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Circadian rhythm of the locomotor activity in *Drosophila melanogaster* and its mutants 'sine oculis' and 'small optic lobes'

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ABSTRACT. The locomotor activity patterns of wildtype *Drosophila melanogaster* and the mutants *so* (sine oculis) and *sol* (small optic lobes) were investigated. In all strains the proportions showing circadian rhythmicity, arrhythmicity and more complex patterns which could not be thus classified were similar. The occurrence of abnormal activity patterns is thus not a property of the mutation as previously claimed (Mack & Engelmann, 1981). In flies with a distinct circadian rhythmicity, the period lengths (τ) varied between strains. τ for wildtype Italy and the mutant *so* was longer than for wildtype Berlin and the mutant *sol*. As different τ's have been reported by others, τ does not seem to be closely determined for *Drosophila melanogaster*. Many flies exhibited two rhythms simultaneously, one with τ shorter and one with τ longer than 24 h, apparently implying two-oscillator control of the locomotor activity. The eyeless *so* mutants were entrained by LD cycles, so the compound eyes are not necessary, and *so* must possess the relevant photoreceptor(s) elsewhere. This may therefore also be true for the wildtype. Histology of the *so* adults revealed no correlation between the degree of reduction in the medulla and the occurrence of abnormal activity patterns. Since the only structures common to the medulla of *so* and *sol* are known to be large tangential cells, it is concluded that either they are of importance for the rhythmic system, or the oscillator(s) controlling locomotor activity is (are) not located in the medulla.

Key words. *Drosophila melanogaster*, circadian rhythm, mutant behaviour, rhythm mutant, genetics, behavioural genetics, optic lobes.

Introduction

Circadian rhythms control development, behaviour and metabolism in many insects (Saunders, 1982). Locomotor activity is easy to record and often used as the hands of the underlying clock. In attempts to localize the pacemaker, the lobula, the most proximal structure of the optic lobe, has been found to be the best candidate in cockroaches. Experiments involving lesions in the optic lobes, severing of the optic tracts, electrocoagulation and transplantation have led to these conclusions (Roberts, 1974; Sokolove & Loher, 1975; Nithiitsutsuji-Uwo & Pittendrigh, 1968; Page, 1978, 1982).

Likewise, in the beetle, *Carabus problematicus*, an aperiodic activity pattern is induced after removing the optic lobes (Balkenohl & Weber, 1981). In the beetle *Anthia sexguttata* severing the optic lobes from the brain does

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not affect the circadian rhythm of sensitivity in the compound eye (Fleissner, 1982), indicating the existence of an oscillator in the optic lobes. The same has been found for the cricket *Gryllus bimaculatus* which has an electroretinogram rhythm even after the optic tracts are severed (Tomioka & Chiba, 1982).

On the other hand, in silkmoths, rhythmic flight activity continues after removal of the optic lobes, but is impaired after excision of the cerebral lobes (Truman, 1974).

In *Drosophila* there are indications that at least the pathway mediating the signals of the circadian pacemaker is different from those in cockroaches. Transplantation of brains from rhythmic donors into the arrythmic (mutant) recipients apparently induces circadian rhythmicity in locomotor activity of the latter (Handler & Konopka, 1979). Humoral output of the pacemaker is thus implied, since neural connections are not formed between implant and host tissue.

Mack & Engelmann (1981) have reported that some individuals of the mutant *so* of *D.melanogaster* which lack ommatidia (Fig. 1) show arrhythmic locomotor activity. The lamina and most of the distal part of the medulla are lacking in this mutant (Fischbach, 1982; Power, 1943). It was therefore speculated that the circadian pacemakers of locomotor activity are located somewhere between the compound eye and the midline of the medulla. In mutants with strong reductions of the nervous structures here, the pacemakers would be absent, and consequently arrythmic behaviour in locomotion would result. This would clearly be in contrast to findings in cockroaches which implicate the lobula.

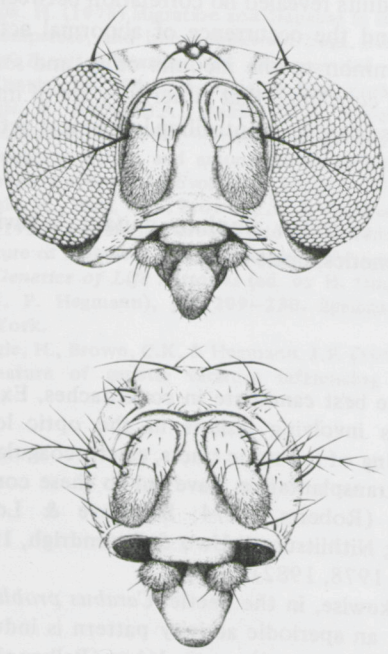


FIG. 1. Frontal view of the head of a wild type fly (above) and mutant *so* (below) with eyes completely absent.

To shed some light on this problem the following work was conducted. Besides studying the *so* mutant, we checked the activity patterns of two wild strains more extensively than has been done before. We also studied the mutant *sol* of *D.melanogaster*. (This mutant affects the medulla and the lobula, but not the lamina; Fischbach & Heisenberg, 1981.)

Material and Methods

Locomotor activity was recorded in the *D.melanogaster* mutants *so* and *sol*, in wild-types Berlin (WT_B) and Italy (WT_I), and in phenotypically wild type, *so*/WT_B crosses. *so* and WT_B were supplied by Dr Götz, Tübingen, *sol* by Dr Fischbach, Würzburg, and WT_I by Dr Sperlich, Tübingen.

so/WT_B was obtained by crossing a single individual of WT_B into the *so* stock about 2 years previously. The wild phenotype persisted in small but constant percentage of the latter population. Because of exchange of the genetic material and the smaller number of WT_B genotypes, flies possessing eyes are expected to have the genetic background of *so*.

The mutant *so* lacks ocelli and the compound eyes are reduced to varying degrees. In fully expressed mutants, ommatidia are completely missing (Fig. 1). The degree of expression depends on the rearing temperature (Milani, 1946). We found completely eyeless flies in about 50% of the offspring if reared at 20°C and in 78% if reared at 27°C. The mutation is localized at 2–57.1 (Milani, 1951).

The outer appearance of the mutant *sol* is identical to that of the wildtype. However, the optic lobes are reduced. This mutation is localized at the X-chromosome –60 (+1) (Fischbach & Heisenberg, 1981).

In most cases the rearing temperature was about 20°C and cultures were kept under a 12 h light:12 h dark (LD 12:12) cycle. In some cases, continuous white light (LL) or continuous red light (RR) was used for rearing two to three generations before recording of activity.

