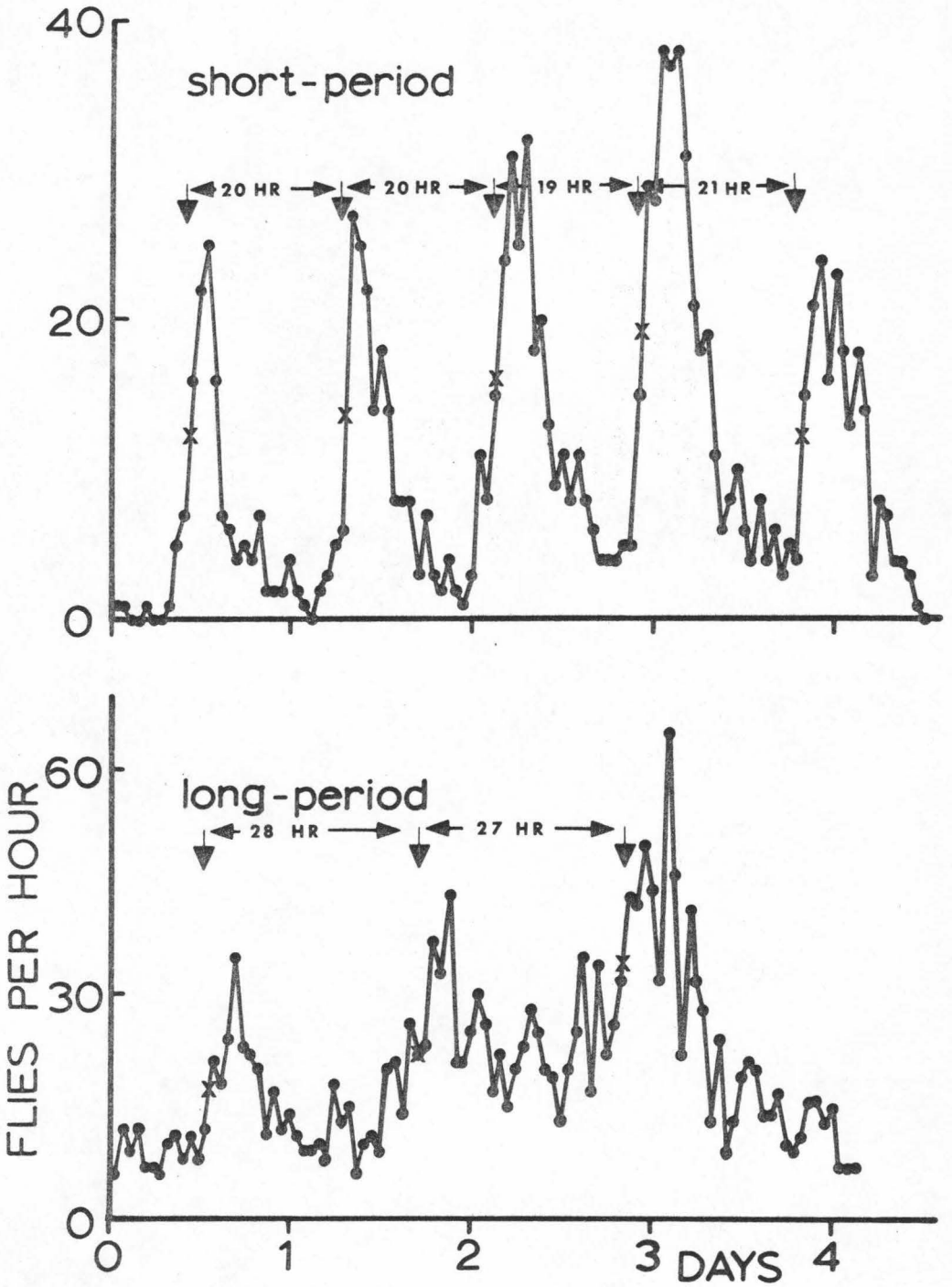
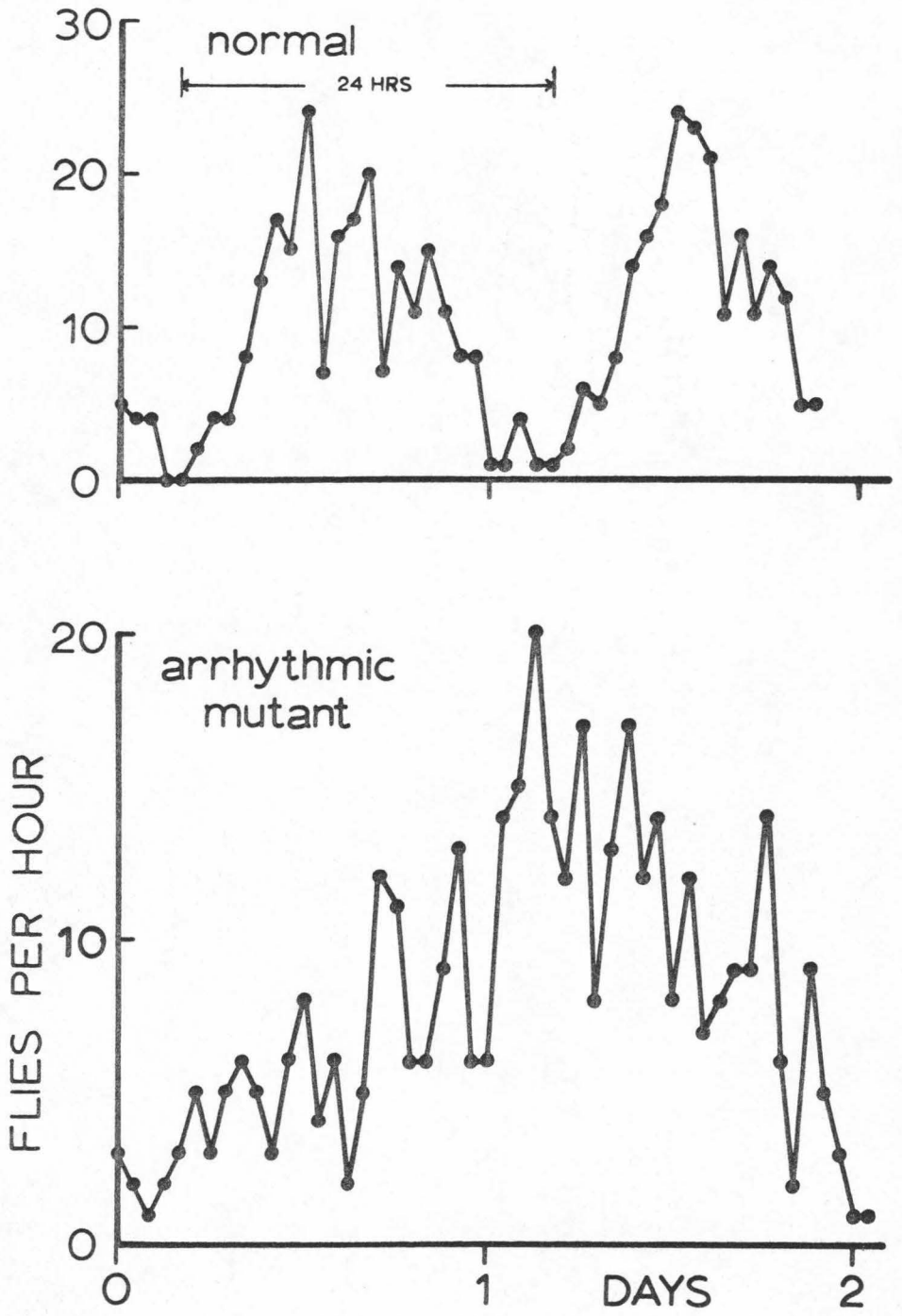

40
20
0
short-period
20 HR
20 HR
19 HR
21 HR
FLIES PER HOUR
60
30
0
long-period
28 HR
27 HR
0
1
2
3
DAYS
4

Figure 4. The eclosion rhythms of rhythmically normal and mutant strains at 25° C. in constant darkness. The rhythmically normal flies are yellow, forked, attached-X females. The short-period, long-period, and arrhythmic mutant data are for males. The pupae were previously raised in LD 12:12 at 25°, then transferred to constant darkness at the end of a light period.


30
normal
24 HRS
20
10
0
20
arrhythmic
mutant
FLIES PER HOUR
10
0
0
1
DAYS
2

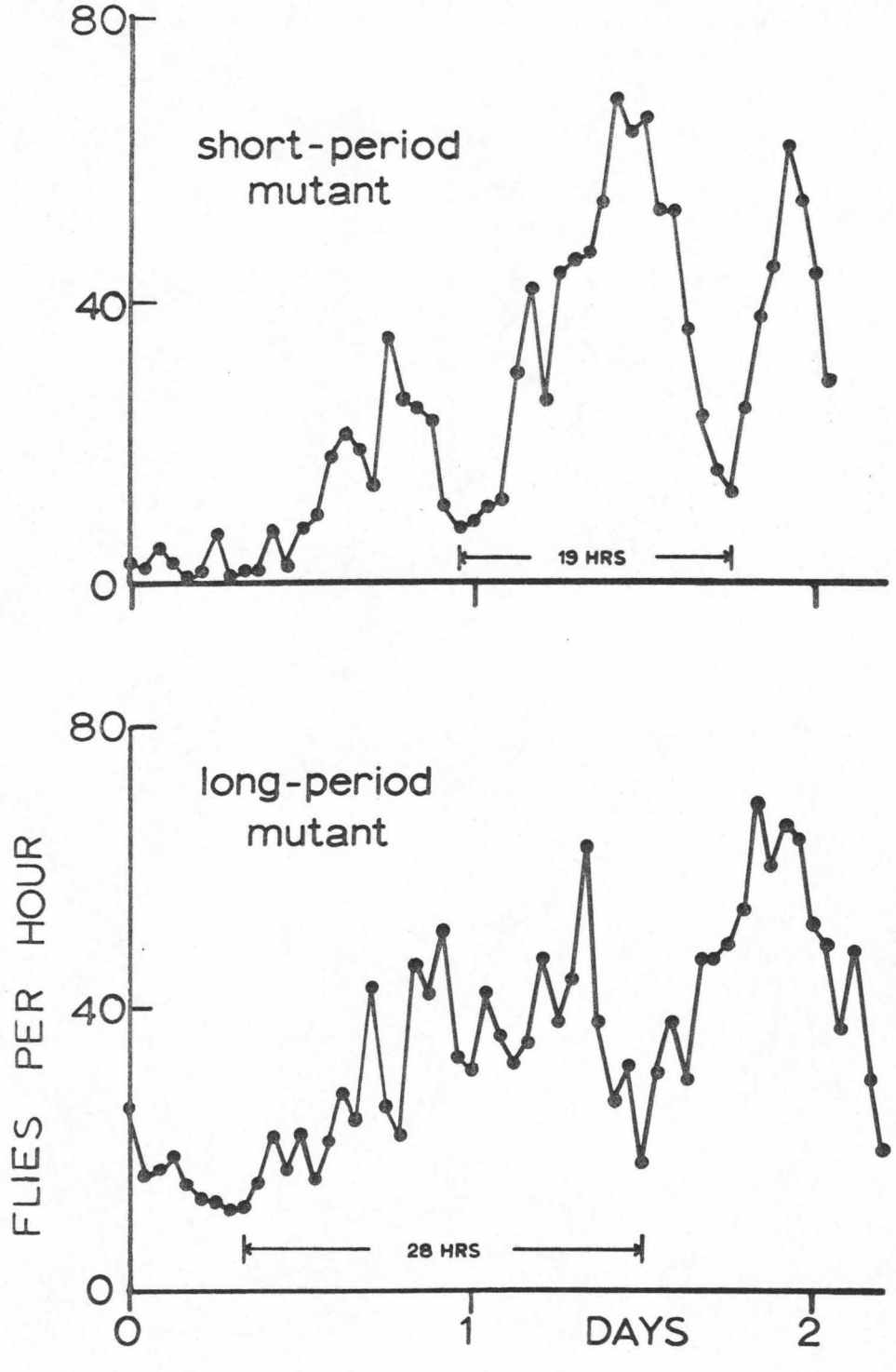

80
40
0
short-period
mutant
19 HRS
80
40
0
long-period
mutant
28 HRS
FLIES PER HOUR
0
1
DAYS
2

TABLE 2.

THE PERIODS OF ECLOSION RHYTHMS OF RHYTHMICALLY NORMAL AND MUTANT STRAINS AT 18° IN CONSTANT DARKNESS

Strain	N	Average period	Number of cycles
Normal (attached-X females)	6	24.0 hrs.	5
		24.4 hrs.	5
		24.0 hrs.	5
		24.6 hrs.	5
		23.8 hrs.	5
		24.5 hrs.	4
Arrhythmic (males)	5	Arrhythmic	
Short-period (males)	3	20.1 hrs.	6
		20.2 hrs.	4
		20.0 hrs.	2
Long-period (males + females)	3	27.5 hrs.	4
(males + females)		28.5 hrs.	2
(males)		29.5 hrs.	2

TABLE 3.

THE PERIODS OF ECLOSION RHYTHMS OF RHYTHMICALLY NORMAL AND MUTANT STRAINS AT 25° IN CONSTANT DARKNESS

Strain	N	Period
Normal (attached-X females)	10	24 hrs. 24 hrs. 24 hrs. 25 hrs. 23 hrs. 24 hrs. 24 hrs. 24 hrs. 23 hrs. 25 hrs.
Arrhythmic (males)	6	Arrhythmic
Short-period (males)	4	20 hrs. 19 hrs. 19 hrs. 19 hrs.
Long-period (males)	2	28 hrs. 27 hrs.

PERIODOGRAM ANALYSES

As an additional monitor of the characteristics of normal and mutant rhythms, locomotor activity data obtained from individual flies were subjected to periodogram analysis. The number of times the fly crossed the center of the cell used in the activity monitor (see Konopka and Benzer (1)) was recorded every 30 or 60 minutes; these data were used for the periodograms, which were determined in the usual way. The data were arranged in order in rows whose width equalled the duration of the trial period. The mean was calculated for each column of data, as well as the standard deviation of the column means. In addition, a mean activity was calculated from the grouped data, along with its standard deviation. Each point on the periodogram represents, for each trial period, the ratio of the standard deviation of the column means to the standard deviation of the mean of the grouped data.

Figure 5a shows a periodogram for activity data obtained from a rhythmically normal (attached-X female) fly. The main peak is at 23-24 hours, as expected. The smaller peak expected at 12 hours (half the period of the main peak) is barely detectable. In the periodogram analysis of data from a long-period mutant male shown in Figure 5b, the major peak is at 30-31 hours, again as expected. The smaller peak at 15 hours (half the period of the main peak) is quite obvious. In Figure 6a, a periodogram from data of a short-period mutant male is shown. The main peak, predictably, is at 19 hours; however, smaller peaks at 10 hours, 13 hours, 26 hours,

and 29 hours are noticeable. Since visual inspection of the data revealed no obvious periodicity at these trial periods, a synthetic 19-hour rhythm was constructed using symmetrical peaks three hours wide every 19 hours (see Figure 6b for details); this "rhythm" was analyzed in the same way as the actual data. The results are shown in Figure 6b. Here the major peak is again at 19 hours, but smaller peaks can be seen at 10 hours, 13 hours, 25 hours, and 29 hours. The smaller peaks in the actual short-period mutant data are, therefore, derived for the most part from the strong 19-hour periodicity. Figures 7a and 7b show periodograms from activity data of arrhythmic mutant males. There are no strong periodicities; small peaks can be seen at 5 hours and 7-8 hours. Figure 8 shows a periodogram from data of a third arrhythmic mutant male. Again, there are no large peaks; small peaks occur at 9-10 hours and at 23 hours. There is a good chance, however, that these represent scatter; the arrhythmic fly whose data is used in Figure 8 was run for less than six days. When the data of the first arrhythmic individual (Figure 7a) are divided into two four-day groups, for example, the scatter is much greater than that of the combined eight-day run, and several "peaks," comprising one point and similar to the 23-hour peak in Figure 8, may be seen (Figure 9).

The periodogram results confirm those obtained by visual inspection of the event-recorder data. The normal fly has an activity rhythm periodicity of about 24 hours, while the short-period mutant has a period of about 19 hours and the long-period mutant 30 hours. The arrhythmic mutant shows no consistent rhythmicity

at trial periods which might be considered circadian (18-30 hours). A slight ultradian rhythmicity exists in the arrhythmic mutant with periods of 5, 7-8, or 9-10 hours which is not found in the normal fly or in the two period mutants. These may be the remnants of a once-circadian oscillator made up of several shorter oscillators (e.g., five 5-hour oscillators or three 8-hour oscillators operating sequentially and repetitively). More likely, they represent some behavior independent of the circadian system and uncovered by its absence.

Figure 5. Periodogram analysis of eight days of locomotor activity data obtained from an individual yellow, forked, attached-X female (5a) and seven days of locomotor activity data obtained from an individual long-period mutant male (5b).

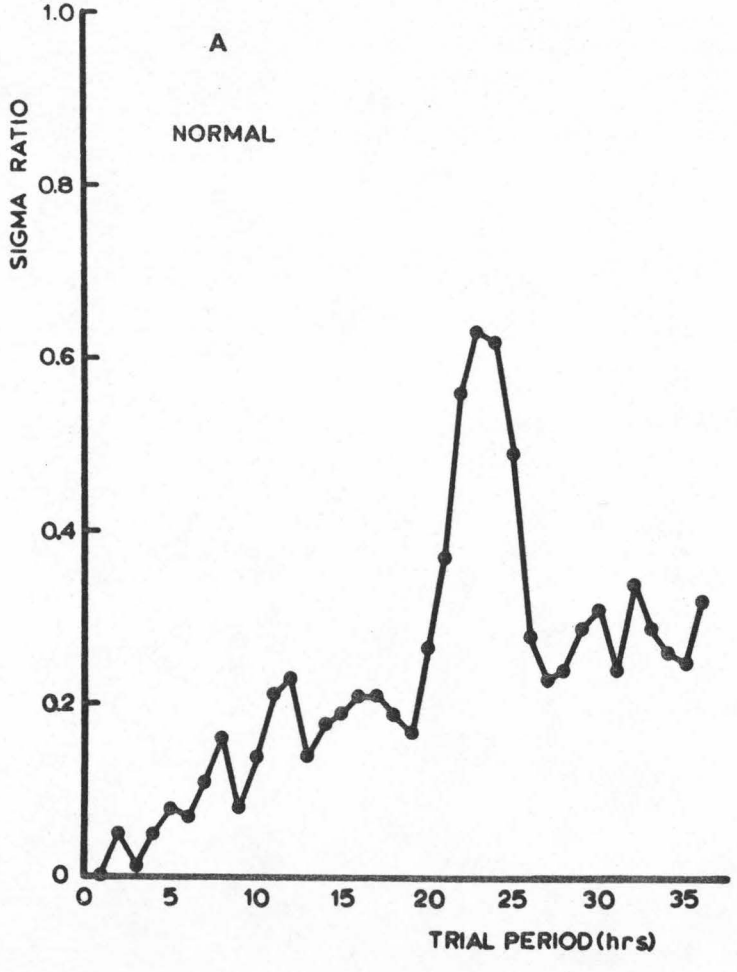
Figure 6. Periodogram analysis of five days of locomotor activity data obtained from a single short-period mutant male (6a) as well as a synthetically produced 19-hour "rhythm" (6b). The synthetic "rhythm" consisted of five days of three-hour-wide activity peaks occurring every 19 hours. Each peak was composed of 25 activity units the first hour, 50 units the second hour, and 25 units the third hour. The activity was zero at all other times.

Figure 7. Periodogram analysis of eight days of locomotor activity data obtained from an individual arrhythmic mutant male (7a) and six days of activity data obtained from a second individual arrhythmic mutant male (7b).

Figure 8. Periodogram analysis of five days, eighteen hours of locomotor activity data obtained from a third individual arrhythmic mutant male.

Figure 9. Periodogram analysis of the first half (9a) and second half (9b) of the eight-day run analyzed in Figure 7a.

