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Role of the Optic Lobes in the Regulation of the Locomotor Activity Rhythm of *Drosophila melanogaster*: Behavioral Analysis of Neural Mutants*

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SUMMARY

The locomotor activity patterns of the *Drosophila melanogaster* brain mutants *optomotor blind (omb), lobula plateless (lop), minibrain (mnb), small optic lobes (sol), sine oculis (so)*, and the double mutants *mnb;so* and *sol;so*, all of which show reductions in the optic lobes, were investigated and compared with those of the wild-type. In none of the mutants was the number of arrhythmic flies significantly higher than in the wild-type, indicating that the optic lobes are not the sole site of a pacemaker controlling the locomotor activity rhythm. However, these mutations greatly influence the stability of the circadian system, in that the number of flies simultaneously showing two or more circadian components increased as the optic lobe defects became more severe. In flies with the strongest reduction of the optic lobes, two free-running circadian components were found almost exclusively. This suggests a two-oscillator control of the locomotor activity. Eyeless mutants also expressing a neural mutation were entrained by light : dark (LD) cycles, but their activity pattern in LD was changed compared to the wild-type and the eyeless mutant *so*.

INTRODUCTION

Evidence has accumulated that, in Diptera, circadian rhythms of locomotor activity are not controlled by the optic lobes: in mosquitoes[17] and houseflies[15] such rhythms continue after lobectomy or severance of the optic tracts. This is in contrast to the situation in cockroaches[20,24–26,28,31,33], *Gryllus*[35], *Teleogryllus*[19,34,40], several beetles[1,10], and *Wetas*[5], where lobectomy or severance of the optic tracts leads to arrhythmicity.

In all these studies different kinds of brain surgery served as the means for locating

* This paper is dedicated to Prof. Dr. E. Bünning on the occasion of his 80th birthday.

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the site of the pacemaker. Working with brain mutants avoids many of the disadvantages of surgery. We have extended previous work on brain mutants of *Drosophila melanogaster*, in which parts of the optic lobes are affected by mutations: the optic lobes of the mutant *sine oculis (so)* are reduced to 20% of its normal volume; the compound eye and the first optic ganglia, the lamina, are often lacking completely; the medulla (2nd order lobe) is reduced to 18%, and lobula complex (higher order lobes) to 40%[6]. In the mutant *small optic lobes (sol)* medulla and lobula complex are reduced to 50%[7]. In *minibrain (mnb)* the whole brain, including optic lobes, is reduced to 52%[8]. All these mutants retain circadian rhythms of locomotor activity[14].

Since each of the mutants still possesses some amount of the optic lobe neuropil, the pacemaker of locomotor activity might reside in the unaffected areas of the optic lobes. In this study we therefore used double mutants carrying *sol* and *so (sol;so)* or *mnb* and *so (mnb;so)*. In *sol;so* and *mnb;so* double mutants, the single mutations have additive effects, with the result that less than 5% of the optic lobes of the wildtype are left[9]. Like *so* mutants the double mutants are eyeless (when *so* is fully expressed). If a pacemaker controlling the circadian rhythm of locomotion is localized in the optic lobes, such a strong reduction would very likely affect the rhythm. We also used the mutants *lobula plateless (lop)* and *optomotor blind (omb)*, which have reductions in the lobula plate[8]. Both mutations affect neurons which are still present in the double mutants *sol;so* and *mnb;so: omb* lacks the giant neurons of the lobula plate which have been shown to exist in *sol;so*. In *lop*, which lacks the small field elements of the lobula plate, more neuronal types of the lobula plate are missing than in *sol;so*. Thus, if the pacemaker resides in the lobula plate, one might expect the *lop* and *omb* mutants to be arrhythmic.

MATERIALS AND METHODS

Locomotor activity was recorded in the mutants of *Drosophila melanogaster* which are listed in Table I. As wild-type the strain "Berlin" (WT_B) was used. For illustration of brain mutations, see Fig. 1.

The flies were kindly supplied by K.F. Fischbach (University of Würzburg) and reared at 20 °C (± 3 °C) in a cycle of 12 h of light and 12 h of dark (LD 12 : 12), on standard medium in which Isabgol replaced agar[32]. Since no sex differences in the expression of the locomotor activity rhythm were found, both males and females were used. All flies used in locomotor activity recordings were less than 5 days old. Prior to recording they were briefly anesthetized with chloroform (to enable the double mutants (see below) to be checked with a binocular microscope for eyelessness), and then transferred with soft forceps to small Petri dishes (35 mm diameter, 11 mm deep) containing a lump of sugar as food. Water was available to the flies via a wet wick from a bottle. For recording locomotor activity, an edge of the Petri dish was inserted into a light beam consisting of a light-emitting diode (LD 271) and a phototransistor (UTP 101). Temperature in the recording chamber was 20 ± 0.5 °C, and illumination consisted of continuous red light (RR) of rather weak intensity, i.e., $6–9 \times 10^{-8}$ W/cm^2,

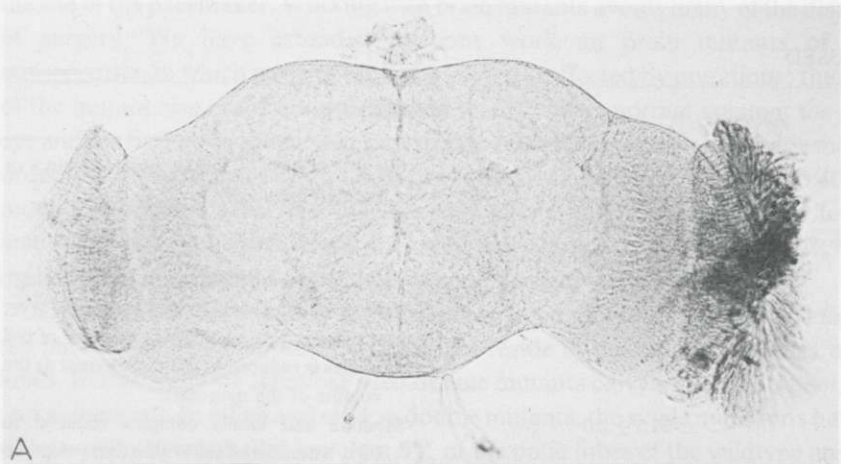
TABLE I

MUTANTS USED

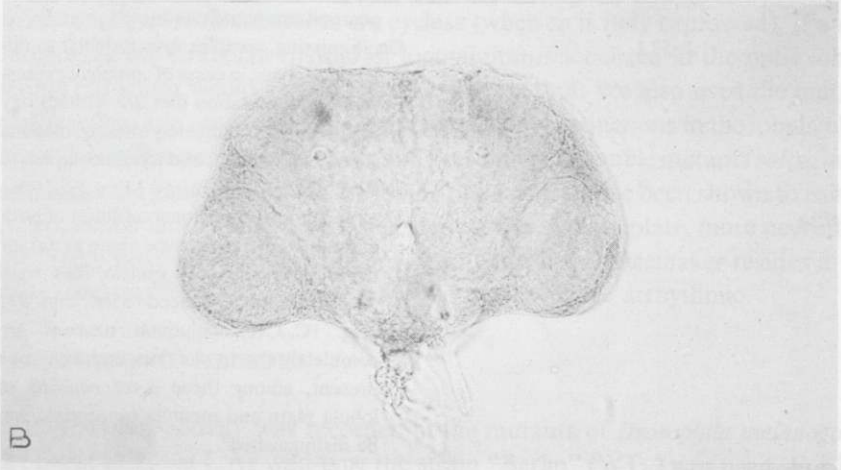
Mutation		Phenotype
omb^{H31}	Inversion on *X* chromosome with breakpoints at 4C4-7 and 12D2-E1	Giant neurons of lobula plate missing or strongly reduced[12].
lop	*2*: 70–72	Small field elements of lobula plate missing[8].
mnb	*x*: 58.5 ± 0.8	Volume of whole brain reduced to 52%[8] (yet it is unkwown whether the number of cell bodies is reduced to the same extent as the volume of the neuropil).
sol^{KS58}	*x*: 67.5 ± 2.0	Medulla and lobula complex reduced to 50%[7] (number of cell bodies and volume of neuropil are equally reduced).
so	*2*: 57.1	Ocelli missing, complex eyes reduced to different degrees; in cases of complete expression of the mutation flies are without any eyes, lamina is completely missing, medulla is reduced to 18%, and lobula complex to 40%[6] (Fig. 1B, H, I).
mnb;so and *sol;so*		Double mutants with linear addition of both mutations; eye phenotype same as *so*; for the experiments only eyeless flies were used; optic lobes reduced to less than 5% (Fig. 1C, J, K); columnar neurons are completely absent; only tangential neurons present, among those giant neurons of lobula plate and medulla tangentials can be distinguished[9].

depending on the distance of the recording channel to the red fluorescence tube (Philips TL 20W/25A 032, with primary red cinemoid filter, Rank Strand). For synchronization experiments, LD 12 : 12 cycles (white Osram L65W/25A fluorescence tubes) were applied. The light intensity was adjusted to 300, 40, 8 or 1 lux with a dimmer. Temperature cycles consisted of 12 h of 22.2 °C and 12 h of 20.2 °C in RR.