Locomotor activity was recorded in individual flies as described by Engelmann & Mack (1978) under (RR) at 20 ± 1°C. The flies were not older than 5 days after eclosion when an experiment started, and activity was then monitored for at least 7 days. The activity of flies which died earlier was not analysed. Some records lasted 40 days or longer. Animals which were sectioned for histological purpose were prepared after recording them for 16 days.

In some cases, locomotor activity was monitored under LD 12:12 (at 6×10^{-4} W cm^{-2}) for 15 days.

The period length (τ_{RR}) of the circadian rhythm of locomotor activity was determined by visually fitting a straight line through the onsets of activity in actograms (or, in cases where onsets were difficult to recognize, through the points of maximum activity). In addition, different methods of time series analyses such as periodogram, signal averaging, and complex demodulation were used (Martin & Brinkmann, 1976).

The histological appearance of some *so* mutants was studied once their activity patterns were known, and compared with wild-types. For histology, animals were narcotized by chloroform and mounted in a special metal block (Heisenberg & Böhl, 1979), fixed for 3 h in Carnoy, dehydrated for 3, 6 and 12 h in isopropanol, and embedded in paraplast (m.p. 58°C). Serial sections (7 μm) were cut, and stained with silver according to Blest (in Witte & Matthaei, 1980) with toludine blue or cresylviolet (Cook, 1974; Witte & Matthaei, 1980).

Results

The mutant *so* and WT_I showed rather unclear separation of activity and rest periods, but in all strains three types of flies could be distinguished: (a) animals which showed a clear circadian rhythm with a constant τ throughout the recording time (Fig. 2a); (b) animals which showed complex rhythmicity (Fig. 2b); (c) animals which were arrythmic (Fig. 2c).

Table 1 shows the distribution of these three types in the five strains. There was no significant difference in the percentages of flies found in each type between any of the different strains (χ^2 test). Fig. 3 superimposes the respective periodograms of each strain, and allows comparison of the variability of each population's rhythmicity.

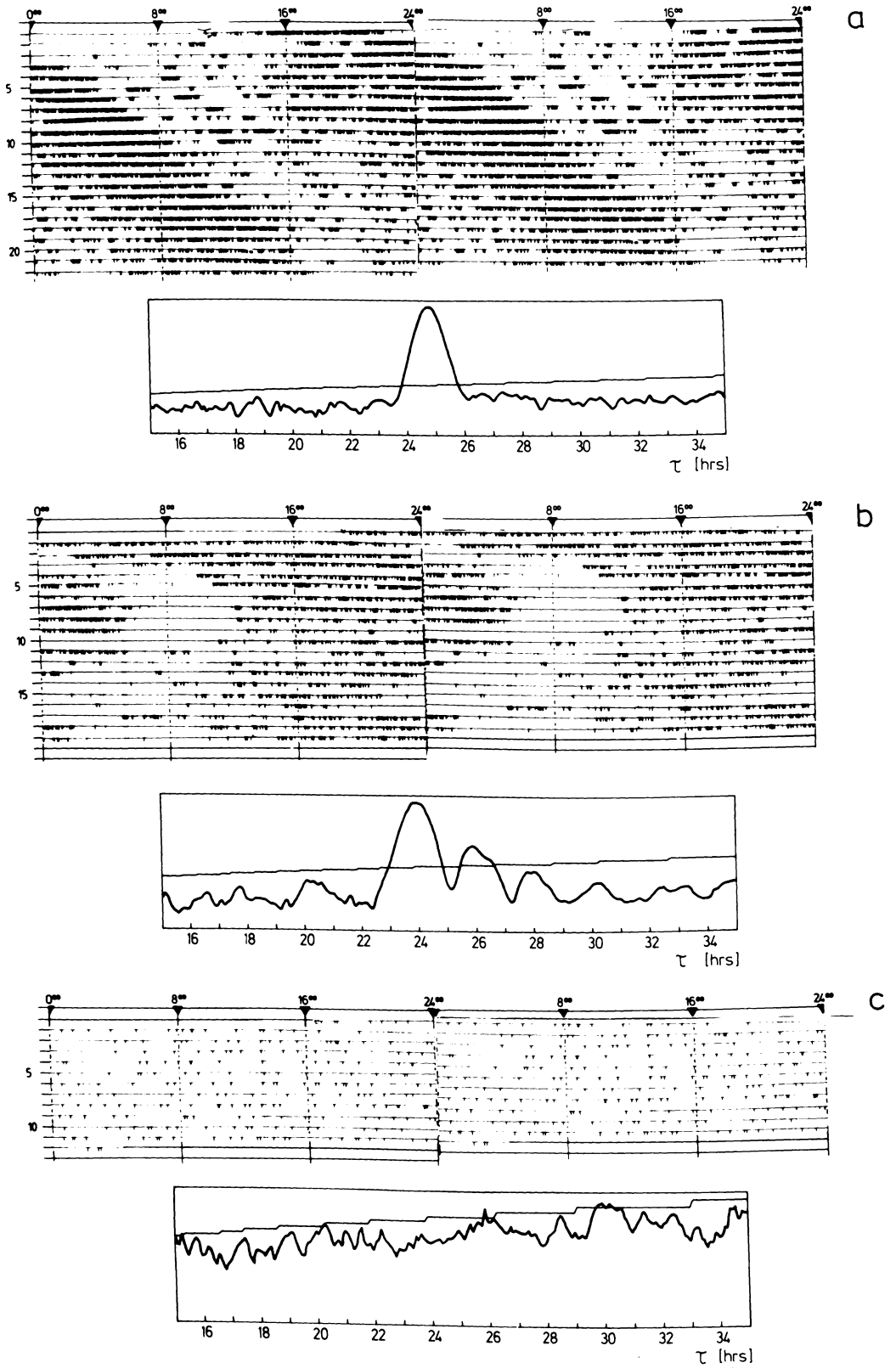
Type (a) animals, showing clear rhythmicity with constant τ

These type (a) activity patterns showed considerable variability of form (Fig. 4). Few files showed the distinct onset of activity and less distinct end of it (Fig. 4a) commonly found in the locomotor activity of animals. Typically, the end of activity was more pro-

TABLE 1. Percentage frequency distributions of the three types of circadian activity patterns found in mutants and wildtype *Drosophila melanogaster*.