FIG. 5


A
NORMAL
SIGMA RATIO
1.0
0.8
0.6
0.4
0.2
0
0
5
10
15
20
25
30
35
TRIAL PERIOD (hrs)

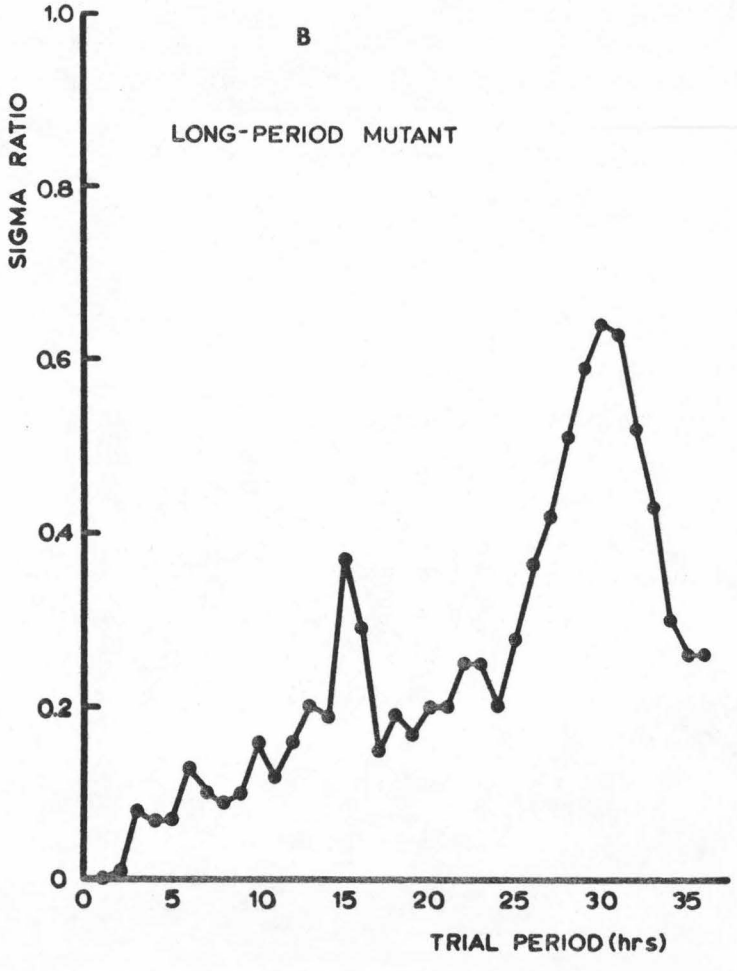
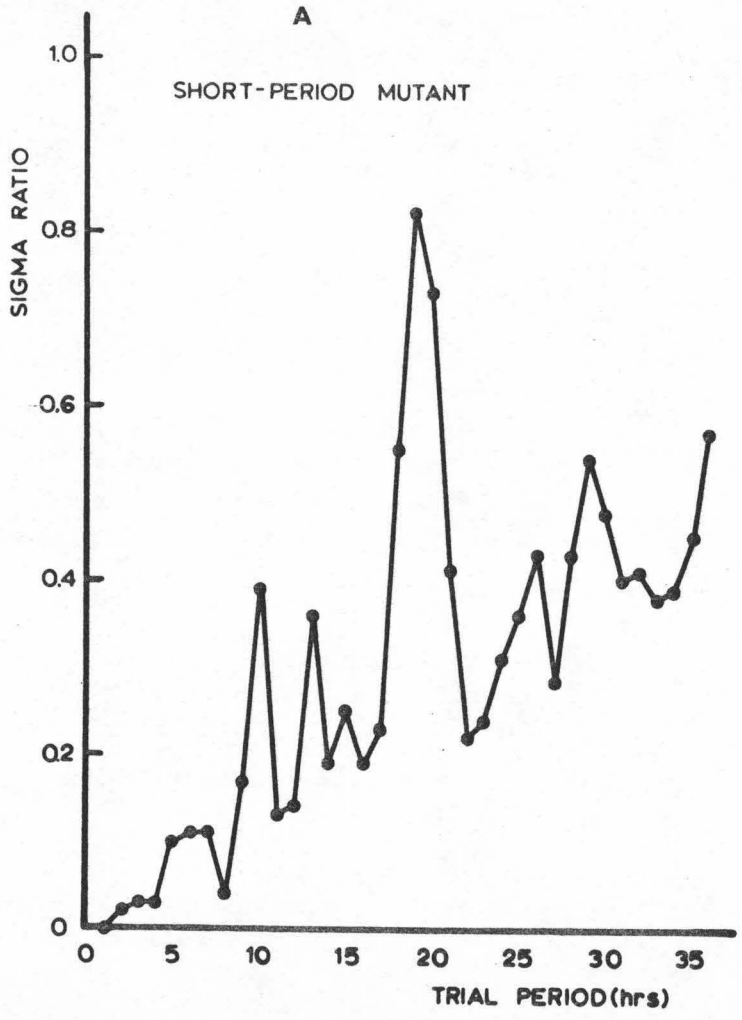

B
LONG-PERIOD MUTANT
SIGMA RATIO
1.0
0.8
0.6
0.4
0.2
0
0
5
10
15
20
25
30
35
TRIAL PERIOD (hrs)

FIG. 6


A
SHORT-PERIOD MUTANT
SIGMA RATIO
1.0
0.8
0.6
0.4
0.2
0
0
5
10
15
20
25
30
35
TRIAL PERIOD(hrs)

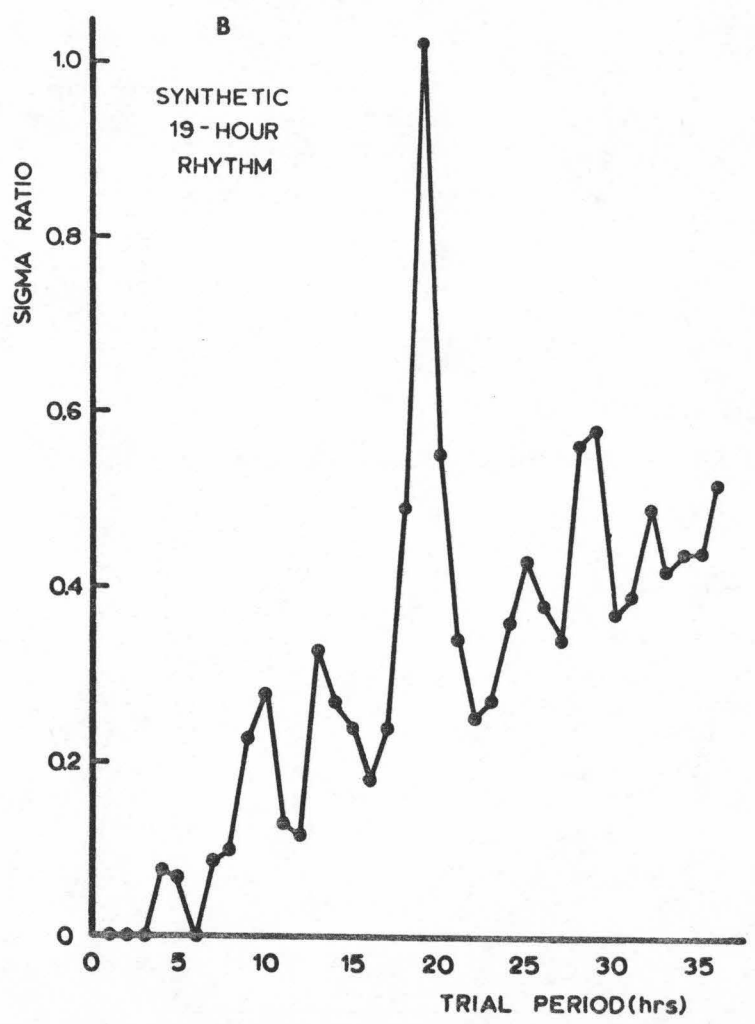
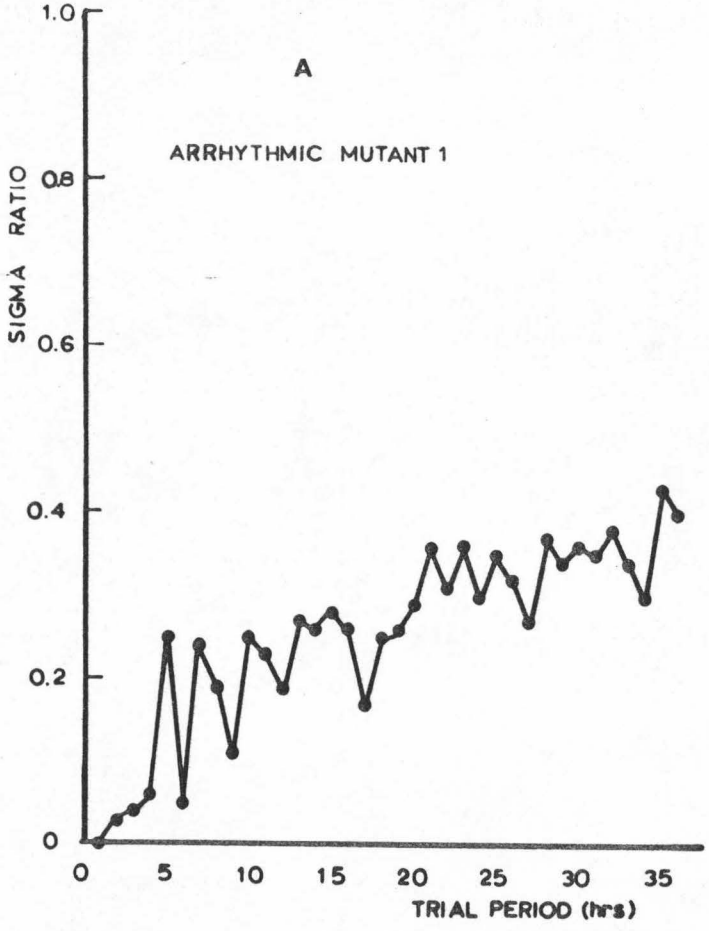

B
SYNTHETIC
19-HOUR
RHYTHM
SIGMA RATIO
1.0
0.8
0.6
0.4
0.2
0
0
5
10
15
20
25
30
35
TRIAL PERIOD(hrs)

FIG. 7


A
ARRHYTHMIC MUTANT 1
SIGMA RATIO
1.0
0.8
0.6
0.4
0.2
0
0 5 10 15 20 25 30 35
TRIAL PERIOD (hrs)

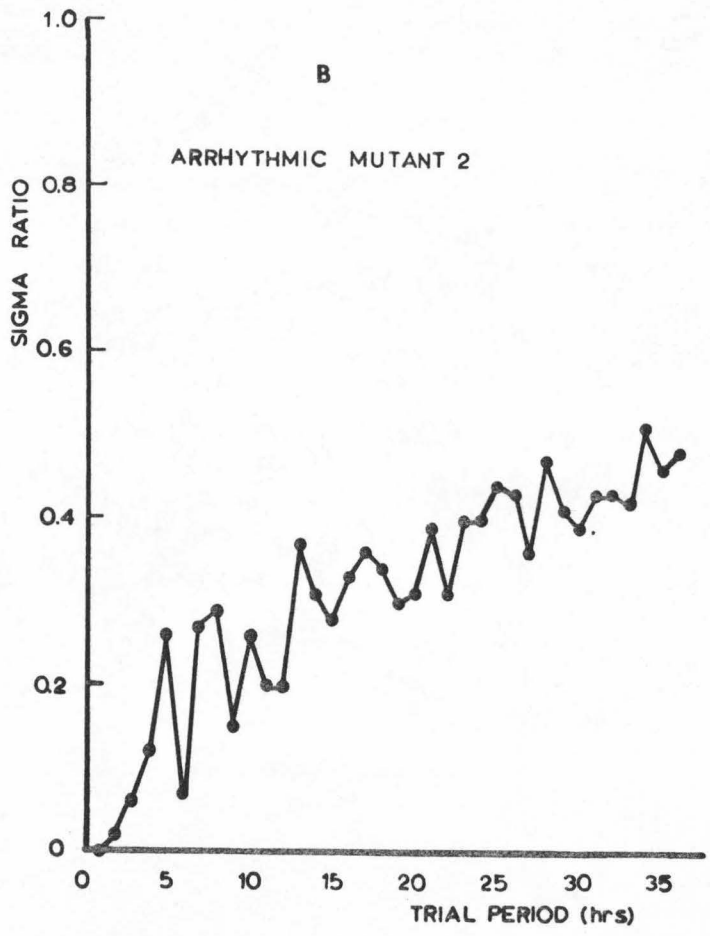

B
ARRHYTHMIC MUTANT 2
SIGMA RATIO
1.0
0.8
0.6
0.4
0.2
0
0 5 10 15 20 25 30 35
TRIAL PERIOD (hrs)

FIG. 8

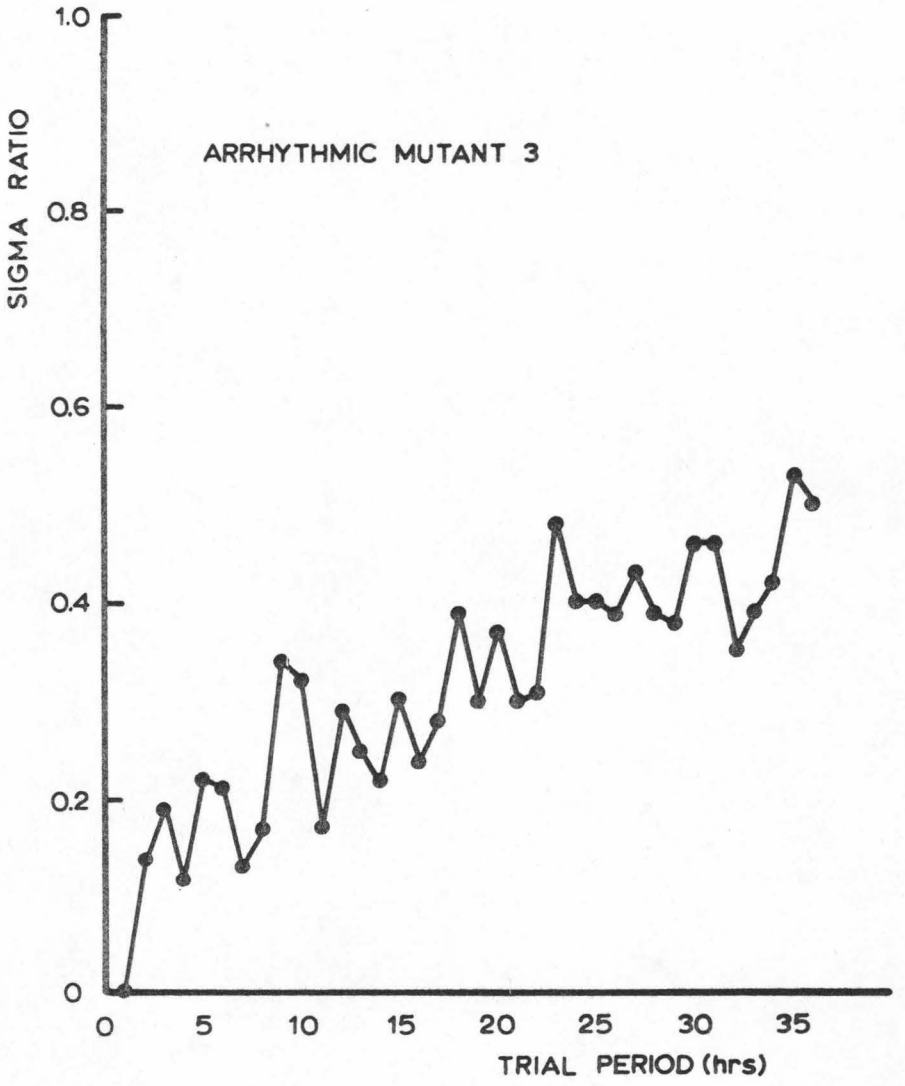
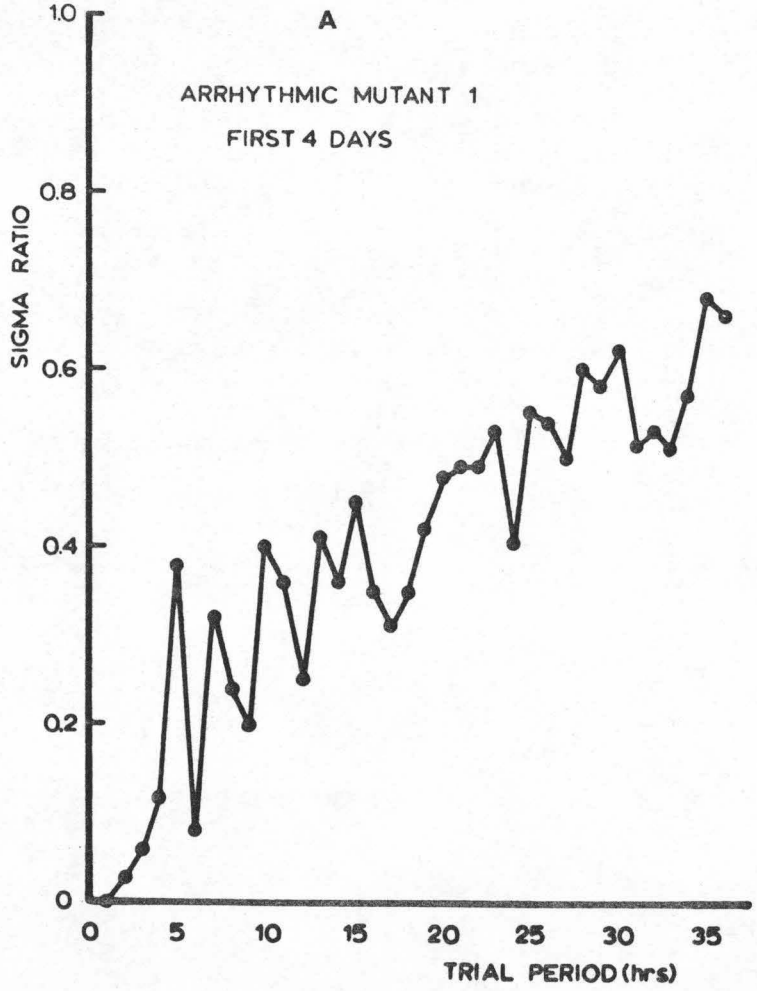
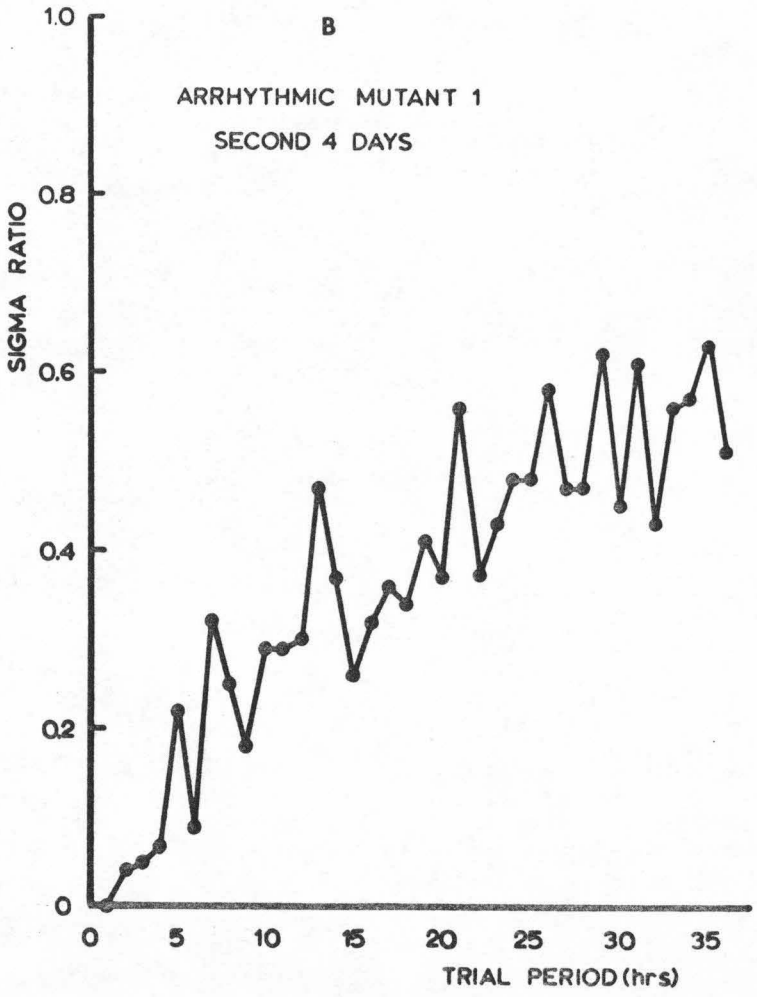

ARRHYTHMIC MUTANT 3
SIGMA RATIO
1.0
0.8
0.6
0.4
0.2
0
0
5
10
15
20
25
30
35
TRIAL PERIOD (hrs)

FIG. 9


A
ARRHYTHMIC MUTANT 1
FIRST 4 DAYS
SIGMA RATIO
1.0
0.8
0.6
0.4
0.2
0
0
5
10
15
20
25
30
35
TRIAL PERIOD (hrs)


B
ARRHYTHMIC MUTANT 1
SECOND 4 DAYS
SIGMA RATIO
1.0
0.8
0.6
0.4
0.2
0
0
5
10
15
20
25
30
35
TRIAL PERIOD (hrs)

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CHAPTER 4.

Localization of the Site of Action of the Short-Period Mutation By Means of Mosaics

INTRODUCTION

In studying the organization of circadian systems, it is desirable to know the location of the components of the system (receptors, the oscillator itself, and its effectors) within the organism. Identification or localization of light receptors for photoperiodic processes has, in the past, been accomplished by the use of ablation, microillumination, or differential shading techniques (1-5). While surgery has been used to provide information concerning the whereabouts of the clock in certain organisms, the technique suffers from the tendency to produce post-operative, often long-lasting trauma, to the point of inducing arrhythmicity and even death (6). These difficulties may be circumvented through the use of genetic surgery by means of gynandromorphs. In a gynandromorph, part of the organism is composed of male tissue and part of female tissue; it is therefore possible to study sex-linked characters or mutations by correlating the phenotype of the trait (mutant or non-mutant, male or female) with the distribution of male and female tissue. An underlying assumption is that the internal structure of the organism is correlated with its external structure, since the sex of most of its internal tissue, including the nervous system, cannot as yet be determined directly. To a first approximation, at least, this assumption appears to be a valid one (see (7) and below). Gynandromorphs have been employed in the study of sex-associated behavior in wasps (8), bees (9), and houseflies (10), and in the anatomical localization of the effects

of mutations involving the nervous system in Drosophila (7,11). Sex-linked mutations affecting the circadian clock, such as those described in this thesis, may be used similarly. Since the organization of the clock system in Drosophila is unknown, it cannot be asserted that these mutations can be used to directly localize the driving oscillator. The mutations may be affecting a cell in one part of the fly (e.g., a nerve cell) which then affects the clock (e.g., through a synapse) in another part of the fly. (The existence of circadian oscillators in single nerve cells is, of course, well established (12)). Nevertheless, determination of the site of action of such a mutation would localize one of the elements in the Drosophila circadian system and might open the route to localizing the others.

