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Localization of the Photoreceptor and Pacemaker for the Circadian Activity Rhythm in the Band-Legged Ground Cricket, *Dianemobius nigrofasciatus*

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ABSTRACT—Anatomical locations of the photoreceptor and circadian pacemaker for the locomotor activity rhythm were investigated by removal of the external photoreceptors, the compound eyes and ocelli, or the optic lobes in the adult cricket, *Dianemobius nigrofasciatus* (Orthoptera: Gryllidae). The activity rhythm of intact crickets freeran under DD with a freerunning period (τ) of 24.6 ± 1.0 h (mean ± SD) and entrained to light-dark cycles of LD 13:13 and LD 12:12. When both compound eyes were removed, some crickets entrained both to LD 13:13 and LD 12:12. Some of the others entrained only to LD 12:12 or showed a complex pattern of the activity. When both compound eyes and all ocelli were removed, some crickets still entrained both to LD 13:13 and LD 12:12. The activity pattern of the crickets receiving a sham operation or unilateral removal of the compound eye was not different from that in the intact crickets. Bilateral removal of the optic lobes caused arrhythmicity both under LD 13:13 and DD, although the activity rhythm in crickets of which the optic lobe was unilaterally removed entrained to LD 13:13 and freeran under DD. These results suggest that *D. nigrofasciatus* possesses its circadian pacemaker in the optic lobe, and uses both extraretinal photoreceptors and compound eyes for its entrainment.

INTRODUCTION

Photoperiod is an important cue to entrain circadian rhythms to the environmental cycles and to induce photoperiodic responses controlling seasonal development in insects. The relationship between circadian rhythms and photoperiodism is an interesting issue to unveil clock mechanisms in animals. Involvement of the circadian clock in the time-measurement of the photoperiodic response has been demonstrated in many insect species by resonance experiments, in which induction of an event controlled by the photoperiod is examined under various light regimes, such as Nanda-Hamner or Bünsow protocols (Saunders, 1982). When the event is induced under the light regime of which the cycle is a multiple of 24 h, the photoperiodic clock shows "resonance" to the circadian clock, suggesting that the photoperiodic response employs the circadian clock for time-measurement. Although many experiments of this kind have resulted in hypothetical models of time-measurement systems, these models are never sufficient to understand the physiological interaction between the circadian and photoperiodic clocks. The importance lies in the parallel examination of physiological mechanisms coordinating the circadian rhythm and photoperiodism, *i.e.*, photoreceptive sites and transduction of photoperiodic signals into neural or neuroendocrine output, by using the same species.

Photoreceptive sites for entrainment of circadian rhythms or photoperiodic responses have been anatomically examined in many insect species covering several orders (Page, 1985; Numata *et al*., 1997) but in most cases, the results for the two phenomena are obtained from different species. The number of species in which photoreceptors for both phenomena have been determined is very few. One reason is that not many species show both clear circadian behavioral rhythmicity and photoperiodism. The adult blow fly *Calliphora vicina* shows overt rhythms in its locomotor activity and a photoperiodic response to control larval diapause in the next generation, and the effect of optic lobe-removal on both phenomena was examined (Cymborowski *et al*., 1994; Saunders and Cymborowski, 1996). Even after bilateral removal of the optic lobes, the fly responds to the photoperiod: entrainment of the locomotor activity rhythm to light-dark cycles and induction of larval diapause under a short-day photoperiod remain as in intact flies, indicating that in *C. vicina* both circadian and photoperiodic clocks use extraretinal photoreceptors probably in the central brain. The Nanda-Hamner experiments showed that the circadian clock is involved in photoperiodic time-measurement to control larval diapause in this species (Kenny

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and Saunders, 1991). These results suggest that circadian behavioral rhythmicity and photoperiodism share common physiological mechanisms. Recently, the role of compound eyes in the circadian rhythm and photoperiodic response was also examined in the adults of a hemipteran, *Graphosoma lineatum*. Deprivation of the compound eyes caused loss of the circadian rhythmicity and photoperiodic regulation of adult diapause (Nakamura and Hodková, 1998).

The band-legged ground cricket *Dianemobius* (= *Pteronemobius*) *nigrofasciatus* (Orthoptera: Gryllidae) is another species showing a clear circadian activity rhythm and a photoperiodic response. In adult crickets, the locomotor activity shows circadian rhythmicity (Shimizu and Masaki, 1997) and the photoperiodic response controls induction of egg diapause in the next generation (Masaki, 1972; Kidokoro and Masaki, 1978). Experiments using light regimes of the Bünsow protocol demonstrated that the circadian clock is involved in this photoperiodic response (Masaki, 1984). In the previous study we revealed that the photoreceptive site for the photoperiodic induction of egg diapause is located in the compound eyes (Shiga and Numata, 1996). In the present study, we examined the photoreceptive site for entrainment of the circadian activity rhythm and also location of the circadian pacemaker in *D. nigrofasciatus* by surgical deprivation of the external photoreceptors or optic lobes, and discuss the physiological interaction between circadian rhythmicity and photoperiodic response.

MATERIALS AND METHODS

Insects

Adult male crickets *Dianemobius nigrofasciatus* were obtained from a laboratory culture originally collected in Toyonaka City, Osaka, Japan (34°50'N, 135°28'E). Nymphs were reared under a cycle of 12 h light and 12 h dark (LD 12:12) at 25±1°C, as previously described (Shiga and Numata, 1996).