		Percentage of:		
	n	Type a	Type b	Type c
so without ommatidia	109	67.9	25.7	6.4
so with remnants of ommatidia	12	75.0	16.7	8.3
so/WT_B	30	80.0	6.7	13.3
WT_B	33	81.8	6.1	12.1
WT_I	25	80.0	4.0	16.0
sol	36	83.3	13.9	2.8

n = total numbers of individuals recorded (excluding a small number of flies (< 10%) which showed almost zero activity).


a
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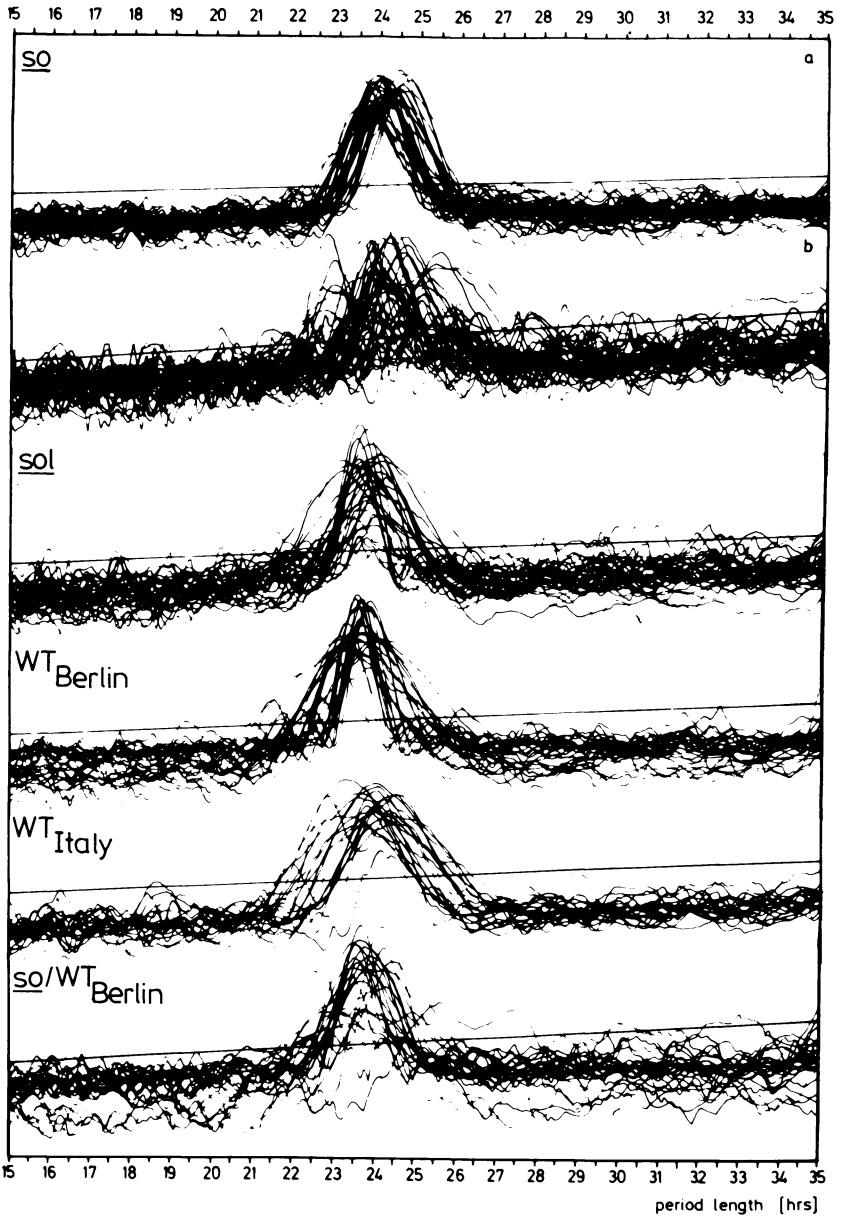


FIG. 3. Superimposed periodograms of all the recordings of the flies of the five strains studied. For the *so* mutant two such figures are given: one contains the periodograms of type (a) flies with a clear rhythm (Fig. 3a), the other contains periodograms of type (b) flies exhibiting more than one period, less clear rhythms, or periods deviating from the average (Fig. 3b).

FIG. 2. Examples of three typical locomotor activity patterns of *Drosophila melanogaster* flies: type (a), showing a single, constant τ_{RR} throughout recording time; type (b), showing equivocal rhythmicity; type (c), showing arrhythmicity. Below, the corresponding periodograms (faint stepped line, 95% CL).