METHODS

The short-period mutation was used since it yields the sharpest rhythms. Although the mutation is not completely recessive, the rhythm of the homozygote or hemizygote, with a period of 19 hours, can be readily distinguished from that of the heterozygote, whose period is 22 hours. To produce gynandromorphs, males whose X chromosome carried the short-period mutation plus the recessive markers y (yellow body) and sn^3 (singed bristles) were mated to virgin females heterozygous for a special ring chromosome known as w^{vC} (white-variegated, Catcheside). A small percentage of the $y\ per^s\ sn^3/w^{vC}$ flies showed mosaicism for the markers y and sn^3, indicating that these were gynandromorphs produced by loss of the

ring-X chromosome early in development (for detailed discussion, see Hotta and Benzer (7)). These gynandromorphs were separated from the rest of the flies and stored in LD 12:12 at 25° for one to several days; individual locomotor activity rhythms were subsequently determined as described in Konopka and Benzer (13). Each fly was then examined under the dissecting microscope to determine the boundaries between male and female parts.

RESULTS AND DISCUSSION

Table 1 summarizes the results for the gynandromorphs tested; the distribution of male and female tissue in each gynandromorph is shown in Figure 1. It can be seen that, for flies whose heads are of uniform genotype, the period of the activity rhythm is strongly correlated with the genotype of the head. Some, if not all, of the arrhythmic flies in this category may be due to the extended storage of the flies in LD (up to two weeks) before measuring their rhythms; the best rhythms are obtained if young, virgin (in the case of females) adults, 2-3 days after emergence, are used. For both split-head and mixed-head flies there are four results: the activity may have a male rhythm (19 hour period), a female rhythm (22 hour period), no apparent rhythm, or a pattern resembling the superposition (sum) of male and female rhythms. An example of a rhythm exhibiting both male and female character is shown in Figure 2. Nothing resembling this pattern was ever seen in the activity of a non-mosaic fly. At this stage, however, the superposition pattern must be treated as preliminary; ideally, the

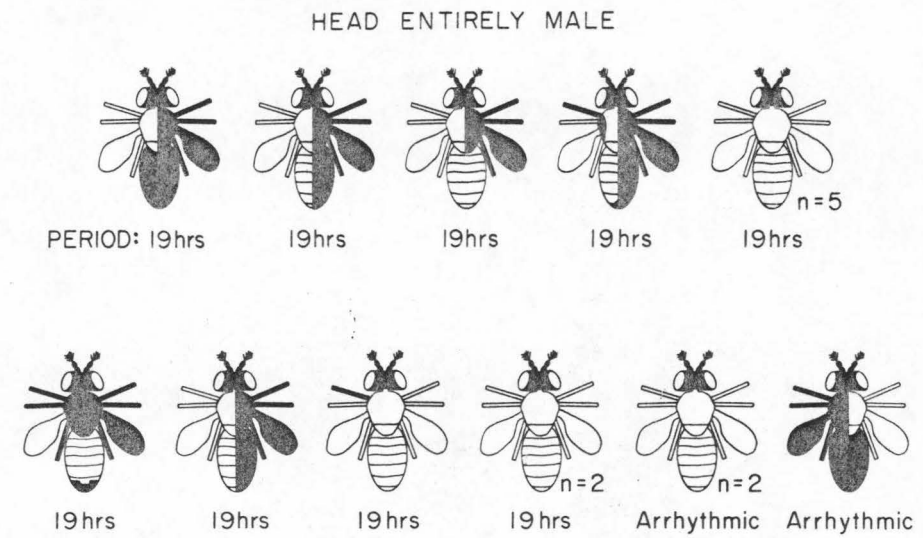
strength of each periodicity should be determined by mathematical analysis of the data. Such an analysis must await future experiments since the data shown were not collected in suitable form. If the interpretation of the pattern is correct, it would imply that two oscillators are present and running essentially independently, each making its own contribution to the observed activity rhythm. Data suggesting that two such independent oscillators are present in vertebrates (controlling an activity rhythm) has been presented by Pittendrigh (14). Since these superposition patterns are found in split-head and mixed-head flies, each oscillator might correspond to a group of cells on one side of the brain. The result that pure male and female rhythms can be obtained with split-head flies implies that the oscillator(s) is located close to the midline of the embryo. The interpretation of the arrhythmic data must be postponed until suitable data collection and mathematical analysis can be made.

TABLE 1.

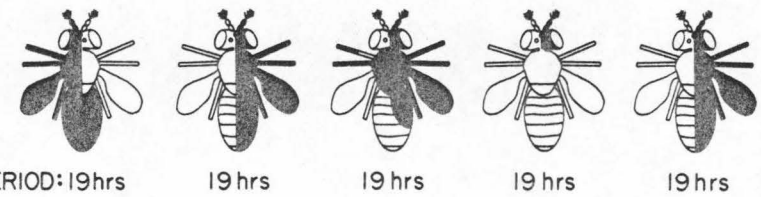
THE ACTIVITY RHYTHM PHENOTYPES OF INDIVIDUAL MOSAIC FLIES DETERMINED IN CONSTANT INFRARED AT 25° C.

	Phenotype of rhythm			
Phenotype of fly	Male	Female	Sum	Arrhythmic
Head entirely male	14	0	0	3
Head entirely female	0	5	0	3
Head split	5	3	3	5
Head mixed	2	1	1	2

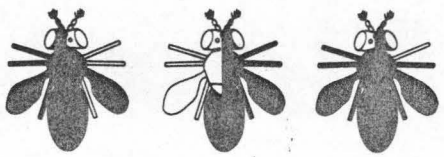
Figure 1. The distribution of male and female tissue on the surface of mosaic flies whose activity rhythms had been measured in constant infrared light at 25°. Areas of male tissue are shown in black. The periods were determined as an average over at least 4 days of data. The periods have been rounded off to the nearest hour.


HEAD ENTIRELY MALE
n=5
PERIOD: 19hrs
19hrs
19hrs
19hrs
19hrs
19hrs
19hrs
19hrs
19hrs
n=2
19hrs
n=2
Arrhythmic
Arrhythmic
HEAD ENTIRELY FEMALE
PERIOD: 22hrs
22hrs
22hrs
22hrs
22hrs
Arrhythmic
Arrhythmic
Arrhythmic

HEAD SPLIT



PERIOD: 19hrs | 19 hrs | 19 hrs | 19 hrs | 19 hrs

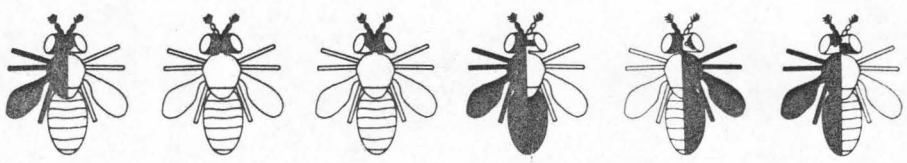


22hrs | 22hrs | 22 hrs

SUM | SUM | SUM

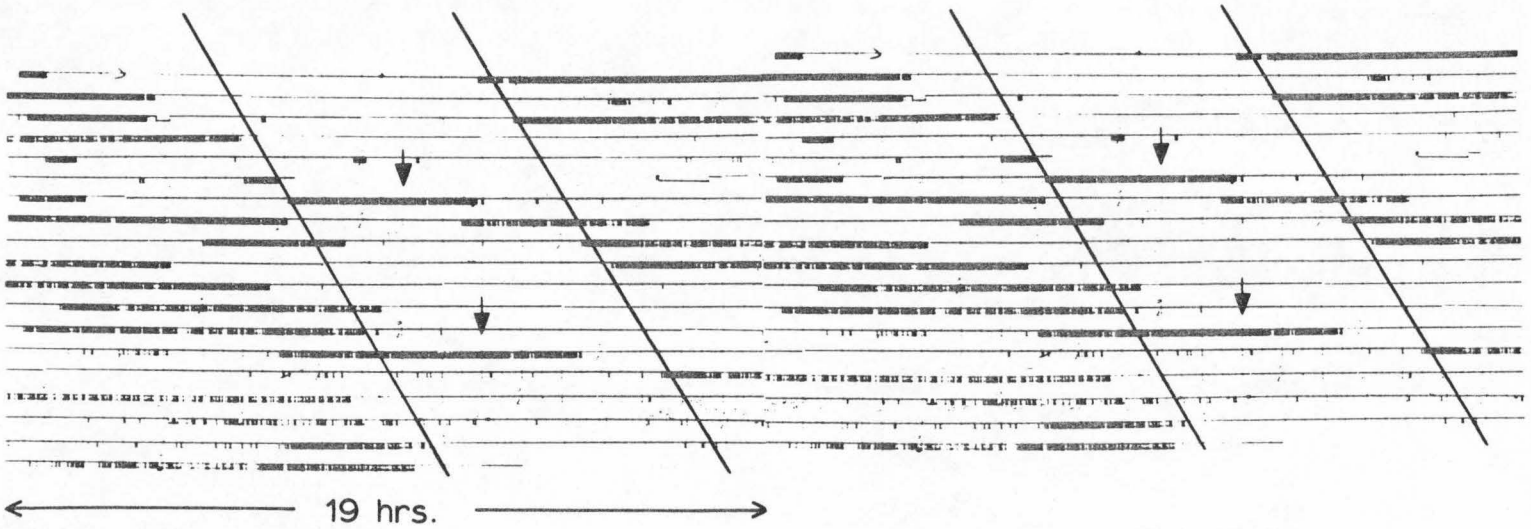
Arrhythmic | Arrhythmic (body not scored)

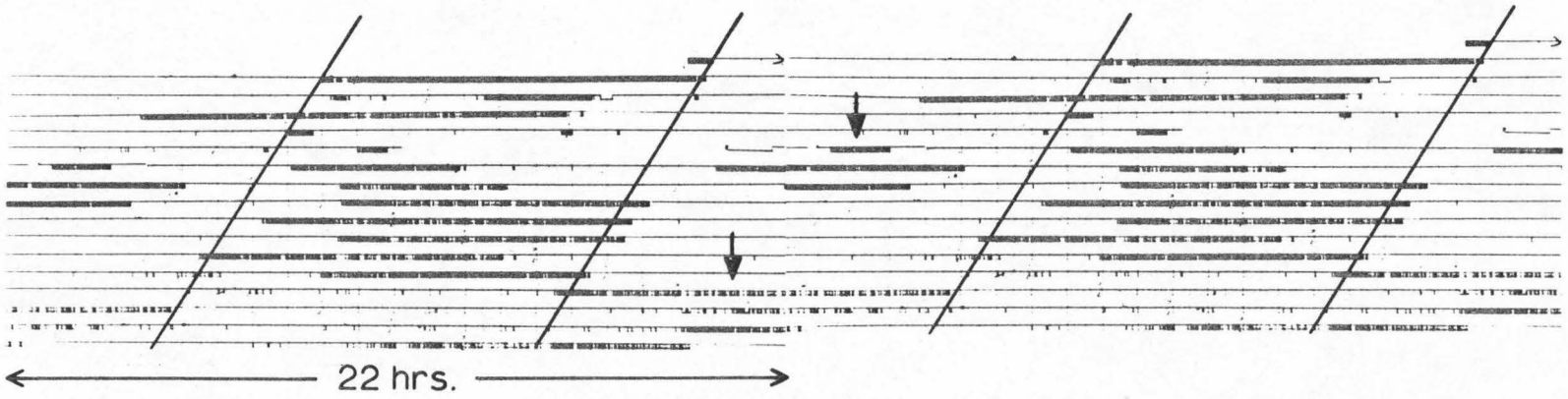
HEAD MIXED



PERIOD: 19hrs | 19hrs | 22hrs | Arrhythmic | Arrhythmic | SUM

Figure 2. An activity pattern resembling the superposition of both male (mutant) and female (heterozygous) rhythms. The longest available run exhibiting such a pattern is shown. The data are plotted on both a 19-hour and a 22-hour scale. When a 19-hour scale is used, some of the activity traces appear to show a period of 19.0-19.3 hrs (19.3 hrs indicated by the slanted lines); in addition, a 22-hour rhythm (indicated by arrows) appears to be moving through the 19-hour cycle. The time interval between successive sweeps of the 22-hour rhythm is approximately that expected for a 22-hour rhythm moving through a 19-hour cycle (about 7 cycles). Similarly, when a 22-hour scale is used, some of the traces appear to have a period of 21.7-22.0 hrs (21.7 hrs indicated by slanted lines). Here again, another rhythm appears to be moving through the data (arrows); this rhythm has a period of about 19 hours. It is apparent that the pattern illustrated by the data is not the exact superposition of a 22-hour rhythm on a 19-hour rhythm; some deviation may, however, be expected due to interactions of the inactive period of one rhythm with the active period of the other. The data do suggest that both 19-hour and 22-hour periodicities are present.


19 hrs.


22 hrs.

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CHAPTER 5.

Phase-Response Curves For Normal and Short-Period Flies

INTRODUCTION

The light response of a circadian oscillator may be conveniently measured by administering a light pulse at various times as the oscillator free-runs in constant darkness and noting the effect on the rhythm in the steady state. A plot of the resulting change in the phase of the rhythm as a function of the time at which the light pulse was given is known as a phase-response curve; it provides an index of the ability of the clock to be reset by light. Response curves of this sort have been determined for several organisms (see Aschoff (1) and Winfree (2) for a summary), including Drosophila pseudoobscura. The parameters of the light stimuli used, however, are quite variable—intensities range from 0.5 foot-candle to 1400 foot-candles, and durations from 0.5 msec to 12 hours. Consequently, it is difficult to draw meaningful conclusions concerning the similarity of clocks in various organisms from the data available (however, see Winfree (2) for a comparative analysis).

In D. pseudoobscura, maximum phase shifts may be induced by relatively low levels of light (15 minutes, 10 lux (3)). Engelmann (4) has shown that, in fact, the extent of the phase shift induced is dependent on the total energy of the light pulse and not directly on its duration or intensity. Long light pulses may, however, induce phase shifts in Drosophila through a different mechanism—by effectively stopping the oscillator midway through its cycle (at the start of the light-sensitive period (5)).

Since sensitivity to light is a fundamental property of circadian oscillators, it is of interest to determine to what extent the short-period mutation, in altering the period of the oscillator, affects its ability to be reset by light. Accordingly, the phase response curve of the mutant was determined and compared with that of normal-rhythm flies.

METHODS

Stocks were reared on cornmeal medium. Eclosion rhythms were determined by means of "superbang" boxes in the laboratory of Dr. Colin Pittendrigh at Stanford University. The principle of operation of "superbangs" is the same as that of "bang boxes"; however, some components and their physical arrangement are altered to enable more measurements to be made in a smaller space. Pupae were reared in constant light and then transferred to constant darkness to initiate the oscillation (5). Light pulses were administered by exposing the pupae to 300 foot-candles of white fluorescent light. At least three cycles occurred between the light exposure and measurement of the phase shift in order for the transients in the rhythm to subside. Phase shifts were determined by noting the shift in time required to superimpose the rhythm of the animals which had received a light pulse upon the control rhythm of animals maintained in constant darkness. Routinely, each "superbang" contained one control group and several experimental (pulsed) groups of pupae. All operations were carried out at 22°.

RESULTS AND DISCUSSION

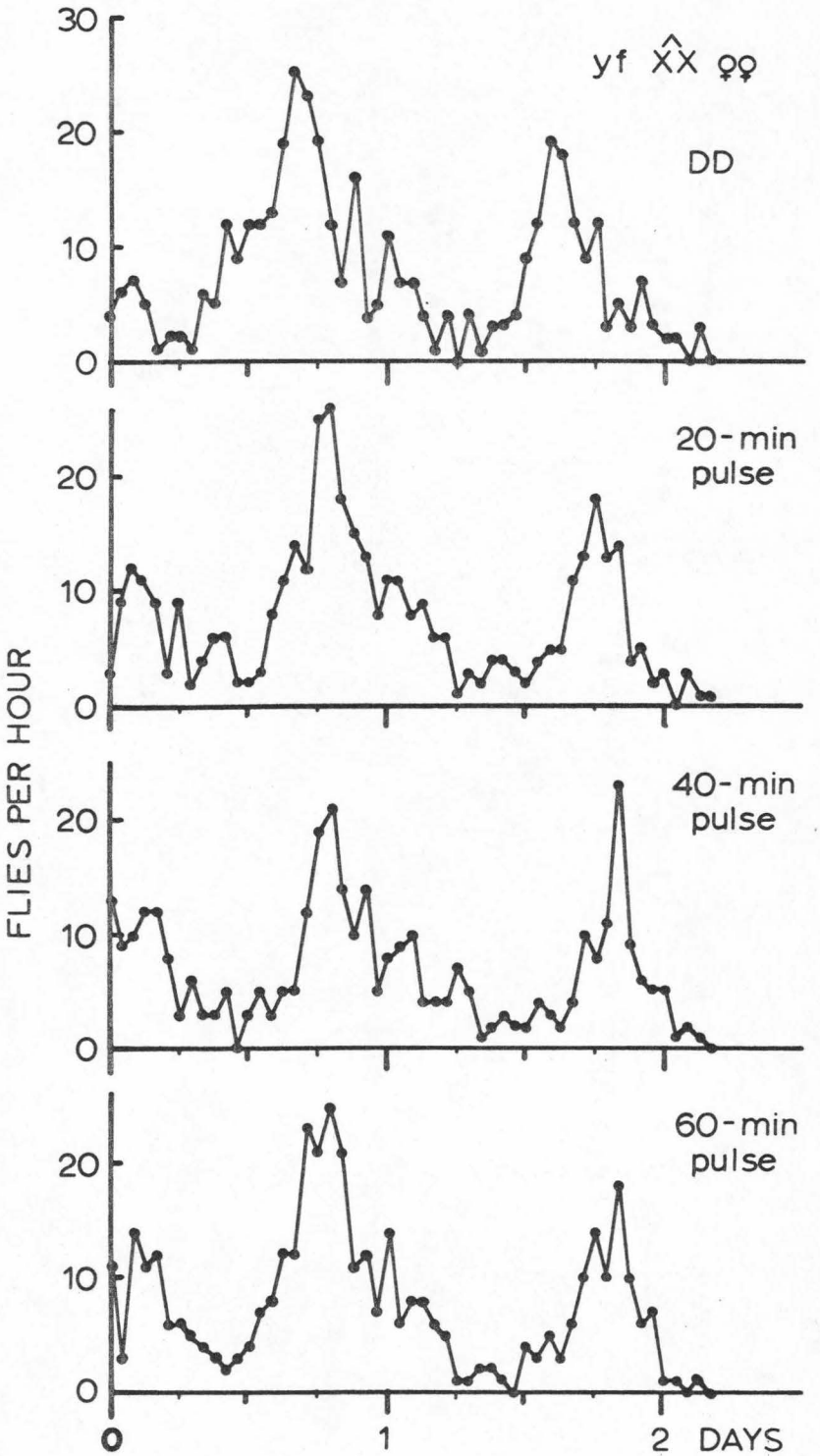
To avoid the difficulties presented by long light pulses, it was necessary to use a stimulus of short duration relative to the period of the oscillation (preferably less than one hour), but intense enough to produce the maximum phase shift. Accordingly, a preliminary experiment was done to determine the relative phase shift produced by 20, 40, and 60 minutes of white light of 300 foot-candles intensity. The pulses were administered four hours after the transition from constant light (LL) to constant darkness (DD). Figure 1 shows the results. For both normal and mutant rhythms, the phase delay induced by the light is already maximal with a 20-minute pulse. As a precaution, however, 40 minute pulses were used in determining the phase-response curves.

Figure 2 shows the phase-response curves for rhythmically normal and short-period mutant flies. Two conventions have been adopted in presenting the data in order to facilitate comparison of the two curves. First, the oscillation is plotted on the abscissa in terms of hours after the LL to DD transition, rather than the customary "subjective circadian time," starting at subjective dawn. This representation was selected because of the large difference in the periods of the two rhythms. It provides for the alignment of the two response curves, time zero being that point at which each oscillation is started by the shift from LL to DD. The second convention is the plotting of the phase shift (ordinate) in terms of

advances and delays. This representation creates an artificial discontinuity in the mutant curve; a delay of 10 hours is equivalent to an advance of 8 hours for a rhythm with an 18 hour period. This discontinuity could have been eliminated by plotting the resultant phase shift (in circadian time) as a function of stimulus time; however, additional difficulties in comparison of the data would have resulted due to the great difference in form between the normal curve and the mutant curve.

The response curve for rhythmically normal flies has a relatively small amplitude (type 1). It is similar to a response curve determined by Pittendrigh for a wild-type strain of D. melanogaster (6), but is quite different from the large-amplitude (type 0) response curve published for D. pseudoobscura (5). The inter-species difference remains unexplained. The amplitude of the short-period mutant response curve, however, is much greater than that of the normal melanogaster curve; the mutant rhythm can be phase-shifted to a far greater extent than can the normal rhythm. This effect, an increase in the ability of the oscillator to be reset by light during the light-sensitive period, is not the only effect on the response curve associated with the mutation. The mutation also shortens the period of the response curve (as one would expect if the driving oscillator were indeed running with a shorter period) by decreasing the length of the light-insensitive period. Thus the gene affected by the short-period mutation has some control over both portions (light-sensitive and light-insensitive) of the oscillation.

