 

# Constant Light, *Pdp1*, and *tim* Exert Influence on Free-running Period of Locomotor Rhythms in the Cricket *Gryllus bimaculatus*

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**Most insects show circadian rhythms of which the free-running period changes in a light-dependent manner and is generally longer under constant light (LL) than under constant dark conditions in nocturnal animals. However, the mechanism underlying this LL-dependent period change remains unclear. Here, using the cricket *Gryllus bimaculatus*, we examined the effects of long-term LL exposure on the free-running period of locomotor rhythms. Initially, the free-running period was considerably longer than 24 h but it gradually became shorter during long-term exposure to LL. The initial lengthening and ensuing gradual shortening under long-term LL exposure were observed even after unilateral removal of the optic lobe. Thus, these changes in the free-running period could be attributable to a single optic lobe clock. RNA interference (RNAi)-mediated silencing of the clock genes *Par domain protein 1* (*Pdp1*) and *timeless* (*tim*) revealed that the treatments eliminated the initial period lengthening by LL without reducing circadian photoreceptor gene expression. However, they did not affect the period shortening during long-term LL exposure. The slopes of the regression line for the period change during long-term LL for *Pdp1*$^{RNAi}$-treated and *tim*$^{RNAi}$-treated crickets were not different from that of the ds*DsRed2*-treated control. These results suggest that the initial period lengthening after transfer to LL requires *tim* and *Pdp1*, while the ensuing period shortening during long-term LL exposure is caused by a mechanism independent of *tim* and *Pdp1*.**

**Key words:** circadian rhythm, cricket, free-running period, constant light, clock gene

## INTRODUCTION

Most insects exhibit a daily activity rhythm that is controlled by an endogenous mechanism called the circadian clock (Saunders et al., 2002; Tomioka and Matsumoto, 2019). The oscillatory mechanism of circadian clocks is well understood in *Drosophila*, and is believed to be based on transcriptional and translational feedback loops consisting of so-called clock genes, including *period* (*per*), *timeless* (*tim*), *Clock* (*Clk*), and *cycle* (*cyc*) (Tomioka and Matsumoto, 2019). In the major loop, the product proteins of *Clk* and *cyc*, CLK and CYC, form a heterodimer which activates transcription of *per* and *tim* during late day to early night. The resultant proteins, PER and TIM, form a heterodimer which enters the nucleus and suppresses *per* and *tim* transcription by inhibiting CLK/CYC (Tataroglu and Emery, 2015). This suppression results in a reduction in PER and TIM levels, releasing CLK/CYC from inhibition by PER/TIM, which leads to the next cycle (Tataroglu and Emery, 2015). In addition, *Clk* is regulated to be expressed during the subjective day by transcriptional repressor VRILLE (VRI) and activator PAR DOMAIN PROTEIN 1 (PDP1) (Cyran et al., 2003). A similar mechanism is thought to play a role in producing circadian rhythms in other insects (Tomioka and Matsumoto, 2015).

The rhythm persists or free-runs under constant conditions of light and temperature, and the free-running period of the rhythm is considerably affected by lighting conditions. The change in the period is described by Aschoff's rule (Pittendrigh, 1960), which states that the period is longer under LL than under DD or lengthens with an increase in light intensity in nocturnal animals, but is shorter under LL or shortens with an increase in light intensity in diurnal animals. Some insects follow this rule, including the cockroach *Leucophaea maderae* (Roberts, 1960) and crickets *Teleogryllus commodus* (Loher, 1972; Sokolove, 1975) and *Gryllus bimaculatus* (Tomioka and Chiba, 1982) as nocturnal insects and *Nasonia* wasps (Bertossa et al., 2013) as diurnal insects. However, many diurnal insects violate this rule, exhibiting longer periods under LL than under DD. These include the dung beetle *Geotrupes sylvaticus* (Geisler, 1961), the blow fly *Calliphora vicina* (Hong and Saunders, 1994), and the mosquito *Aedes aegypti* (Geisler, 1961; Taylor and Jones, 1969; Clopton, 1984; Hong and Saunders, 1994). The rhythm sometimes becomes arrhythmic in high-intensity LL but free-runs under lower intensity of light (Tomioka and Chiba, 1982; Clopton, 1984; Hong and Saunders, 1994; Yoshii et al., 2005).