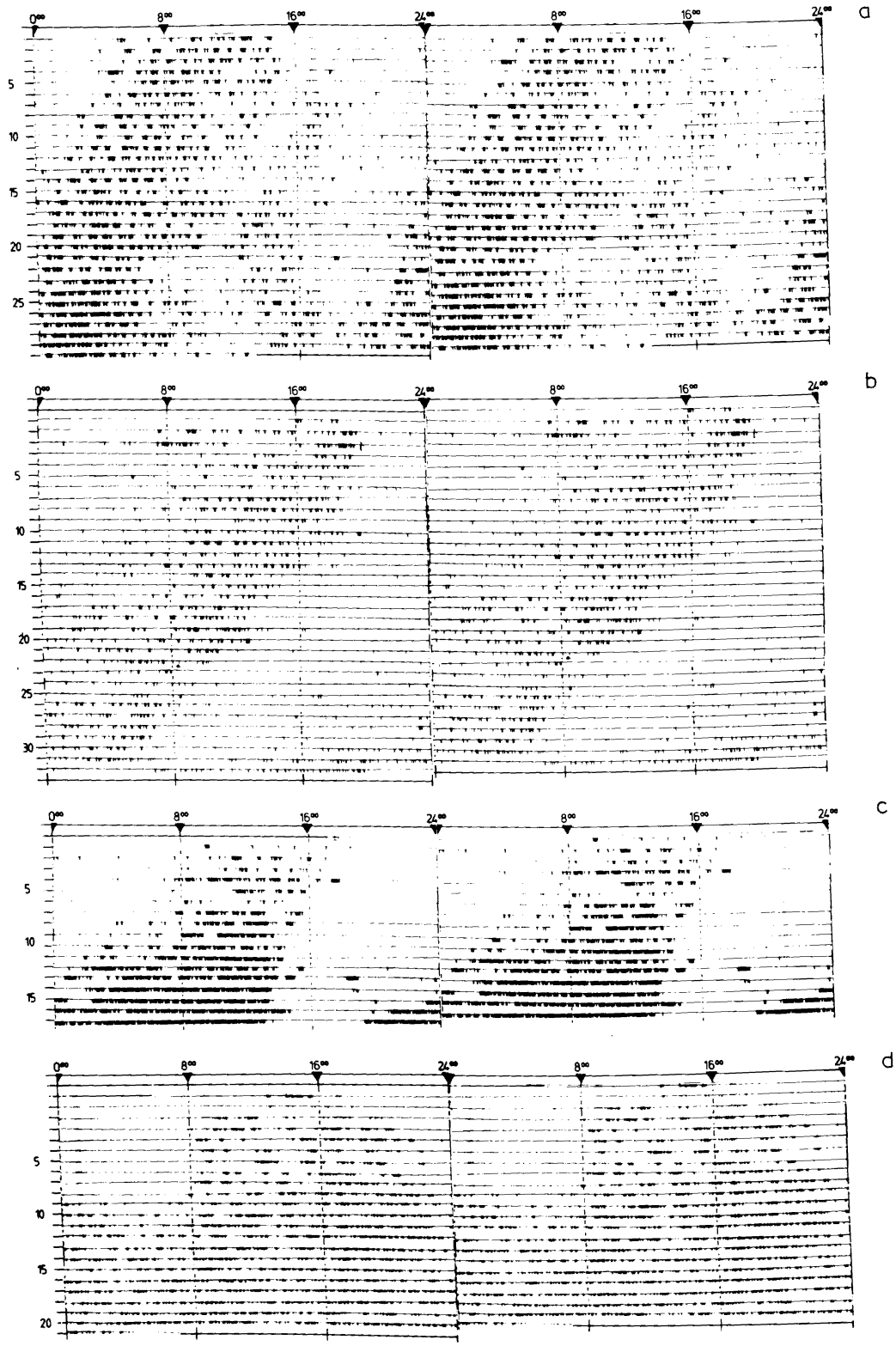


FIG. 4. Typical examples of the type (a) locomotor activity patterns: a and b are wildtype (Berlin), c and d *so* mutants. These patterns were found in all forms studied.

TABLE 2. Mean (± SE) free-running periods (in RR) of the different strains of *D.melanogaster*, determined by periodogram analysis.

	n	Period length
so without ommatidia	65	24.15 ± 0.04^{c}
so with remnants of ommatidia	9	24.10 ± 0.10^{c}
so/WT_B	23	23.60 ± 0.09^{c}
WT_B	25	23.60 ± 0.05^{a}
WT_I	15	24.05 ± 0.13^{b}
sol	27	23.80 ± 0.05^{c}

n = numbers of flies tested. Figures in each column followed by a different letter have significantly different variances (F-test).

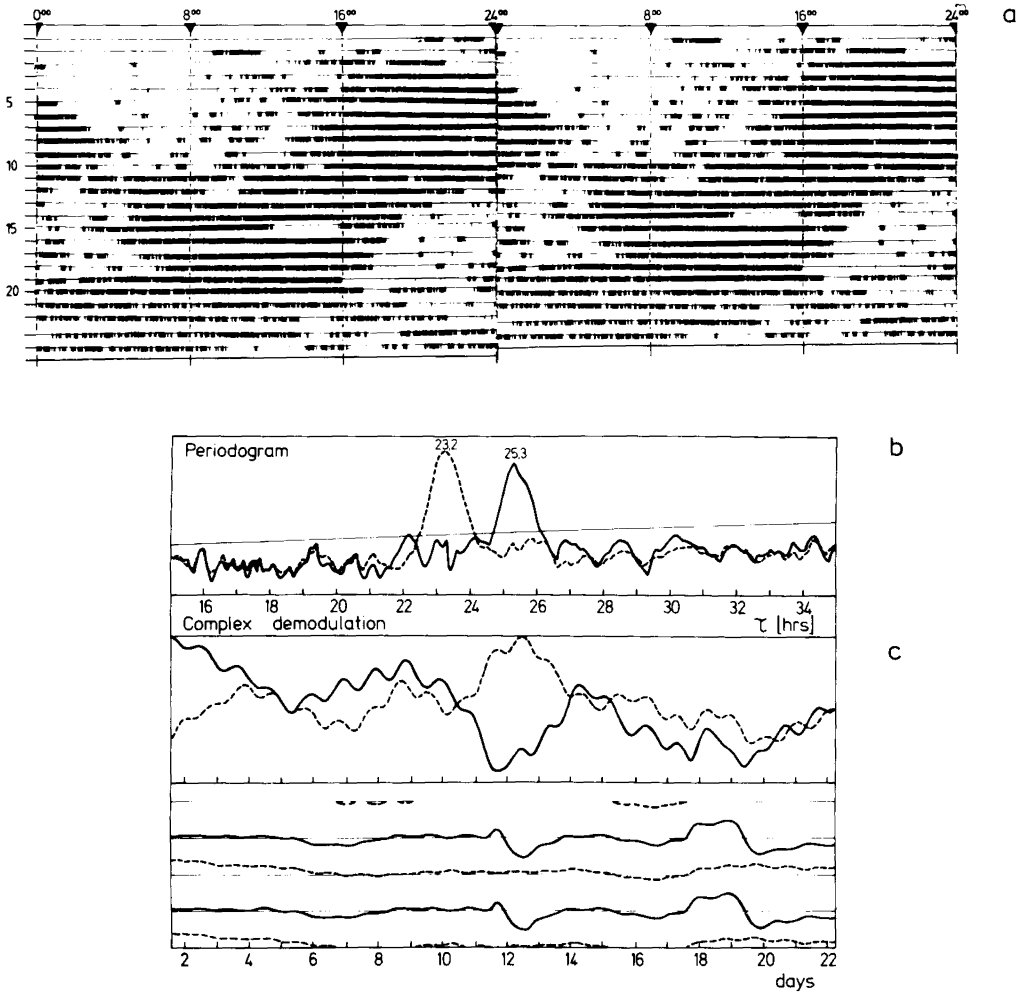


FIG. 5. Example of locomotor activity of animals showing two circadian rhythms simultaneously; (b) periodogram analysis, (c) complex demodulation (the broken curve refers to the residues after eliminating the 25.3-h component).

nounced (Fig. 4b), both in the wildtypes and in the mutants. Some flies had a constant activity : rest ratio throughout; others increased their activity time (Fig. 4c) often leading to a hyperactive arrhythmia (Fig 4d). Mean period τ of the different strains as determined by periodogram analyses are shown in Table 2.

τ for *so* flies did not differ according to rearing conditions: reared under RR at 20°C, τ was 24.2 ± 0.13 h (SE); reared under LL at 20°C, τ was 23.9 ± 0.08 h, reared under LD at 27°C, τ was 24.1 ± 0.05 h. These means are thus combined for Table 2. The variances of the mean τ were significantly different between the wildtypes Berlin and Italy, which also differed from the other groups. The variances of periods of *sol*, *so*/WT_B and *so* with and without ommatidia were, however, not different.

A comparison of the mean τs, revealed that τ of *so* was significantly longer than that of *sol*, WT_B or *so*/WT_B, but identical to τ of WT_I.

Type (b) animals, showing complex rhythmicity

Twenty-eight *so* mutants without ommatidia showed neither a clear free running rhythm of constant τ, nor certain arrythmicity. Ten had an activity pattern revealed by periodogram analysis to be composed of two distinct rhythm components (Fig. 5); four others appeared from visual inspection to be the same but were not proved to be so by mathematical analysis.