Figure 1. Phase shifts in the eclosion rhythms of rhythmically normal and short-period mutant flies induced by light pulses. The light pulses were of 300 foot-candles intensity and varying duration, as indicated in the figure. Light pulses were applied 4 hours after the pupae had been transferred from LL to DD, initiating the rhythm.


yf XX ♀♀
DD
20-min pulse
40-min pulse
60-min pulse
FLIES PER HOUR
30
20
10
0
0
1
2 DAYS

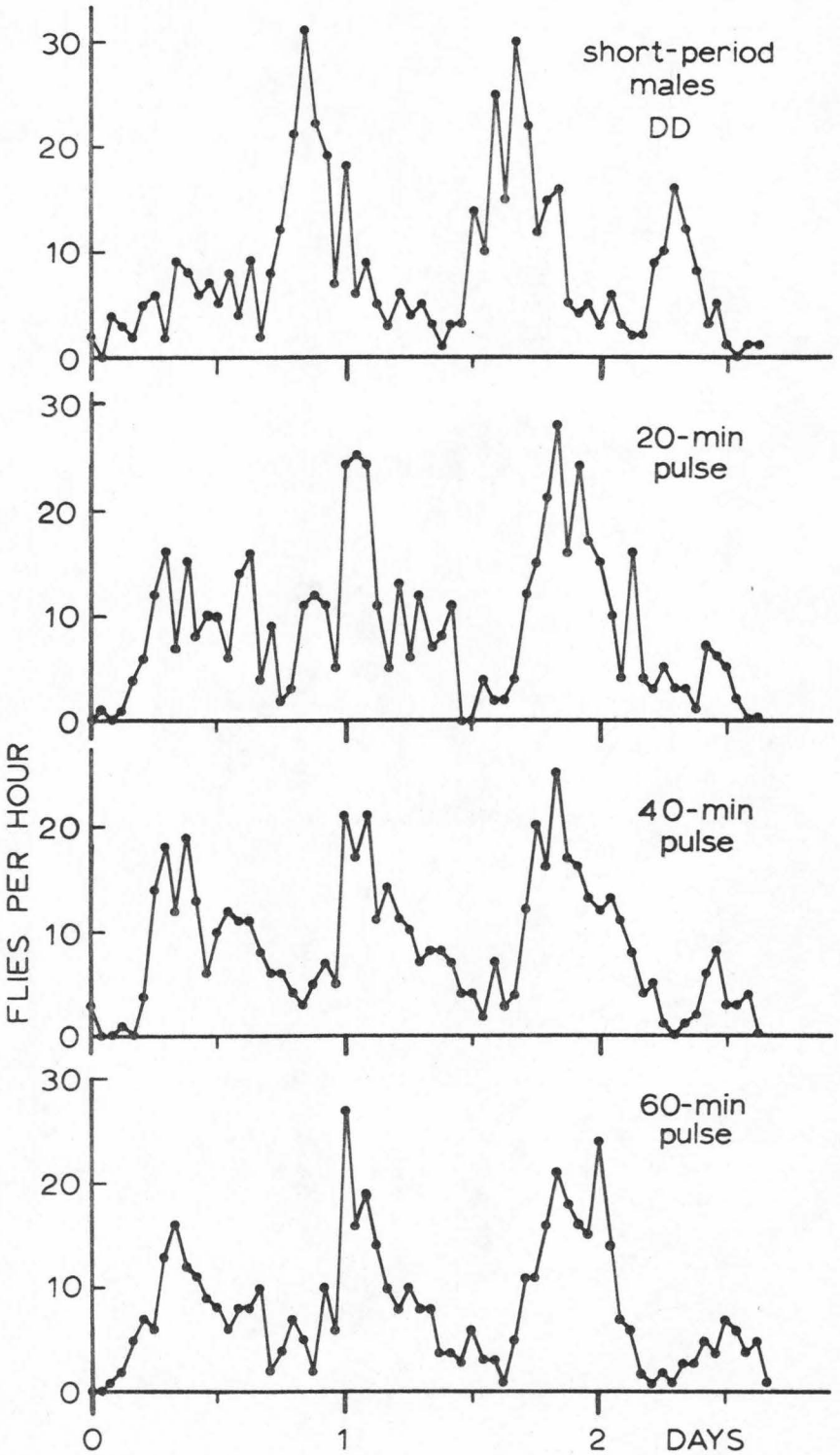
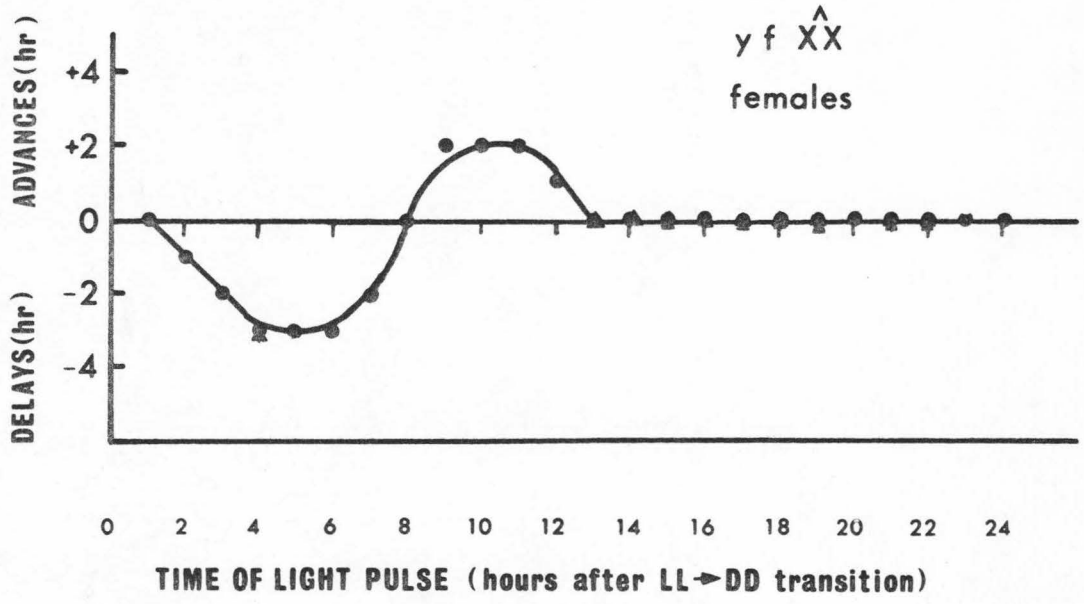
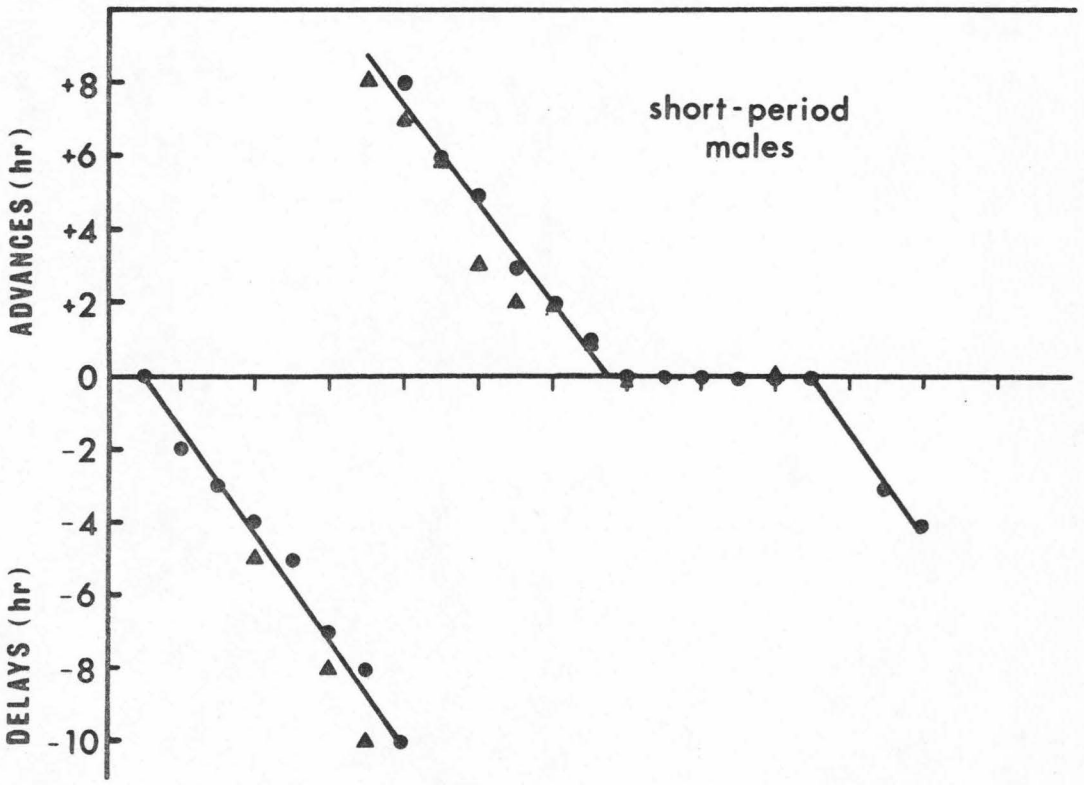

short-period
males
DD
20-min
pulse
40-min
pulse
60-min
pulse
FLIES PER HOUR
30
20
10
0
0
1
2
DAYS

Figure 2. Phase-response curves for steady-state phase shifts in normal and short-period mutant eclosion rhythms induced by short light pulses. The light pulses consisted of 300 foot-candles of white fluorescent light for 40 minutes. Pupae were reared in LL, then transferred to DD to initiate the oscillation; light pulses were administered at hourly intervals after the LL to DD transition. The triangles indicate results of duplicate experiments.


y f XX
females
ADVANCES(hr)
DELAYS(hr)
+4
+2
0
-2
-4
0 2 4 6 8 10 12 14 16 18 20 22 24
TIME OF LIGHT PULSE (hours after LL→DD transition)


short-period
males
ADVANCES (hr)
DELAYS (hr)
+8
+6
+4
+2
0
-2
-4
-6
-8
-10

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CHAPTER 6.

The Entrainment of Eclosion Rhythms by Light and Temperature; Support for the Two-Oscillator Model of the *Drosophila* Clock

INTRODUCTION

It is characteristic of a circadian rhythm to persist with a defined period length under constant conditions. By introducing a cyclic change in some environmental variable, it is possible to entrain the rhythm to the period of the environmental cycle (1). Temperature and light cycles, with either complete or "skeleton" photoperiods (in which case the "photoperiod" is actually made up of two brief light pulses, one at the start and one at the end of the "photoperiod," with darkness in between), have commonly been used as entraining agents, or "Zeitgeber." (1-5). Previous investigations have shown that the circadian eclosion rhythm in D. pseudoobscura can be entrained by these agents administered independently as well as concurrently (5-8). Single brief light pulses can, under certain conditions, permanently reset the eclosion rhythm in D. pseudoobscura, advancing or delaying it by as much as 12 hours. Single temperature pulses or steps, on the other hand, produce spectacular temporary perturbations in the rhythm (known as transients), but the phase of the rhythm eventually stabilizes at a point only 1-3 hours away from its original position.

Pittendrigh and his colleagues interpreted these observations to mean that there are two oscillators in the Drosophila clock, a light-sensitive driving oscillator and a temperature-sensitive driven oscillator (6,9,10). If a light pulse is given to a population of pupae during the dark period of a 12:12 light-dark cycle, the driving

oscillator is reset immediately; transients are observed in the rhythm as the driven oscillator is gradually re-entrained by the driver. If a single temperature pulse or step is administered under the same conditions, the driven oscillator is affected, producing large transients. The driving oscillator, however, is not very sensitive to temperature, so it is only slightly affected, and the rhythm eventually returns to a phase close to its original phase. In this section, some light and temperature responses of the eclosion rhythms of rhythmically normal and mutant D. melanogaster will be described and discussed with respect to the two-oscillator model.

METHODS

Flies were reared in controlled environment chambers under the appropriate light and temperature conditions. After the pupae had been harvested, they were transferred to "bang boxes." The temperature of the pupae in the "bang boxes" was controlled by means of cold water circulated from a water bath through a brass plate onto which the pupae were mounted. Temperature cycles were constructed by turning the water bath on and off; since the temperature rise was due to the plate warming to room temperature (25°), the rising phase of the cycle was slower than the falling phase. The profile of a typical temperature cycle is shown in Figure 1. Exposure of the pupae to light was accomplished by a fluorescent tube either inside a sealed "bang box" or outside an open box. The light intensity was at least 10 foot-candles in the entrainment experiments

and 100 foot-candles in all other experiments.

RESULTS

I. Entrainment by Light

The eclosion patterns of rhythmically normal flies as well as the short-period, long-period, and arrhythmic mutants in a 12:12 light-dark cycle are shown in Figure 2. The normal flies, as well as the short-period and long-period mutants entrain to the 24-hour cycle, but the arrhythmic mutant does not. The light intensity used (10 foot-candles) is at least 100 times the minimum required to entrain the normal rhythm; Figure 3 shows the rhythm of normal flies entrained to a 12:12 light-dark cycle in which the light intensity is 0.05-0.1 foot-candles.

The entrained rhythm of the short-period mutant differs from that of normal flies in two respects: the eclosion peak anticipates lights-on in the case of the mutant, whereas it coincides with lights-on in the case of the normal flies; in addition, the width of the eclosion peaks is slightly less in the mutant than in the normal case. In the case of the long-period mutant, the eclosion peaks are slightly wider than in the normal case.

II. Entrainment by Temperature

A. Temperature Entrainment in Constant Light and Constant Dark

Figure 4a shows the eclosion rhythm of rhythmically normal flies entrained to a temperature cycle in constant light. Constant light

of this intensity (100 foot-candles) is sufficient to damp out the rhythm in constant temperature (Figure 4b). With a temperature cycle, however, a rhythm becomes apparent. Figure 4c shows the rhythm entrained by temperature in constant darkness. The onset of the first entraining cycle was 10 hours after the LL to DD transition, the flies having been previously raised in LL. In this case, the phase of the rhythm is the same as in LL—the eclosion peaks coincide with the time of the temperature rise. If the onset of the first entraining cycle is close to the time of the LL to DD transition, however, the phase is such that the peaks anticipate the temperature rise by about 3 hours (Figure 4d). Thus there are two stable phases for the temperature-entrained rhythm in DD.

In order to further demonstrate that the rhythm is actually entrained by the temperature cycle, two samples of pupae were transferred from LL to DD at different times, 7.5 hours apart but close to the onset of the first temperature cycle. In both cases, the phases of the rhythms are identical; as expected, both anticipate the temperature rise (Figures 4e and 4f).

In subsequent experiments, unless otherwise stated, the pupae were treated as in Figures 4e and 4f, starting the first entraining cycle close to the LL to DD transition time.

Figures 5a and 5b show the eclosion pattern of short-period males in a temperature cycle and either constant light (5a) or constant darkness (5b). In constant light, the rhythm is entrained by temperature, since the period is 24 hours instead of the usual

20 hours in constant darkness and constant temperature. The phase of the rhythm is such that the eclosion peak occurs about 5 hours before the temperature begins to rise. In constant darkness, however, the rhythm is not entrained to the 24 hour temperature cycle. The rhythm has a period of 21 hours (slightly longer than the usual 20 hours), unless the projected eclosion peak coincides with the step-down portion of the temperature cycle; in this case, an additional delay of 9 hours is produced, causing the next peak to appear 30 hours after the preceding peak. In Figure 5b, for example, the temperature step-down occurs 21 hours after the second peak; consequently, the next peak is delayed 9 hours.

Figures 6a and 6b show the eclosion pattern of arrhythmic males induced by a temperature cycle in constant light (6a) or constant dark (6b). The arrhythmic mutant shows no rhythm in constant temperature, either in constant darkness (see Chapters 2 and 3) or in a light-dark cycle (see above); it does, however, show a rhythm in a temperature cycle. Although the rhythm is not as sharp as that of normal-rhythm flies, the phase is approximately the same; the first eclosion peak corresponds to the time of the temperature rise. The temperature-entrained rhythm in DD does not differ significantly from that in LL for this mutant. In contrast, for rhythmically normal flies under similar conditions, there is a 3 hour phase difference between the rhythm in LL and the rhythm in DD (see above, Figure 4).

B. Ontogeny of the Rhythm

In order to determine how many entraining temperature cycles were necessary for the development of a rhythm in a population of pupae of the arrhythmic mutant, groups of pupae were exposed to temperature cycles after eclosion had begun. The results are shown in Figure 7. For the arrhythmic mutant and for rhythmically normal flies, the rhythm is very well developed by the end of the second temperature cycle. For the short-period mutant, the eclosion peak at the start of the second cycle is already fairly sharp.

C. Persistence of the Rhythm in LL and DD

To determine whether the eclosion rhythm of the arrhythmic mutant persisted after temperature entrainment, groups of pupae were exposed to at least 3 temperature cycles and then transferred to constant conditions. Figures 8a and 8b show the results. In both LL and DD, the eclosion profile reverts to an arrhythmic pattern after the temperature cycles are discontinued; the oscillation which had been induced by the temperature cycle is not self-sustaining. The only apparent difference between the LL and DD patterns is a small transient peak which sometimes appears in the DD patterns before the eclosion becomes arrhythmic.

Similar experiments were performed with attached-X females and short-period mutant males to illustrate the behavior of the normal and short-period clocks. The results are shown in Figures 8c-8f. In contrast to the arrhythmic mutant, the short-period mutant and

the normal flies continue to be rhythmic in DD; their rhythms free-run with their characteristic periods under these conditions. In LL, of course, they damp out.

III. Support for the Two-Oscillator Model of the Drosophila Clock

If there are indeed two oscillators in the Drosophila clock—one light-sensitive driver and one temperature-sensitive driven oscillator—it should be possible to stop one with constant light and keep the other going with a temperature cycle. This situation corresponds to temperature entrainment in constant light, which has been described above. That an oscillation is in progress during temperature entrainment in LL is apparent from the data presented above. The question remains: is there an oscillation which is stopped under these conditions? It is known that, in the case of the Drosophila pseudoobscura clock, constant light stops the clock approximately halfway through the cycle (8). It is also known that if the eclosion rhythm of pseudoobscura is driven by simultaneous temperature and light cycles, and both cycles are then discontinued, the rhythm will revert to the phase determined by the light cycle—the phase of the rhythm in the steady state is determined by a light-sensitive oscillator (6). It should therefore be possible to test for the presence of an inhibited oscillation during temperature entrainment in LL by releasing two groups of pupae into DD at different times during the second (cold) portion of the temperature cycle, thereafter maintaining both groups at that temperature. (The

cold part of the cycle was chosen since it allows the flies to eclose over a longer duration of time.) If there is only one oscillation, releasing it into DD should make no difference in the rhythm (since it will merely free-run), and both groups should have the same phase in the steady state. If, however, the light-sensitive oscillator was stopped by the light while the temperature-sensitive oscillator was being driven by the temperature cycle during entrainment in LL, there should be a resulting phase difference equal to the length of time between the insertion of the two groups of pupae into DD.

The results of this experiment, using rhythmically normal and short-period mutant flies, are shown in Figure 9. For both normal and mutant flies, transferring two samples of pupae into darkness at different times during the cold portion of the temperature cycle after temperature entrainment in constant light results in a difference in phase between the eclosion rhythms of the two populations in the steady state. The phase difference is equal to the number of hours separating the transfer of the two groups of pupae into DD, within the limits of experimental error.

DISCUSSION

The eclosion rhythms of the short-period and long-period mutants can be entrained to a 24-hour period with a 12:12 light-dark cycle; the arrhythmic mutant, however, remains arrhythmic under these conditions, even though the light intensity used is at least

two orders of magnitude higher than the minimum required for entrainment of the normal rhythm.

Temperature cycles may also be used to entrain the eclosion rhythm of D. melanogaster; during entrainment in DD, the eclosion peaks are synchronized to the temperature cycle rather than the LL to DD transition time.

The eclosion rhythm observed in a temperature cycle appears to be due to the entrainment of an oscillation within the pupae rather than a direct effect of temperature. In normal-rhythm flies, for example, it takes two cycles to fully develop the rhythm; the first temperature rise has only a slight effect on the eclosion pattern (Figure 7). In constant darkness, the peak of eclosion anticipates the temperature rise rather than coinciding with it. Finally, the rhythm does not damp out immediately upon exposing the pupae to constant light and temperature, a procedure which should stop the driving oscillator within one cycle (8). Instead, there are one or more small transient peaks, the spacing of which may be different from the period of the temperature cycle (24 hours). The rhythm of the short-period mutant shows both the requirement for two entraining cycles and the transient peaks before damping in LL. Although this mutant does not entrain to a temperature cycle in DD, the peak of eclosion anticipates the temperature rise by 5 hours during temperature entrainment in LL. The arrhythmic mutant likewise requires at least two cycles for full development of the rhythm in a temperature cycle. Its peak eclosion coincides with the onset of the

temperature rise in both LL and DD, but occasionally a small transient peak is apparent in DD after the temperature cycles have been discontinued, before the rhythm reverts to its arrhythmic phenotype.