The mechanism underlying the light-dependent change in the free-running period remains largely unknown. Considering that rhythm synchronizes with environmental light

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cycles, the changes in the free-running period under LL may be caused by the light entrainment pathway. In *Drosophila*, the effect of LL is often explained in relation to CRYPTOCHROME (CRY), which is a blue light receptor expressed in cerebral clock neurons and plays an important role in photic entrainment of the clock (Emery et al., 1998, 2000; Stanewsky et al., 1998; Yoshii et al., 2008). Under LL with low light intensity, the rhythm free-runs for a period longer than 24 h, whereas it disappears under LL of high intensity (Konopka et al., 1989). The arrhythmic activity in high-intensity LL is explained by the fact that the circadian clock is arrested at around CT12 by continuous degradation of the TIM protein, which is induced by light-activated CRY with JETLAG (Pittendrigh, 1960; Yoshii et al., 2005; Koh et al., 2006; Peschel et al., 2009). Under LL with low light intensity, incomplete degradation of the TIM may allow the rhythm to persist, but with a longer period.

However, many insects use the compound eye for photic entrainment, and the mechanism of their photic entrainment is still not fully understood (Saunders et al., 2002; Tomioka and Matsumoto, 2019). Therefore, the mechanism underlying LL-dependent changes in free-running periods in these insects remains to be elucidated. The light entrainment mechanism via the compound eye has been studied in detail in the cricket *G. bimaculatus*. In this cricket, light information is perceived by opsin-Long Wavelength (opLW) in the compound eye (Komada et al., 2015), sent to the circadian clock located in the optic lobe via the neural pathway (Tomioka and Chiba, 1984, 1992), and shifts the clock via intracellular pathways, including *Pdp1* and *c-fos* (Kutaragi et al., 2016, 2018). In addition, we recently found that *tim*$^{RNAi}$ prevents photic entrainment of the clock in delay direction in this species (Moriyama et al., 2022). The cricket *G. bimaculatus* shows period lengthening under LL an intensity-dependent manner (Tomioka and Chiba, 1982); it is likely the clock components involved in photic entrainment may play an important role in this LL dependent period lengthening.

In addition, LL or constant red light sometimes causes the activity rhythm to split into two components. This rhythm splitting is known for the cricket *Teleogryllus commodus* and the fruit fly *Drosophila melanogaster* (Helfrich and Englemann, 1983; Wiedenmann, 1983; Yoshii et al., 2004). In the split situation, emerged two rhythms run with different free-running periods. For example, in *Drosophila cry*$^{b}$ mutant, two components run with a period longer and shorter than 24 h, respectively (Yoshii et al., 2004). These facts suggest that the circadian system controlling activity rhythms includes two clocks with different period responsiveness to light. However, it is unknown how LL affects the two clocks differentially.

The aim of this study was to understand the mechanism by which LL affects the clock and clock systems that control the free-running period of locomotor rhythms. Using the cricket *G. bimaculatus*, we first investigated the effect of long-term exposure to constant light and found that LL initially lengthened the free-running period, although the period gradually shortened during prolonged exposure to LL, which was not associated with a reduction in circadian photoreceptor gene expression. LL also caused splitting of the locomotor rhythm into two components with different free-running periods, with both exhibiting period shortening. We then examined the role of *tim* and *Pdp1*, which are involved in light-induced phase delay, using RNAi and found that *tim*$^{RNAi}$ and *Pdp1*$^{RNAi}$ significantly shortened the free-running period under LL and the period further shortened during long-term LL exposure. The results are discussed in relation to photic entrainment and clock oscillatory mechanisms.

