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Activity Rhythm of *Drosophila* Kept in Complete Darkness for 1300 Generations

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Locomotor activity rhythms of dark stock flies of *Drosophila melanogaster* kept in complete darkness for 700 to 1340 generations were examined. The stock was established by the late Prof. S. Mori in November 1954 to investigate long-term effects of darkness on organisms. The activity of flies was recorded under three types of light conditions: DD after LD12:12, and DD after exposure to a 3.5 h (P3) or 7.5 h (P7) light pulse. In all of these conditions, the experimental dark flies exhibited clear circadian rhythms similar to those of control light flies. We compare our results with those of various studies on troglobites.

Key words: circadian, darkness, *Drosophila*, endogenous rhythm, fruit fly, generation

INTRODUCTION

Animals and plants living on the surface of the earth tend to limit their locomotor or physiological activities to a relatively narrow part of the day, synchronizing them with environmental day and night cycles. Adaptive aspects of such fluctuating activities have been discussed by Cloudsley-Thompson (1961). Most fluctuating activities are sustained in so-called constant conditions with respect to light and temperature, and thus are thought to be controlled by an endogenous timing mechanism or biological clock (Bünning, 1963). Possession of such a mechanism would be advantageous, as organisms could predict and prepare for coming environmental changes. Owing to its "time-sense" based on a biological clock, a bee can visit flowers at the species-specific time when they offer food, even after a few days of confinement in the hive due to bad weather (Bünning, 1960).

In contrast to organisms exposed to natural day and light cycles, animals living in an environment without cyclic changes, such as in caves or arctic regions, are known to be arrhythmic, or to have weak rhythms (Bünning, 1963; Imafuku, 2008). In constant conditions, possession of a biological clock should be redundant, or in some cases harmful, because rhythmically active cave animals will have a larger chance to miss opportunities to obtain food resources, which are non-periodically supplied in such conditions (Paulson and White, 1969). This predicts that long-term experience without environmental cycles would lead organisms to lose endogenous rhythms. What happens to the organism at the initial phase of entering such constant conditions? To address this question, Mori carried out a long-term experiment in which strains of a fruit fly, *Drosophila melanogaster*, were maintained over many generations under the condition of complete darkness at a constant temperature (Mori and Yanagishima, 1959a). In his earlier reports, he presented data that the experimental flies ("dark flies") showed diurnally less clear patterns of eclosion from the pupal case than the control flies ("light flies") did, from 26 to 135 generations, but that thereafter, the tendency was reversed until 238 generations (Mori et al., 1964).

By 2009, the dark flies had experienced 55 years (corresponding to at least 1300 generations) of complete darkness. We examined their locomotor activity rhythm to see if there had been any changes in their behavior.

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MATERIALS AND METHODS

Flies

Two types of stocks, the dark stock (D stock) and the light stock (L stock), were established from Oregon RS strain of *Drosophila melanogaster* in November, 1954 (Mori and Yanagishima, 1959a). D stock was maintained in complete darkness in a light-tight can (24 cm high, 24.5 cm in diameter) placed in a temperature-controlled room at 25°C in the Department of Zoology, Kyoto University. L stock was maintained at the same temperature in an incubator with glass windows through which natural daylight entered, or in a light- and temperature-controlled room with a 12 or 14 h light phase (LD12:12 or LD14:10).

Flies were cultured in ordinary milk bottles (14 cm high, 5.5 cm in diameter) with Pearl's synthetic medium (Ohsawa et al., 1958), and transferred to fresh medium to produce a new generation every two weeks. All transfer procedures were performed in complete darkness for D stocks. The population size of the flies in the bottle fluctuated largely between 50 and 200.

In Experiment 1, flies of D and L stocks from 710 to 918 generations were tested. As L stock unfortunately became extinct at the 1224th generation on 6 August 2002, a new L stock (K series) was re-established on 11 November 2005 as a derivation of the same strain kept in the Drosophila Genetic Resource Center, Kyoto Institute of Technology, where flies had been reared with the standard cornmeal-glucose-yeast-agar medium (Hirai et al., 2004) under the condition of LD12:12 at 23–24°C. Before coming to the center on 18 December 2002, the stock flies had been maintained under roughly LD11:13 at 22°C at the Bloomington Drosophila Stock Center, Indiana University. The newly established L stock was reared with Pearl's medium in our laboratory, and used for Experiment 2

from 42 to 44 generations in our laboratory, together with D stock flies of 1330 to 1334 generations in darkness.