In all cases, one component had $\tau < 24$ h and one $\tau > 24$ h. The mean τ of the shorter component was 22.9 ± 0.09 h (SE) and that of the longer 25.5 ± 0.10 h ($n = 14$).

In a few *so* mutants, there were indications of more than two rhythm components (Fig. 6). Either one period was dominant (Fig. 6b) or the components were of more or less equal strength (Fig. 6a). Other *so* mutants showed activity patterns that were unclear or with τ changing or with the rhythm disappearing and becoming arrhythmic.

Such equivocal rhythmicity was also found in the other strains, including the wildtypes. An interesting case is shown in Fig. 7. Here the wildtype Italy suddenly exhibited activity during the dark phase of LD 12:12 after having shown the normal pattern for 4 days. This then changed back into the normal pattern after 8 days. Under RR the same animal showed two rhythms with periods of 22.9 and 24.4 h.

Table 3 indicates the frequency distribution of activity patterns with two rhythm components for the different strains.

Type (c) animals showing arrhythmia

Arrhythmic activity patterns were found as frequently in the two wildtypes and *so*/WT_B as in *so* and *sol* (Table 1). Often arrhythmic flies were less active than the rhythmic flies. The percentage of arrhythmic *so* flies was independent of their rearing conditions (LD, LL, RR and temperature).

Rhythm patterns in LD cycles 12:12

Fifteen flies of WT_I and fifteen *so* mutants were recorded for 10–15 days under RR conditions and then transferred to LD 12:12 set a few hours out of phase with the activity pattern of the majority of the flies. All flies,

TABLE 3. Percentage frequency distribution of flies showing activity patterns containing two circadian components of differing frequencies.

	n	% by periodogram analysis	% by visual inspection
so	109	9.2	12.8
so/WT_B	30	3.7	3.7
WT_B	33	0.0	6.5
WT_I	25	4.0	4.0
sol	36	2.8	5.6

n = total number of flies recorded.

including the *so* mutants, re-entrained almost immediately and without transients (Fig. 8); On return to RR from LD, τ was often changed, as here.

During entrainment to LD 12:12, a bimodal distribution of activity was often visible with activity more pronounced after lights-on and for several hours before lights-off. Some animals, which were almost inactive in RR nevertheless entrained normally to LD 12:12,

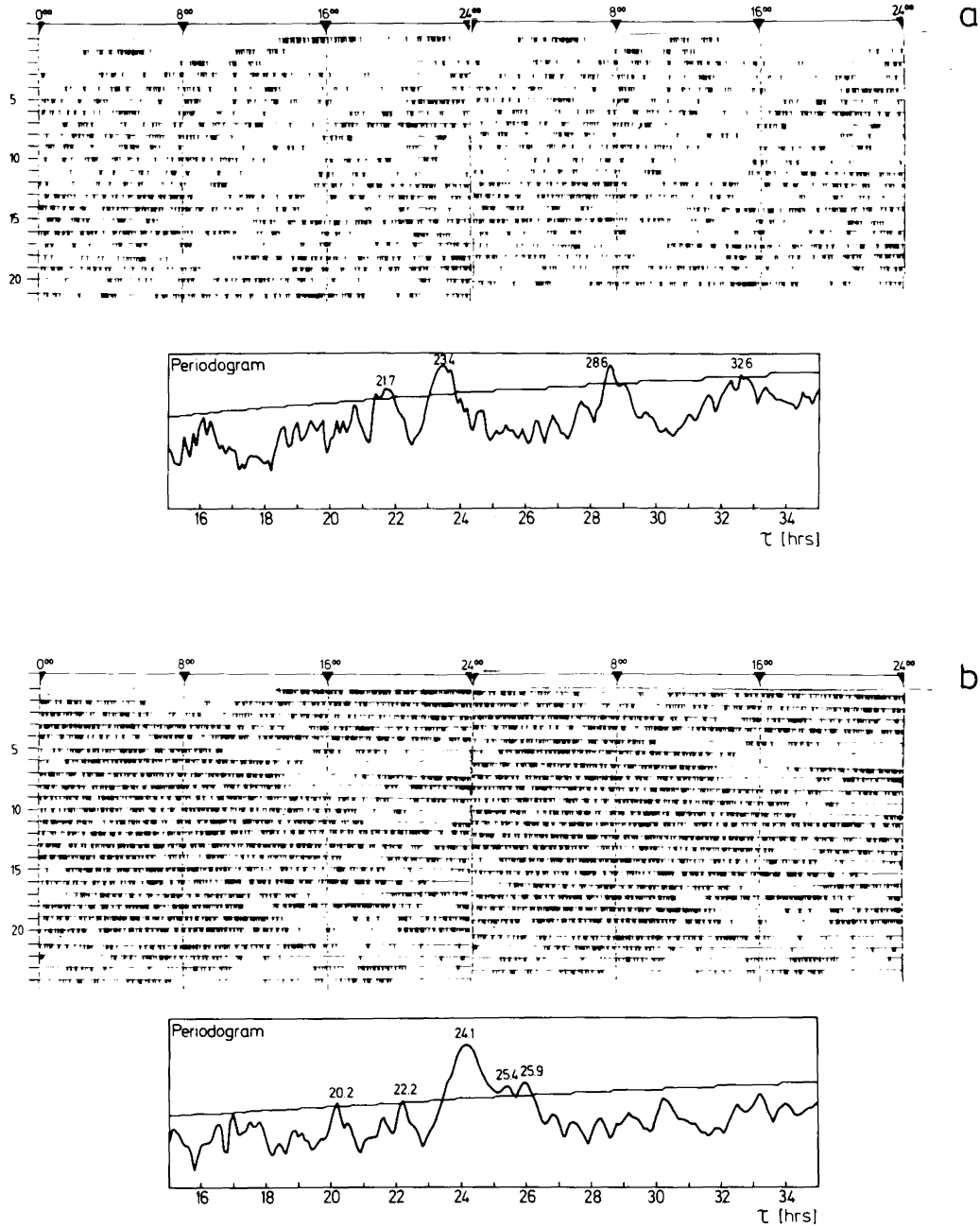


FIG. 6. Two examples of locomotor activity patterns with more than two rhythm components (corresponding periodograms shown below).