## MATERIALS AND METHODS

### Animals

Adult male crickets *G. bimaculatus*, within 1 week after the imaginal molt, were used for this study. They were taken from a laboratory colony kept under 12 h light: 12 h dark cycle (LD12:12, L: 06:00–18:00, Japan Standard Time) at a constant temperature of 25.0 ± 1.0°C. Animals were fed laboratory chow (CA-1, CLEA Japan, Tokyo, Japan) and water.

### Surgical operation

For removal of a single optic lobe, a cricket anesthetized with $CO_2$ was placed on a specially designed platform, and its head was fixed with plastic clay. The head cuticle around the compound eye was cut with a razor knife, and the compound eye was slightly opened with tweezers to expose the optic lobe. The optic lobe in crickets includes only the outer two neuropils, the lamina, and medulla. The third neuropil, the lobula, is located proximal to the brain, separate from the lamina and medulla (Honegger and Schurmann, 1975). The optic nerve and the optic stalk, which connects the optic lobe including the two outer neuropils and the lobula, were cut with small scissors, and the optic lobe was removed using tweezers. The lobula remained intact. The compound eye was then replaced to its original position, and the wound was closed with hemolymph coagulation. The optic lobe removal was performed 7 days before transfer of the cricket to LL.

### RNAi

Double-stranded RNAs (dsRNAs) from *G. bimaculatus Pdp1* (GenBank/EMBL/DDBJ accession No. LC512908), *tim* (BAJ16356), and *DsRed2* derived from a coral species (*Discosoma* sp.) were synthesized using the CUGA in vitro transcription kit (Nippon Gene, Tokyo, Japan) according to the method described by Moriyama et al. (2022) with primers 5′-CTAATACGACTCACTATAGGGAGAGTAAAGAAGATAGAGAGTAT-3′ and 5′-CAATTAACCCTCACTAAAGGGAGATTGGAGAGAACTGAAGAGGT-3′ for *tim* (519 bp); 5′-CTAATACGACTCACTATAGGGAGATCAACGGACACGTCAAGGT-3′ and 5′-CAATTAACCCTCACTAAAGGGAGAAGCCCCATGTTCTCCTTCTC-3′ for *Pdp1* (475 bp); and 5′-CTAATACGACTCACTATAGGGAGATCATCACCGAGTTCATGCG-3′ and 5′-CAATTAACCCTCACTAAAGGGAGACTACAGGAACAGGTGGTGGC-3′ for *DsRed2* (659 bp). After the yield and quality of the RNA were assessed, the same amount of sense and antisense RNA were mixed. RNA was denatured for 5 min at 100°C and annealed by gradual cooling to room temperature. After ethanol precipitation, the dsRNA was suspended in ultrapure water (Invitrogen, Tokyo, Japan) and adjusted to a final concentration of 20 μM. The dsRNA solution was stored at −80°C until use. The dsRNA solution (760 nl) was injected with a nanoliter injector (WPI, Sarasota, FL) into the abdomen of adults anesthetized with $CO_2$. Injection was performed within a week after the imaginal molt.

### mRNA measurement

*opLW* mRNA levels in the compound eye were measured by quantitative real-time PCR (qPCR). The crickets were transferred to LL at 6:00 on Day 1. The crickets treated with dsRNAs were transferred to LL on the day of injection (Day 0). The compound eyes of adult male crickets were collected at around 12:00 under LD (before transfer to LL, Day 0), and on Day 10, 20, and 30 after transfer to LL. Total RNA was extracted and purified from single adult male compound eyes using TRIzol reagent (Invitrogen). RNA was treated

with DNase I to remove contaminating genomic DNA. qPCR was performed using the CFX Connect Real-time system (Bio-Rad, Tokyo, Japan) with Thunderbird Next SYBR qPCR Mix (Toyobo, Osaka, Japan) containing SYBR Green with primers 5′-CAGTTCCCTCCCATGAACCC-3′ and 5′-GATGTAGACGACCATGCCGT-3′ for *opLW* (LC004297); and 5′-GCTCCGGATTACATCGTTGC-3′ and 5′-GCCAAATGCCGAAGTTCTTG-3′ for *rpl18a* (DC448653). Quantification was performed based on a standard curve obtained using a known amount of the template. The results were analyzed using the software provided with the instrument. *rpl18a* was used as an internal control, and the values of *opLW* were normalized to the value of *rpl18a* at each point. The results of five to eight independent experiments were used to calculate the mean ± standard error of the mean (SEM).