Recording of activity

A fly placed in a transparent plastic cell (1 × 1 cm, 4.5 cm high) was monitored with a combination system of an infrared light emitting diode (TLN103A, peak wavelength 940 nm, Toshiba) and a phototransistor (TPS601A, Toshiba), and the on/off information was recorded on a counter IC (MSM5511RS, Oki) for Experiment 1, or directly on a personal computer (OptiPlex GX100, DELL) through an interface (PCI-2703A, Interface Corp.) for Experiment 2.

Experiments were performed under three light conditions: DD, P3 and P7. In DD conditions, flies of both D and L stocks were reared from the egg through the adult stage in LD12:12, and on the 2nd to 4th day of adult life they were shifted to continuous darkness (DD) and their locomotor activity was recorded. In P3 and P7 conditions, D stock flies were maintained in complete darkness, and then in the early adult stage, they were suddenly exposed to a single light pulse of 3.5 h (P3) or 7.5 h (P7) when they were placed into the cell to start activity recording. L stock flies were kept in darkness from the egg stage and exposed to a light pulse in the adult stage, as for D stock flies. In some tests of DD conditions, flies were grown and maintained with corn-yeast medium from the egg to adult stages, including the entire recording period (see Table 1).

Data analysis

For data analysis, flies for which records were obtained for at least seven days were used. Firstly, a possible long-term trend was removed by application of the moving average (Tomioka et al., 2003) and then subjected to periodgram analysis (Enright, 1965; Sokolove and Bushell, 1978). Numbers of flies with a significant rhythm were compared between D and L stocks by a Fisher's exact test or a χ^2 test. The statistical power of each (Liu et al., 1991), as an indicator of the strength of rhythmicity, was compared between the D and L stocks by a *t*-test.

RESULTS

Two example actograms are shown in Fig. 1. A fly kept in complete darkness for 715 generations and suddenly exposed to a 3.5 h light pulse showed a significant circadian rhythm of 23.4 h period in the following darkness. Another fly kept in complete darkness for 1339 generations exhibited a clear rhythm of 24.2 h period in darkness after exposure to the light condition of LD12:12 from the egg to the 3rd day of the adult stage.

All data are summarized in Table 1. In Experiment 1, D stock flies showed endogenous rhythms as clearly as L stock flies did in the DD and P3 conditions, whereas D stock flies exhibited clearer rhythms than did L stock flies in P7 conditions. In Experiment 2, D stock flies reared with corn-yeast medium showed significantly clearer rhythms than the control L flies in DD conditions.

DISCUSSION

Mori and colleagues reported some aspects of the dark flies, such as diurnal eclosion patterns and photokinesis during the period of 94 to 630 generations (ref. Mori and Imafuku, 1982). In the present study, circadian rhythms of the dark flies were first examined with the result that the dark flies exhibited detectable circadian rhythms of activity, and occasionally exhibited clearer rhythms than the light flies did. This indicates that a period of 1340 generations of complete darkness does not attenuate this circadian rhythm. Occasional exhibition of clearer rhythms by the dark flies may be attributable to a higher sensitivity of the dark flies to light stimulation (Mori and Yanagishima, 1959b). However, the examined number of batches, seven, was too small to allow a conclusion. Apart from persistency of the rhythm, other aspects, such as activity forms and readiness to re-entrainment, may be different between the light and dark stocks. These aspects should be compared in future.

Persistence of circadian rhythms over a long period of constant conditions has been reported for other stocks of *Drosophila melanogaster* that were kept in continuous light for 600 generations and showed eclosion rhythms in both continuous dark (DD) and continuous light (LL) conditions (Sheeba et al., 1999). Our results, together with these earlier findings indicate that circadian rhythms of fruit flies are not altered by such a short-term experience of constant conditions as 600 to 1340 generations.