The behavior of the short-period mutant in a temperature cycle may be explained as follows. In DD, the driving oscillator is allowed to run. Normally, it has a period of about 20 hours; the effect of the temperature cycle is to lengthen the period slightly to 21 hours (except, of course, when the step-down coincides with the projected eclosion peak, in which case there is an additional delay). In LL, the driving oscillator is stopped, and the temperature-sensitive driven oscillator can be directly entrained by the external temperature cycle; consequently, the period of the observed rhythm is 24 hours. It is not surprising that in DD, the short-period mutant does not entrain to the 24-hour temperature cycle. In D. pseudoobscura, for example, the maximum phase delay that can be produced by a temperature step-down or pulse is about 2.5 hours (7). If there is any similarity between the responses of the pseudoobscura and melanogaster clocks to temperature, it would be impossible to induce the necessary 4 to 5 hour delay per cycle needed to entrain the short-period mutant to a 24-hour period by means of temperature.

The results of the experiment described in the third section of the results offer additional support for the two-oscillator model of the Drosophila clock. They suggest that during temperature entrainment in LL, the light-sensitive driving oscillator is indeed stopped, and

only the temperature-sensitive driven oscillator is entrained by the temperature cycle.

The experiments involving the arrhythmic mutant show that the light sensitivity of the clock has been virtually abolished by the arrhythmic mutation. The mutant does not entrain to a 12:12 light-dark cycle, nor is there any evidence of the phase difference between the LL and DD temperature-entrained rhythms exhibited by rhythmically normal flies. These results indicate that, at the least, there are separate receptors for light and temperature. The insensitivity to light in this mutant, however, is correlated with a loss in the ability of the rhythm to persist under constant conditions, suggesting that a light-sensitive function is necessary for the self-sustaining property of the clock. Nevertheless, an oscillation exists which can be entrained by temperature. These observations can be interpreted in terms of the two-oscillator model (5,6,8-10), in which the driving oscillator is light-sensitive and the driven oscillator (normally driven by the light-sensitive oscillator), which determines the immediate phase of the rhythm, is temperature-sensitive. For this mutant, it appears that the driving oscillator is stopped or missing, while the temperature-sensitive driven oscillator is intact. This oscillator cannot sustain itself, but it can be driven by an exogenous temperature cycle or the endogenous driving oscillator, when present. The effective elimination of the driving oscillator by the mutation, however, does not imply that the mutation necessarily affects a component of the oscillator directly.

The mutation may, instead, affect an external receptor or other structure or process which, in turn, causes an alteration in the properties of the oscillator.

Figure 1. The profile of a typical temperature cycle during the 24 hours of one cycle. The cycle was constructed as described in Methods. The temperature was monitored by means of a thermistor probe attached to the brass plate of the "bang box" as though it were a pupa; the thermistor was connected to a recording thermometer.

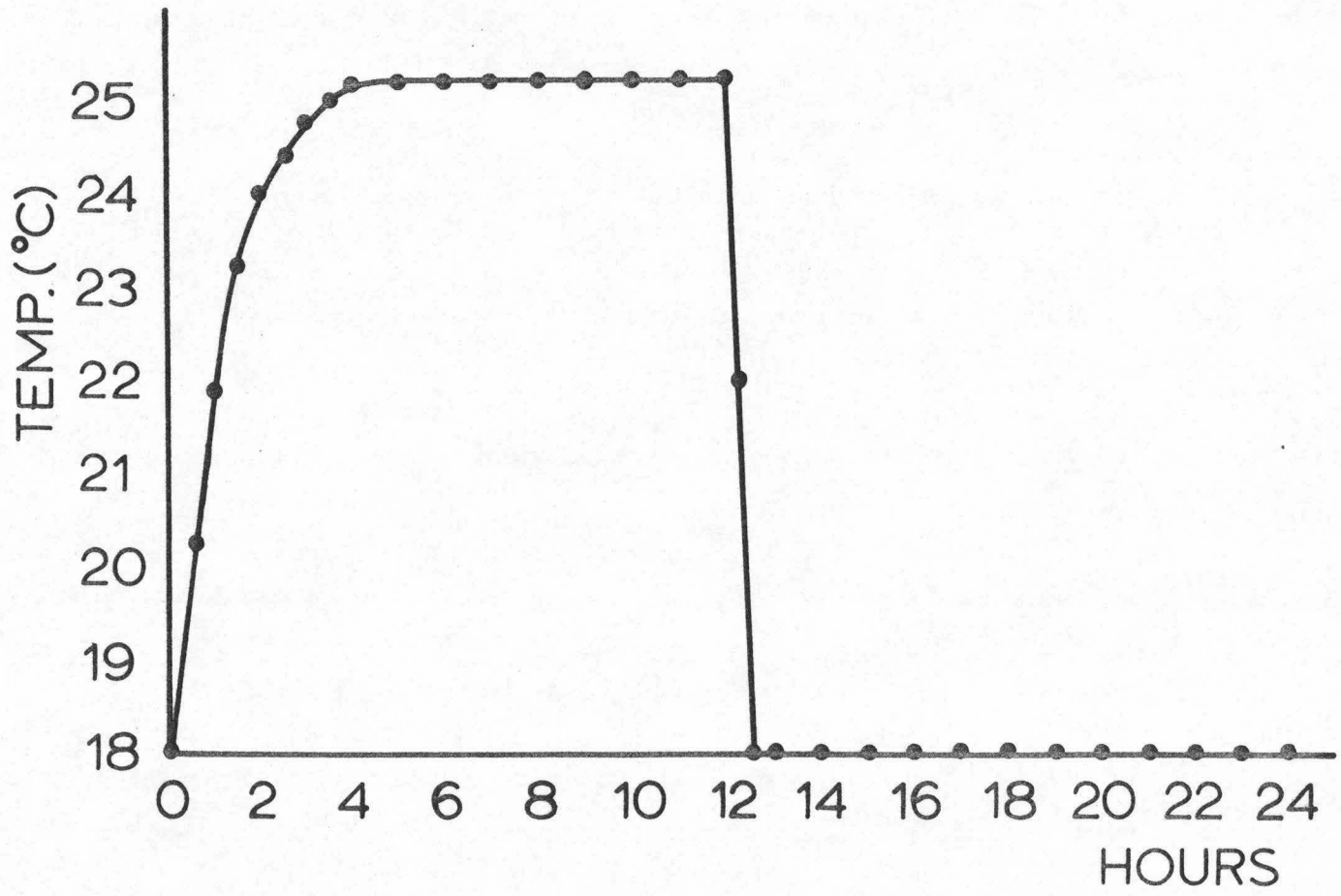

TEMP.(°C)
25
24
23
22
21
20
19
18
0 2 4 6 8 10 12 14 16 18 20 22 24
HOURS

Figure 2. Entrainment of normal and mutant eclosion rhythms to a 12:12 light-dark cycle. The light intensity was 10 foot-candles; the temperature was 18°. The time at which the light came on is shown by the arrows.

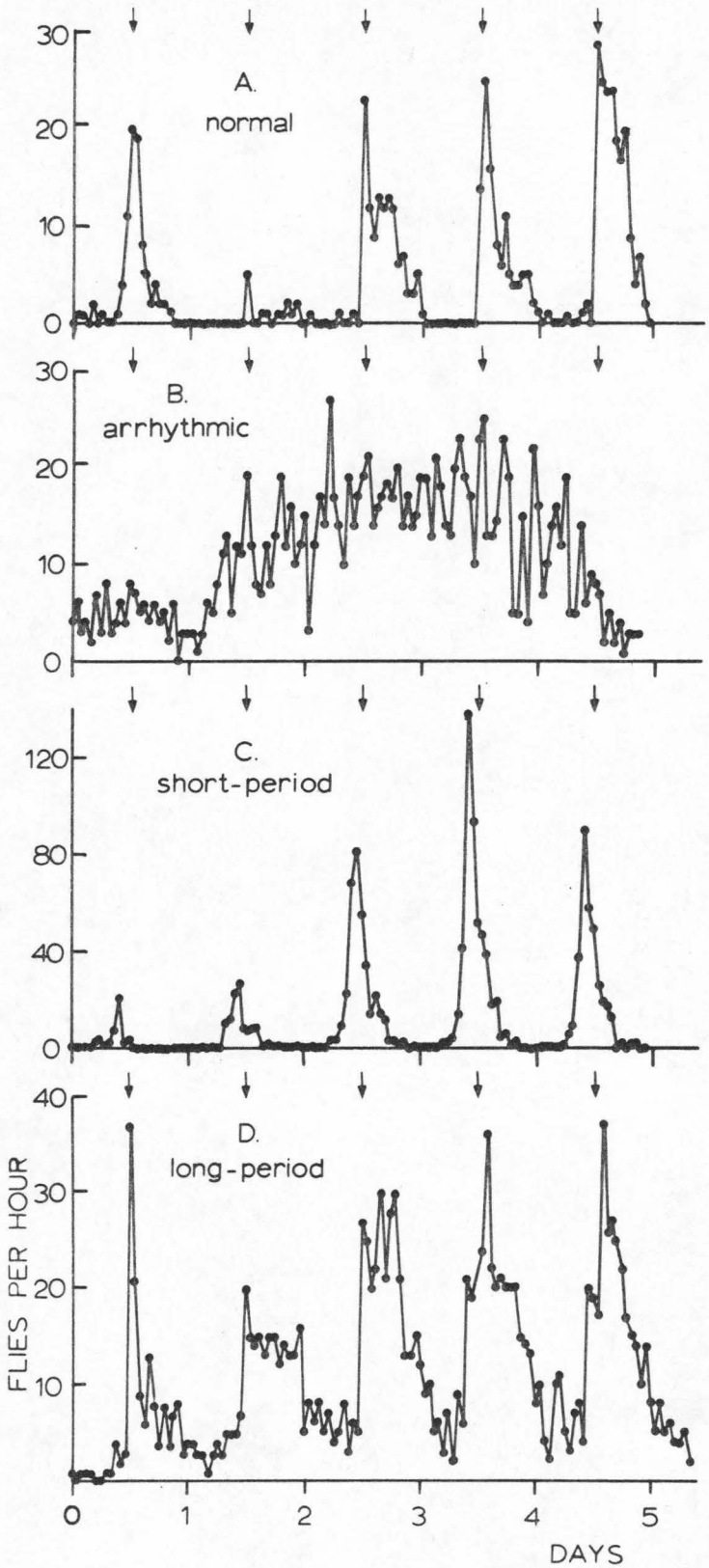

A.
normal
B.
arrhythmic
C.
short-period
D.
long-period
FLIES PER HOUR
DAYS
0
1
2
3
4
5
10
20
30
40
80
120

Figure 3. Entrainment of rhythmically normal flies (attached-X females) to a 12:12 light-dark cycle. The light intensity was 0.05-0.10 foot-candles. Two populations of pupae were grown in LL; they were then transferred to the light-dark cycle 5 hours (B) and 10 hours (A) after the lights came on. The rhythms were assayed 3 days after the introduction of the pupae into the light-dark cycle. In both cases, the peaks correspond to the time of lights-on (arrows). The experiment was conducted at 20°.

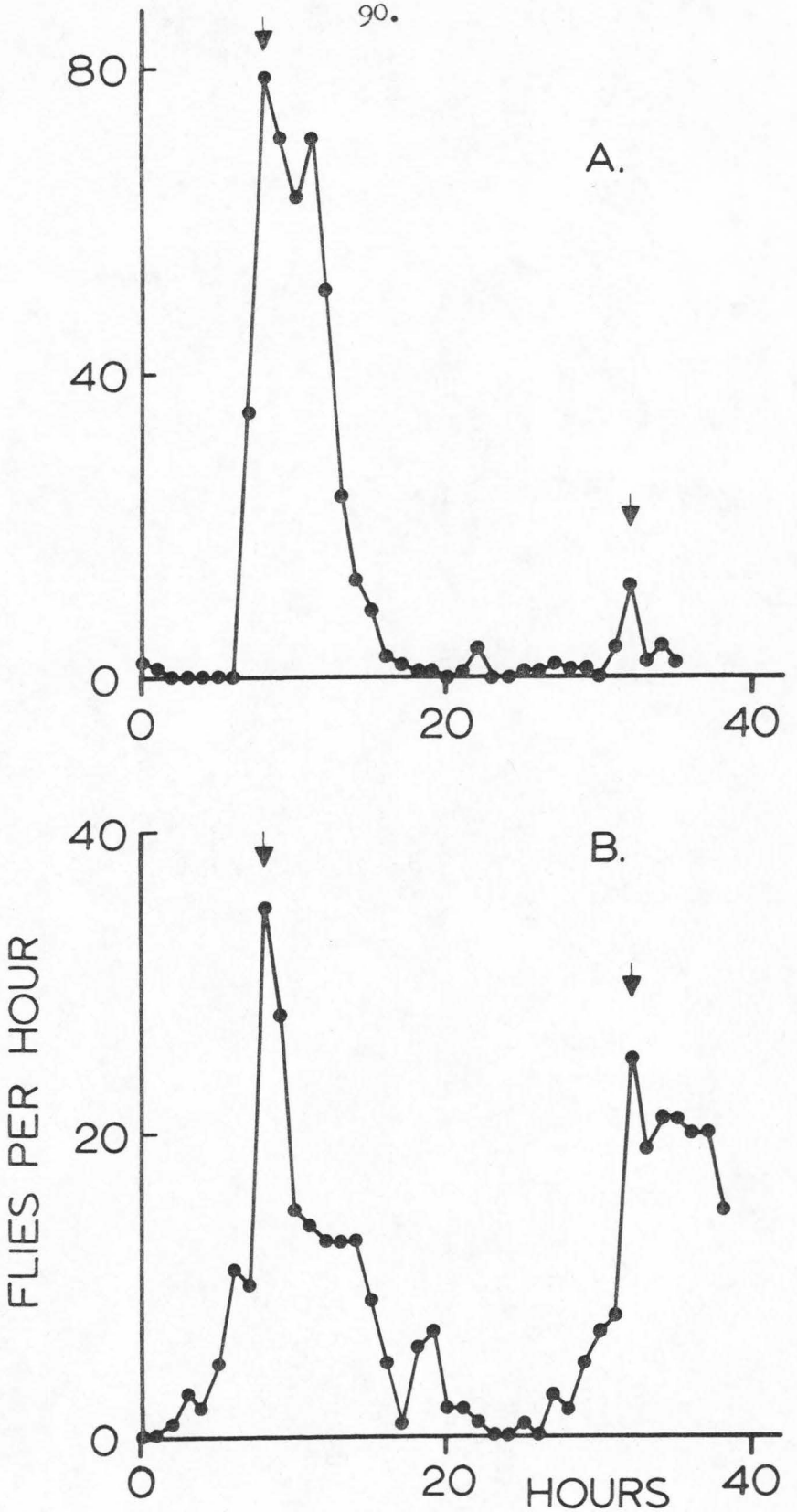
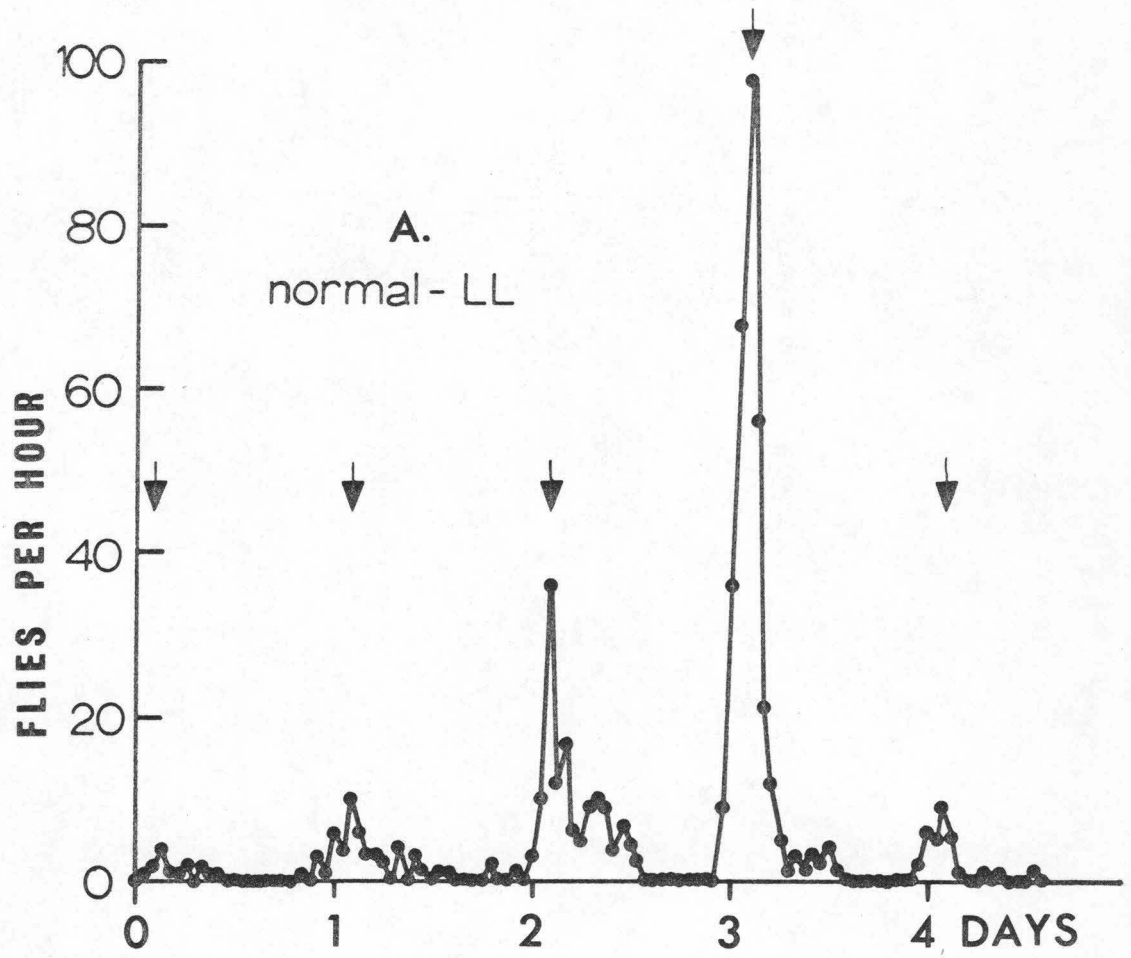

80
40
0
A.
0
20
40
40
20
0
B.
FLIES PER HOUR
0
20
HOURS
40

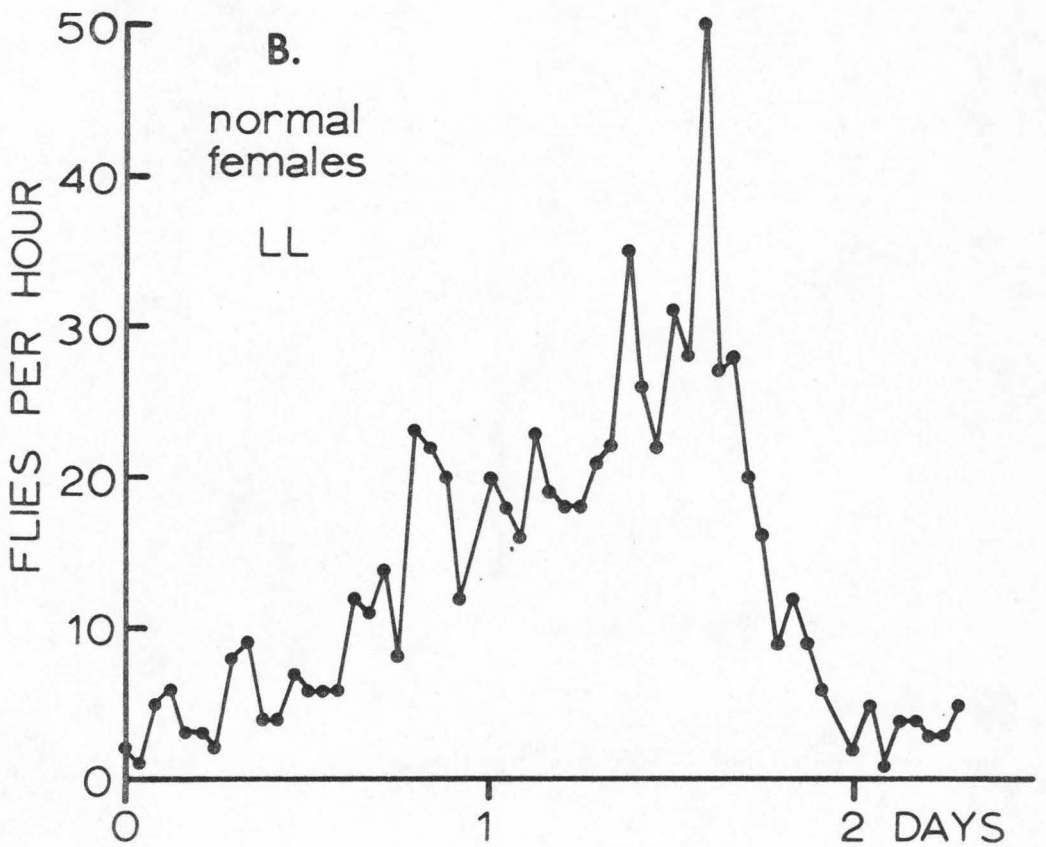
Figure 4. Entrainment of the eclosion rhythm of rhythmically normal flies (attached-X females) by a temperature cycle, and damping out of the rhythm in constant light and constant temperature. The arrows indicate the time at which the temperature began to rise (hour zero in Figure 1).