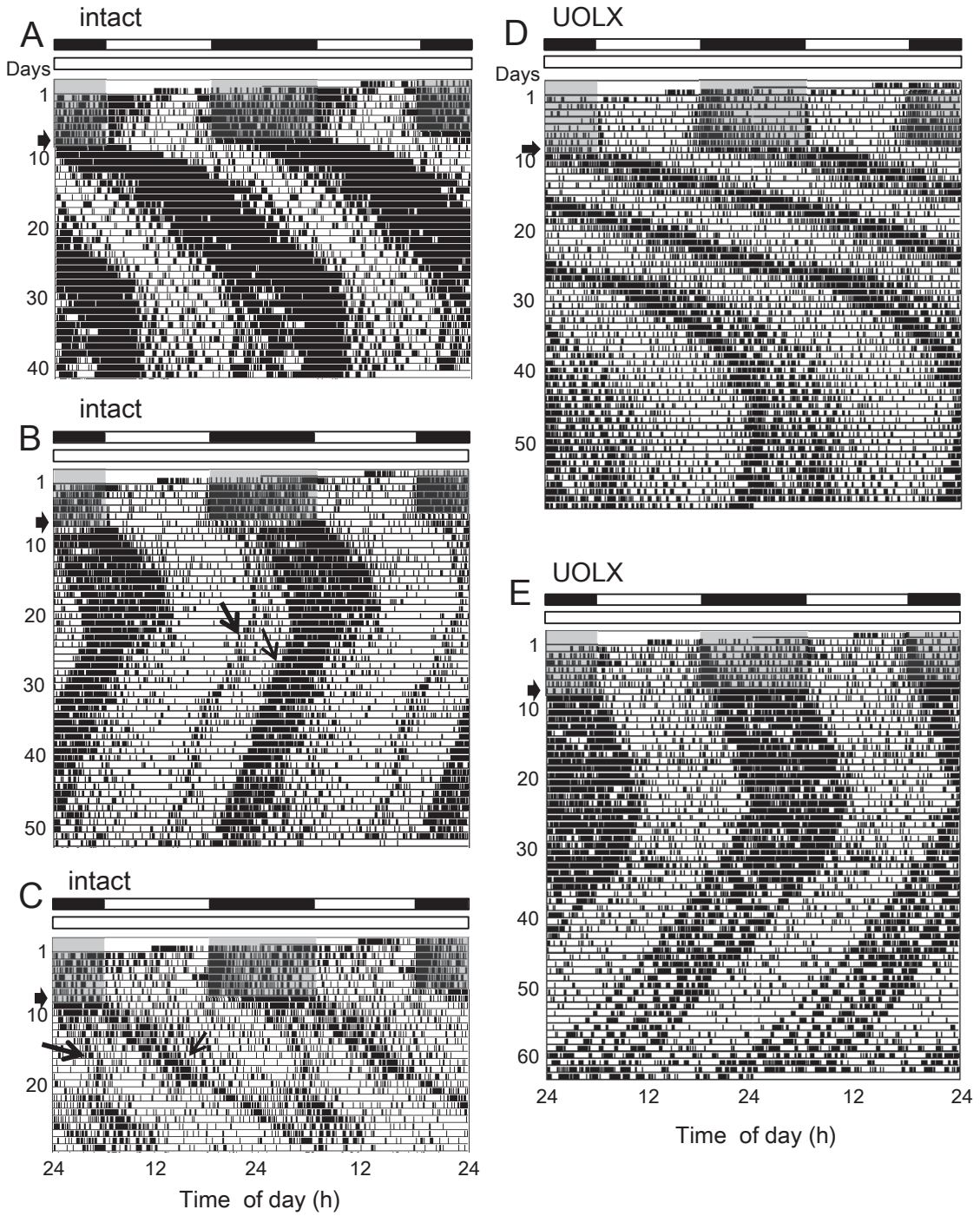


**Fig. 1.** Double plotted actograms of locomotor rhythms of intact adult male crickets (*Gryllus bimaculatus*) **(A–C)** and crickets with unilateral optic lobe removed (UOLX) **(D, E)**. Crickets were kept under LD12:12 for the first several days, then transferred to constant light on the day indicated by an arrow on the left side of the actograms. White and black bars indicate light (white) and dark (black) conditions. Gray area in actograms indicates the dark phase. Temperature was kept constant at 25°C. A gradual decline in the free-running period is observed in **(A, B, D, E)**. Rhythms split into two peaks, with a leading peak (thick arrow) and a lagging peak (thin arrow) in **(B, C)**.

**Recording of locomotor activity and visual inspection of actograms**

The locomotor activity of crickets was recorded as described previously (Moriyama et al., 2008). Briefly, adult crickets were individually housed in a transparent plastic box (18 × 9 × 4.5 cm) with a rocking substratum. For the crickets treated with dsRNA, the recording was started immediately after dsRNA injection. Movement of the substratum caused by a moving cricket was sensed by a magnetic reed switch placed at the bottom of the actograph and recorded every 6 min using a computerized system. Food and water were provided ad libitum. The actographs were placed in an incubator (MIR-154, Panasonic, Osaka, Japan or MIR-153, Sanyo, Osaka, Japan), in which lighting conditions were provided by a cool white fluorescent lamp connected to an electric timer, and the temperature was kept at 25.0 ± 1.0°C. The light intensity ranged from 2.04 to 9.27 W/m$^2$, which varied depending on the distance from the light source.

The raw data were displayed as conventional double-plotted actograms using actogramJ (Schmid et al., 2011) to determine activity patterns. The onset of locomotor activity was used as the reference phase. When the onset split into two distinct components soon after transfer to LL and they ran with different free-running periods, we recognized it as rhythm splitting. The splitting state was characterized by weaker activity when the peak of a component was in the subjective day of the other, and conversely, stronger activity when it was in the subjective night. This activity modulation is caused by activity suppression during subjective day of the optic lobe clock (Tomioka et al., 1991).