Activity recording

Crickets were individually housed in a chamber of plastic petri dishes (diameter = 3 cm). An infrared beam (EE SPW-321, Omron, Kyoto) was passed across the chamber and the number of interruptions of the beam was recorded at 6 min intervals on a personal computer (Yoshioka, 1992). The lateral side of the chamber was covered with black tape to ensure visual isolation, except for two windows for the beam transmission. A small piece of dried food made of insect pellet (Oriental Yeast, Tokyo), agar and propionic acid was placed at one end and a water source at the other end of a line diagonal with the infrared beam. Twelve recording chambers were set on a plate in one incubator equipped with a 15W fluorescent lamp (*ca*. 500 lx). Lighting schedules were also controlled by the computer for activity recording. For intact crickets, the locomotor activity was recorded under constant darkness (DD) for the first 12 days after adult emergence and under LD 12:12 for the subsequent 11 days. The operated adults were kept separately under LD 12:12 for 2–3 days after operation. Then, the activity was recorded under LD 13:13 for the first 7–16 days and under LD 12:12 or DD for the next 7–13 days. The file region of either forewing was amputated to ensure acoustical isolation in advance of recording. All experiments were performed at a constant temperature of 25.0±0.5°C. The difference in temperature between the light and dark periods was about 0.04°C. Rhythmicity was determined by chi-square periodogram analysis and eye-fitting methods.

Operation

We performed three kinds of operation, removal of 1) the compound eyes, 2) the compound eyes and ocelli and 3) the optic lobes, 1–2 days after adult emergence. In advance of surgery, crickets were anaesthetized by immersion in ice water (*ca*. 0°C) for 10 min and mounted in a sealing compound with the frontal surface of the head exposed under a stereoscopic microscope. Procedures for operation 1) were described by Shiga and Numata (1996). For operation 2), after removal of the compound eyes, all of the three ocelli were scraped off with a razor blade and the opening was sealed with the dental wax. For operation 3), after removal of the compound eye, connection of the optic lobe and central brain was severed with small scissors and the optic lobe was taken off. For the sham operation, the frontal margin of the compound eye was incised bilaterally. At the end of the experiment the operated crickets were sacrificed for histological examination by staining with reduced silver impregnation in accordance with the Holmes-Blest techniques (Blest and Davie, 1980).

RESULTS

Locomotor activity in intact crickets

Locomotor activity in intact crickets revealed circadian rhythmicity (Fig. 1). Under DD, the activity rhythm in 34 of 40 crickets freeran. The freerunning period (τ) was 24.6±1.0 h (mean ± SD, n = 34). Of 34 crickets, 29 showed a unimodal activity pattern (Fig. 1A) and 5 showed two or more peaks of the activity in a circadian day (Fig. 1B). Six out of 40 crickets were arrhythmic under DD. All the crickets (n = 40) showed entrained rhythmicity to LD 12:12 with the activity concentrated around light-off (Fig. 1). Usually, the active period after light-off was longer than that before it. Another minor peak of the activity was observed around light-on under LD 12:12 in 2 out of 40 crickets (data not shown).

Histological examination

The optic lobe in *D. nigrofasciatus* is closely connected to the central brain (Fig. 2A). Three distinct neuropiles of the lamina, medulla and lobula underlie the retina. In the crickets of which the compound eye had been removed, the retina was completely absent and the lamina was partly or completely destroyed (Fig. 2B). The medulla and lobula remained (Fig. 2B). In the optic lobe-removed crickets, the retina, lamina and medulla were completely absent. The lobula was also removed or only its remnant remained (Fig. 2C). The ratios of crickets histologically examined were 26/29 (2 compound eyes and 3 ocelli-removal), 8/11 (2 compound eyes-removal), 3/3 (1 compound eye-removal), 10/12 (2 optic lobes-removal) and 3/25 (1 optic lobe-removal).

Effects of removal of the external photoreceptors

The activity pattern was determined under each light cycle of LD 13:13 and 12:12 (Table 1). The activity with the period of 26±0.5 h under LD 13:13 and 24±0.5 h under LD 12:12 was classified into the entrained pattern. When the period under either photoperiod was out of this range, the activity was classified as a complex pattern. Under these photoperiodic conditions there was no crickets that freeran with the