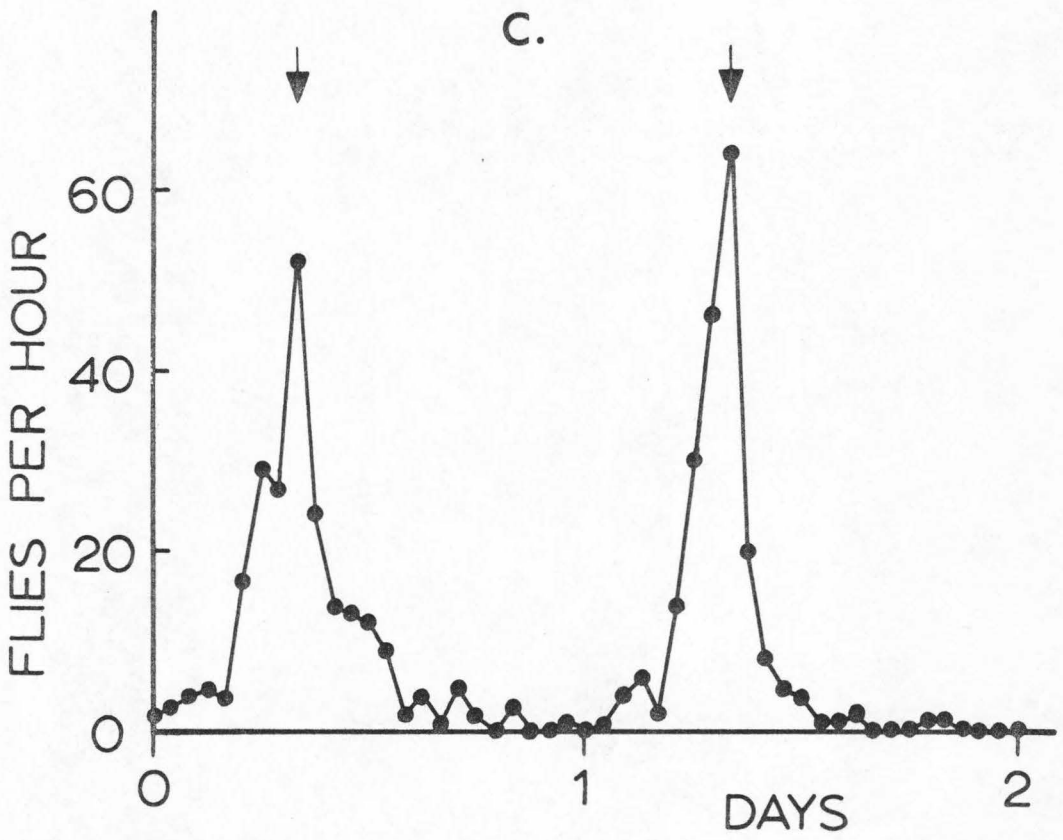
4A: Temperature entrainment in constant light of 100 foot-candles intensity, at 18°.

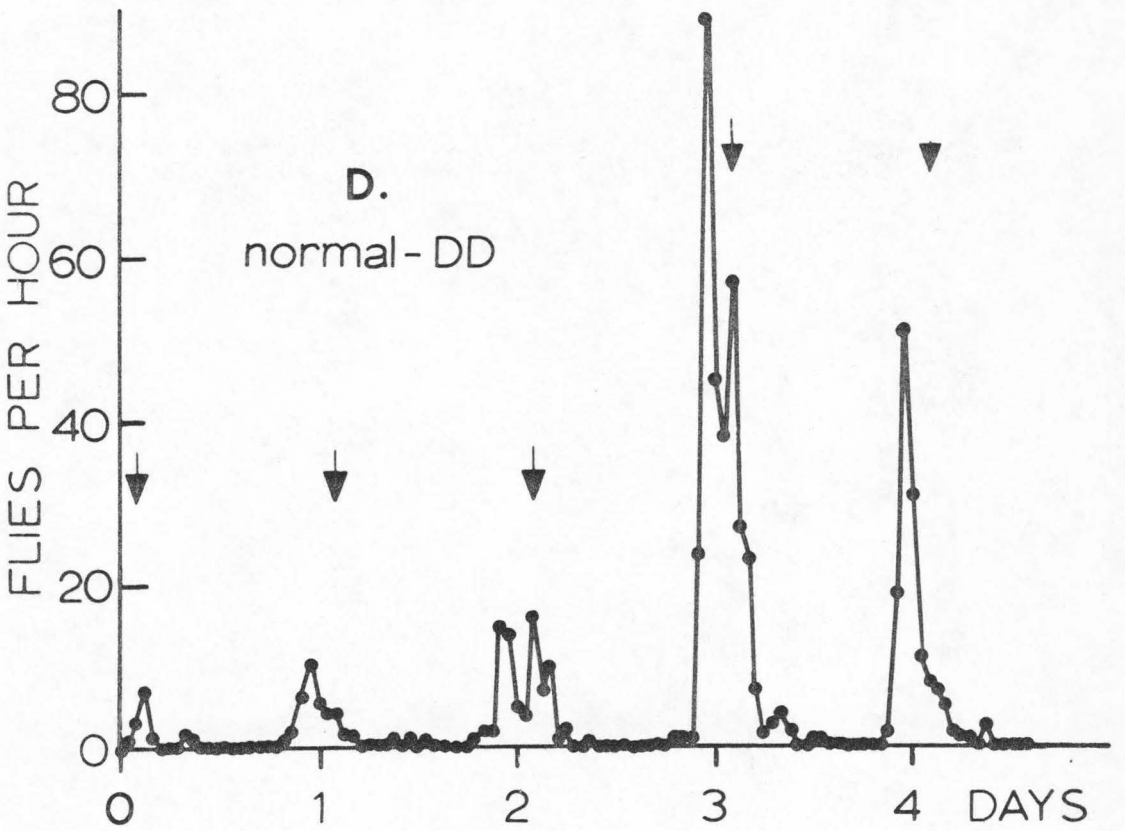
4B: The eclosion pattern in constant light (100 foot-candles) and constant temperature (25°). There is no apparent rhythm.

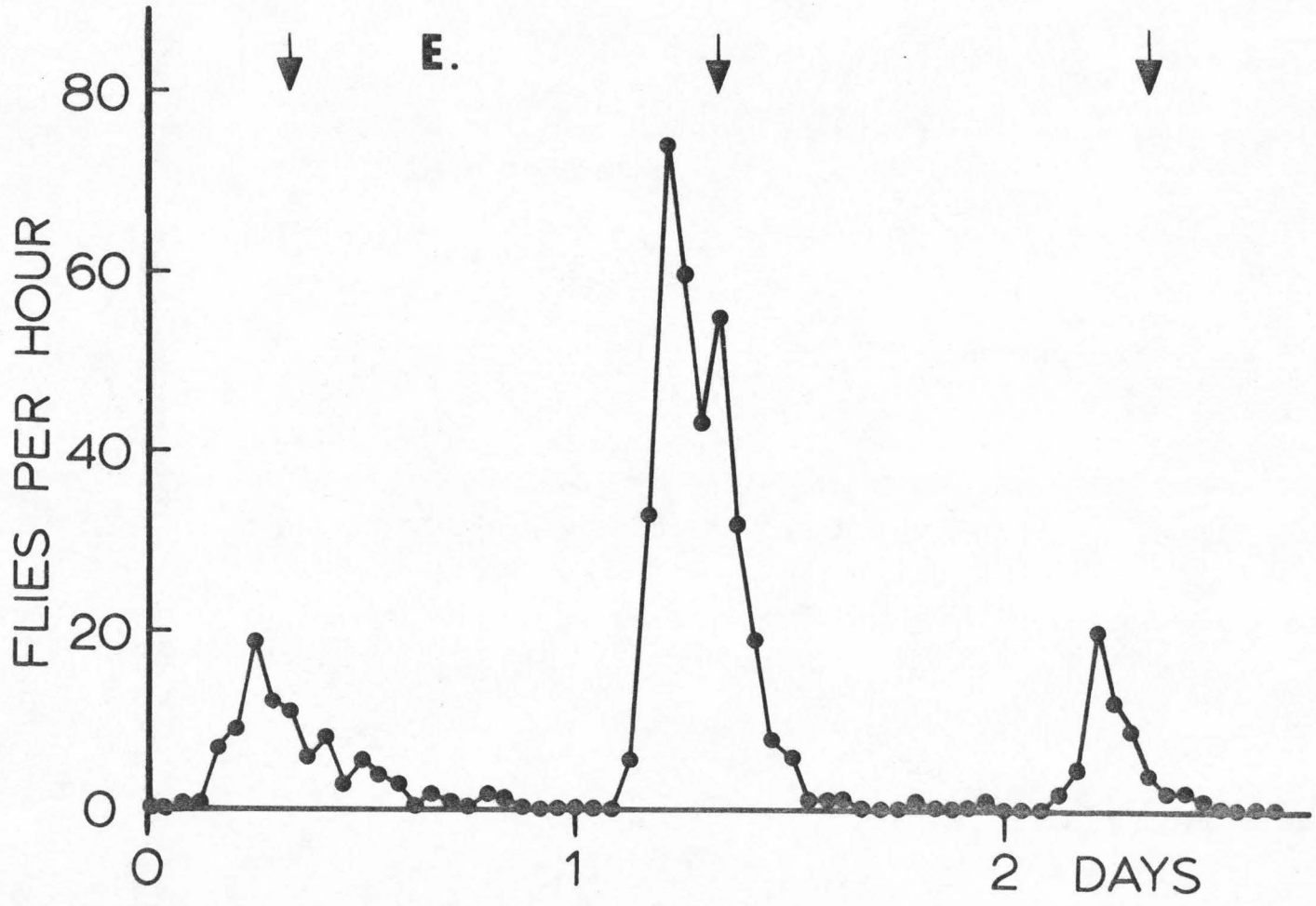
4C-4F: Temperature entrainment in constant darkness. Flies were raised at 18° in LL before the start of the experiment. In 4C, the LL-DD transition occurred 10 hours before the start of the temperature rise. In 4D-4F, the LL-DD transition occurred close to the time of the first temperature rise (in 4E, 5.5 hours before the temperature rise; in 4F, 2 hours after the temperature rise).


A.
normal - LL
FLIES PER HOUR
100
80
60
40
20
0
0
1
2
3
4 DAYS


B.
normal
females
LL
FLIES PER HOUR
50
40
30
20
10
0
0
1
2 DAYS


C.
FLIES PER HOUR
60
40
20
0
0
1
2
DAYS


D.
normal - DD
FLIES PER HOUR
80
60
40
20
0
0
1
2
3
4
DAYS


E.
FLIES PER HOUR
80
60
40
20
0
0
1
2
DAYS

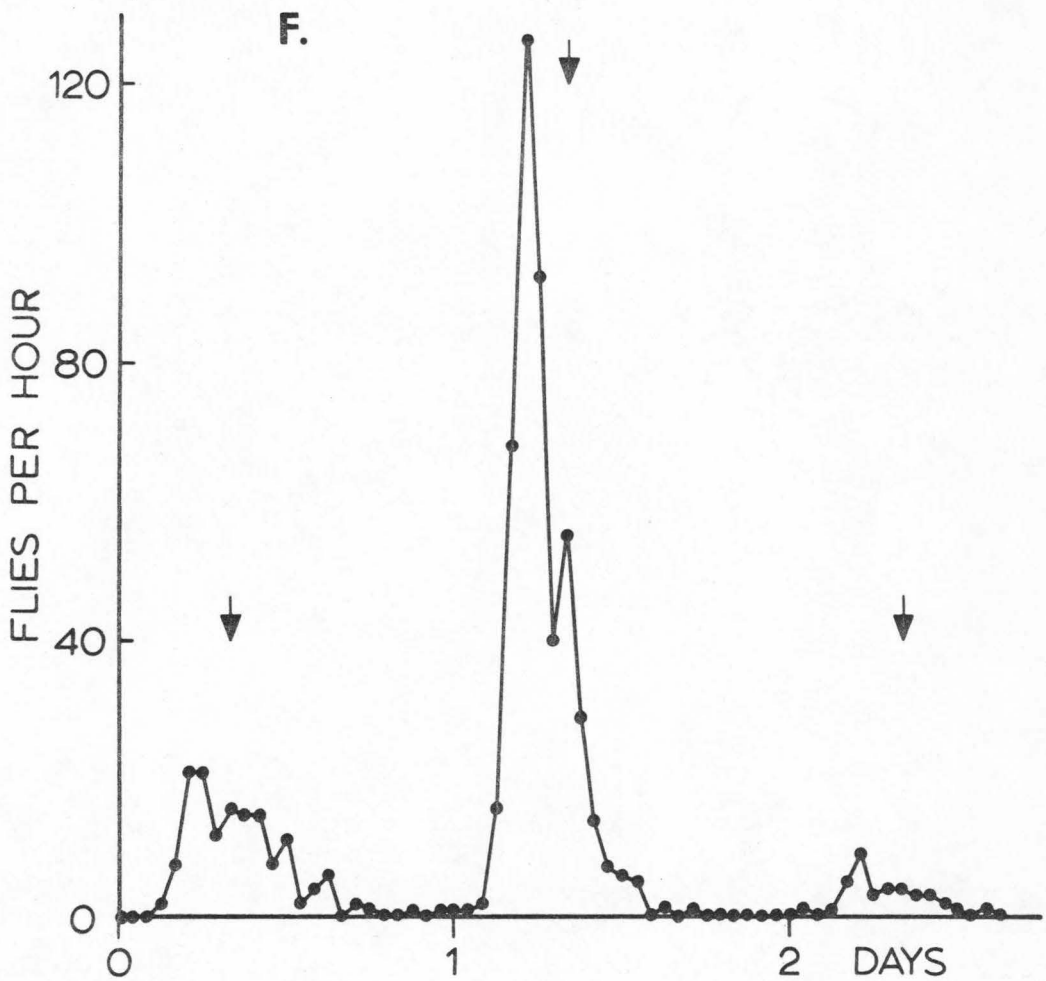

F.
120
80
40
0
FLIES PER HOUR
0
1
2
DAYS

Figure 5. The eclosion pattern of short-period mutant flies in a temperature cycle and constant light (5A) or constant darkness (5B). Flies were raised in LL at 18°, then transferred to the appropriate conditions. The time at which the temperature began to rise is shown by the arrows. The first temperature rise shown represents the start of the third entraining cycle. In 5A, the rhythm is entrained to a 24-hour period. In 5B, the rhythm is not entrained to the temperature cycle; the period of the rhythm is 21 hours except when the projected eclosion peak coincides with a temperature step-down, in which case an additional 9-hour delay is produced (see text).

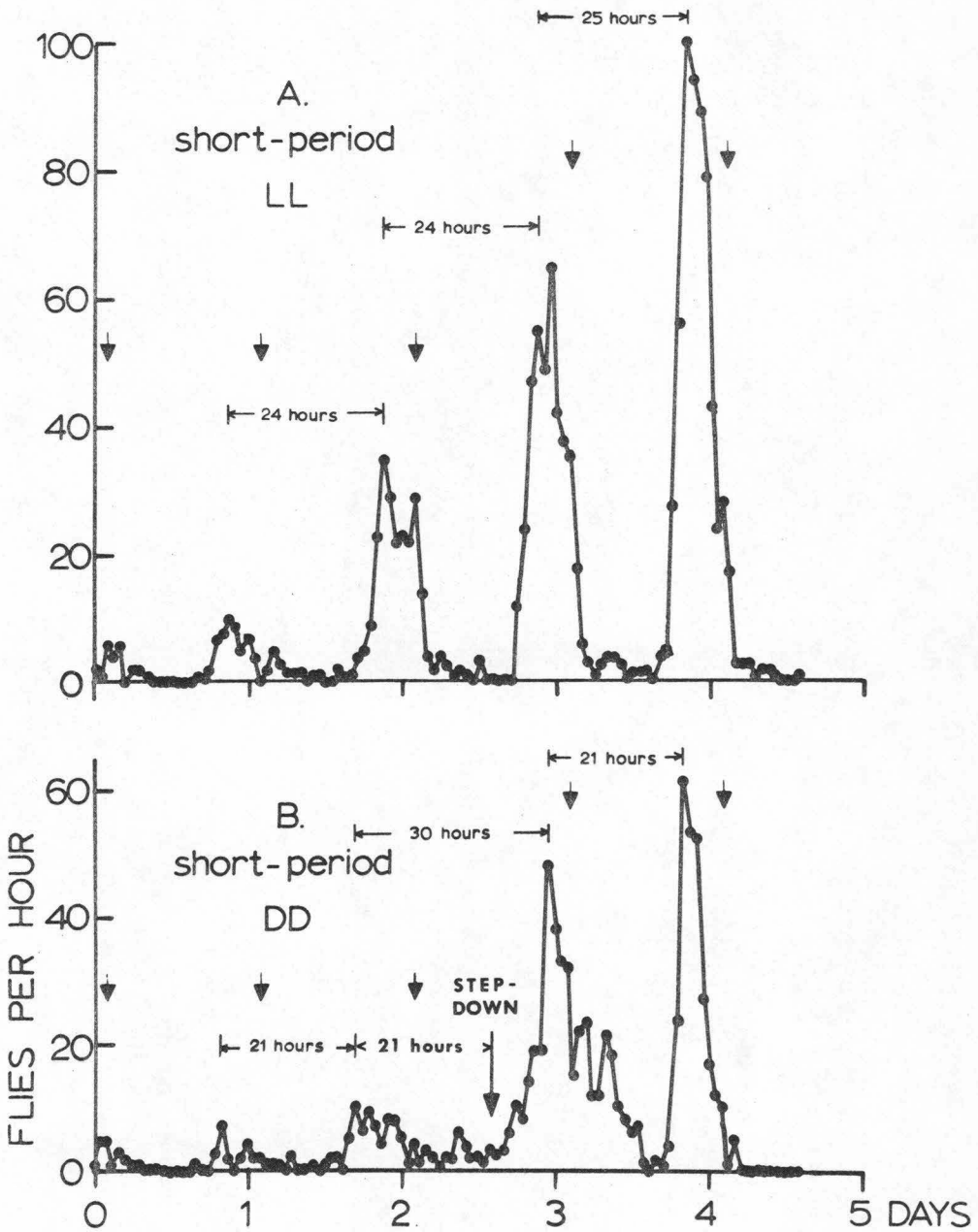

A.
short-period
LL
25 hours
24 hours
24 hours
B.
short-period
DD
21 hours
30 hours
STEP-
DOWN
21 hours
21 hours
FLIES PER HOUR
100
80
60
40
20
0
0
1
2
3
4
5 DAYS

Figure 6. The eclosion rhythm of arrhythmic mutant flies in a temperature cycle in constant light (6A) and constant dark (6B). Flies were raised in LL at 18°, then transferred to the appropriate conditions. The time at which the temperature began to rise is shown by the arrows; the first arrow shown represents the start of the third entraining cycle.