**Statistical analysis**

To detect long-term changes in the free-running period, the periods for every 10 days were calculated by the chi-square periodogram using actogramJ (Schmid et al., 2011) for each individual. Individuals whose activity could not be recorded for 5 days or more during each period were excluded from the analysis. When two components were observed, the period of the stronger component was adopted. Differences in the free-running period between the first 10 days of LL and subsequent 10 day periods were tested by ANOVA followed by Dunnett's test. Free-running periods and their long-term changes were compared between groups of different treatments by factorial ANOVA followed by Tukey's test, according to Dalla Benetta et al. (2019). Comparison of the slopes of regression lines for long-term changes in free-running periods in crickets treated with various dsRNAs was performed using analysis of covariance (ANCOVA).

For comparison of *opLW* mRNA levels of differently treated groups, one-way ANOVA followed by post hoc Tukey's test was used.

## RESULTS

### Locomotor rhythms of intact crickets under LL

Locomotor activities of 29 intact adult male crickets were recorded under constant light. The crickets were placed under LD12:12 for the first several days and then transferred to LL. Under LD conditions, they showed nocturnal rhythms with a prominent peak at lights-off. After transfer to LL, two became arrhythmic, and the rest showed a free-running rhythm (Table 1). The rhythmic crickets initially showed a free-running rhythm with a period longer than 24 h (Fig. 1A–C). More than half of the rhythmic crickets showed two peaks that ran with different periods (Table 1). One peak (the leading peak) ran with a slightly shorter period than the other (the lagging peak), and the leading peak was always weaker than the lagging peak (Fig. 1B, C). The two peaks often ran parallel (Fig. 1B), whereas in some crickets, the leading peak merged with the lagging peak (Fig. 1C).

