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# Circadian Locomotor Rhythms in the Cricket, *Grylloides sigillatus*

## II. Interactions between Bilaterally Paired Circadian Pacemakers

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**ABSTRACT**—The optic lobe is essential for circadian locomotor rhythms in the cricket, *Grylloides sigillatus*. We examined potential interactions between the bilaterally paired optic lobes in circadian rhythm generation. When one optic lobe was removed, the free-running period of the locomotor rhythm slightly but significantly lengthened. When exposed to light-dark cycles (LD) with 26 hr period, intact and sham operated animals were clearly entrained to the light cycle, but a large number of animals receiving unilateral optic nerve severance showed rhythm dissociation. In the dissociation, two rhythmic components appeared; one was readily entrained to the given LD and the other free-ran with a period shorter than 24 hr, and activity was expressed only when they were inphase. The period of the free-running component was significantly longer than that of the animals with a single blinded pacemaker kept in LD13:13, suggesting that the pacemaker on the intact side had some influence on the blinded pacemaker even in the dissociated state. The ratio of animals with rhythm dissociation was greater with the lower light intensity of the LD. The results suggest that the bilaterally distributed pacemakers are only weakly coupled to one another but strongly suppress the activity driven by the partner pacemaker during their subjective day. The strong suppression of activity would be advantageous to keep a stable nocturnality for this cricket living indoors.

### INTRODUCTION

It is generally accepted that the circadian system of many animals is composed of multiple oscillatory constituents (Pittendrigh, 1974). In insects the famous two oscillator model based on the *Drosophila* eclosion rhythm (Pittendrigh, 1981) provides a general view of the pacemaker-slave structure of the system. However, there are lines of evidence indicating that the system includes two bilaterally paired pacemaking systems. For example, the pacemakers are located one in each of the paired eyes in mollusks (Roberts *et al.*, 1987), in the optic lobes of some insects (Page, 1978, 1981; Wiedemann, 1983; Tomioka *et al.*, 1991) and in the suprachiasmatic nuclei of rodents (Inouye and Kawamura, 1982). In general the paired pacemakers interact to keep stable temporal structure in animal's behavior.

The orthopteran and dictyopteran insects such as crickets and cockroaches are one of the most suitable animals to analyze mechanisms of the interaction, since their pacemakers are well separated morphologically so that the two pacemakers can be subjected to separate surgical manipulations. In the cockroach *Leucophaea maderae*, the pacemakers are so tightly coupled to one another that the two pacemakers can not be desynchronized unless the coupling path-

way is destroyed by a certain surgical manipulation (Page, 1983). They have a pathway through which they inhibit the activity driven by their partner pacemaker (Page, 1983). In the crickets, however, the two pacemakers are desynchronized by making one pacemaker blind by optic nerve severance and the inhibitory effect is rather weak (Wiedemann, 1983; Tomioka *et al.*, 1991). Detailed analysis in *Gryllus bimaculatus*, provided important insights into the mechanism of the pacemaker interaction. The coupling is achieved by mutual phase shifts caused not only by temporal but also by photic information being exchanged between the optic lobes, and the pacemakers mutually suppress the activity driven by their partner pacemaker during their subjective day (Tomioka, 1993; Tomioka *et al.*, 1994; Tomioka and Yukizane, 1997).

In the present experiment, we used the cricket, *Grylloides sigillatus*, which showed a clear nocturnal rhythm to investigate the pacemaker interaction. It has been shown that the optic lobe is indispensable for persistence of the rhythm and the compound eye is the principal photoreceptor for the photic entrainment (Abe *et al.*, 1997). *G. sigillatus* mostly occurs indoors so that the light it receives in its habitat is only weak and sometimes unreliable as a zeitgeber because of the artificial light. The purpose of the present study was to determine whether this cricket could exhibit pacemaker interaction and whether it possessed specific advantages for the study of the interaction mechanism. We assayed the locomotor activity of the crickets with their optic nerve unilaterally severed under

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the various lighting conditions. The results show that the pacemakers are only weakly coupled to one another, but they strongly inhibit activity driven by the partner pacemaker during their subjective day so that the activity occurs only when the subjective nights of the two pacemakers crisscrossing.

## MATERIALS AND METHODS

### Activity recording and data analysis

All experiments were performed with adult males, *Grylodes sigillatus*, obtained from laboratory colonies. They were maintained in LD12:12 at a constant temperature ( $25 \pm 0.5^\circ\text{C}$ ). Recording methods of locomotor activity of individual crickets were identical to those described previously (Abe *et al.*, 1997). The light intensity was 75-540 lux except dim LD experiments where the light intensity was lowered to 1.5-8.1 lux by shading fluorescent lamps.

Event records of locomotor activity were double plotted in a conventional manner by computer with a resolution of 6 min. The free-running period was objectively determined by the chi-square periodogram (Sokolove and Bushnell, 1978). The phase angle difference between the activity onset and the lights-off was determined by fitting a straight line through activity onsets of several consecutive days. If the activity onset preceded lights-off, the value was expressed as positive value, whereas it expressed as negative value when the onset began later than the lights-off. Values are shown as mean  $\pm$  SD.