The infrared light-emitting diodes of the 55 recording channels were switched on by microprocessor control every 20 ms for a few μs, and automatic determination was made whether light reached the phototransistor (i.e., no fly in the light beam, 0) or not (i.e., light beam interrupted by a fly, 1). If the light beam was interrupted once or more during a 4-min interval, the fly was considered to be active. Thus fifteen 0 or 1 conditions were recorded every h and stored on a floppy disk of a microprocessor, and simultaneously on a PDP11 in our University's computing center. Recordings lasted at least 7 days. Shorter records were discarded.



A



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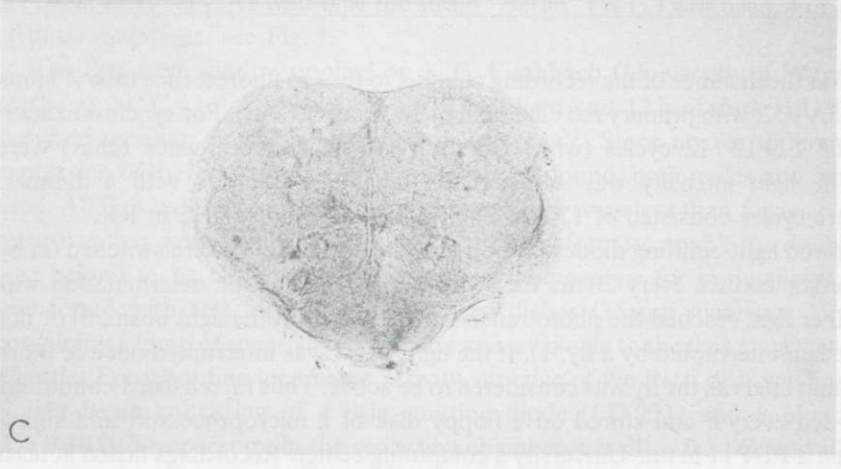

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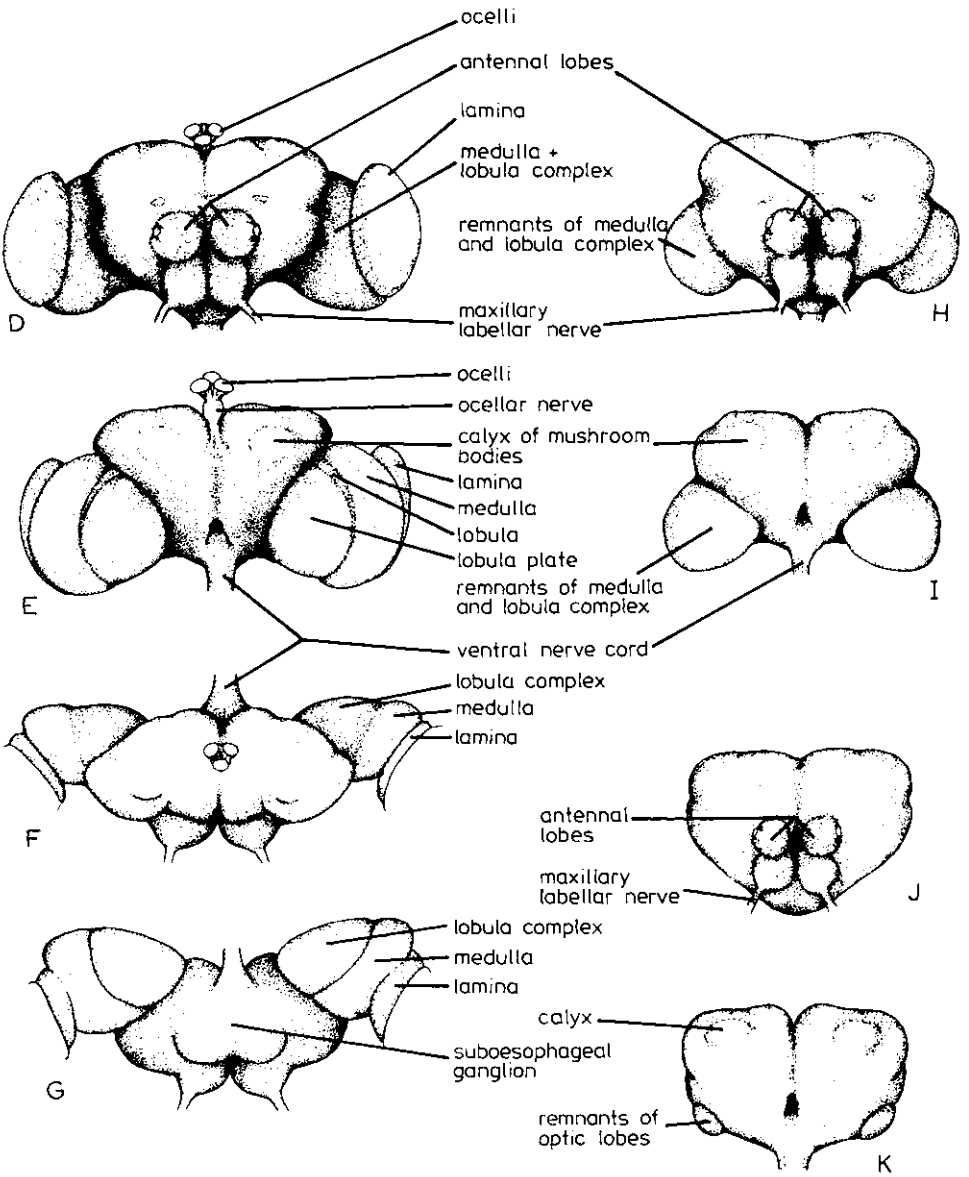


Fig. 1. Comparison of the wild-type brain (A and D–G) with the brain of the mutant *so* (B and H,I) and the double mutant *sol;so* (C and J,K). The photographs show the prepared brains, and the drawings illustrate the different parts of the wild-type and mutant brain. The wild-type brain is depicted from the front, the back, from above, and below; and the mutants from the front and the back.

The data were analyzed with the time-series analysis program package TIMESDIA[22] on a TR440 computer. Period length was determined with periodogram analysis (95% confidence limits). To test for stability of the period and phase jumps, complex demodulation was used. The occurence of a second circadian period was determined by using a signal average program, which allows the elimination of one period and the determination of the period length of the other in the residues.

RESULTS

Locomotor activity under free-run conditions

The locomotor activity patterns of normal and mutant flies fell into 3 categories (see ref. 14). Table II shows the percentages of wild-type and mutant flies in each category. One category of flies displayed clear and persistent rhythms, as judged by visual inspection. Periodogram analysis showed a single significant period which was stable throughout the recording time. Flies in category 2 showed complex rhythmicity consisting of either an unstable single rhythm, or several periodicities occurring simultaneously (Fig. 2). In flies showing multiple periodicities, the individual periods usually differed by more than 2 h and very often two periods were found (Figs. 3 and 4). Category 3 included flies with arrhythmic activity patterns. Periodogram analysis showed no significant periodicity.