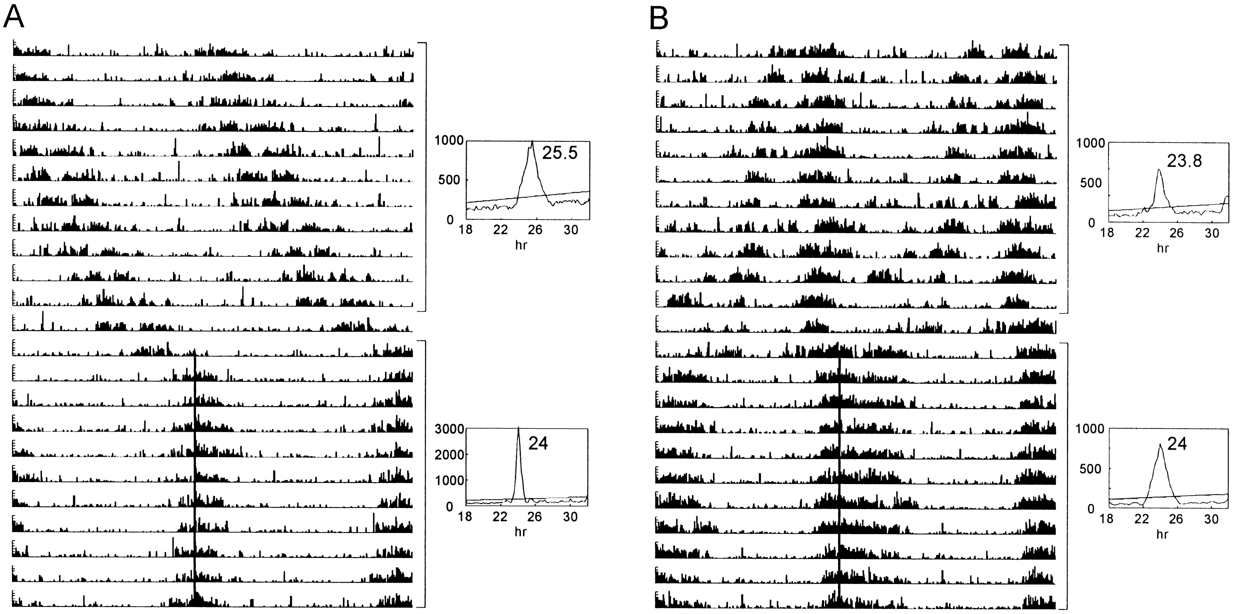

Fig. 1. Two examples of the locomotor activity rhythm presented by a double plotted actogram and associated periodogram analyses in intact adults of *Dianemobius nigrofasciatus*. The crickets were held under DD for the first 12 days and under LD 12:12 for the subsequent 11 days. The solid line on the actogram indicates the light-off. Under DD, unimodal activity pattern appeared in (A). Two peaks of the activity were found in a circadian day in (B). Under LD 12:12 the activity occurred around the time of the light-off.

period far from 26 or 24 h. The activity of most crickets in the sham operation and all in the unilateral compound eye-removal entrained to both photoperiods similar to intact crickets (Table 1; Fig. 3A, B). Under LD 13:13 the activity was concentrated around light-off as under LD 12:12. In some crickets, however, the active component before light-off under LD 13:13 was less than that under LD 12:12 or disappeared. When the light-off was advanced at the photoperiodic change from LD 13:13 to LD12:12, the activity peak was also advanced without a transient (Fig. 3A, B).

We removed both compound eyes in 11 crickets. The activity rhythm in 4 crickets entrained both to LD 13:13 and LD 12:12 (Table 1; Fig. 3C). In Fig. 3C the activity peak did not occur strictly at light-off under LD 13:13 but appeared mainly before light-off. After the photoperiod was changed to LD 12:12, the activity peak moved to light-off. Two crickets showed arrhythmicity under LD 13:13 but entrainment to LD 12:12 (Table 1). We classified the activity of another 3 crickets into the complex pattern (Table 1). Fig. 3D shows one example. The activity was arrhythmic at first and rhythmicity appeared later with the period of 25.6 h. The activity peak occurred at the beginning of the photophase. After the photoperiod was changed to LD 12:12, the activity rhythm still kept the period close to 26 h for several days. Then, the period became close to 24 h and the peak moved to light-off (Fig. 3D). In the other 2 crickets of the complex pattern the activity was arrhythmic in one photoperiod and freeran under the other photoperiod. Two crickets showed arrhythmicity throughout the recording period.

Next we removed three ocelli in addition to the bilateral compound eyes in 14 crickets. The activity rhythm in 3 crickets entrained to both photoperiods (Table 1; Fig. 4A). The activity shown in Fig. 4A was rather scattered around light-off. The characteristics of the activity were not different from those in the crickets of which only the compound eyes were removed. Two crickets were arrhythmic under LD 13:13 but showed entrainment to LD 12:12. The activity in 9 crickets was classified into the complex pattern. Two examples of the complex pattern are shown in Fig. 4B and C. The activity in Fig. 4B shows two rhythmic components under LD 13:13 and the period of one of them was close to 26 h. As in Fig. 4C the activity was scattered around light-off without a substantial change in the period between the two photoperiods, being 25 and 24.4 h under LD 13:13 and LD 12:12, respectively. In this case, we could not determine whether the activity rhythm completely freeran or responded to the photoperiod.

We also examined rhythmicity under LD 13:13 and subsequent DD in other 15 crickets of which both compound eyes and ocelli were removed (Table 2). Six showed freerunning rhythmicity under DD. Their τ was 23.9 ± 1.5 h (mean ± SD, n = 6), which was not significantly different from that in the intact crickets (*t*-test, $P > 0.05$). Among them, one entrained (Fig. 5A), 3 were arrhythmic and 2 freeran under LD 13:13. The other 9 crickets were arrhythmic under DD but 5 of them entrained to or freeran under LD 13:13.