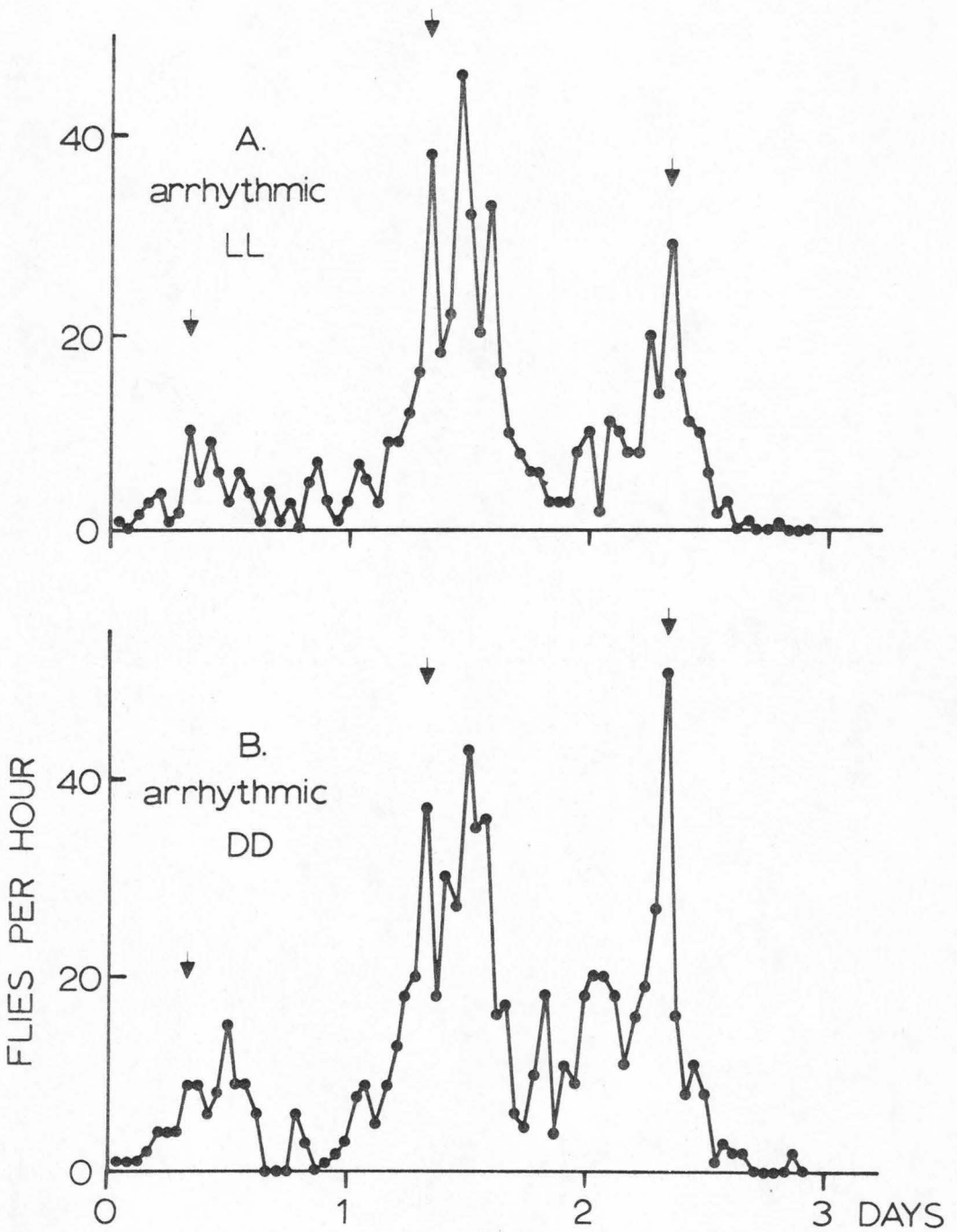
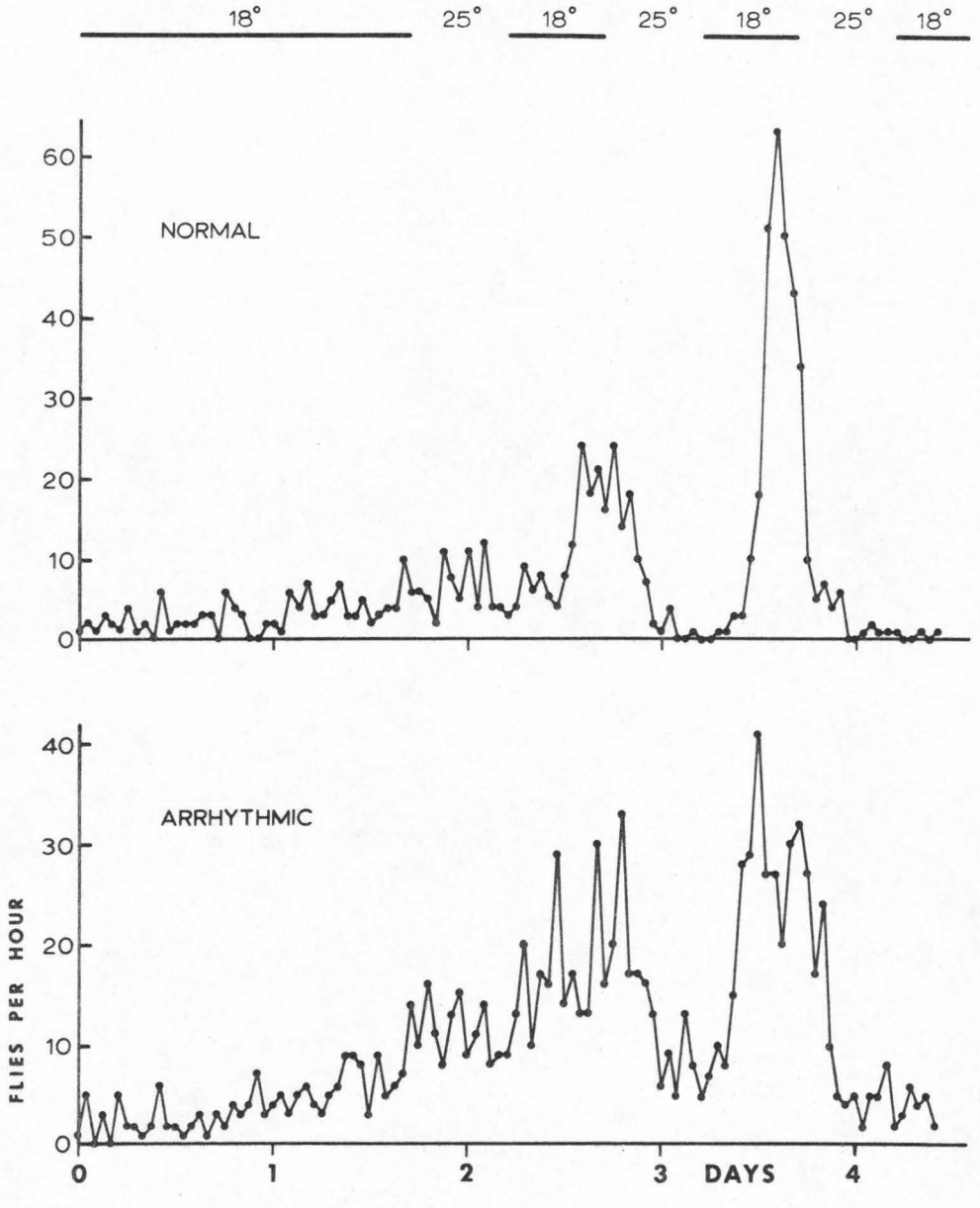

A.
arrhythmic
LL
B.
arrhythmic
DD
FLIES PER HOUR
40
20
0
0
1
2
3 DAYS

Figure 7. The eclosion rhythm of normal-rhythm (attached-X females), arrhythmic mutant, and short-period mutant flies in constant light and constant temperature, followed by constant light and a temperature cycle. The temperature regime is shown at the top. The light used throughout the experiment was 100 foot-candles in intensity. Flies were raised in constant light at 18°.


18°
25°
18°
25°
18°
25°
18°
NORMAL
ARRHYTHMIC
FLIES PER HOUR
DAYS
0
10
20
30
40
50
60
0
1
2
3
4

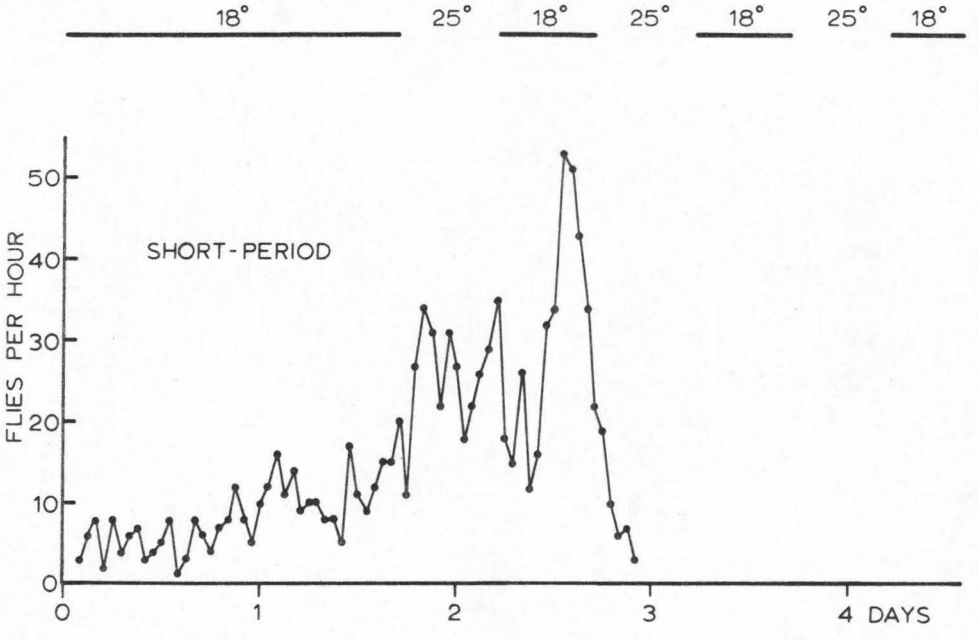
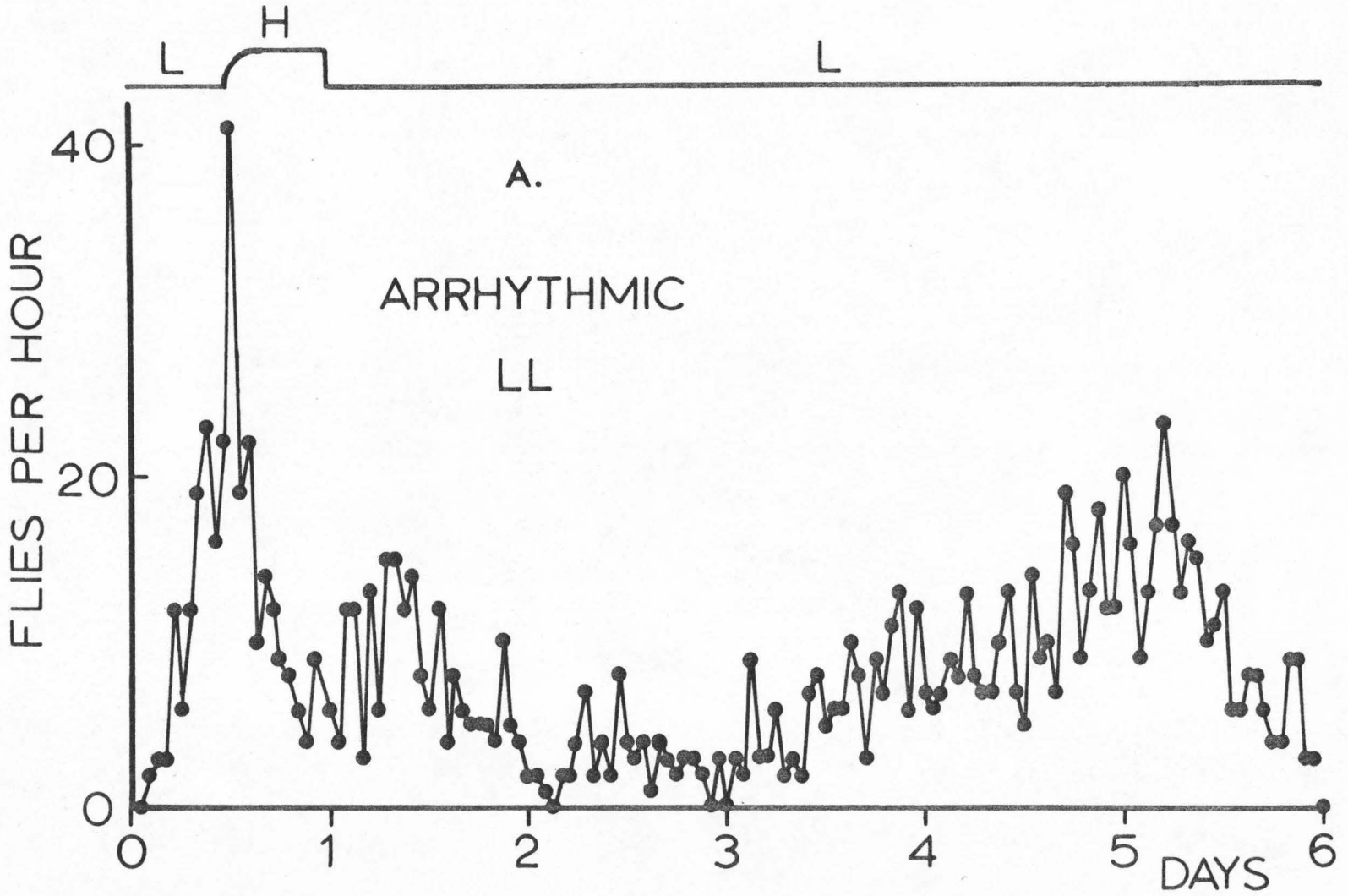
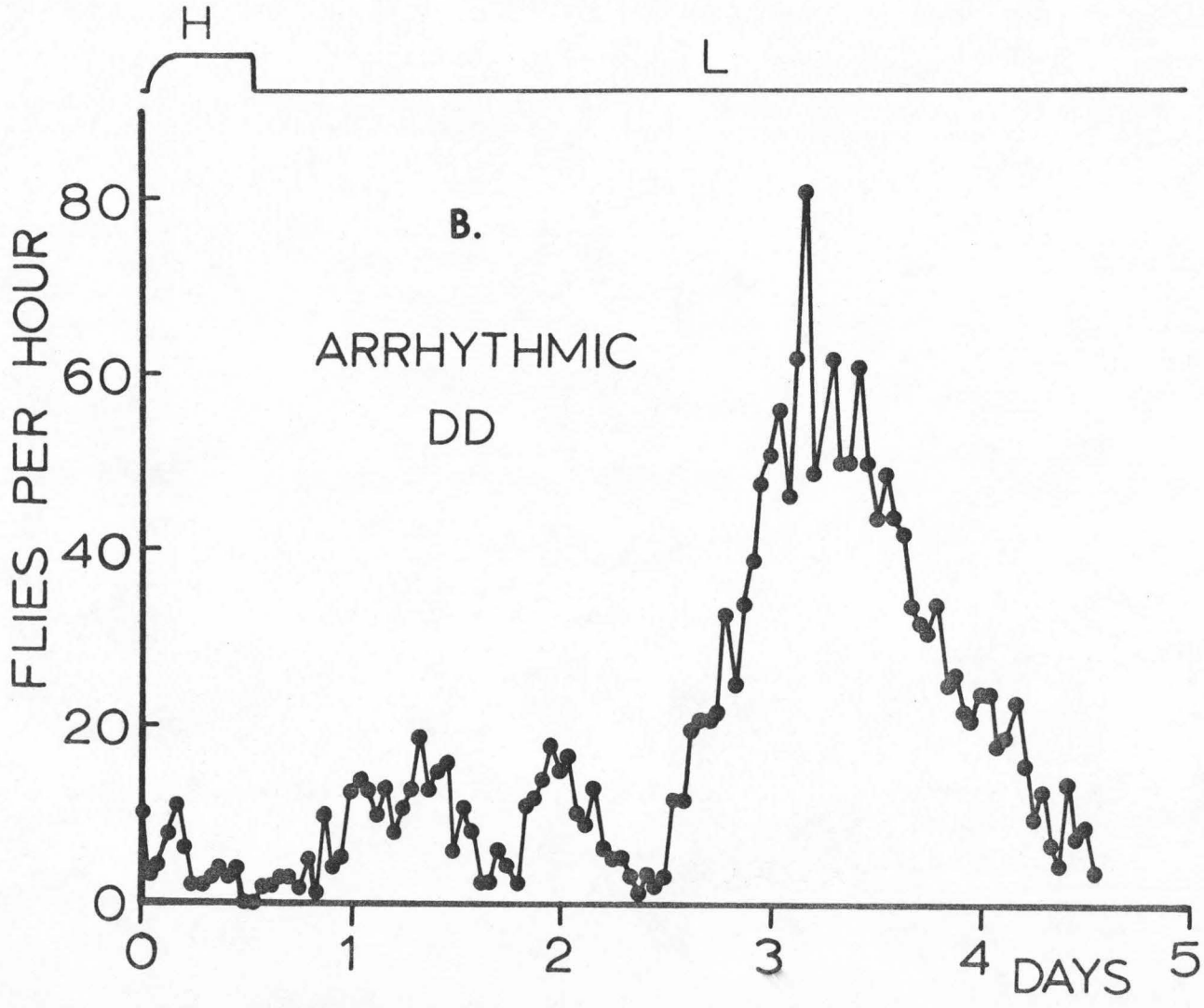
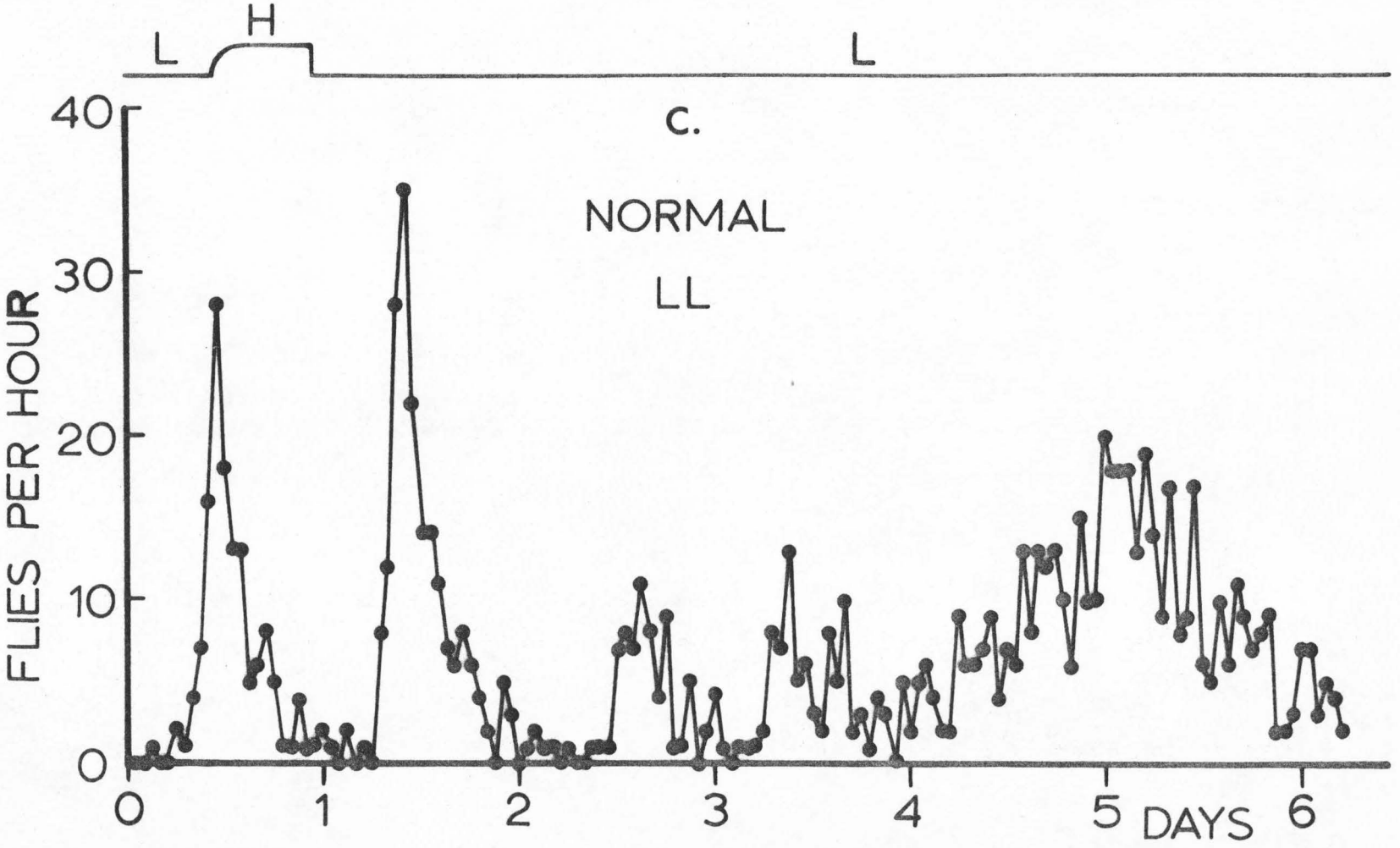
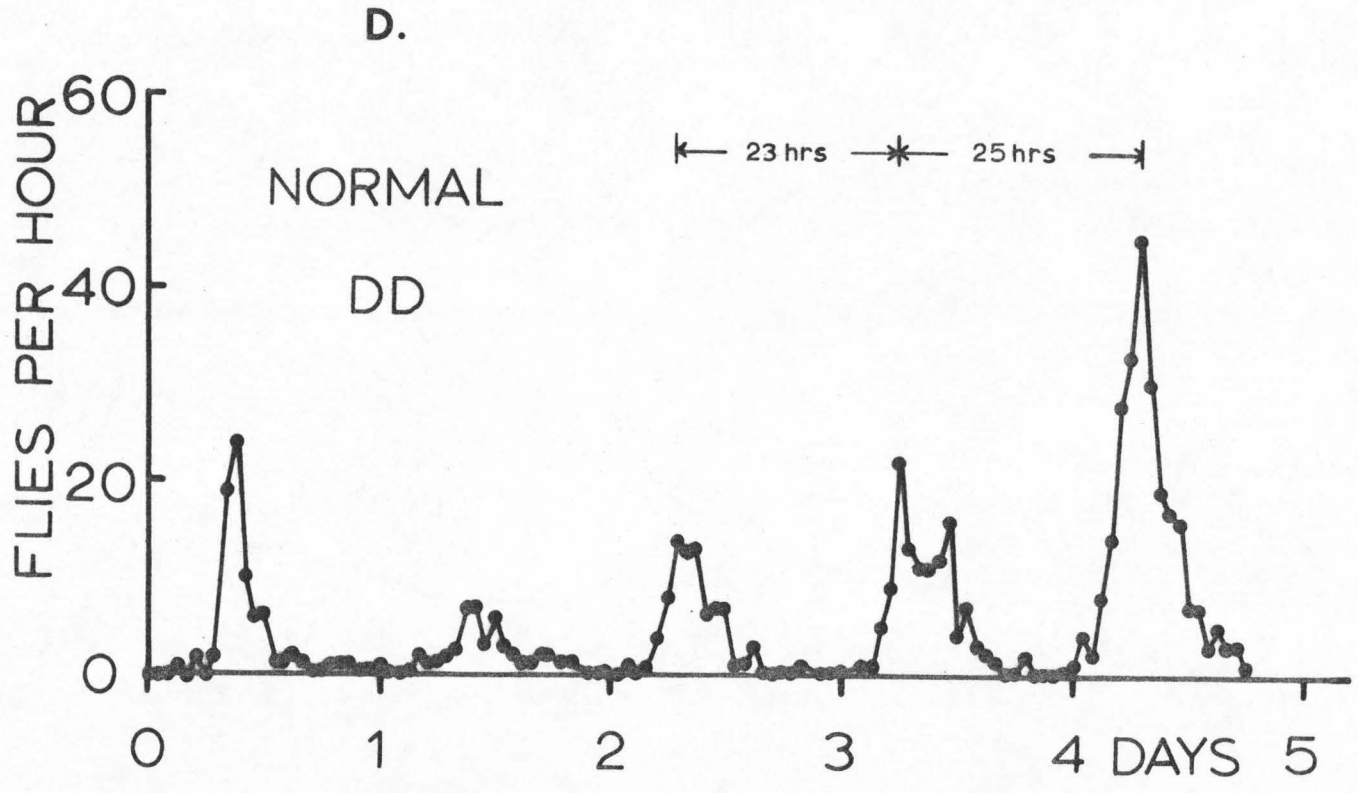