Flies with a complex pattern of activity were found in all strains, in the wild-type as well as in the mutants (Table II). The percentage of those was slightly higher in the mutant *so* and significantly higher in the double mutants. Table III shows the percentage

TABLE II

DISTRIBUTION OF ACTIVITY PATTERNS FOUND IN MUTANT AND WILD-TYPE *Drosophila melanogaster*

The first column shows the number of flies recorded, all other figures are percentages of each group (rows).

	n	Rhythmic pattern	Complex rhythmicity	Arrhythmic pattern
Wild-type (Berlin)	66	80%	14%	6%
omb	19	84%	0%	16%
lop	26	92%	8%	0%
mnb	16	81%	6%	13%
sol	36	83%	14%	3%
so	109	68%	26%*	6%
mnb;so	32	0%	81%**	19%
sol;so	49	0%	86%**	14%

* Significantly higher percentage compared to the wild-type at $\alpha' = 0.1$ (χ^2-test).

** Significant at $\alpha' = 0.01$.

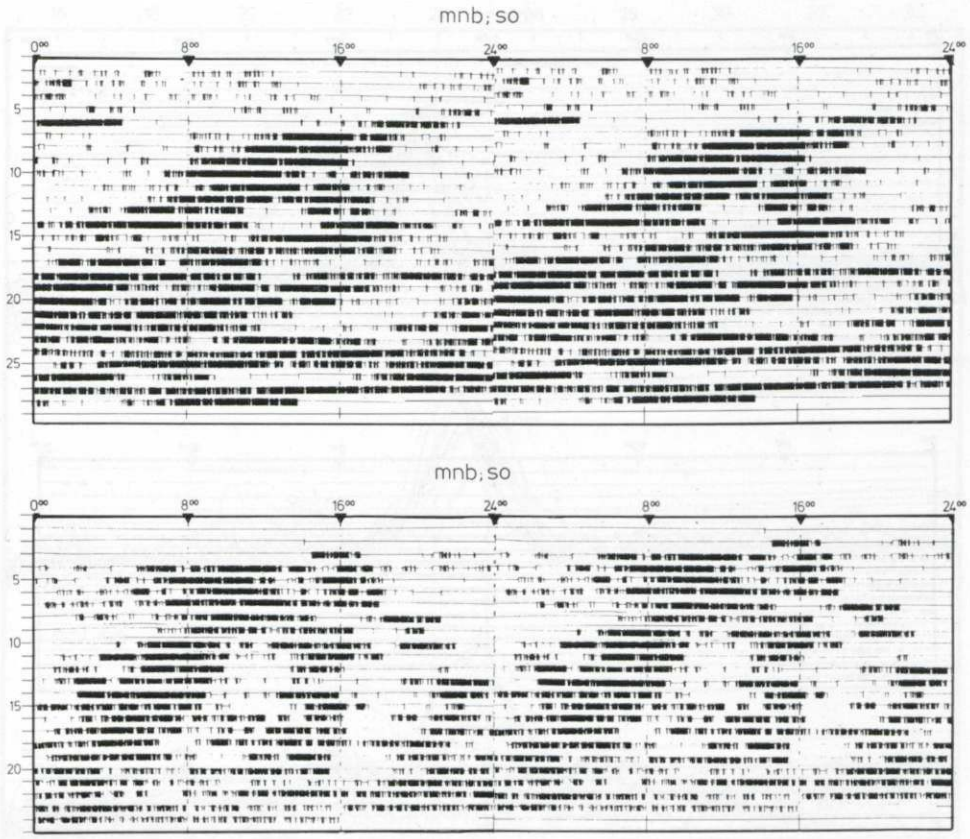


Fig. 2. Activity records of a *mnb;so* mutant in which several free-running components were detected by visual inspection and by periodogram analysis.

TABLE III

NUMBER AND PERCENTAGE OF WILD-TYPE FLIES, *so*, AND DOUBLE MUTANTS, WITH COMPLEX RHYTHMICITY SHOWING TWO RHYTHMS

The first column shows the number of flies with complex rhythmicity.

	n	Flies with two τ's	
		Number	Percentage
Wild-type (Berlin)	9	2	22
so	28	14	50
mnb;so	26	22	85
sol;so	42	38	91

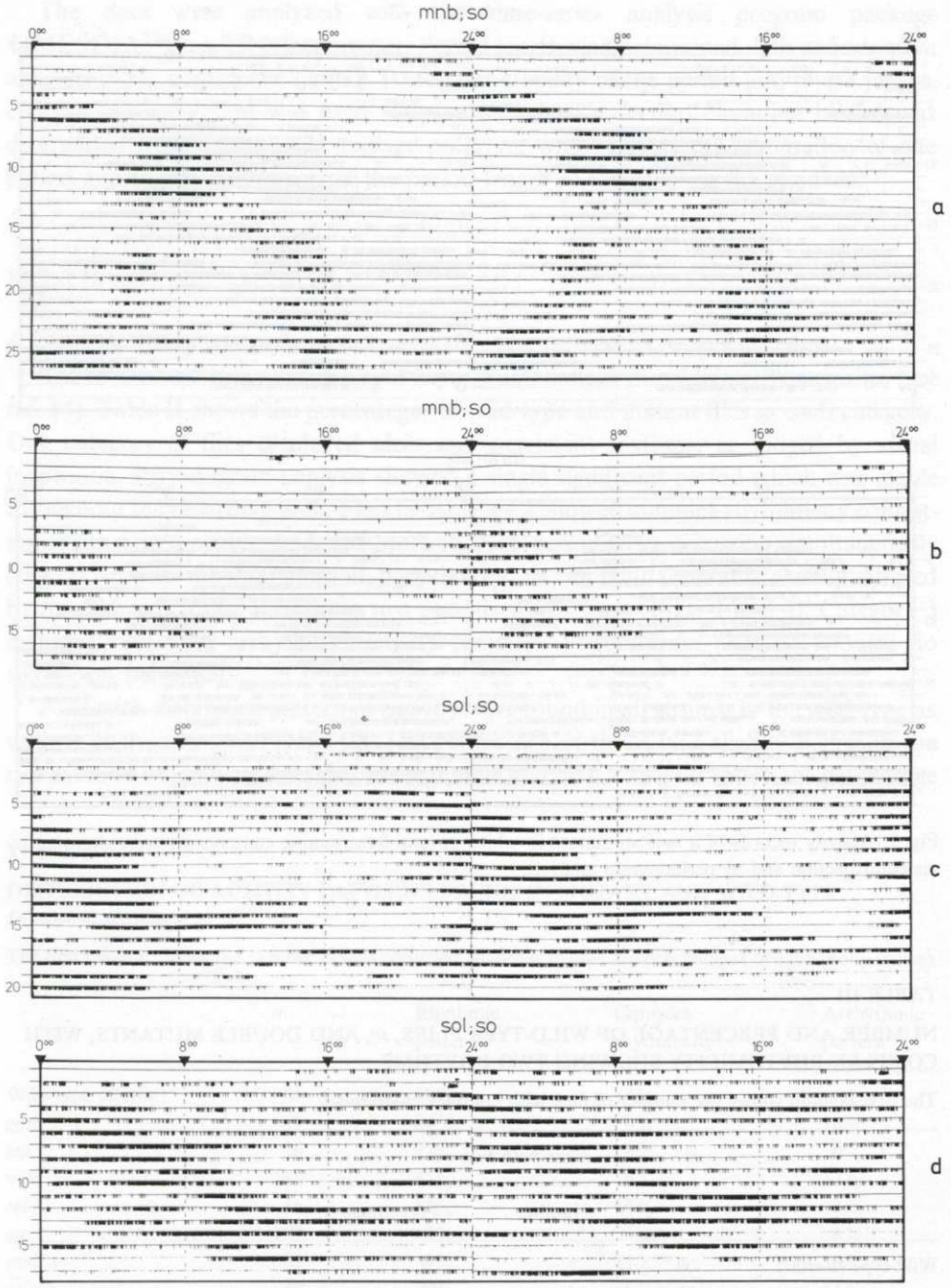


Fig. 3. Actograms of flies which show two free-running circadian components (τ_1 and τ_2) simultaneously: a: splitting of a circadian rhythm in the double mutant indicated. b: τ_1 (24.9 h) is dominant; τ_2 (21.3 h) is especially observable at its crossing points with τ_1. c: τ_1 (25.2 h) is somewhat more pronounced than τ_2 (21.4 h). d: τ_1 (25.6 h) and τ_2 (21.2 h) are of equal strength.