Effects of removal of the optic lobes

The optic lobe was unilaterally removed in 25 crickets. The activity rhythm in 21 crickets entrained to LD 13:13 and freeran under DD (Fig. 5B; Table 2). Two crickets were ar-

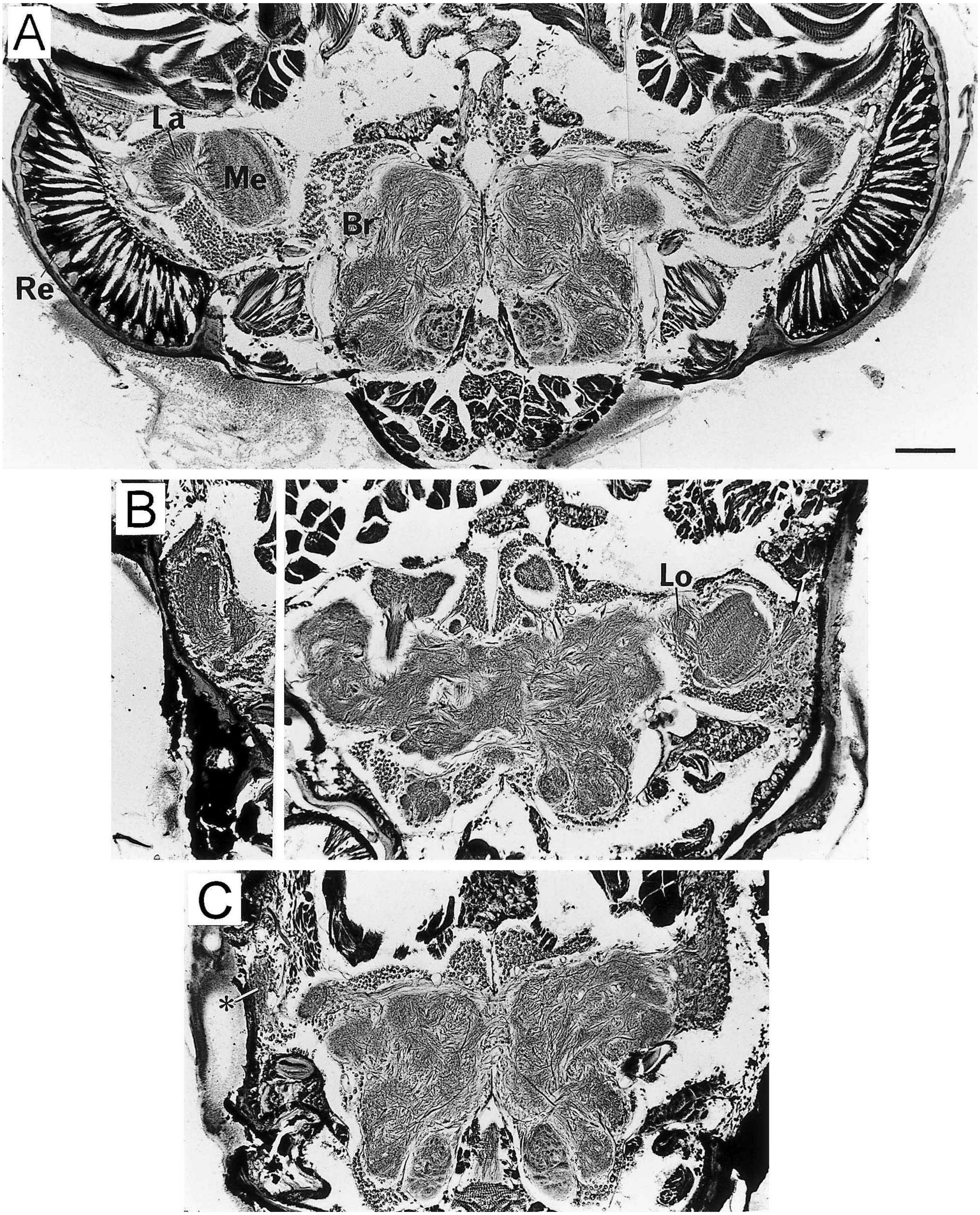

Fig. 2. Examples of histological examination of the optic lobes and central brain in the adults of *Dianemobius nigrofasciatus*. (A) Intact. The optic lobe is closely connected to the central brain (Br). The lamina (La) and medulla (Me) underlie the retina (Re). The lobula is not seen in this section. (B) Bilateral removal of the compound eyes. The retina was completely removed and the small lamina (an arrow) remained. The lobula (Lo) is seen in this section. The microphotograph of the left optic lobe was obtained from a different section of the same individual. (C) Bilateral removal of the optic lobes. The retina, lamina and medulla were completely removed. The remnant of the lobula (*) remained in the left side. Scale, 100 µm.

Table 1. Effects of surgical operations on activity patterns in the adults of *Dianemobius nigrofasciatus* under LD 13:13 and LD 12:12.

Removal of	n	Rhythmic pattern				Arrhythmic pattern
		LD 13:13	entrained	arrhythmic	complex*	
		LD 12:12	entrained	entrained		
intact	6		6	0	0	0
sham	21		20	0	1	0
1 compound eye	3		3	0	0	0
2 compound eyes	11		4	2	3	2
2 compound eyes and 3 ocelli	14		3	2	9	0

* Rhythmic components were obviously detected but their entrainability was not clear. See text for details.