### Surgery

Surgical procedures were identical those described previously (Abe *et al.*, 1997). At the end of experiments, animals receiving the optic nerve severance or optic lobe removal were dissected to verify

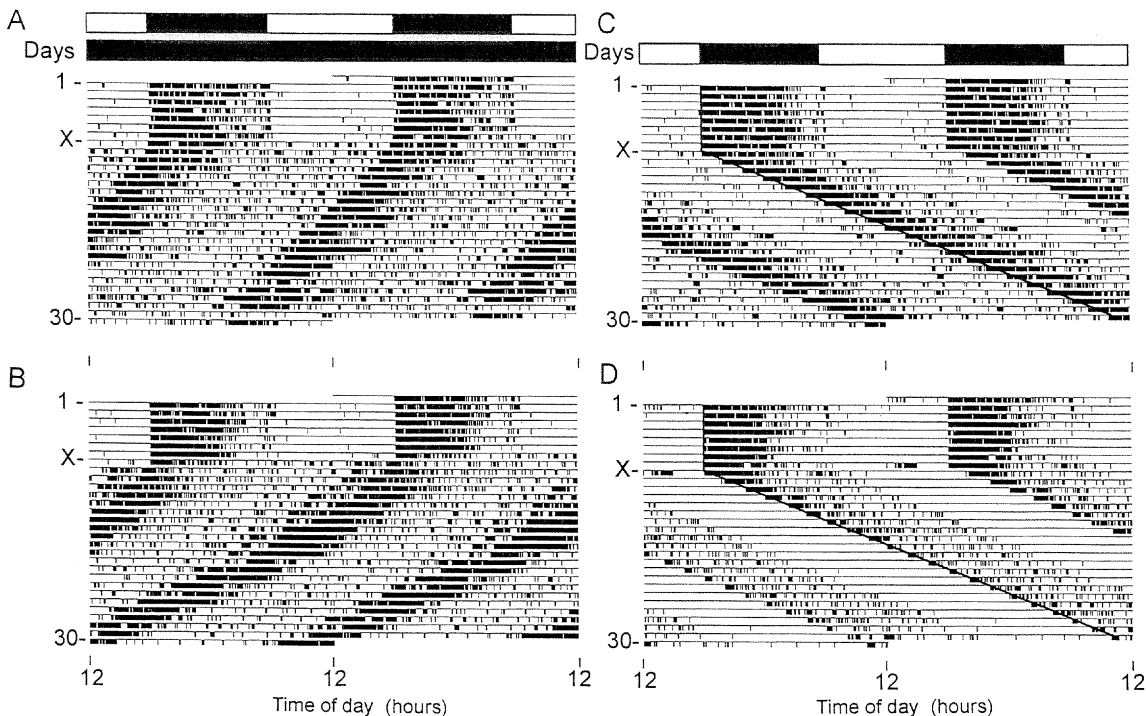
the success of the surgical operation under a dissecting microscope.

## RESULTS

### Locomotor rhythms of intact and sham operated animals

We first examined the locomotor activity rhythms of intact and sham operated crickets under 13 hr light and 13 hr dark cycle (LD13:13) and constant darkness (DD). They were held in LD12:12 for the first several days, and then received the operation and were transferred to either DD or LD 13:13. Figure 1 shows representative results. The animals showed clear nocturnal activity in LD12:12 sometimes with sporadic activity before lights-off. The nocturnal component occurring just after the lights-off in LD12:12 clearly persisted in DD with its onset sometimes fluctuating (Fig. 1A, B). The free-running period of the intact and the sham operated animals were  $23.3 \pm 0.2$  (n=10) and  $23.2 \pm 0.3$  hr (n=12), respectively (Table 1). There was no significant difference between those values ( $P > 0.24$ , t-test).

In LD13:13, the animals were steadily entrained to the light cycle with the activity concentrating during the dark phase (Fig. 1C, D). Although the activity onset usually occurred just after the lights-off, a large day to day fluctuation ( $\sim 2$  hr) in it was observed in some animals. These were commonly observed both in intact and sham operated animals. Phase angle differences between activity onset and lights-off were  $1.0 \pm 1.1$  hr and  $0.6 \pm 0.9$  hr in the intact (n=14) and the sham oper-



**Fig. 1.** Entrained and free-running circadian locomotor rhythms of intact (A, C) or sham operated crickets (B, D) kept either in DD (A, B) or LD13:13 (C, D) from the day indicated by X. The crickets were placed in LD12:12 for the first several days then transferred to the different lighting conditions. Sham operation was performed on the day of transfer. In DD, the crickets showed a clear free-running rhythm with a period shorter than 24 hr, whereas, in LD13:13, they were clearly entrained to the light cycle. A thick line in C and D indicates lights-off. White and black bars indicate the light (white) and dark (black) phases.