In most crickets, the free-running period changed during long-term LL exposure (Figs. 1A, B; 2). The period for the initial 10 days was 25.53 ± 1.00 h (mean ± SD, $n$ = 26), which gradually shortened to less than 24 h (23.57 ± 1.32 h, $n$ = 9) during 31–40 days of LL (ANOVA, $F_{4,75}$ = 7.5539, $P$ < 0.001) (Fig. 2).

**Table 1.** Summary of results of locomotor rhythms under LL in the cricket *Gryllus bimaculatus*.

| Treatment | $n$ | Rhythm[1] | | Splitting[2] | | |
|---|---|---|---|---|---|---|
| | | R+ | R– | S+ | S± | S– |
| Intact | 29 | 27 | 2 | 14 | 0 | 13 |
| Right OLX | 18 | 18 | 0 | 0 | 0 | 18 |
| ds*DsRed2* | 20 | 16 | 4* | 8 | 0 | 8 |
| *Pdp1*$^{RNAi}$ | 11 | 11 | 0 | 4 | 1 | 6 |
| *tim*$^{RNAi}$ | 13 | 12 | 1 | 5 | 0 | 7 |

1: R+, rhythmic; R–, arrhythmic. 2: S+, split; S±, faintly split; S–, not split. *These crickets were rhythmic during the first 10 days.

### Locomotor rhythms of crickets with the optic lobe unilaterally removed

In the cricket, the overt locomotor rhythm is governed by two circadian clocks located one in each of the two optic lobes, which include the two outer neuropils, the lamina, and medulla (Tomioka and Chiba, 1984, 1992). The free-running period is determined by an interaction between the two clocks (Okada et al., 1991; Tomioka et al., 1991). Therefore, changes in free-running period under LL might involve changes in this interaction. To examine whether the mutual interaction between clocks is required for the period lengthening and subsequent shortening under LL, we recorded the locomotor activity of 18 adult male crickets with the optic lobe, including the lamina and medulla, unilaterally removed

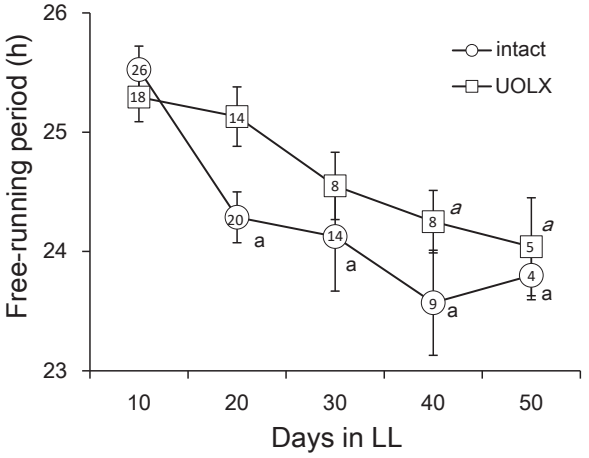


**Fig. 2.** Changes in free-running period under prolonged LL in intact and uninalteral optic lobe removed (UOLX) adult male crickets *Gryllus bimaculatus*. Abscissa indicates the period of every 10 days. Values indicate average at each 10-day period and vertical bars indicate SEM. Number in each symbol indicates number of crickets used. Lowercase letters a and *a* indicate significant differences compared to the first 10-day period (ANOVA followed by Dunnett's test, $P$ < 0.05). There was no significant difference between intact crickets and UOLX crickets (factorial ANOVA, $P$ > 0.05).