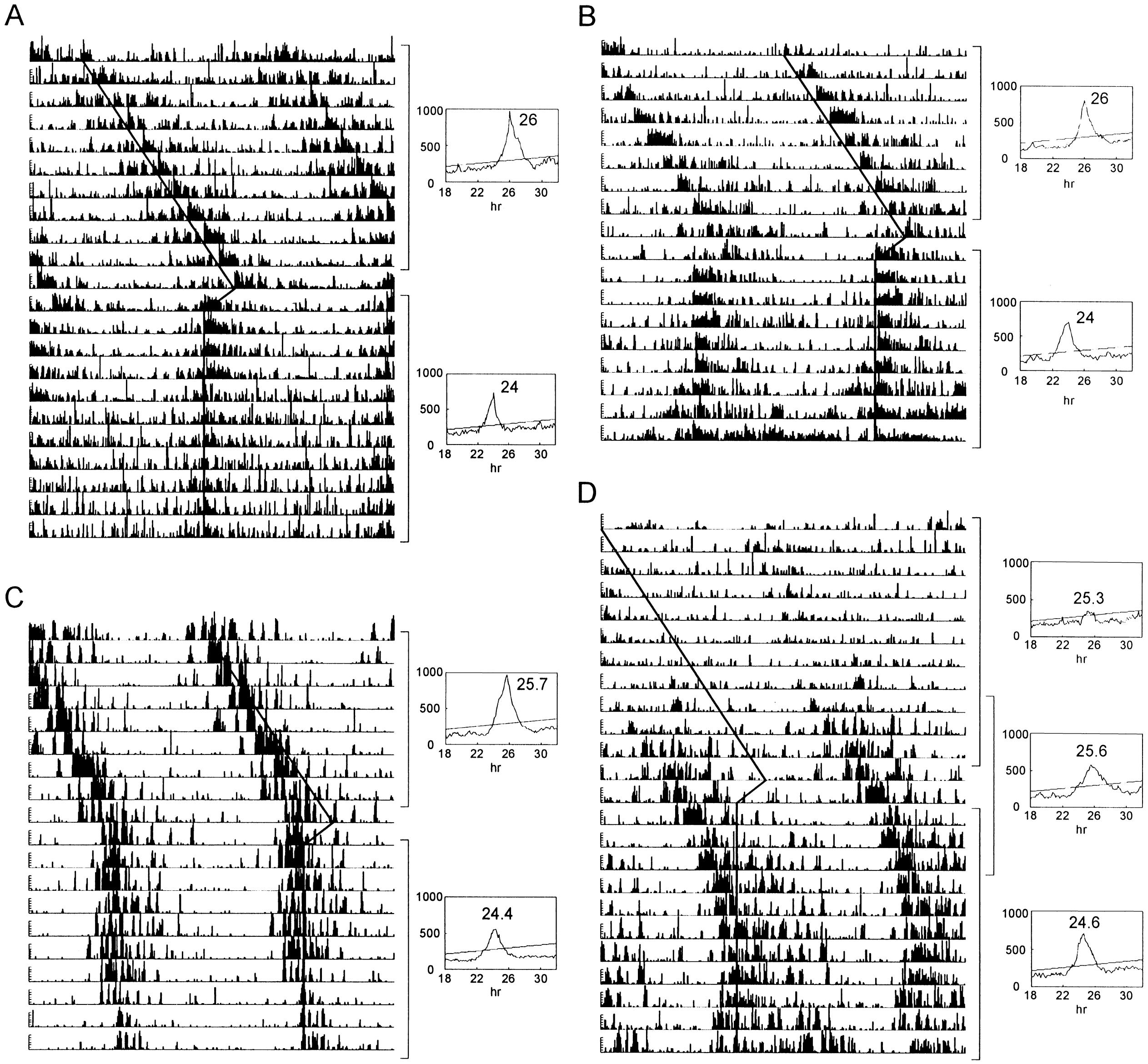

Fig. 3. Locomotor activity rhythms under LD 13:13 and subsequent LD 12:12 in the adults of *Dianemobius nigrofasciatus* receiving the sham operation (A), unilateral removal of the compound eye (B) and bilateral removal of the compound eyes (C and D). When the light-dark cycle was changed from LD 13:13 to LD 12:12, the light-off (solid line) was advanced by 4 hr. For further explanations see Fig. 1.