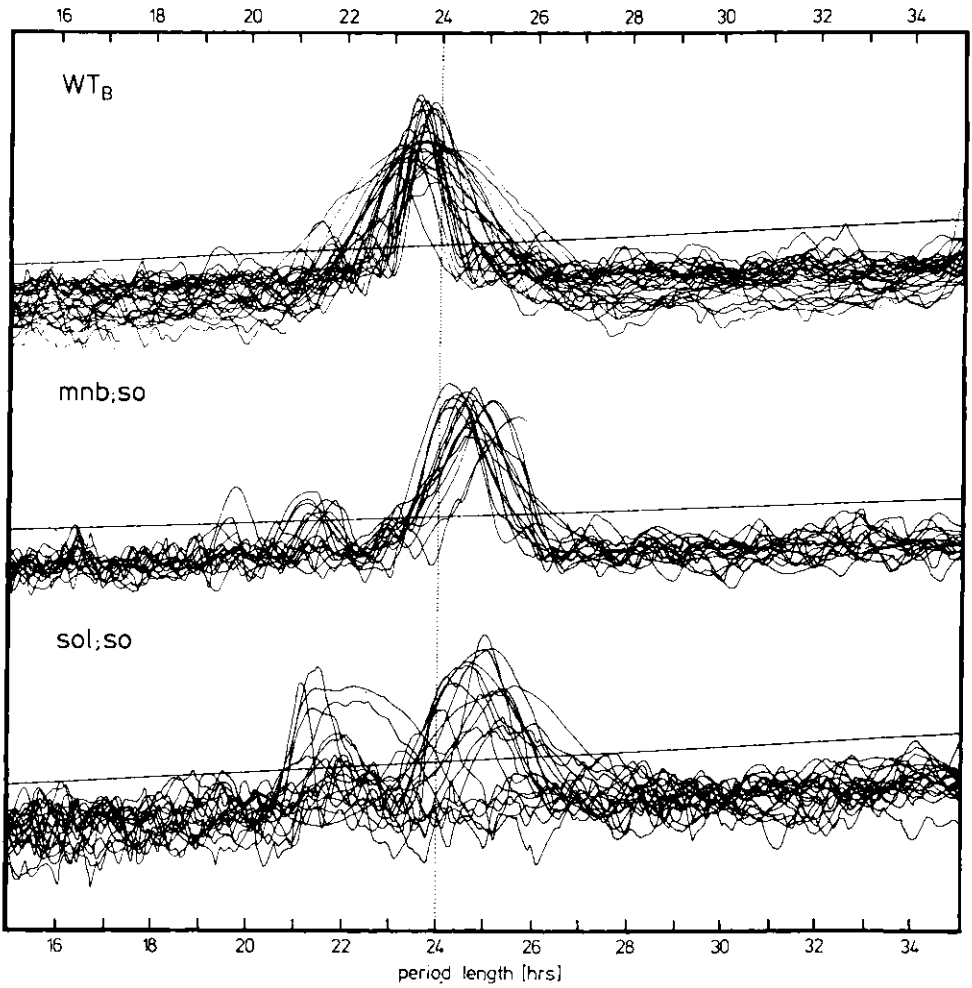


Fig. 4. Superimposed periodograms of all the recordings of wild-type (WT_B) flies and of the mutants *mnb;so* and *sol;so*. The confidence limits (95%) of the periodograms are given as an inclined line in each graph. In *mnb;so* and *sol;so* two significant period lengths are found. In *mnb;so* the longer period is clearly dominating; *in sol;so* both periods are of almost equal strength.

of flies showing two rhythms among those with complex rhythmicity. There was a high incidence of such flies in the double mutants *mnb;so* and *sol;so*. In *mnb;so* double mutants the long-period rhythm always dominated the records. The shorter-period rhythm was not always clearly visible and seldom found to occur throughout the whole record (Fig. 3b). Likewise, in *sol;so* the longer component was usually more pronounced (Fig. 3c), but animals that had a dominating shorter period or rhythms with equal strength were also found (Fig. 3d). Flies with "split" activity, in which the components had the same period but were out of phase by 180°, were found (Fig. 3a) among the

TABLE IV

MEAN (± S.E.M.) FREE-RUNNING PERIODS (IN RR) OF MUTANT AND WILD-TYPE *Drosophila melanogaster*

For flies showing two rhythms the period length of each is given. All period lengths are determined by periodogram analysis.

	n	Simple periodicity	*n*	Complex periodicity
Wild-type (Berlin)	53	23.6 ± 0.1	2	22.9 ± 0.1 24.7 ± 0.3
omb	16	23.5 ± 0.1		
lop	24	23.6 ± 0.1	1	22.5 24.8
mnb	13	23.5 ± 0.1		
sol	27	23.7 ± 0.1	2	23.1 ± 0.2 24.7 ± 0.2
so	74	24.1 ± 0.1*	14	22.9 ± 0.1/25.2 ± 0.1*
mnb;so			22	21.4 ± 0.2/25.1 ± 0.1*
sol;so			38	21.3 ± 0.1/25.5 ± 0.1*

* Compared to wild-type, period length significantly longer or shorter, respectively, at $\alpha' = 0.01$ (Mann–Whitney *U*-test).

flies with two components. This splitting was frequently found in *mnb;so* mutants and always originated from the longer rhythm. It is uncertain whether the shorter rhythm is involved in splitting. Fig. 3a shows signs of a continuation of the shorter rhythm after the rhythm with longer period had split.

Period length of the different mutants, as determined by periodogram analysis, is shown in Table IV. *so* Mutants with clear circadian rhythmicity have a significantly longer period length than the wild-type, *lop*, *omb*, *sol* and *mnb*. The period lengths of the short and long rhythms of flies which show two rhythms are quite similar in *so*, *sol;so*, and *mnb;so*.

Synchronization by LD cycles

Synchronization of locomotor activity rhythms after transfer from constant conditions to a LD 12 : 12 occurred rapidly in the wild-type (n = 15) and *so* mutant (n = 15), without any transients (cf. ref. 14). In LD most of the flies showed a bimodal activity pattern (compare Fig. 9). Activity began immediately after lights-on, decreased somewhat thereafter and increased again 3 h before lights-off. During the dark period the flies were almost inactive. The mutant *so* showed the same activity pattern as the wild-type[14]. These experiments were conducted with 400 lux during the light period. In this study, 3 wild-type flies were recorded as controls at 300 lux and 5 flies at 40 lux. All showed the same behaviour as described for 400 lux.

In 24 of the *sol;so* mutants activity was recorded in LD cycles with 300 lux. All flies were synchronized but showed, with 3 exceptions, a pattern different from that of the wild-type. Of two activity components found, one began some time before lights on (the

phase angle $\psi = 2.55 \pm 0.32$ h), the other between 3 h and 0 h before lights-off ($\psi = 1.66 \pm 0.17$ h), extending 1–4 h into the dark period (Figs. 5, 6, 7a). Mean activity time (α) was 4.60 ± 0.21 h for the lights-on component and 4.10 ± 0.23 h for the lights-off component. The latter was always more pronounced. Activity was almost absent between both components, with the exception of a few flies which were active preferentially during the light period or, more frequently, during the dark period.

At 40 lux all of the 28 flies studied were synchronized. The lights-on component was not always found at this intensity, and the lights-off component was broader ($\alpha = 6.00 \pm 0.35$ h) and started about 2 h earlier ($\psi = 3.68 \pm 0.24$ h) than at 300 lux (Figs. 7b, 8). These differences were significant (Mann–Whitney U-test, $\alpha' = 0.001$).

The observed activity patterns in LD are compiled in Table V. Further reduction of light intensity in steps down to 1 lux did not change the pattern in 3 out of 6 flies. Two flies which synchronized like the wild-type at 40 lux exhibited two components of activity at 1 lux, a weaker one at lights-on and a more pronounced one at lights-off. One animal which showed two components of activity at 40 lux had only a broadened lights-off component remaining at 1 lux.