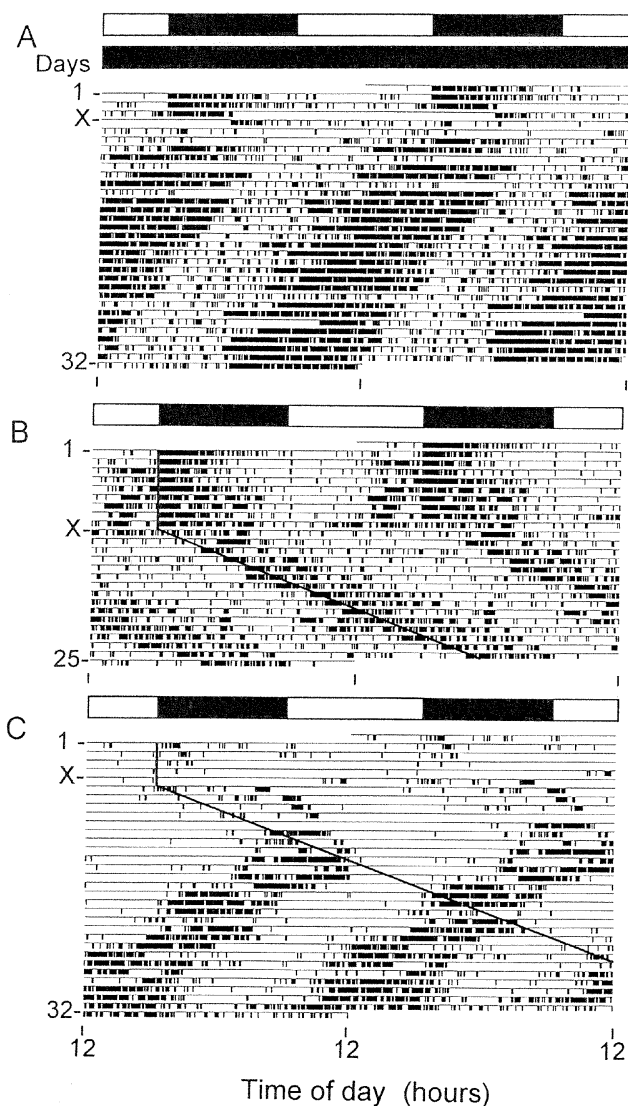
**Table 1.** Free-running periods ( $\tau_s$ ,  $\tau_L$ ) and phase angle differences ( $\Psi$ ) between lights-off and activity onset in crickets with various surgical operations

operation	light cycles	$\Psi^a$ (mean $\pm$ SD hr)	$\tau_s$ (mean $\pm$ SD hr)	$\tau_L^b$ (mean $\pm$ SD hr)
none (intact)	bright LD13:13	1.0 $\pm$ 1.1 (14)	–	–
	DD	–	23.3 $\pm$ 0.2 (10)	–
sham	bright LD13:13	0.6 $\pm$ 0.9 (13)	–	–
	DD	–	23.2 $\pm$ 0.3 (12)	–
unilateral optic lobe removal	bright LD13:13	1.2 $\pm$ 1.8 (17)	–	–
	DD	–	23.5 $\pm$ 0.4 (16)	–
unilateral optic lobe removal and optic nerve severance	bright LD13:13	–	23.5 $\pm$ 0.4 (13)	–
	DD	–	–	–
unilateral optic nerve severance	bright LD13:13	1.2 $\pm$ 1.0 (46)	24.1 $\pm$ 0.7 (11)	26.0 $\pm$ 0.1 (11)
	dim LD13:13	6.2 $\pm$ 2.6 (13)	23.6 $\pm$ 0.7 (11)	26.0 $\pm$ 0.0 (11)
	dim LD12.5:12.5	4.5 $\pm$ 3.9 (13)	23.7 $\pm$ 0.3 (16)	25.0 $\pm$ 0.1 (16)

<sup>a</sup> phase angle difference was determined only for animals with a single entrained rhythm.

<sup>b</sup> calculated period of longer period component detected in unilaterally blinded crickets with infradian rhythms.

Numbers in parenthesis show number of animals used.



ated animals ( $n=13$ ), respectively (Table 1). There were no significant differences between the intact and the sham operated animals ( $P>0.16$ , t-test).

### Effects of unilateral optic lobe removal on the free-running and entrained rhythms

*G. sigillatus* has two circadian pacemakers probably located in the optic lobes (Abe *et al.*, 1977). To test whether each pacemaker can regulate the rhythm, the effects of unilateral removal of the optic lobe on the free-running and entrained rhythms were examined in various lighting conditions. When the activity of 20 animals was measured in DD after the optic lobe was unilaterally removed, 16 of them showed a free-running rhythm with a period of  $23.5 \pm 0.4$  hr. The remaining 4 animals became arrhythmic. The rhythm is often unstable showing a day to day fluctuation in activity onset (Fig. 2A). The period was significantly longer than that of the sham operated animals kept in DD ( $P<0.05$ , t-test) (Table 1).