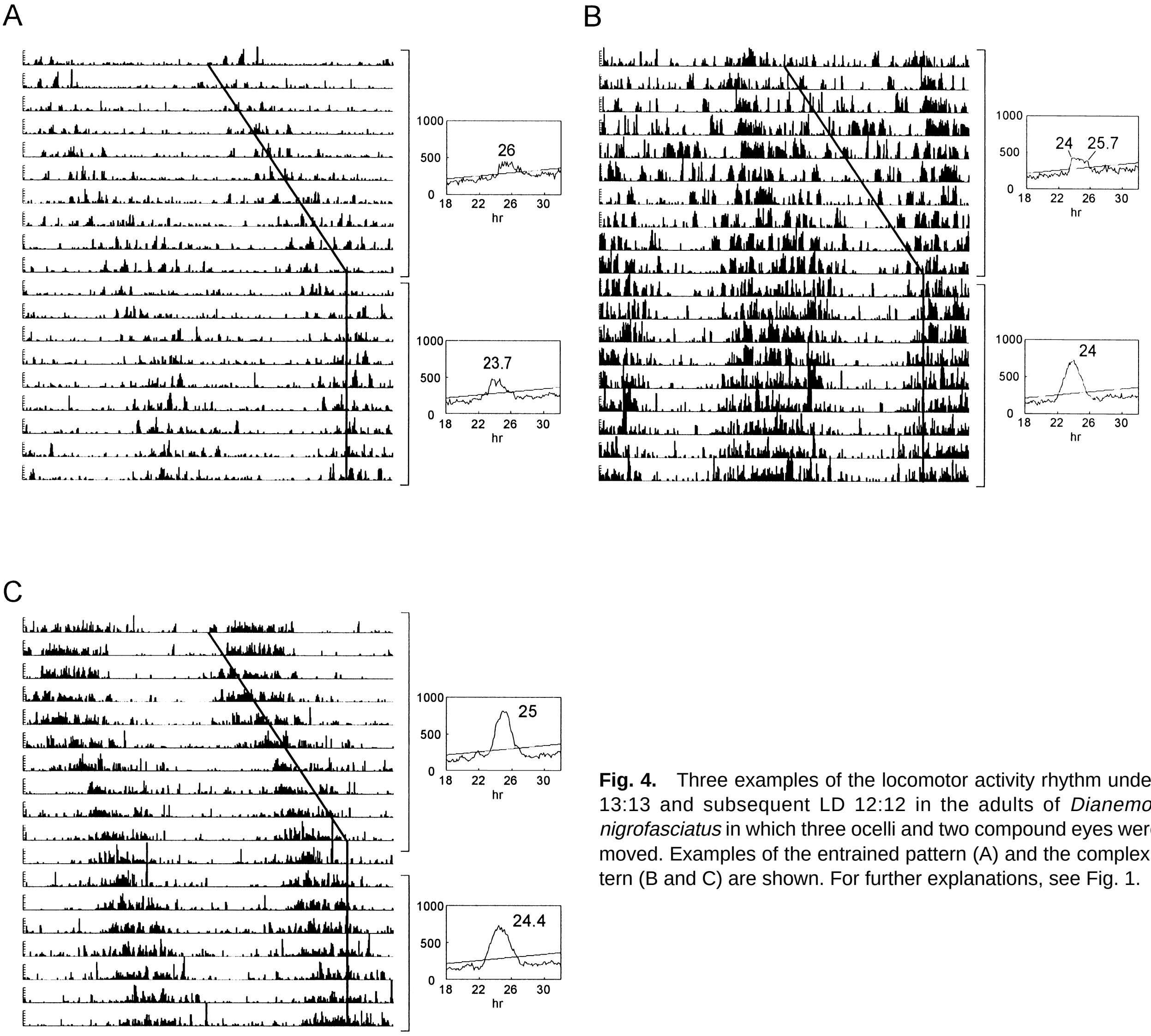

Fig. 4. Three examples of the locomotor activity rhythm under LD 13:13 and subsequent LD 12:12 in the adults of *Dianemobius nigrofasciatus* in which three ocelli and two compound eyes were removed. Examples of the entrained pattern (A) and the complex pattern (B and C) are shown. For further explanations, see Fig. 1.

Table 2. Effects of surgical operations on activity patterns in the adults of *Dianemobius nigrofasciatus* under LD 13:13 and DD.

Removal of	n	Rhythmic pattern						Arrhythmic pattern
		LD 13:13	entrained	entrained	arrhythmic	freerunning	freerunning	
		DD	freerunning	arrhythmic	freerunning	arrhythmic		
2 compound eyes and 3 ocelli	15		1	1	3	4	2	4
1 optic lobe	25		21	0	2	0	1	1
2 optic lobes	9		0	0	0	0	0	9

rhythmic under LD 13:13 and freeran under DD. One freeran under LD 13:13 and DD. The τ under DD was 25.1 ± 0.8 hr (mean ± SD, n = 24), which was not significantly different from that in the intact crickets (*t*-test, $P > 0.05$). After the optic lobes were bilaterally removed, all the crickets (n = 9) were arrhythmic throughout the recording period (Fig. 5C; Table 2).

DISCUSSION

The present study reveals that removal of the optic lobes deprives *D. nigrofasciatus* of the circadian activity rhythm and removal of the compound eyes and ocelli does not completely prevent *D. nigrofasciatus* from the entrainment to light-dark cycles. Some crickets without the external photoreceptors obviously responded to the change of light cycles. However,