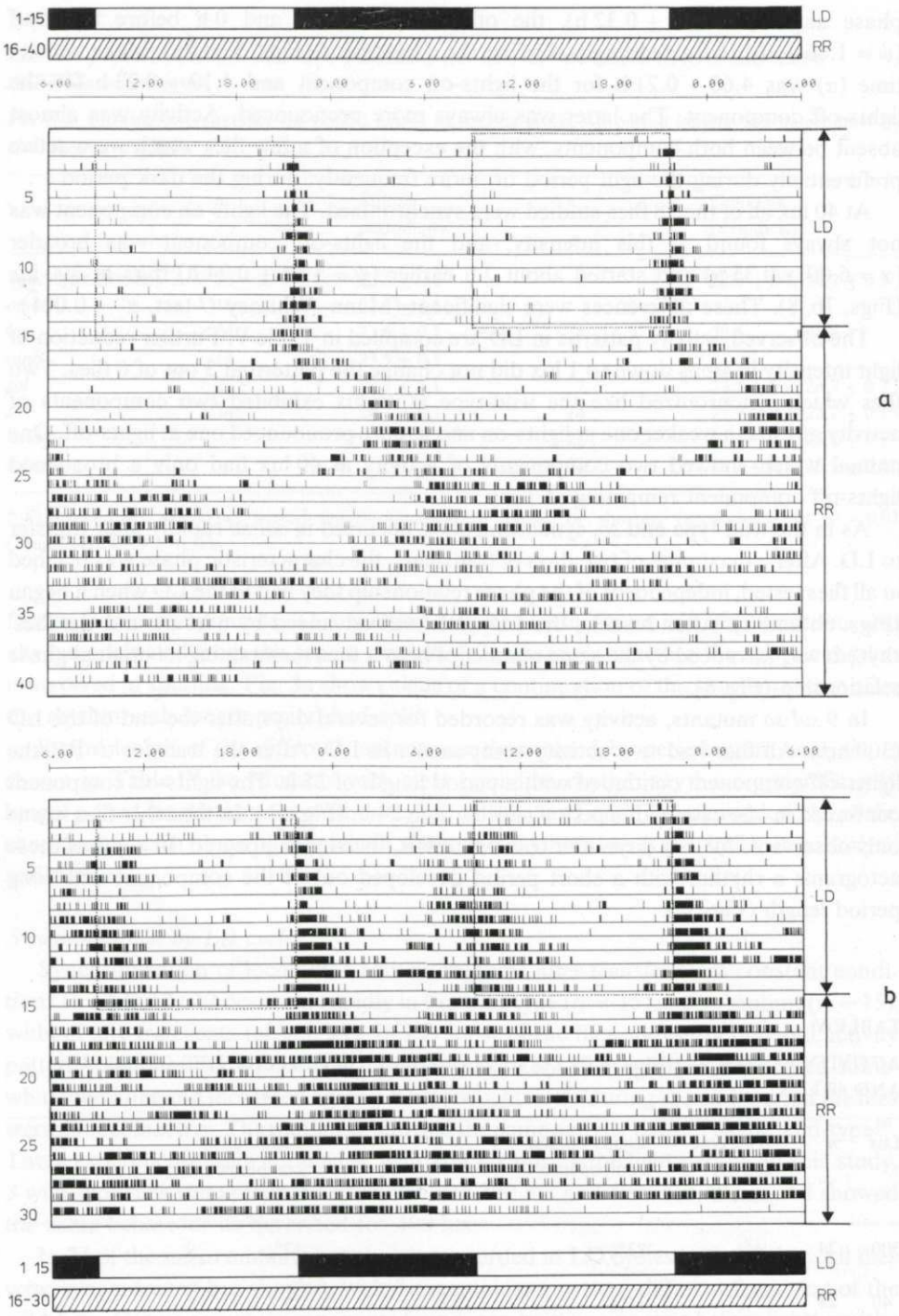
As in the wild-type and *so*, synchronization occurred in *sol;so* rapidly after transfer to LD. After a maximum of two days of transients, the characteristic phase was reached in all flies tested, independent of the phase relationship they had to the LD when it began (Figs. 6b and 7). After 2 weeks the LD cycle was advanced by 6 hours and the flies' rhythm was advanced by the same number of hours, thus maintaining its original phase relationship (Fig. 8).

In 9 *sol;so* mutants, activity was recorded for several days after the end of the LD (300 lux). All flies had two activity components in LD. After the transfer to RR the lights-off component continued with a period length of 25 h. The lights-on component continued in one case with a period shorter than 24 h. (Fig. 5b). In the other flies it was only observable for 1–2 days after transfer to RR, then it disappeared. In some of these actograms a rhythm with a short period developed out of the component with long period length (Fig. 5a).

TABLE V

ACTIVITY PATTERNS OF *sol;so* IN LD 12 : 12 UNDER LIGHT INTENSITIES OF 300 LUX AND 40 LUX

Lux	n	Wild-type activity pattern	Lights-on and -off components	Lights-off component only
300	24	4%	83%	12%
40	28	11%	11%	79%


1-15
16-40
LD
RR
6.00
12.00
18.00
0.00
6.00
12.00
18.00
0.00
6.00
5
10
15
20
25
30
35
40
LD
RR
a
6.00
12.00
18.00
0.00
6.00
12.00
18.00
0.00
6.00
5
10
15
20
25
30
LD
RR
b
1 15
16-30
LD
RR

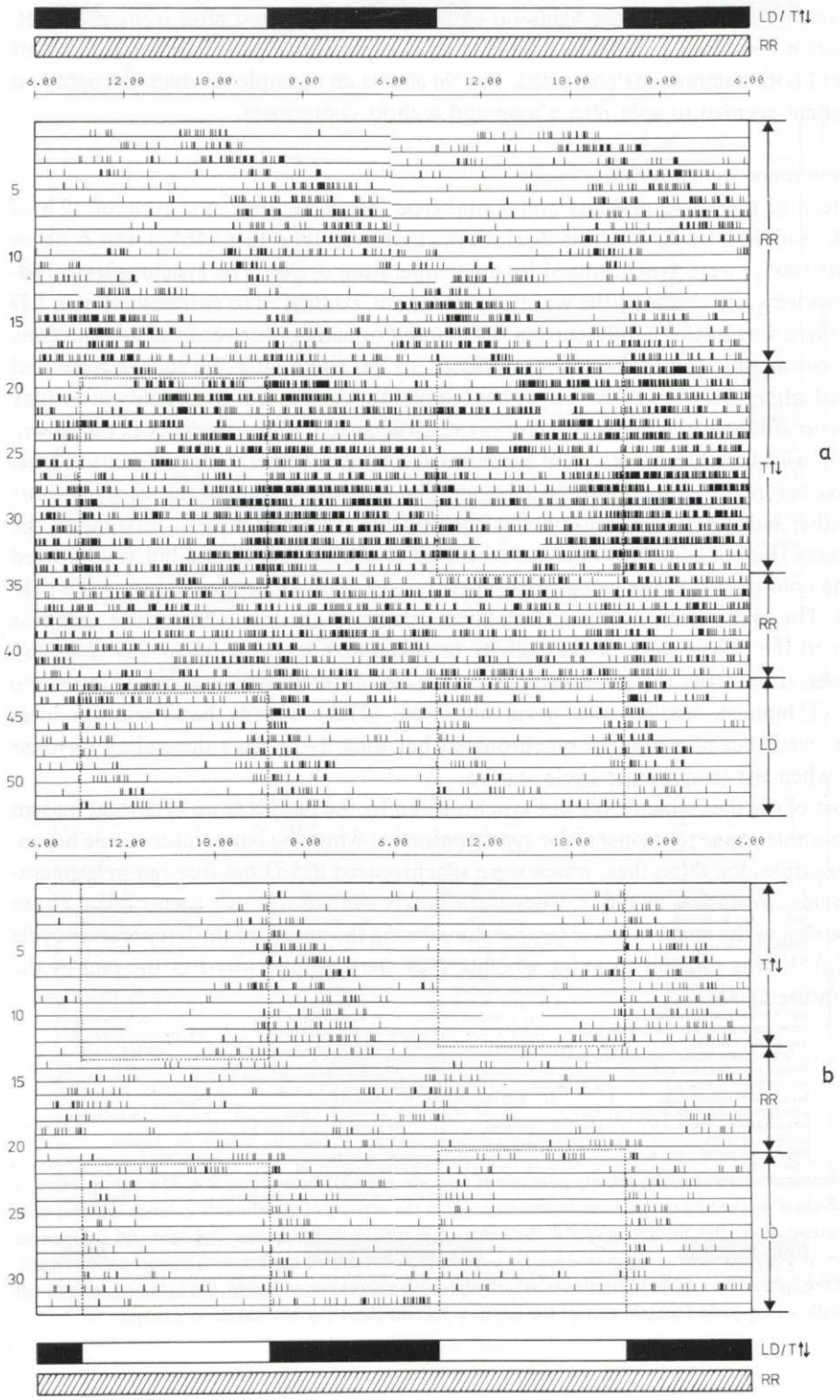
In some wild-type flies the lights-on component disappeared after transfer to RR. In others it continued parallel to the lights-off component (Fig. 9a), or countercurrent to it until both components coincided. Fig. 9b shows an example in which the lights-on component seemed to split into a long and a short component.