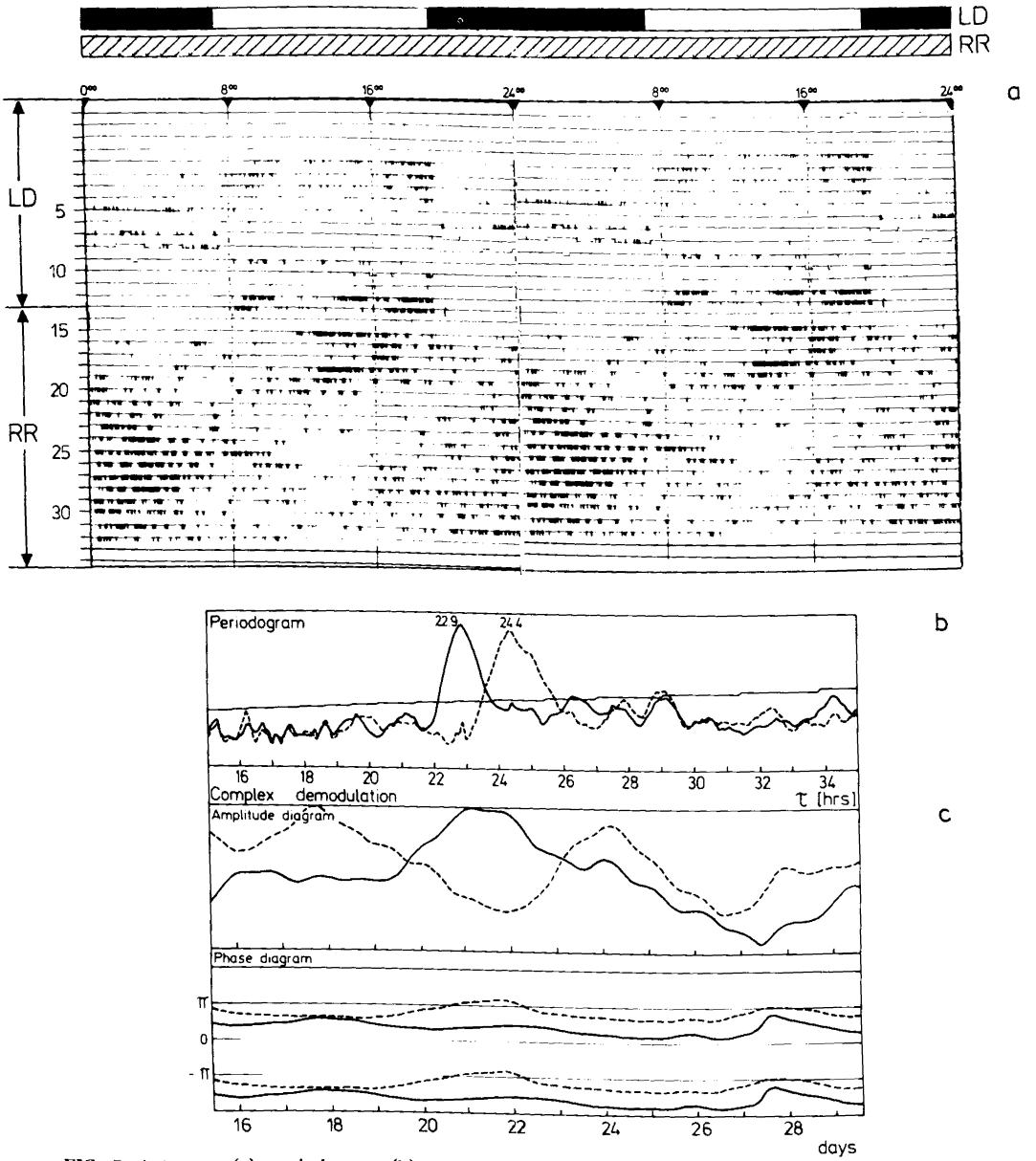


FIG. 7. Actogram (a), periodogram (b) and complex demodulation (c) of a wildtype Italy fly showing two periods simultaneously under constant red light (RR) after LD 12:12. In (b) and (c), solid line shows periodogram analysis and complex demodulation after elimination of the 24.4 h component, broken line after elimination of the 22.9 h component. The phase diagram of complex demodulation shows the course of the two periods during the free-run.

but reverted to their previous inactivity on return to RR (Fig. 9)

Neural anatomy of the mutants

In *so* mutants, the lamina and the first optic chiasma are absent (Fischbach, 1983), and the remnants of the optic lobes, especially medulla and lobula plate, are fused. In the wildtype flies, the different parts of the optic lobe are quite distinct. The distal part of the medulla, characterized by its columnar organization in the wildtype, was not recognizable in *so*, and

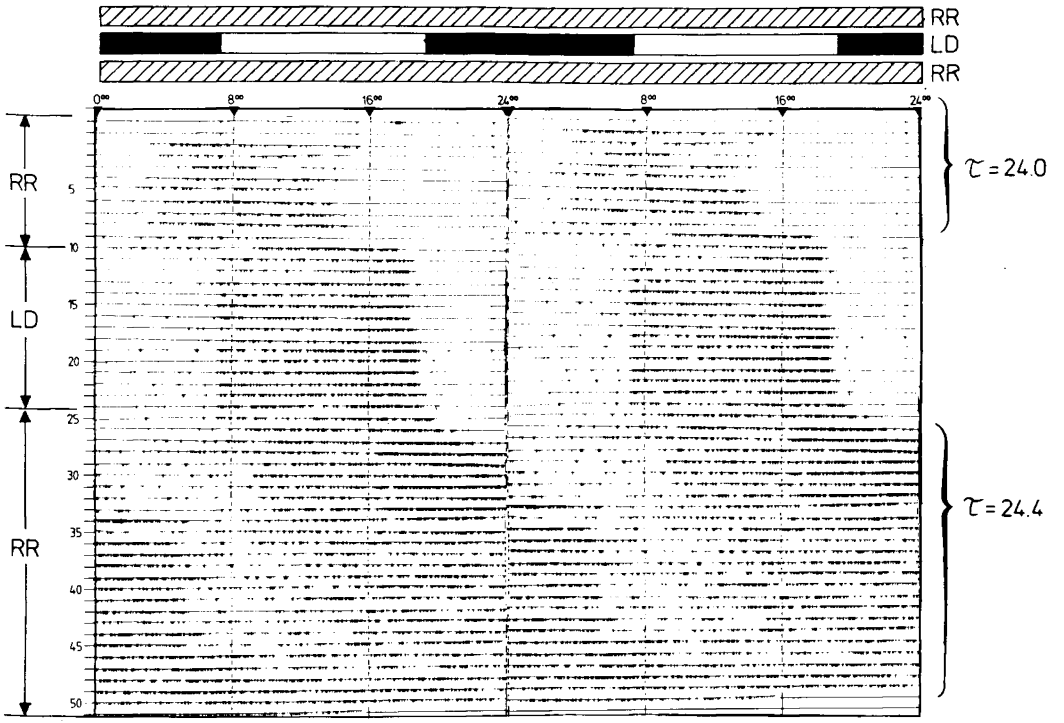


FIG. 8. Typical example of locomotor activity pattern showing entrainment of an eyeless *so* mutant transferred from continuous red light (RR) to LD 12:12 (days 11–24), and then back to RR. Note change in τ before and after RR.