In contrast to fruit flies subjected to artificial environmental conditions, various genuine cave animals ("troglobites") are known to be arrhythmic or to have weak rhythms. These include crayfish, *Cambarus pellusidus* (Park et al., 1941), amphipods, *Niphargus puteanus* (Günzler, 1964), and fish, *Astyanax mexicanus* (Erckens and Martin, 1982b). Crayfish are inferred to have entered Mammoth Cave in the late Pliocene to early Pleistocene, 1 to 4 million years ago (Park et al., 1941). Species of the genus *Niphargus* are inferred to have extended their distribution to underwater

Table 1. Experimental results of circadian rhythms of *Drosophila* kept in darkness for 700 to 1300 generations.

Condition	Medium	Generation[a]	Stock	n[b]	Period (h)	Power	*P*[c]
Experiment 1							
DD	Pearl	710–918	D	26/27	23.7 ± 0.3	82 ± 48	0.9999
			L	15/16	23.5 ± 0.5	68 ± 38	0.3724
P7	Pearl	740–897	D	16/16	23.7 ± 0.2	67 ± 31	0.0277*
			L	5/8	23.2 ± 0.1	20 ± 18	0.0006**
P3	Pearl	715–739	D	11/12	23.5 ± 0.4	40 ± 34	0.9999
			L	11/11	23.7 ± 1.2	58 ± 26	0.1396
Experiment 2							
DD1	Pearl	1335–1338	D	5/10	24.2 ± 0.4	18 ± 31	0.1827
		K44–47	L	9/11	24 ± 0.4	34 ± 28	0.1283
DD2	Corn	1340	D	8/8	24.1 ± 0.2	80 ± 28	0.4667
		K49	L	6/8	24.2 ± 0.3	21 ± 16	0.0008**
DD3	Corn	1342	D	12/12	24.2 ± 0.2	104 ± 35	0.0081**
		K51	L	8/15	23.5 ± 1.4	9 ± 17	0.0001**
P3	Pearl	1339	D	7/9	23.8 ± 5.2	21 ± 23	0.1140
		K48	L	1/3	23.6	0.3	0.0738

[a]Generation numbers of the L stock in Experiment 2 are those after the transfer from the Drosophila Genetic Resource Center.

[b]Number of individuals with a significant rhythm divided by the number examined.

[c]*P*-values for comparison of numbers of individuals with a significant rhythm (upper row) and of powers (lower row). *$P < 0.05$, **$P < 0.01$.