Synchronization by temperature cycles

Fifteen of the *sol;so* mutants and 9 wild-type flies were kept in a cycle of 12 h of 20.2 °C and 12 h of 22.2 °C for 7–16 days. Five wild-type flies (56%) and 6 *sol;so* mutants (40%) were synchronized by the temperature cycles. The synchronized wild-type flies were active during the warmer period; but, compared to entrainment in a LD cycle, there was less of a difference in the amount of activity between the two portions of the entraining cycle. Often flies continued to be active after the temperature had dropped and began to be active before the temperature increased. Bimodality of activity was never observed. Whether synchronization occurred with transients is unknown, since all wild-type flies which were synchronized by the temperature had already been in phase before the temperature cycle began. The synchronization of the *sol;so* mutants was rather indistinct, and free-running components were still present in some of the actograms (Fig. 6a). Activity was not restricted to the warmer period but was delayed into the cold period to the extent that, in two cases, activity occurred during the cold period. The phase angle of the activity onset relative to the low temperature shift was similar to the phase angle of the activity onset relative to lights-off in LD (Fig. 6b). However, the activity rhythm in temperature cycles differed from that in LD in several ways: (1) biphasic activity was never observed; (2) the activity band was broader; (3) flies were not immediately synchronized but took 5–10 days depending on their phase when the temperature cycle started.

Most of the flies which were not synchronized by the temperature cycle had had an unfavourable phase relationship for synchronization when the temperature cycle began. Two examples for *sol;so* flies, which were synchronized in LD but free-ran in temperature cycles, are shown in Fig. 7. They were slowly shifting towards their typical phase relationship to the onset of lower temperature during the course of the temperature cycle (Fig. 7a). It was difficult to judge whether they were synchronized at the end of the temperature cycles.

Fig. 5. Actograms showing the activity patterns of *sol;so* in LD (300 lux) and in RR. The light portion of the LD cycle is marked by dotted lines to emphasize that the activity of the mutants extends into the dark period. a: two days after beginning of RR the lights-on component disappears; the lights-off component continues as a rhythm with a long period length, out of which develops a rhythm with a short period length. b: in RR the lights-on component continues as a rhythm with a short period length, the lights-off component as one with a long period length. Out of the latter, a second short rhythm seems to emerge.


LD/ T↑↓
RR
6.00
12.00
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45
50
RR
T↑↓
RR
LD
a
6.00
12.00
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0.00
6.00
12.00
18.00
0.00
6.00
5
10
15
20
25
30
T↑↓
RR
LD
b
LD/T↑↓
RR

DISCUSSION

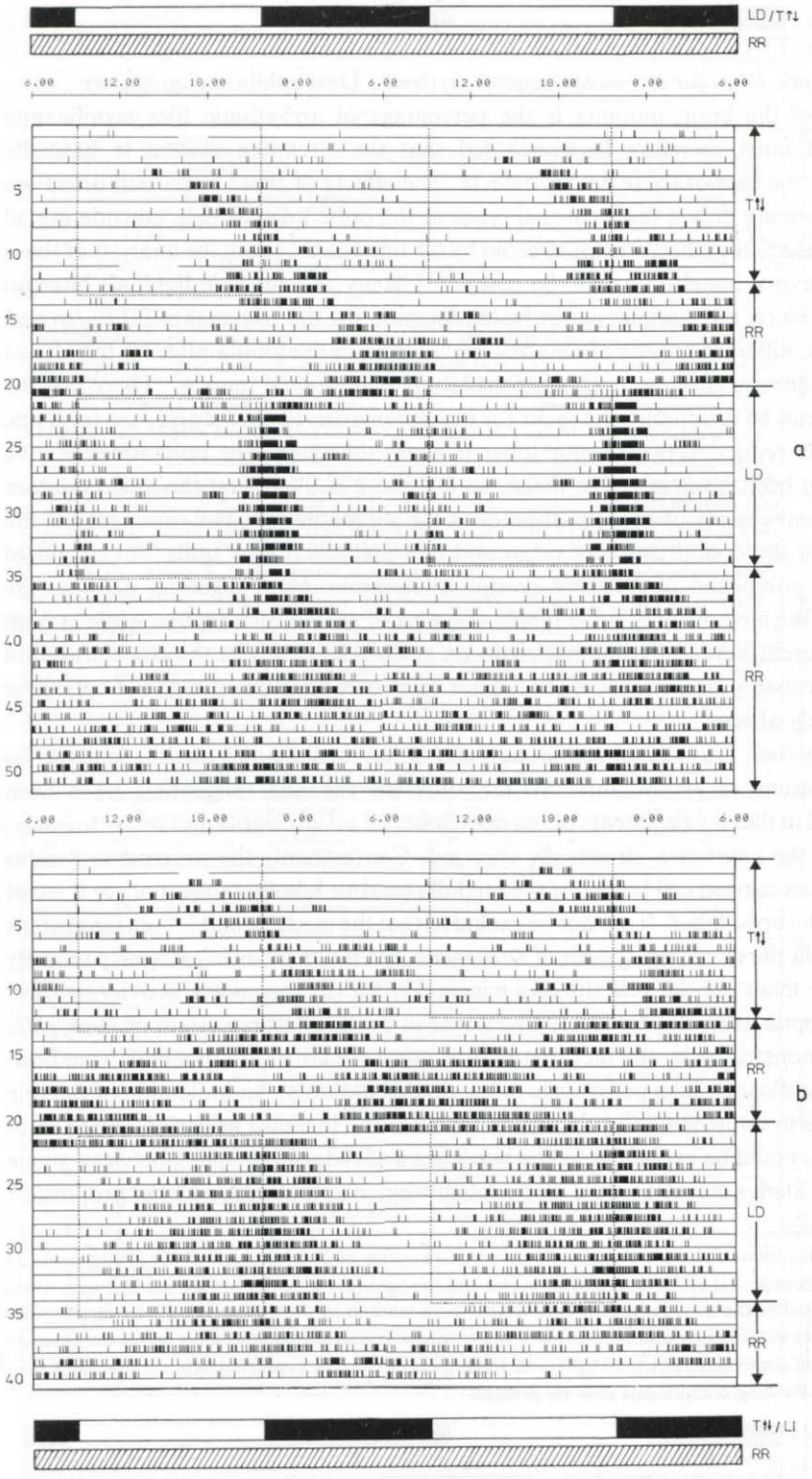
Role of the optic lobes for locomotor activity rhythm in Drosophila melanogaster

In none of the brain mutants is the percentage of arrhythmic flies significantly increased. It must therefore be concluded that the circadian system is basically functional in the face of these neural defects. The effects of these brain mutations are such that there are only a few neuronal types in the optic lobes which, considering all the genetic defects together, are not affected by the mutations. From the analysis of these mutations, several conclusions can be made. (1) Many *so* mutant individuals have no lamina. Therefore, the lamina cannot be the location for the pacemaker.(2) In *lop* and *omb* mutants, different groups of neurons are missing in the lobula plate. If the effects of the mutations are additive, nothing of the lobula plate should remain[8]. Therefore, the lobula plate can be dismissed as a locus for the pacemaker. (3) In *sol* and *mnb* mutants, the amount of reduction of the optic lobes is about the same. The optic lobes of *mnb* flies have not been investigated in detail, so far, but it is likely that the *mnb* mutation affects different classes of neurons than does the *sol* mutation[8]. The rudiments of the optic lobes of the double mutants *sol;so* and *mnb;so*, which look quite similar, might therefore be composed of different groups of neurons. (4) Tangential neurons are preserved in the medulla and in the lobula complex of *so* and *sol* mutants, while in both mutants different kinds of columnar neurons are missing[9]. (5) In the double mutant *sol;so* (and probably also in *mnb;so*), columnar neurons with cell bodies in the optic lobe are completely absent[9].