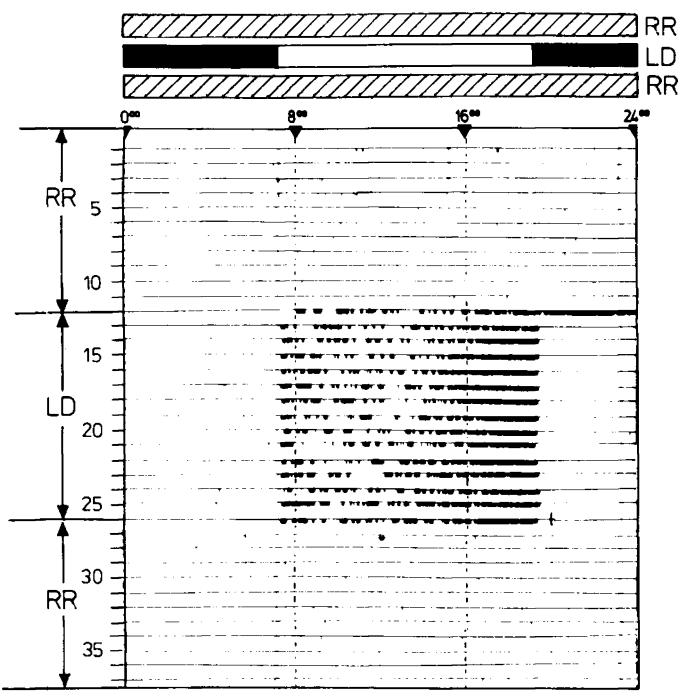


FIG. 9. Actogram of an eyeless *so* mutant which showed extremely low activity under continuous red light (RR), but normal entrained activity under LD 12:12.

the remaining medulla showed a diffuse structure: its dorsal extensions was shortened and the distal part surrounded by a reduced number of cell bodies.

The serpentine medial layer of the medulla was unrecognizable as an inserted layer and seemed to form an outer layer covering the rest of the medulla (Fig. 10). However, it could not be determined histologically whether some neurones of the distal part were still present. The degree of reduction of the medulla was the same in animals reared at 27°C.

All the mutants completely lacking the compound eyes showed the same reduction of the medulla. No histological differences could be found between the structure of the medulla of the rhythmic and arrhythmic flies, nor of those possessing two or more rhythms. *so* mutants with some ommatidia present showed a local formation of the distal part of the medulla but a columnar organization was not recognizable; and even some of these animals were arrhythmic.

Discussion

Our most important finding is the lack of any major difference between the wildtypes and the mutants *so* and *sol*. All strains include flies which exhibited a clear circadian rhythmicity, or were arrhythmic, or showed neither of these patterns. This diversity of activity patterns was expected for the *so* mutant and described by Mack & Engelman (1981). It came as a surprise, however, that the wildtype showed about the same amount of arrhythmic patterns and equivocal rhythms as the mutants. The number of wildtypes studied by Mack & Engelmann (1981) was small, however, so this apparently passed unnoticed.

Flies with a clear circadian rhythmicity

The wildtype Italy was kept for years under constant light. Its relatively weak rhythmicity could therefore imply a connection between its long term environmental experience and its rhythmicity. Without a light cycle there may have been no strong selection for the flies to retain clear rhythmicity in their genome. That, indeed, is the implication of the work by Clayton & Paietta (1972).

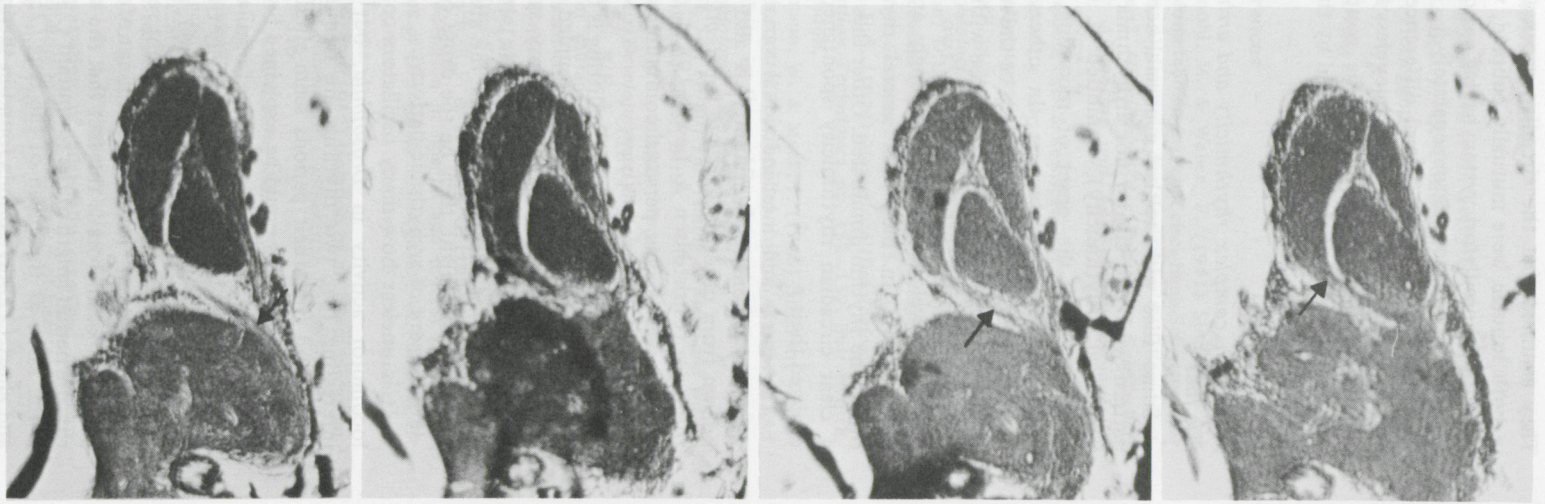
However, this argument cannot be pursued in the same way for the *so* mutant which was reared under a LD cycle, but likewise had relatively poor rhythmicity, since our LD entrainment experiments demonstrated that they do perceive light. This seems to imply a genetic background to the differing rhythmicity of the different strains.

Genetics could also account for the differences found in τ between the strains. Konopka & Benzer (1971) found τ for the strain Canton Special to be 23.8 h for males and 24.5 h for females. We found no such difference between the sexes. It is unlikely that the differences in τ between these and our findings are due to different methods of determination. It is more likely that τ in *Drosphila* is variable and depends on the genetic strain as well as on environmental factors.