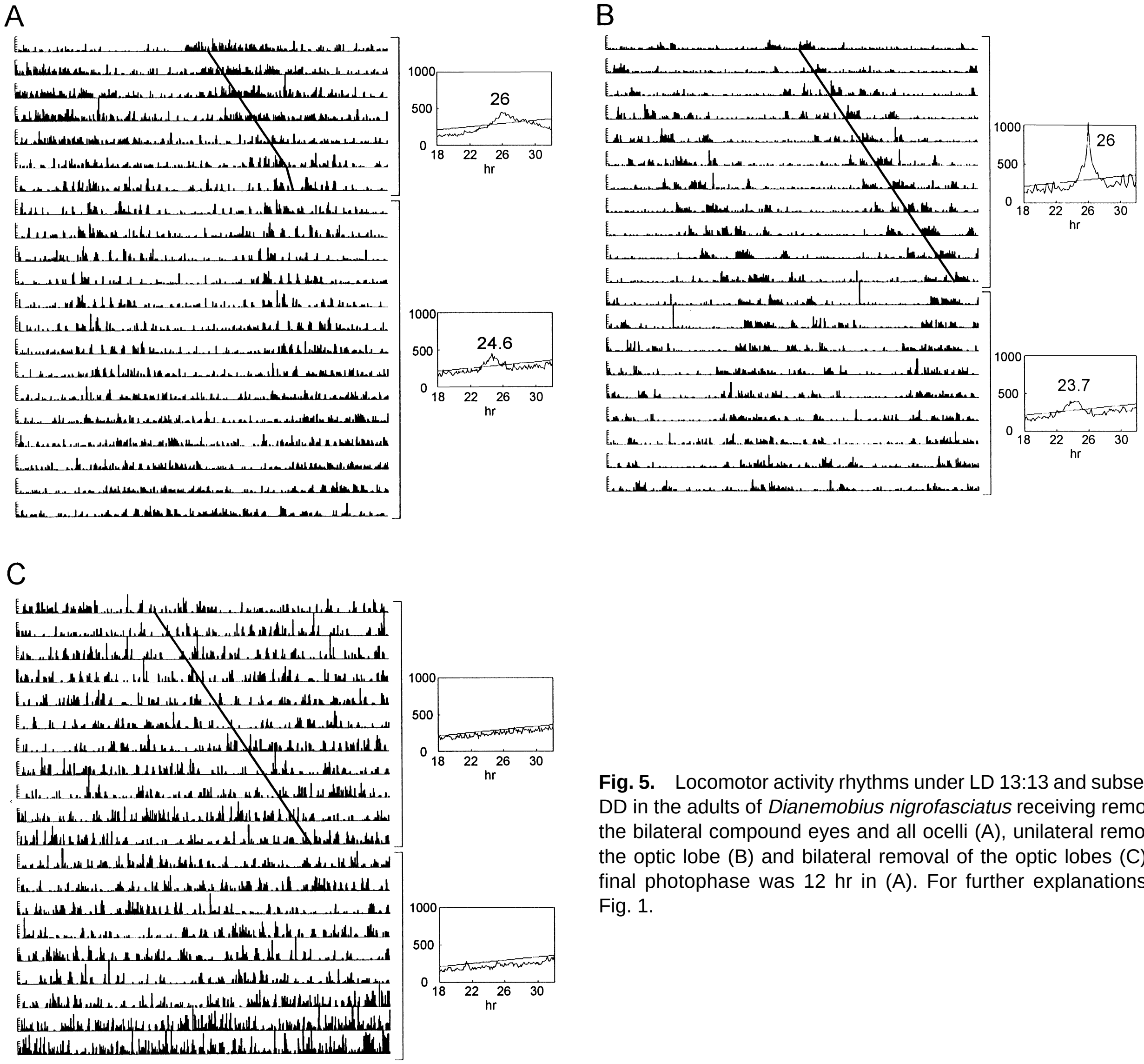

Fig. 5. Locomotor activity rhythms under LD 13:13 and subsequent DD in the adults of *Dianemobius nigrofasciatus* receiving removal of the bilateral compound eyes and all ocelli (A), unilateral removal of the optic lobe (B) and bilateral removal of the optic lobes (C). The final photophase was 12 hr in (A). For further explanations, see Fig. 1.

some differences in the activity pattern from intact crickets were observed. These results show that *D. nigrofasciatus* possesses the pacemaker in the optic lobe and uses both of extraretinal photoreceptors and compound eyes for entrainment of the rhythm. Anatomical location of the circadian pacemaker and the photoreceptor for its entrainment has been examined in several orders of insects (Chiba and Tomioka, 1987). *D. nigrofasciatus* agrees with the other orthopteran species with respect to the pacemaker location, e.g., *Teleogryllus commodus* (Loher, 1972; Sokolove and Loher, 1975), *Gryllus bimaculatus* (Tomioka and Chiba, 1984), *Hemideina thoracica* (Waddell *et al.*, 1990) and *Gryllodes sigillatus* (Abe *et al.*, 1997). Moreover, arrhythmicity in insects of which the optic lobes are removed has been shown in the cockroach *Leucophaea maderae* (Nishiitsutsuji-Uwo and Pittendrigh, 1968a) and the beetle, *Anthia sexguttata* (Fleissner, 1982). Disappearance of rhythmicity by surgical deprivation of the optic lobe is not sufficient to demonstrate that the optic lobe is the real site of the pacemaker as it might be an output pathway from the pacemaker. In *L. maderae* and *G. bimaculatus*, however, transplantation and culture experiments have unequivocally demonstrated that the optic lobe contains the pacemaker (Page, 1982; Tomioka and Chiba, 1992). With respect to such rigorous evidence, it is conceivable that the optic lobe in *D. nigrofasciatus* contains the circadian pacemaker.