Taken together, only the tangential neurons of the medulla and the lobula could be common neurons in all mutants. So far, only the medulla tangentials have been distinguished in the tiny rudiments of the optic lobes of *sol;so* double mutants. However, the shape of the neurons is drastically changed. Consequently the pacemaker resides either in the unchanged cell bodies of the medulla (and/or lobula) tangentials or it is not localized in the optic lobes. It cannot be excluded that the medulla and lobula tangentials are involved in the circadian system of *Drosophila*, but it would seem extremely unlikely that the optic lobes are the sole site of a pacemaker for the locomotor activity rhythm.

That the optic lobes do, however, play a role in the oscillatory system of *Drosophila* is clearly demonstrated by the fact that two or more circadian components have been found in a significantly high percentage of the double mutants. An indication of the role of optic lobes in influencing the circadian system is the increase in period length in *so* mutants. This could be explained by the hypothesis of reduced coupling between single oscillators[14]. Such a surmise would also explain why, in *so* mutants, a higher proportion

Fig. 6. Synchronization of *sol;so* mutants with temperature cycles of 20.2 °C : 22.2 °C (T↑↓) and with LD (300 lux). The warm period of the temperature cycles and the light period of the LD are marked with white bars above and below the actograms. They are furthermore marked by dotted lines in the actograms. The activity of the flies in the temperature cycles has a similar phase relationship to the onset of low temperature than the lights-off component in LD to lights-off. In temperature cycles synchronization is less clear than in LD, and free-running components may be present.


LD/T↑↓
RR
6.00
12.00
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a
6.00
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RR

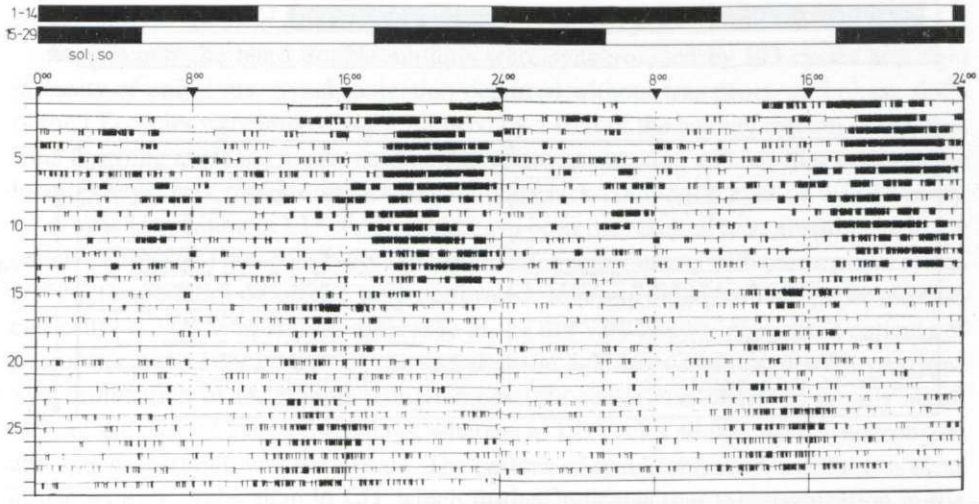

Fig. 8. Activity records of a *sol;so* mutant in LD (40 lux). On day 15 the LD was advanced by 6 h. The fly advanced its activity by the same number of hours, while transients were practically absent.

of flies exhibit two rhythms simultaneously than in the wild-type. Finally, the double mutants consisted almost exclusively of such flies.

Since the period becomes longer and the rhythms more loosely coupled with increasing reduction of the volume of the optic lobes (wild-type > *so* > *sol;so* = *mnb;so*), it seems to be the sum of intact neurons rather than a small localized area of neurons which is responsible for a normal rhythm and a normal period.

The manner in which the optic lobes influence the period of the rhythm and its tendency towards splitting into two or more components still remains speculative. Two hypotheses are proposed here: (1) The locomotor activity could be controlled by a population of oscillators, each of which consists of single neurons in the brain and in the optic lobes. A reduction of optic lobe neuropil might change the interaction between the remaining neurons and could lead to a change in period length and in stability of the rhythm. In the isolated eye of *Aplysia*, the free-running period of the compound action potential (CAP) is indeed dependent on the number of intact neurons: period length becomes shorter with increased reduction of the tissue[16]. (2) The optic lobes could act as a kind of coupling device on oscillators located in the central brain. A reduction of the optic lobes would reduce the coupling strength between these oscillators. A reduction in coupling strength would first lead to a lengthening of the period and finally to an uncoupling of component oscillators (compare ref. 15).

Fig. 7. Activity records of *sol;so* mutants which were synchronized in LD (300 lux, in a and 40 lux, in b, but not in temperature cycles (20.2 °C : 22.2 °C). Labelling is as in Fig. 8. a: the fly's activity is shifted towards the onset of low temperature during the course of the temperature cycle and might be synchronized towards the end of the temperature cycles. b: the fly is clearly free-running in the temperature cycle.

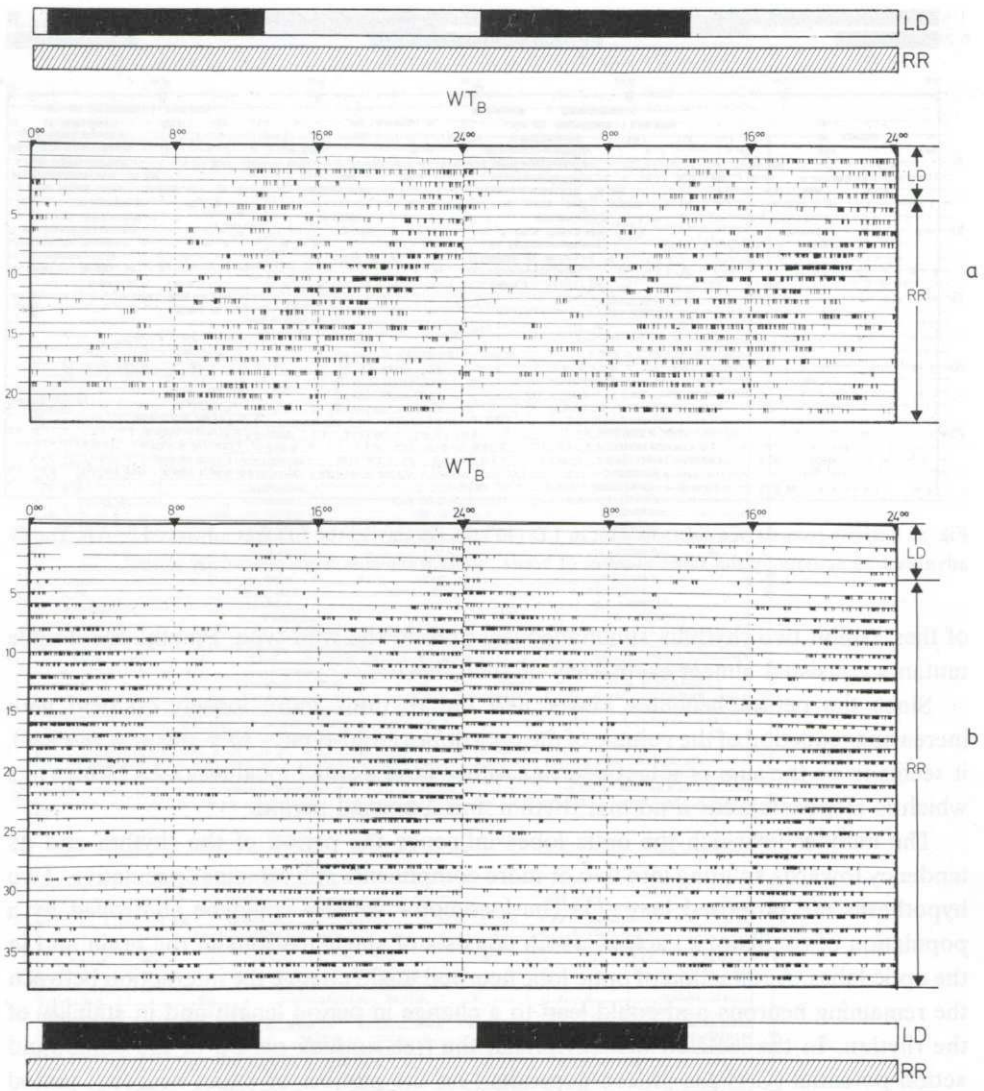

Fig. 9. Actograms of wild-type (WT_B) flies in LD (400 lux) and RR. In LD activity is restricted to the light period, and the flies show bimodal activity patterns. a: in RR both activity components free-run parallel to each other. b: in RR the lights-on component splits into a short and a long period rhythm.