18°
25°
18°
25°
18°
25°
18°
SHORT-PERIOD
FLIES PER HOUR
0
10
20
30
40
50
0
1
2
3
4 DAYS

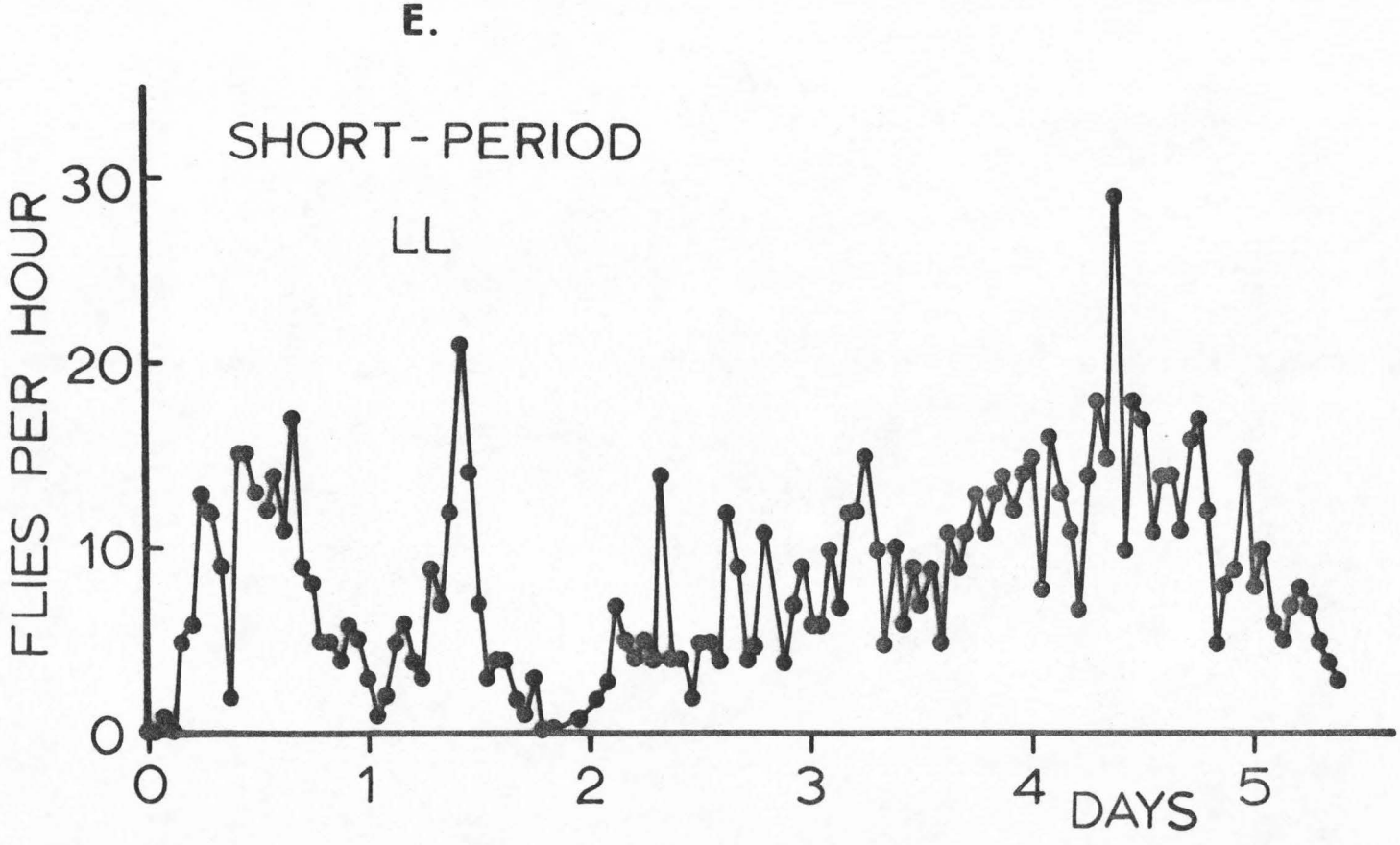
Figure 8. The eclosion rhythms of arrhythmic mutant flies, as well as rhythmically normal flies (attached-X females) and short-period mutant flies in constant conditions (18°, and constant light or constant dark) after entrainment in a temperature cycle. All populations were entrained by at least three successive temperature cycles. In some cases, the last cycle is shown at the top of the figure; in the cases where the temperature regime is not shown, the transfer to constant conditions was made before the start of the record. The light intensity was 100 foot-candles. The normal and short-period mutant rhythms damp out in constant light (8C, 8E) and free-run in constant darkness, as expected (8D, 8F). The arrhythmic mutant damps out in both constant light and constant darkness (8A, 8B).


L
H
L
A.
ARRHYTHMIC
LL
FLIES PER HOUR
40
20
0
0
1
2
3
4
5
6
DAYS


H
L
B.
ARRHYTHMIC
DD
FLIES PER HOUR
80
60
40
20
0
0
1
2
3
4
5
DAYS


L
H
L
C.
NORMAL
LL
FLIES PER HOUR
40
30
20
10
0
0
1
2
3
4
5
DAYS
6


D.
FLIES PER HOUR
60
40
20
0
NORMAL
DD
23 hrs
25 hrs
0
1
2
3
4 DAYS
5



110.

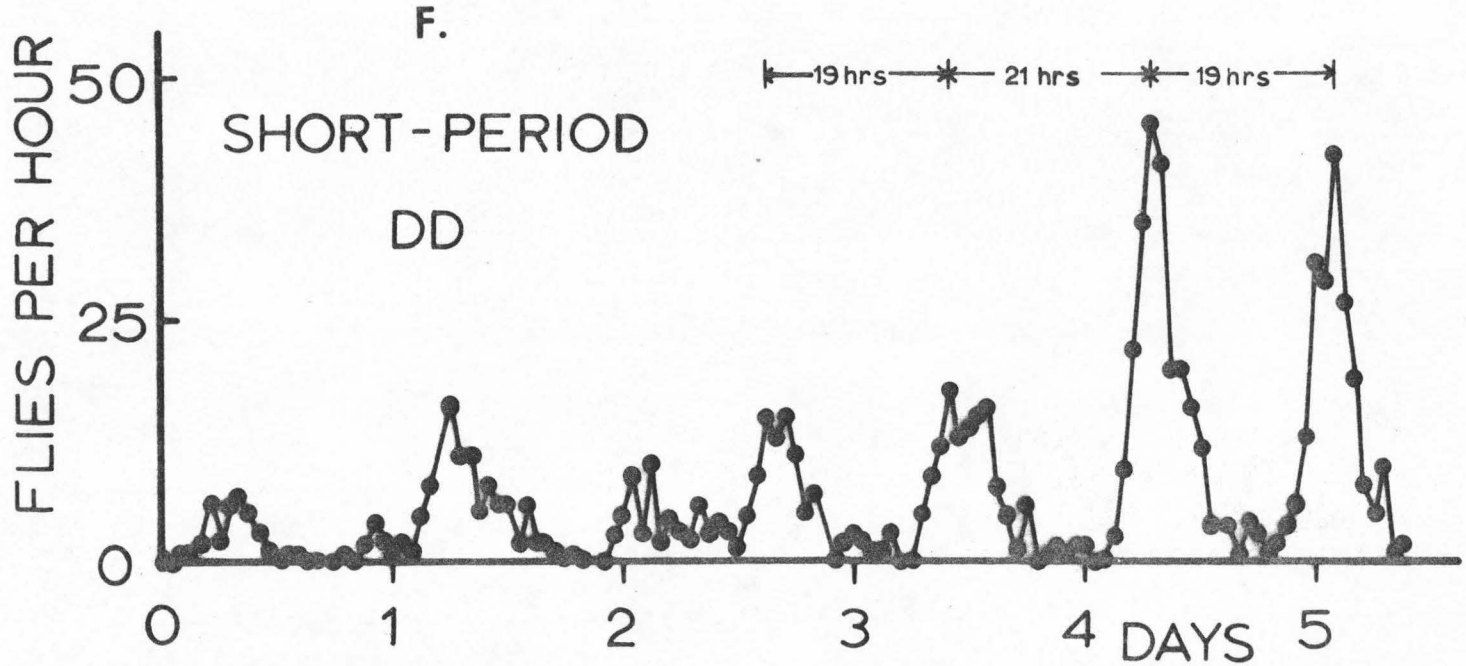
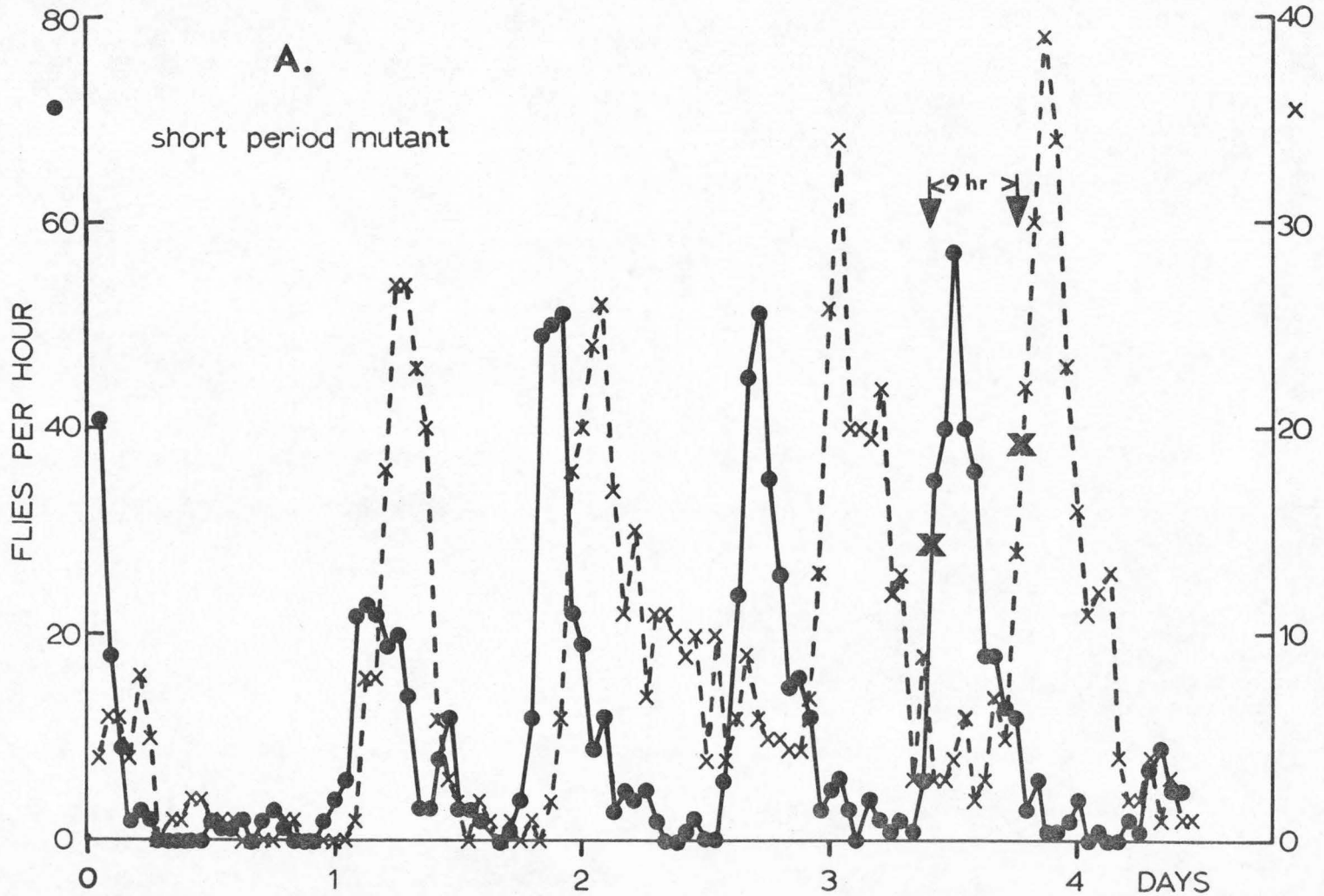

F.
SHORT-PERIOD
DD
19 hrs
21 hrs
19 hrs
FLIES PER HOUR
50
25
0
0
1
2
3
4
DAYS
5

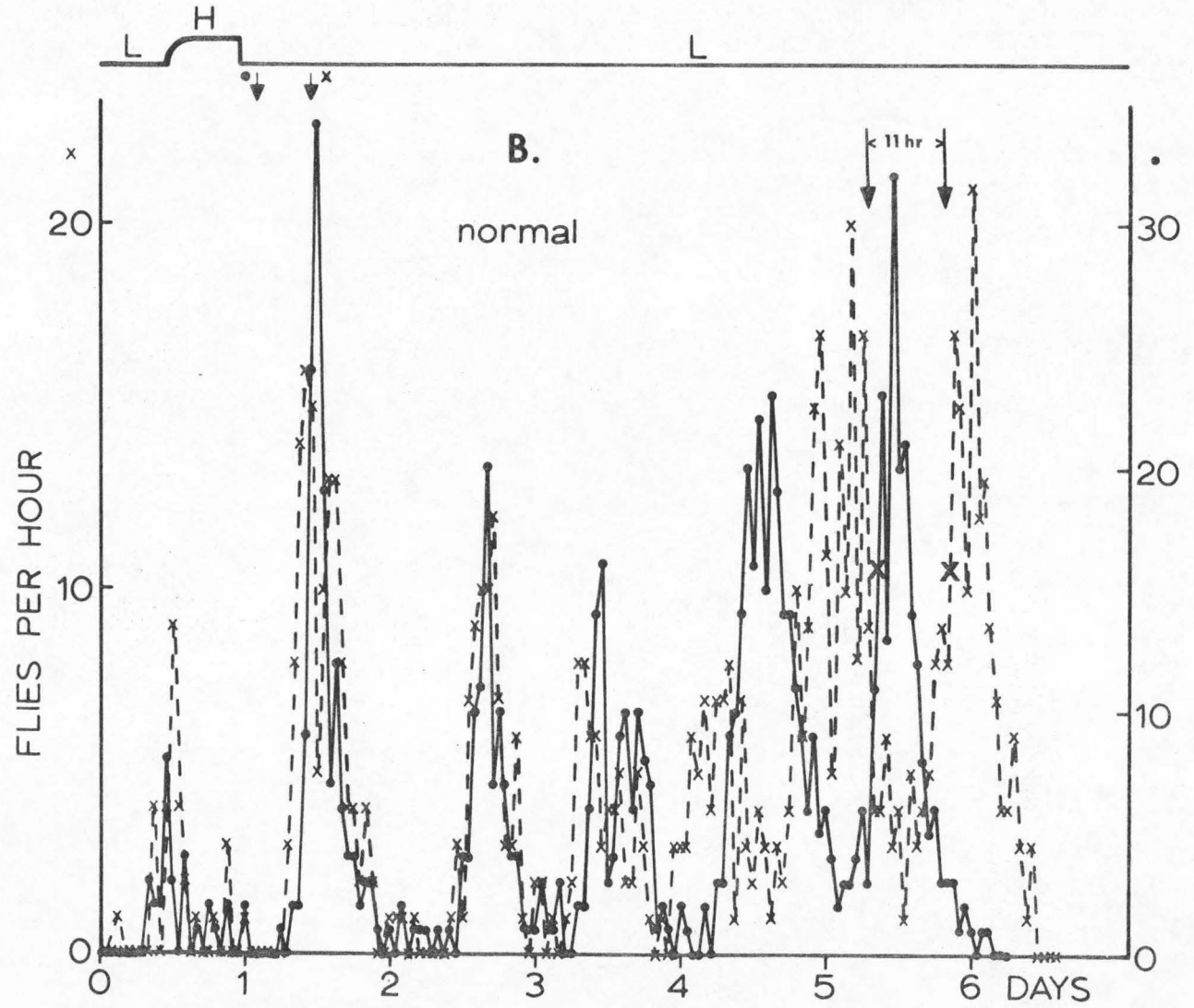
Figure 9. Eclosion rhythms of rhythmically normal (attached-X females) and short-period mutant flies in constant darkness at 18° after entrainment by a temperature cycle in LL. The light intensity was 100 foot-candles.

9A: Short-period mutant pupae were entrained in LL in the temperature cycle; during the low-temperature portion of the sixth entraining cycle, one hour after its start, a sample of pupae (•) was transferred to DD. Eight hours later, still during the low temperature portion of the cycle, the rest of the pupae (x) were transferred to DD. The pupae were maintained at 18° from this time on. The figure shows the eclosion rhythm of the two groups of pupae at 18° in DD. By the fourth day of the record, the sample transferred into darkness later (x) lags the sample transferred first (•) by about 9 hours, which is close to the 8-hour delay in transfer of the two groups.

9B: Rhythmically normal pupae were entrained in LL by the temperature cycle; during the low-temperature portion of the fourth entraining cycle, three hours after its start, a sample of pupae (•) was transferred to DD. Nine hours later, at the end of the low-

temperature portion of the cycle, the rest of the pupae (x) were transferred to DD. The figure shows the time of transfer of the two samples (arrows). After the transfer to DD, both samples were kept at 18°. The figure shows the eclosion rhythm of the pupae in DD at 18°. By the fourth day after the transfer into DD, the sample transferred later (x) lags the sample transferred first (•) by about 11 hours, which is close to the 9-hour delay in transfer of the two samples.


A.
short period mutant
9 hr
FLIES PER HOUR
80
60
40
20
0
40
30
20
10
0
0
1
2
3
4
DAYS


L
H
L
B.
normal
11 hr
FLIES PER HOUR
20
10
0
30
20
10
0
0
1
2
3
4
5
6
DAYS

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CHAPTER 7.

WHERE DO WE GO FROM HERE?

The experiments discussed in the preceding chapters show that mutations in one genetic locus can profoundly affect the properties of a circadian clock. The period of the clock in constant darkness can be altered, the ability of the clock to be reset by light can be changed, and the observed rhythm can even be abolished completely. Certainly much more can be learned by studying mutant clocks and comparing their behavior with that of a normal clock. The greatest contribution of the mutations, however, will probably result from their use as markers by means of which a defect may be localized and identified and its effects studied on the molecular, cellular, or organismic level. Some of the potential of the use of these mutations as markers is evident in the mosaic studies which roughly localize the site of action of one mutation to the head of the fly. In the near future, when it becomes possible to histochemically distinguish male and female tissue (1,2), it should be possible to carry the localization even further, perhaps to a single cell or group of cells.

Ultimately, the clock mutations can be employed as markers in searching for the gene product affected by the mutations. One might expect that the arrhythmic mutation would produce no product or a product with little or no activity; the short- and long-period mutations might produce a gene product with altered activity. By separating and quantitating various species of protein and RNA from normal and mutant strains by chromatographic techniques, it may be possible to isolate the gene product responsible for producing the

mutant phenotypes. If the gene product is a molecule found in many cells, it might be sufficient to use whole flies or fly heads for the isolation. Alternatively, the mutant molecule may only be found in one or at most a few cell types; in this case further localization of the cells involved in determining the rhythm might facilitate the sorting out of the altered gene product from the many similar gene products.

A fine-structured localization might be accomplished by the histochemical experiments involving mosaics mentioned previously; a rough localization could be carried out by means of mosaics or transplant experiments akin to those of Truman and Riddiford (3). By transplanting brains into headless Saturniid pupae, Truman and Riddiford were able to show that the photoreceptor and clock controlling the eclosion rhythm in these moths was contained within the brain. In Drosophila, one might attempt a similar demonstration using a 19-hour donor brain implanted into the abdomen of an arrhythmic mutant fly and measuring the locomotor activity of that fly. The appearance of a 19-hour activity rhythm in the recipient would unequivocally show transfer of at least one element of the clock system with the transfer of the brain and would indicate a hormonal link in the control of the activity rhythm. Failure of the donor brain to induce a rhythm in the recipient would imply that either the clock is located in another structure or that control of the rhythm is effected by direct neural connections rather than hormonally.

Histological examination of the nervous system of the arrhythmic mutant may reveal a morphological abnormality. It is possible that certain neurosecretory cells may be atrophied due to their inability to release the substance (say, an eclosion inhibitor) which they synthesize. King et al. have found, for example, that the corpus allatum and corpus cardiacum of a female-sterile Drosophila mutant, fs(2)B, are atrophied due to an accumulation of a vitellogenic hormone caused by lack of oocytes in the mutant (4). On the other hand, a group of cells might be reduced in size or completely missing due to the absence of the substance which these cells normally secrete (e.g., an eclosion hormone). In one lethal Drosophila mutation, l(2)gl, a reduction in the size of the ring gland is correlated with a deficiency in the hormones it normally produces (5).

Since the genetic location of the clock mutations is known fairly accurately, it would be a simple task to screen for a temperature-sensitive (ts) clock mutation at the locus in the form of a temperature-sensitive lethal, using either a deficiency or a point lethal, such as one of those isolated by Judd in that region (6), to uncover the ts mutation. A ts clock mutation would be very valuable in determining the time of action of the gene during development as well as whether the gene itself is active during the course of the oscillation. Such data might yield an insight into whether the affected gene product is part of the basic oscillator, and whether sequential gene induction is involved in the mechanism

of the oscillation. A ts mutation would also aid in the isolation of the gene product, since the ts gene could be turned on and off at will.

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