To reveal whether a single pacemaker can synchronize to LD 13:13, we assayed locomotor activity of 17 animals, of which optic lobe was unilaterally removed, under the LD. As shown in Fig. 2B, all animals entrained to the light cycle but with marked fluctuation of the activity onset. The average  $\Psi$ ,  $1.2 \pm 1.8$  hr, was similar to the intact and the sham operated animals (Table 1).

To further examine whether blinded pacemaker was affected by LD13:13, locomotor activity was assayed with 17

**Fig. 2.** Circadian locomotor rhythms of animals with optic lobe unilaterally removed (A, B) or with the optic nerve unilaterally severed in addition to the removal of the contralateral optic lobe (C). The animals were held in LD12:12 for the first several days, then received surgical operation and were transferred to either DD (A) or LD13:13 (B, C) on the day indicated by X. The operation resulted in a large phase delay in C. For further explanations see text and Fig. 1.

animals with their optic nerve unilaterally severed in addition to removal of the contralateral optic lobe; they thus had a single blinded pacemaker. They frequently showed a large phase delay just after the operation; this was probably caused by the severance of the optic nerve and was often observed also in the other cricket, *G. bimaculatus* (Tomioka *et al.*, 1991). The unstable period of the first 5 days was excluded from the analysis. Thirteen of them exhibited free-running rhythm with a period of  $23.5 \pm 0.4$  hr (Fig. 2C). The value was quite similar to that of the animals with a single intact optic lobe and kept in DD (Table 1). The remaining 4 animals became arrhythmic.

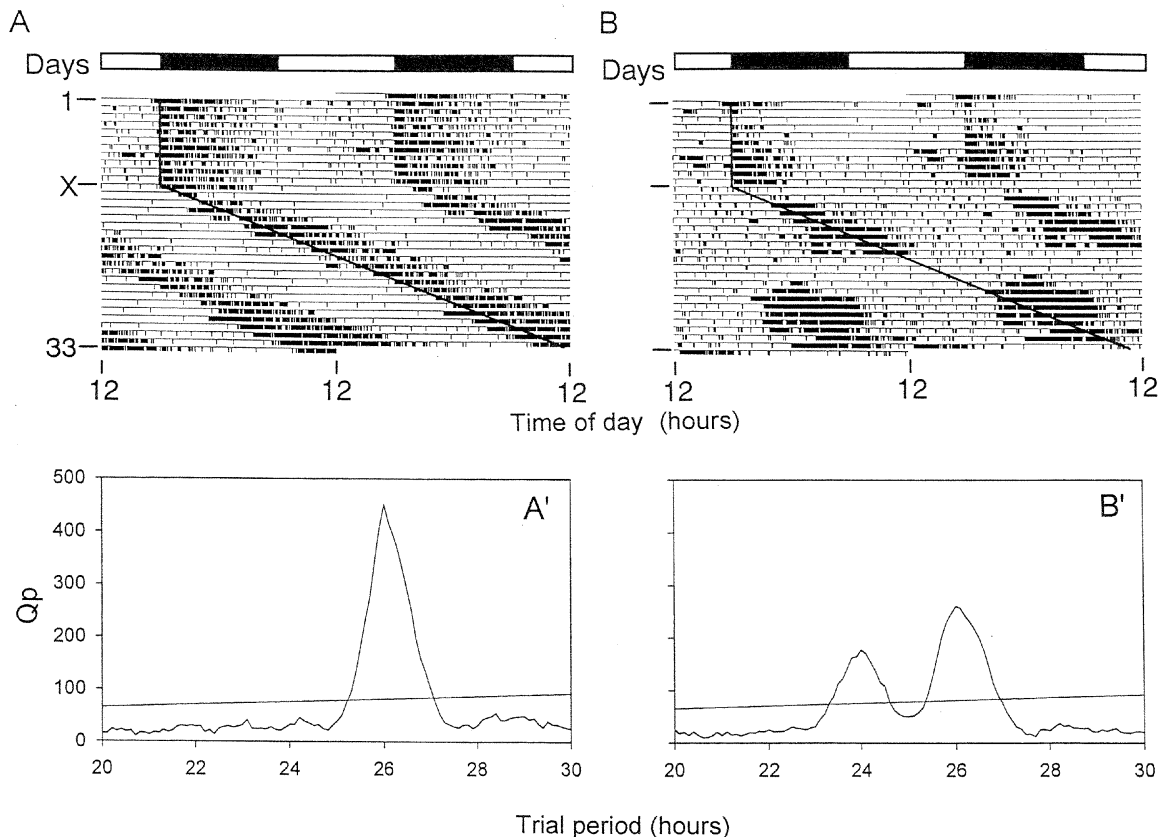
### Effects of unilateral optic nerve severance on the locomotor activity