Neither hypothesis can presently be excluded. Both hypotheses assume the existence of more than one oscillator controlling locomotor activity in *Drosophila*. The presence of multiple periodicities in occasional wild-type flies strengthens this interpretation. Thus, the mutants might be characterized by a reduced stability in the whole circadian system and may serve as tools to study multioscillator systems (compare ref. 14).

Synchronization of blind flies in LD and in temperature cycles

Surprisingly the blind double mutants were synchronized by LD cycles at a light intensity of only 1 lux. Synchronization occurred without transients, and phase shifts of the LD cycles were immediately followed by a shift of the activity rhythm of the flies. This is strong evidence for an extraocular photoreceptor (cf. ref. 36) that controls the locomotor activity rhythm, since these mutants lack compound eyes as well as ocelli.

A synchronization in LD cycles could have been the result of heat absorbance in the cuticle. To exclude this possibility synchronization experiments with temperature cycles of 2 °C temperature difference were performed. It seems unlikely that light absorption can cause a 2 °C temperature difference in the not very heavily pigmented cuticula of *Drosophila*, especially in view of the fact that the light intensities used were very low. Synchronization of the rhythm using temperature cycles was observed in only about 50% of the flies, and took 5–10 days, whereas in LD cycles all flies were synchronized and synchronization was immediate. The pattern of synchronization was far less clear in temperature cycles than in LD, which further indicates that synchronization in the LD cycles is the result of the light and not of a temperature change.

Extraocular photoreceptors are frequently found for synchronization of rhythms in invertebrates[23,27,36]. It has been shown that compound eyes and ocelli do not contribute to the synchronization of the eclosion rhythm in *Drosophila*[4,42,43]. An indication that compound eyes and ocelli are not necessary for synchronization of the locomotor activity rhythm comes from the observation that the eyeless mutant *so* could be synchronized by LD cycles[14].

Whereas *so* and the wild-type show a morning and evening peak in locomotor activity coinciding with the light period[11,21], in the double mutants the lights-on component begins before the light period, and the evening activity extends into the dark period. Since in both *so* and the double mutants compound eyes and ocelli are lacking, this difference must be due to the further reduction in the size of the optic lobes. Two explanations are possible: either the optic lobes can perceive light directly and therefore *sol;so* perceives less light than *so*, or the circadian system in *sol;so* has changed more than in *so* due to the larger reduction in the size of the optic lobes, and this change in the circadian system causes the changed synchronization behaviour. The first explanation is unlikely, since absorbing pigments have not been found in the optic lobes as yet. In accordance with the second explanation is the finding that *sol;so* mutants show similar entrainment patterns in temperature and LD cycles. Activity bouts extend into the cold period of the temperature cycle and into the dark portion of the LD cycle. In contrast, the activity of the wild-type was restricted to the subjective day in both temperature and LD cycles. Thus, the changed phase relationship of the lights-on and lights-off components seem to be caused by the reduced size of the optic lobes. The next point to be clarified is the nature of the lights-on and lights-off components. Do they correspond to the short and long rhythms respectively, found under free-running conditions? If so, the unusual phase relationship of both components to lights-on and lights-off is understandable. The phase relationship of an oscillator to an external

Zeitgeber depends on the frequency of the oscillator. The activity of an oscillator with a long period length is shifted to the end of the light period and may also extend into the dark period if the period length of the oscillator is very long. Similarly, an oscillator with a short period length is synchronized at the beginning of the light period and activity may already begin before lights-on. If this explanation is correct, the lights-off component should continue with a long period after transfer to RR conditions and the lights-on component with a short period. The lights-off component did indeed continue always as a rhythm with long periods. The lights-on component, on the other hand, continued only in one case as a rhythm with short periods; in the other cases it disappeared one or two days after onset of RR. This was not surprising, since under free-running conditions the short period rhythm was often weakly expressed and sometimes only visible around its "crossing points" with the long period rhythm. Thus, after the transfer from LD to RR it might free-run without being expressed in activity until it crosses the long period component. This explains the fact that in some flies the short-period rhythm seems to develop out of the long-period rhythm.

The lights-on component tends to disappear not only under free-run conditions, but also if the Zeitgeber is rather weak, as it was the case in the temperature cycles employed here and in LD with light intensities of 40 lux and less. Under these conditions the evening peak began earlier and became broader. Mean activity stayed the same. This suggests that the lights-on and lights-off component fused to form a common evening peak. Apparently the Zeitgeber was too low to synchronize the lights-on component. Instead this component free-ran without being expressed until it coincided with the synchronized lights-off component. Under free-run conditions a coupling of the lights-on component (short periods) with the lights-off component (long periods) is impossible because a period of 25 h lies outside the range of entrainment for the lights-on component. However, under synchronized conditions the lights-off component has a period of 24 hours, and since this is within the range of entrainment of the short period, both remain coupled. This explains the broadened activity and its phase shift towards lights-on.

More experiments are necessary to clarify whether separate oscillators with different period lengths govern morning and evening bouts of activity as was proposed for vertebrates[29,30]. Probably, the circadian system of *Drosophila* controlling locomotor activity is even more complicated. More than two components seem to exist, as shown by the occasional occurrence of more than two free-running components in RR and the splitting of the lights-on component (wild-type fly; Fig. 7b) or lights-off component *(sol;so* mutant; Fig. 5b) immediately after transfer from LD to RR. The circadian system might be composed of two groups of oscillators rather than of two single oscillators.

As mentioned before, the lights-on and lights-off components are also found in the bimodal activity pattern of the wild-type, although with a different phase relationship to the LD. In RR the lights-off component always continued, as did the lights-on component in many cases. This similarity in the behaviour of the wild-type and the mutants with heavily reduced optic lobes indicates, again, fundamentally identical structures of the oscillatory system in flies of these different genotypes.

Comparison of the multioscillator system of Drosophila *with other insects*

Locomotor activity in *Drosophila* is apparently not controlled by a single pacemaker: the occurence of flies with two or more free-running components is better explained by the participation of several oscillators in the control of locomotor activity. The frequent presence of two activity components with a similar free-running period in all mutants indicates the existence of two oscillator groups: one with a mean period length of about 25 h and a second with a period length of about 22 h. These two components are apparently not specific to *Drosophila*. They are also observed in *Musca domestica*[15], *Calliphora stygia*[37], *Calliphora erythrocephala* (my unpublished results) and *Culiseta incidens*[3]. However, the nature of these two populations of oscillators is still unknown.

In other insects splitting into two components is also taken as a sign for control by two oscillators. Under certain conditions splitting was observed in the locomotor activity rhythm of *Leucophaea maderae*[38] and *Hemideina thoracica*[2], in the singing rhythm of *Teleogryllus commodus*[39–41], and in the ERG rhythm of *Blabs gigas*[18].

These two components are usually explained as being the result of the bilateral organization of the brain[18,39]. In the case of *Drosophila*, *Musca*, and *Calliphora* this explanation is, however, unlikely since here the period lengths of the two components are quite different and the mutations (in *Drosophila*) and surgical manipulations (in *Musca*) affect both halves of the brain to the same extent.

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