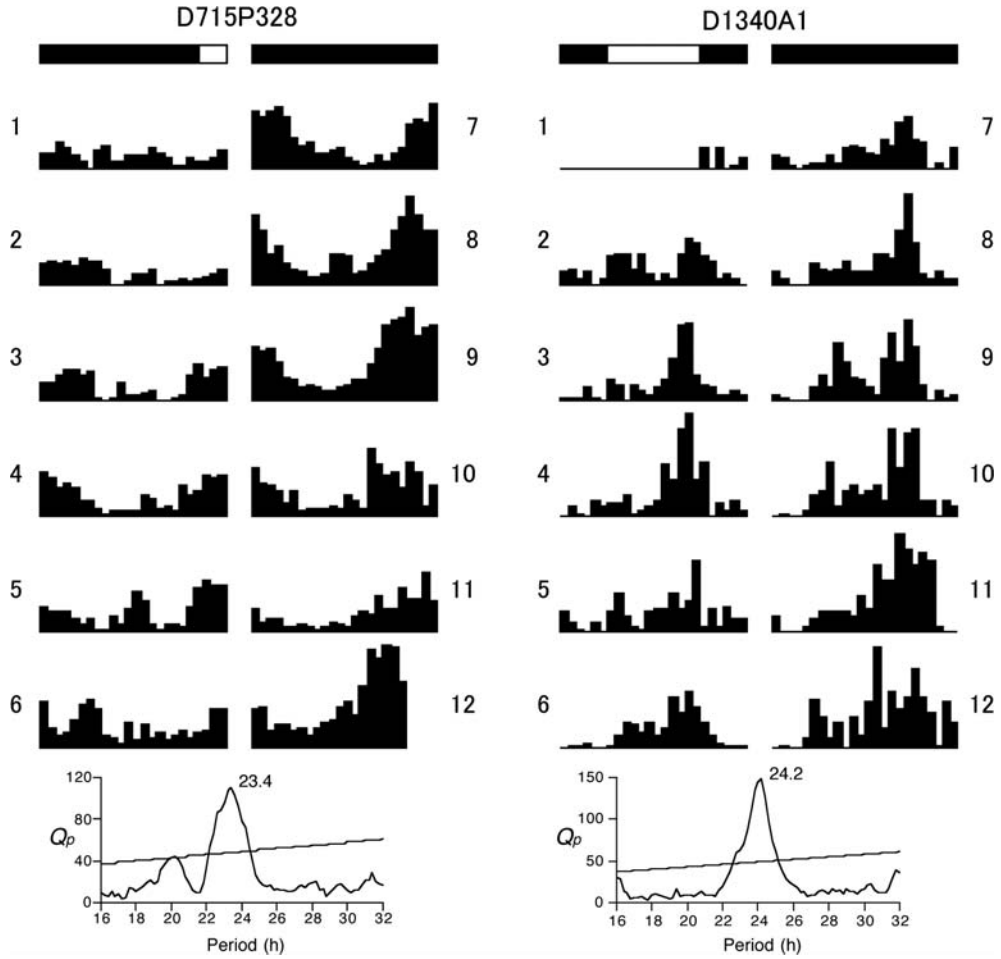

Fig. 1. Two sample actograms of dark stock flies obtained under constant dark conditions. The top bars indicate the light condition. One fly (D715P328) was the progeny of D stock flies that were kept in continuous darkness for 714 generations, and was suddenly exposed to a 3.5 h light pulse in the early adult stage. The recording was started immediately after the end of the light pulse (20:30–24:00 on Day 0). A circadian rhythm of 23.4 h period was confirmed by the periodgram analysis (shown at the bottom). Another fly (D1340A1) was the progeny of D stock flies kept in darkness for 1339 generations, and was subjected to LD12:12 throughout all growth stages from the egg. The recording was started after the end of the last light phase (6:00–18:00 on Day 1) on the third day of the adult stage. A circadian rhythm of 24.2 h period was confirmed.

habitats already in the Pliocene (Günzler, 1964), more than 1.7 million years ago. Assuming these small animals attain reproductive age in one year, evolutionary changes in them have occurred during the course of 1–4 $\times$ 10^6 generations. Most individuals of *Astyanax* obtained from Pachon Cave were arrhythmic (Erckens and Martin, 1982b), in contrast to river individuals with clear circadian rhythms (Erckens and Martin, 1982a). The cave population is eyeless and unpigmented. The cavernicolous population of this species is inferred to have separated from the epigean river population 0.7 million years ago, as shown by analysis of genetic divergence (Chakraborty and Nei, 1974). The generation time of *Astyanax* is estimated to be six years and thus, morphological and behavioural changes of this species have been attained in the course of 10^5 generations in the cave. From the information on these troglobites, the number of generations needed for evolutionary changes in circadian rhythms is from 1 $\times$ 10^5 to 4 $\times$ 10^6, which is approximately 100 to 1000 times longer than our fruit fly generation numbers of 1 $\times$ 10^3.

A gene controlling the circadian activity rhythm is known in *Drosophila melanogaster* (Konopka and Benzer, 1971). Mutation of such a gene will occur in our *Drosophila* population, and the probability of displacement by a mutant allele in the population can be calculated. Possession of rhythmicity in a non-periodic environment may be costly, as mentioned in Introduction, but the cost is inferred to be not so large for our flies, as the stay in darkness for more than 1000 generations did not diminish their rhythmicity. Thus, the Kimura's neutral theory could be applied to our flies. The mutation rate in *Drosophila* is known to be roughly 10^{-5} (Maynard Smith, 1989). The fixation rate of a neutral gene is same as the mutation rate, and the fixation time of the gene is a generation number of four times of the effective population size ($4N_e$) (Kimura, 1983). N_e is a harmonic mean of fluctuating population sizes; here defined as 90, an approximation to the harmonic mean of 50, 100 and 200 (see Method). Based on these data, the generation number necessary for occurrence and fixation of a mutant responsible for arrhythmia is calculated to be approximately 3000 ($(10^5 + 10^5)/90 + 4*90 = 2582$). This probabilistic calculation indicates further efforts are required.

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