Most crickets with bilaterally paired pacemakers showed a unimodal rhythm like animals with a single pacemaker. This fact is interpreted as that the bilaterally distributed pacemakers are coupled to one another to move in phase. To examine whether the pacemakers could move separately when different photic information was given to each pacemaker, we recorded the locomotor activity, under LD13:13, of 57 animals with the optic nerve unilaterally severed. As shown in Fig. 3A, 46 animals showed a unimodal rhythm entrained to the given LD. However, the activity phase often became shorter. The

phase angle difference between the activity onset and the lights-off was  $1.2 \pm 1.0$  hr ( $n=46$ ). In the remaining 11 animals, the rhythm started to freerun from the subjective night, but the rhythm soon disappeared for a few days, then reappeared at several hours earlier than the previous activity phase (Fig. 3B). This infradian rhythm persisted with a period of 8 to 10 days. The chi-square periodogram analysis revealed two circadian components involved in the activity rhythm: one component freerun with a period of  $24.1 \pm 0.7$  hr ( $n=11$ ) and the other seemed to be entrained to the light dark cycle since its period was  $26.0 \pm 0.1$  hr ( $n=11$ ) (Table 1). Thus, it seemed that the activity appeared only when the subjective night of the two components overlapped.

### Locomotor rhythm of unilaterally blinded animals in dim LD13:13 and 12.5:12.5

It has been suggested that, in *Gryllus bimaculatus*, photic information from the contralateral eye is involved in the mutual coupling mechanism between the bilaterally paired pacemakers (Tomioka, 1993). To examine this hypothesis, locomotor rhythms of 24 unilaterally blinded animals were recorded under LD13:13 with lower light intensity of 1.5-8.1 lux. In 11 animals, two rhythmic components were detected by the chi-square periodogram analysis. Although a minor peak was of-



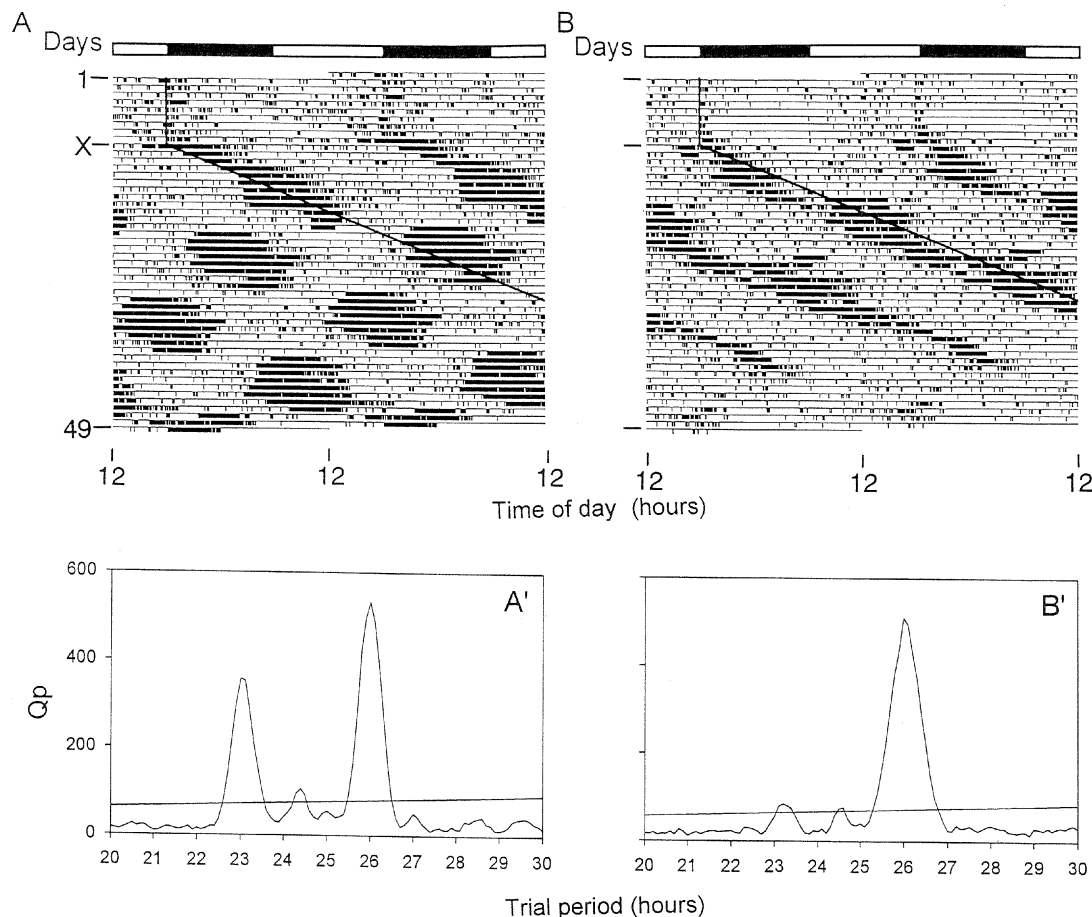
**Fig. 3.** Two examples of locomotor rhythms of animals receiving unilateral optic nerve severance and being transferred to LD13:13 on the day indicated by X. In **A**, only a single rhythmic component persisted, being clearly entrained, while in **B** dense activity bouts appeared around lights off interrupted with a period of almost no activity. **A'** and **B'** show results of periodogram analysis for **A** and **B**, respectively, after the operation. Note that two rhythmic components were detected in **B'**. For further explanations see text and Fig. 1.