In all the above species, except *H. thoracica*, the photoreceptors for entrainment to light-dark cycles are located solely in the compound eyes, because the activity rhythm freeruns under light-dark cycles in compound eye-removed insects as in intact ones under DD (Loher, 1972; Sokolove and Loher, 1975; Tomioka and Chiba, 1984; Abe *et al.*, 1997; Nishiitsutsuji-Uwo and Pittendrigh, 1968b; Fleissner, 1982). In *D. nigrofasciatus*, however, the results were different. The activity rhythm in some of the eyeless crickets seemed to entrain to light-dark cycles and responded to a change in the light-dark cycles from LD 13:13 to LD 12:12. The temperature difference between the light and dark periods was only 0.04°C. This temperature cycle is too small to entrain the activity rhythm. Entrainment to light-dark cycles in the compound eye-removed insects is also reported in *H. thoracica* (Waddell *et al*., 1990). In both *D. nigrofasciatus* and *H. thoracica*, insects still responded to light signals after both ocelli and compound eyes were removed, indicating that these species have extraretinal photoreceptors. Although extraretinal photoreceptors for entrainment have been identified to mainly in dipteran and lepidopteran species in which pacemakers are not in the optic lobe (Truman, 1974; Helfrich *et al*., 1985; Dushay *et al*., 1989; Cymborowski *et al*., 1994), Waddell *et al*. (1990) and the present study have demonstrated that an extraretinal photoreceptor is also operative in the insects having the pacemaker in the optic lobe.

Under artificial zeitgebers, pacemakers can be entrained to periods that deviate from their own τ and this range decreases with a reduction in the zeitgeber strength (Aschoff, 1981). In *D. nigrofasciatus*, some of the compound eye-removed crickets showed entrainment to LD 12:12 but lost entrainability to LD 13:13. Lack of the compound eyes might weaken the capacity of photoreception and this could be equivalent to a reduction in the zeitgeber strength for the pacemaker. Therefore, it is possible that the range of entrainment decreases and some of the operated crickets lose entrainability to 26 h. The activity rhythm in the beetle, *Carabus cancellatus* (Lamprecht and Weber, 1973) and the eclosion rhythm in *Drosophila pseudoobscura* (Bruce, 1960) entrain to artificial periods with a range of about 20 hr. Considering this range, the period of 26 hr is not far from the τ in *D. nigrofasciatus* and is included in the entrainable range for intact crickets. We interpret the present results in that the compound eyes of *D. nigrofasciatus* also function as photoreceptors for entrainment with the extraretinal photoreceptors. In *Manduca sexta*, *Musca domestica* and *C. vicina*, in which the extraretinal photoreceptors have been demonstrated to be involved in the circadian rhythm, the role of the compound eyes for entrainment might be found when the activity is examined under artificial photoperiods different from 24 hr (Truman, 1974; Helfrich *et al*., 1985; Cymborowski *et al*., 1994). Only in *M. domestica* among these species the importance of the compound eyes was discussed as a coupling factor of bilateral pacemakers due to the difference in the activity pattern between the sham-operated and the eye-removed flies (Helfrich *et al*., 1985). In *Drosophila melanogaster*, genetic ablation of eyes or mutations in the visual phototransduction pathway do not block the entrainment of the circadian rhythm to light-dark cycles (Dushay *et al*., 1989; Yang *et al*., 1998), indicating that the relevant extraretinal photoreceptors exist. Recently a member of the photolyase/cryptochrome family *cry* was genetically and physiologically identified in *D. melanogaster* (Emery *et al*., 1998). Null mutant of this gene, *cry*^{b}, is rhythmic in constant darkness but shows no response to light pulses which cause phase shifts in the wild type, suggesting that a blue light receptor, cryptochrome is circadian photoreceptor in *D. melanogaster* (Stanewsky *et al*., 1998). Although *cry*^{b} flies synchronize to photoperiods and respond to photoperiodic changes, double mutants of *norpA*^{P41};*cry*^{b}, in which the compound eyes and ocelli are completely unresponsive to light, fail to entrain to changed photoperiods (Stanewsky *et al*., 1998). These results also indicate that both extraretinal and retinal photoreceptors are involved in photoreception for circadian clocks.

Considering the fact that the photoperiodic response controlling the induction of egg diapause shows clear circadian periodicity under light-dark schedules of the Bünsow protocol (Masaki, 1984), the circadian pacemaker seems to be involved in the photoperiodic time-measurement system in *D. nigrofasciatus*. When the compound eyes were bilaterally removed, *D. nigrofasciatus* lost responsiveness to photoperiod in the photoperiodic response (Shiga and Numata, 1996). Taken together with the present results, the compound eye is a common photoreceptor for both the circadian behavioral rhythm and photoperiodic response in *D. nigrofasciatus*. However, some differences are evident. The extraretinal photoreceptors are not effective in the photoperiodic response when both compound eyes were removed. Moreover, when either compound eye was removed, some crickets failed to perceive a short-day photoperiod to produce diapause eggs (Shiga and Numata, 1996) but the activity rhythm entrained to light-dark cycles. Our results show that *D. nigrofasciatus* has acquired a robust photoreceptive system for entrainment of the circadian behavioral rhythm compared with that for induction of the photoperiodic response. Answers to the question why such differences exist in the two related systems, are not currently available. We will have to wait for further studies to answer this question and approach the transduction mechanisms of photoperiodic information into neural or neuroendocrine output involved in the circadian rhythm and photoperiodic response.

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