The longer τ in the *so* mutant might, likewise, be explained genetically on the basis that the wildtype from which *so* was derived had a τ of 24.1 h. This, however, is unlikely: the 2% *so*/WT_B flies which we found in our population (normal phenotype in respect to ommatidia, but otherwise with the genetic make-up of *so*), should also then have exhibited the longer period, but they did not. Their τ was 23.6 h, near the value found by Mack & Engelmann (1981) for *so* flies with partly expressed eyes (23.5 h) and the τ for WT_B (23.6 h) found by us. Only flies lacking ommatidia or possessing minute remnants of ommatidia showed the longer τ of 24.1 h. Period length thus seems more likely to be correlated with the development or size of the compound eyes and/or optic ganglia.

Another explanation for the longer τ of *so* might be a changed coupling between groups of circadian oscillators. Thus Page (1978) and Page *et al.* (1977) found an increase in τ of locomotor activity in cockroaches (from 23.7 to 24.0 h) after destruction of one lobula.

FIG. 10. Right optic lobes of a wildtype (left) and a completely eyeless *so* mutant (right) of *D.melanogaster*. Some scale and Blest silver staining in all figures. Arrows in top left figure mark the Cuccati-bundle; all other figures, arrows point to the posterior optical tract.



They assumed that the circadian pacemakers in both lobulae were mutually coupled, with the coupled oscillatory system having a shorter τ than each single oscillator separately.

We never observed bilateral differences in the reduction of the optic lobes in eyeless flies, but it is still conceivable that the coupling between single oscillators is changed by the *so* mutation.

Flies with complex rhythmicity and arrhythmic flies

Some flies with neither a normal activity pattern with one clear τ nor arrhythmia (i.e. 'equivocal' rhythmicity), exhibited a clear rhythmicity at the onset of recording, but became arrythmic later on. In others the apparent arrythmicity seemed to be the result of two rhythms drifting out of phase with one another. In yet others one or two clear rhythms developed out of seemingly arrythmic pattern. In flies with several rhythms often one rhythm dominated. Other flies with a clear circadian rhythmicity spontaneously changed their τ.

The intra- and inter-individual variability was thus extensive. Variability is also well known from other insects, e.g. for *Hemideina thoracica* (Christensen & Lewis, 1982), *Calliphora stygia* (Lewis, personal communication) and cockroaches (Page, 1982).

The occurrence of rhythm splitting is taken as evidence for a population of coupled oscillators rather than a single oscillatory system (Christensen & Lewis, 1982; Enright, 1980). Uncoupling of these rather than the absence of oscillators might then be the cause of the development of arrhythmic patterns.

It is unclear how many oscillators control locomotor activity in *Drosophila*, but it is interesting that two rhythms are frequently found and that their periods seem similar in most animals. This could be explained by two oscillators, or rather two groups of oscillators, with the individual oscillators normally tightly coupled. If this coupling were weakened, more than two periods of arrythmicity might result.

An argument for a decoupling of two groups of oscillators is the fact that the single rhythm period length of the *so* mutant (24.1 h) is the same as the mean of the long (25.2 h) and short (22.9 h) periods found in the individuals exhibiting two rhythms. And Koehler & Fleissner (1978) have found for *Blaps gigas* that the two bilateral oscillators controlling the sensitivity of the compound eyes can differ greatly in period (Koehler, personal communication). It is unclear at present whether in *Drosophila* the locomotor activity is likewise, under control by bilateral structures in the brain, with their rhythms differing in τ by over 2 h.

The question remains, whether this decoupling of oscillators is characteristic of the mutant *so* and whether it is the result of a defect in the circadian system, There are certainly more cases in *so*, but examples are also found in the wildtype (Fig. 7). Arguing against a disturbed circadian system in *so* is the fact that although the number of completely eyeless flies increased at higher rearing temperatures, the percentage of arrhythmic flies and flies with equivocal rhythms did not.

The mutants might thus be characterized only by a somewhat reduced coupling strength between single oscillators and may therefore be a suitable model in which to study multioscillatory systems.

LD cycles

LD 12:12 entrains the eyeless flies of *so* just as well as in the wildtype (Mack, 1980), and the bimodal activity pattern of *so* is identical with that of the wildtype, which suggests that the same oscillator underlies the activity rhythm in both strains.

It is remarkable that animals with weak activity were also entrained by LD and showed bimodality. This could indicate that it is not the oscillator which is disturbed but rather the expression of the overt rhythm being suppressed by external factors (the RR conditions).

Whether this explanation also holds for arrhythmic flies we do not know as we have not yet subjected them to LD cycles. If arrhythmicity is the result of oscillator decoupling, one would expect the oscillators in LD to recouple and remain coupled for some time in subsequent RR.

Whether the locomotor activity of the wildtype of *Drosophila melanogaster* is controlled by extraocular photoreceptors, as it is in other insects (Truman, 1976), is not known. If the compound eyes are the only receptors in the

wildtype, as they are in cockroaches and crickets, the *so* mutant must use a photoreceptor for entrainment different from the one used by the wildtype. It is of course also possible that the wildtype uses both the compound eyes and extraocular photoreceptors and that the *so* mutant can manage with only the latter.

Localization of the oscillators

We have no evidence of a missing oscillator in *so* and *sol*. Mack (1980) has suggested that from a certain level of reduction in the distal part of the medulla, normal circadian locomotor activity is lost and replaced by arrhythmia. Our histological studies show, however, that the reduction of the medulla is the same in all eyeless flies. We could not determine whether the distal part of the medulla is completely lacking in eyeless flies, but Fischbach (1982) has demonstrated that some neurones are still present.

Nevertheless, we found no correlation between the reduction of the medulla and the occurrence of rhythmic patterns, arrhythmic patterns, or patterns with several rhythms simultaneously. In the *sol* mutant, 50% of the cells of the medulla and the lobula complex are missing (because the normal cell degeneration at the pupal stage is amplified; Fischbach & Technau, 1981), but we still found it to have the same proportions of the three rhythm types as in the other strains.

In both *so* and *sol* mutants, large tangential cells are found in the medulla as almost the only common structure (Fischbach, personal communication). We conclude that the oscillator(s) for the control of the locomotor activity is (are) either not localized in the medulla or that the large tangential cells are of importance.

Thus it may be possible that the oscillators are located in the lobula, as in cockroaches. Alternatively, they may be sited elsewhere completely outside the optic lobes. This latter is perhaps indicated by the brain transplantation experiments of Handler & Konopka (1979), which suggest humoral circadian control. Furthermore, Konopka & Wells (1980) have demonstrated in the arrhythmic mutant per° a significantly increased percentage of abnormally located brain neurosecretory cells.

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