ten detected around 24 hr, it seems a harmonics of the two rhythmic components. Ten of the 11 animals showed an activity rhythm that intermittently occurred only when the subjective night phases of the two components were crisscrossing (Fig. 4A). The remaining one showed a unimodal rhythm that was rather clearly entrained to LD cycles but with a marked infradian rhythm, in which activity level was changed with a period of several days (Fig. 4B). The duration of activity phase gradually increased for the first few days, then gradually decreased in the next several days and the activity again disappeared. The average period of the free-running component in these animals was  $23.6 \pm 0.7$  hr (Table 1). The percentage of animals that showed rhythm dissociation was significantly greater than in bright light ( $P < 0.01$ ,  $\chi^2$ -test). In the remaining 13 animals only a unimodal rhythm that was entrained to the LD was observed. Their phase angle difference between the activity onset and the lights-off was  $6.2 \pm 2.6$  hr ( $n = 13$ ). This value is significantly greater than that under bright light conditions (t-test,  $P < 0.01$ ) (Table 1).

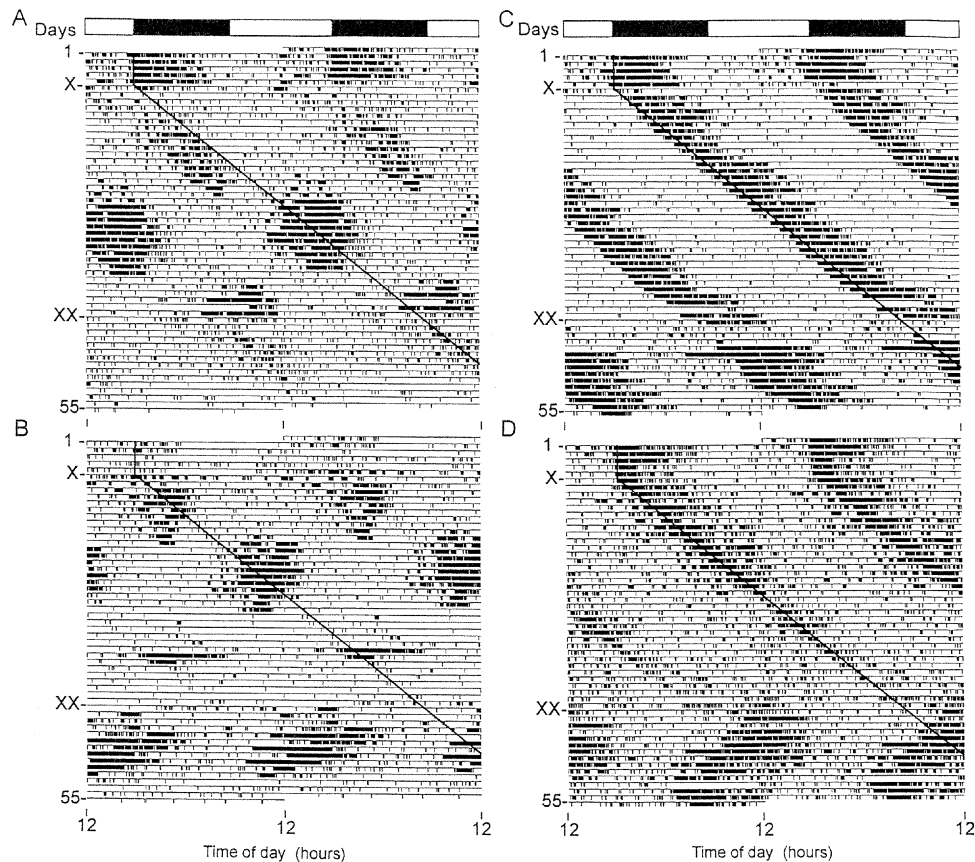
Considerable number of animals showed a split activity pattern also in LD12.5:12.5. We assayed locomotor activity in

29 crickets with unilateral optic nerve severed in this condition and found that 16 showed an infradian rhythm characteristic to the dissociation of the two rhythmic components (examples, Fig. 5). The chi-square periodogram analysis revealed that the period of the free-running component was  $23.7 \pm 0.3$  hr ( $n = 16$ ), whereas that of the entrained components was  $25.0 \pm 0.1$  hr ( $n = 16$ ) (Table 1). In the animals with unimodal entrained rhythms, the phase angle difference between the activity onset and the lights-off was relatively large ( $4.5 \pm 3.9$  hr,  $n = 13$ ) (Table 1).

To examine which pacemaker controls which component, unilateral optic lobe removal was additionally performed on 20 animals: 10 of them were with two rhythmic components and the rest with only an entrained component. In animals with two rhythmic components, the removal on the blinded side resulted in immediate disappearance of the free-running component (Fig. 5A,  $n = 5$ ), while the entrained component was immediately abolished after the optic lobe on the intact side was removed (Fig. 5B,  $n = 5$ ). These facts clearly indicate that the shorter and the longer period components are driven by the pacemaker on the operated and on the intact side, re-



**Fig. 4.** Two examples of locomotor rhythms of animals receiving unilateral optic nerve severance and being transferred to dim LD13:13 on the day indicated by X. In **A**, dense activity bouts appeared around lights off, being interrupted with a period of almost no activity, while in **B** only a single rhythmic component persisted, synchronizing to the LD, but the length of active phase changes with a period of several days. **A'** and **B'** show results of periodogram analysis for **A** and **B**, respectively, after the operation. Note that two rhythmic components were detected in both cases. For further explanations see text and Fig. 1.



**Fig. 5.** Four examples of locomotor rhythms of animals receiving unilateral optic nerve severance and being transferred to dim LD12.5:12.5 on the day indicated by X. In **A** and **B**, dense activity bouts appearing around lights off were interrupted with several days of inactive period, while in **C** and **D** only a single rhythmic component persisted, being entrained to the LD. After removal of the optic lobe on blinded side (**A**, **C**) on the day indicated by XX, the entrained component persisted, while the free-running component occurred when the intact side was removed (**B**, **D**). For further explanations see text and Fig. 1.

spectively. In animals with a single entrained component, either it persisted when the blinded optic lobe was removed (Fig. 5C), or immediately disappeared and a new component suddenly appeared and freeran with a shorter period when the intact optic lobe was removed (Fig. 5D). The free-running component always appeared near the activity phase of the entrained component and extrapolation of its activity onsets seldom corresponded to the dark phase of the original LD12:12 (Fig. 5D).

## DISCUSSION

Our previous study suggested that the principal photoreceptor for photic entrainment of this cricket resides in the compound eye (Abe *et al.*, 1997), like other crickets and cockroaches (*Leucophaea maderae*, Nishiitsutsuji-Uwo and Pittendrigh, 1968b; *Teleogryllus commodus*, Loher, 1972; *Gryllus bimaculatus*, Tomioka *et al.*, 1990). The present results further confirm the earlier conclusion by showing that the animals with a single blinded pacemaker freeran even in LD13:13 (Fig. 2) and that the unilateral severance of the optic nerve often resulted in a loss of synchronization of the ipsilateral pacemaker to light cycles (Fig. 5). Our present results

also support the hypothesis that the optic lobe is the locus of the circadian pacemaker (Abe *et al.*, 1997; Nishiitsutsuji-Uwo and Pittendrigh, 1968a; Page, 1978; Sokolove and Loher, 1975; Tomioka and Chiba, 1984), since the unilateral removal of the optic lobe in the animals with split activity patterns resulted in a loss of one of the two rhythmic components (Fig. 5).

These facts also imply a complex nature of the cricket circadian system that consists of bilaterally paired structure including the compound eyes and the optic lobes. In the natural conditions they seem mutually coupled to move in synchrony through some active mechanisms, since even in DD the locomotor rhythm is consistently unimodal in intact and sham operated animals (see also Abe *et al.*, 1997). Our present experiment revealed some features of the coupling and interaction mechanisms between the bilaterally paired structures.

### Pacemaker coupling

The present study showed that considerable percentage of animals exhibited dissociation of two rhythmic components by exposing the right and the left pacemakers to different lighting schedules (Figs. 3 and 4). The rhythm dissociation is explained that each pacemaker is affected by differential photic

information so that the mutual entrainment of the two pacemakers could not be maintained. The period difference between the pacemakers may be out of allowable range for mutual coupling, since the period of a single pacemaker under DD is close to 23.5 hr. It is thus suggested that the coupling between the two pacemakers is not so tight as in the cockroach *Leucophaea maderae*, in which the two pacemakers keep synchronous movement under any external conditions (Page, 1983). Similar weak coupling relationship has been reported for other cricket species, *Teleogryllus commodus* (Wiedenmann, 1983), and *Gryllus bimaculatus* (Tomioka *et al.*, 1991, 1993). Thus it seems that the weak pacemaker coupling is a common feature in the cricket circadian system. The coupling strength is still enough even for this indoor living cricket to keep stable pacemaker coupling.

It has been reported that, in rhythm dissociation of *G. bimaculatus*, the period of two pacemakers changes dependent on the mutual phase angle relationship (Tomioka *et al.*, 1991, 1993). Such changes in period were not observed in the present study, since the activity in dissociated states occurred only intermittently. Yet there are two facts indicating that the interaction affects the free-running period. First, the period of the free-running component was longer than the animals with a single blinded optic lobe (Table 1). Second, the free-running period of animals with an optic lobe unilaterally removed was significantly longer than that of the intact or sham operated animals (Table 1). It is known that the coupling of pacemakers often makes the free-running period of the circadian system shorter or longer than that of individual pacemakers in some insects (Page *et al.*, 1977; Waddel *et al.*, 1990; Okada *et al.*, 1991) and mollusks (Roberts *et al.*, 1987). Probably, the pacemakers mutually phase shifts their partner in a phase dependent manner and it is likely that phase advance shifts are greater than phase delays in *G. sigillatus*.

A substantial portion of the unilateral blinded animals showed a unimodal entrained rhythm (Fig. 3A). In *T. commodus*, Wiedenmann (1983) showed that, even in unilaterally blinded animals, which exhibited a unimodal rhythm in constant light, the pacemaker on the blinded side freeran as if it were in DD. However, this seems unlikely in *G. sigillatus*, since that after the removal of intact optic lobe the free-running rhythm always appears near the activity phase of the entrained rhythm and that the extrapolation of the onset of free-running rhythm does not lead back to the dark phase of the original LD12:12 (Fig. 5D). It is thus likely that the animals that exhibited an entrained unimodal activity may have relatively stronger coupling force than those showing the dissociated rhythms. This also indicates that light received by either eye can act as an entraining agent for the ipsilateral and the contralateral pacemakers as suggested for cockroaches and other cricket species (Page, 1978; Tomioka and Yukizane, 1997). There were some animals that showed only entrained components but with a marked infradian rhythm in the amount of daily activity (Fig. 4B). An interpretation for this is that the free-running component appears only when it is closely overlapping the other. It is thus suggested that the overt rhythm is

influenced by both underlying components even though only one of them is the main source.

### Light intensity modulates the coupling strength

The number of animals that showed two rhythmic components was significantly less under bright LD 13:13 than under dim LD13:13. The free-running period of the free-running component was also significantly longer in bright light than in dim light (Table 1). These facts support the assumption that the light intensity influences on the coupling strength of the pacemaker (Daan and Berde, 1978; Tomioka, 1993). An important question to be addressed is whether the light affects the contralateral pacemaker directly or indirectly through the ipsilateral pacemaker. In the cricket *G. bimaculatus*, it has been revealed that the light affects the contralateral pacemaker through a neural pathway interconnecting bilateral medulla (Yukizane and Tomioka, 1995; Tomioka and Yukizane, 1997) and that the dorso-caudal area of the compound eye mediates the photic information for coupling (Tomioka and Yukizane, 1997). Further critical study is required to solve this question.

### Mutual inhibition of the activity

The second significant conclusion of our studies is that an optic lobe pacemaker strongly suppresses the activity driven by the contralateral pacemaker. The suppression was reflected in complete absence of an activity component when the subjective night of one pacemaker overlapped with the subjective day of the other (Figs. 3-5). Inhibition between pacemakers has been reported for the cockroach, *L. maderae* and crickets, *T. commodus* and *G. bimaculatus* (Page, 1983; Wiedenmann, 1983; Tomioka *et al.*, 1991). In this cricket, inhibitory signal seemed so strong, in comparison with *T. commodus* and *G. bimaculatus*, that the activity occurs only when the subjective nights of the two pacemakers overlap. This view is consistent with the previous suggestion that the suppression of activity occurs during the rest phase of activity rhythm both in nocturnal and in diurnal animals to keep the stable temporal structure (Meijer *et al.*, 1990; Tomioka, 1993). In the animals that showed two rhythmic components, it is thought that the suppression equally occurred on both components. However, the animals that exhibited entrained component whose amounts of activity changed in an infradian manner imply that the suppression occurred only in the free-running component. This domination of the intact side has been reported for *G. bimaculatus* and *T. commodus* (Wiedenmann, 1983; Yukizane and Tomioka, 1995), suggesting that some photic information is involved in the inhibitory effect.

The strong mutual inhibition of activity during the subjective day might have biological significance in this indoor species. In the field the light plays a major role in keeping the stable temporal structure in cricket's behavior, but in doors the light signal is so weak and labile because of artificial lights associated human activity that the strong inhibition during the subjective day by the pacemaker would be advantageous to



keep a stable nocturnality. The likelihood of this hypothesis should be examined with other indoor species.

### Circadian system in *Gryllobates*

Taken together, it is suggested that the pacemaker has three functionally distinct output pathways, for driving the locomotor activity, for the coupling to the contralateral pacemaker, and for the suppression of the activity driven by the contralateral pacemaker during its subjective day. The mutual suppression of activity by the pacemakers suggests that the output signals from the two pacemakers are integrated at the driven system that regulates the locomotor activity. The strong phase dependent suppression of activity would be advantageous to investigate the neural mechanism regulating the locomotor activity. Elucidation of the neuronal elements involved in the mechanisms would allow us a direct comparison of the coupling mechanism with other insect species.

### ACKNOWLEDGMENT

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