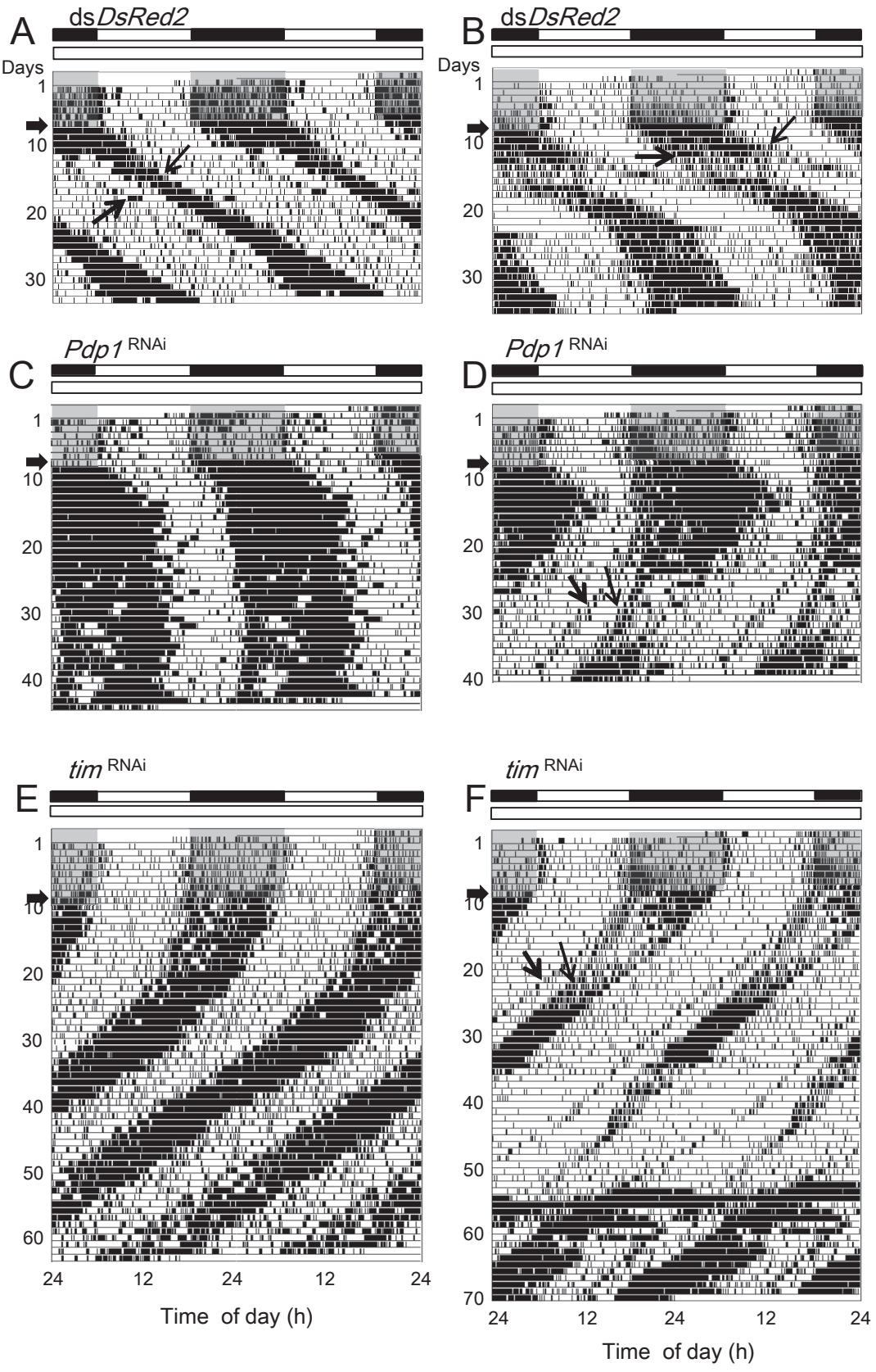


**Fig. 3.** Double-plotted actograms of locomotor rhythms of adult male crickets (*Gryllus bimaculatus*) treated with ds*DsRed2* **(A, B)**, *Pdp1*$^{RNAi}$ **(C, D)**, and *tim*$^{RNAi}$ **(E, F)**. Crickets were kept under LD12:12 for the first several days, then transferred to constant light on the day indicated by an arrow on the left side of the actograms. A gradual decline in the free-running period is observed. **(A, B, D, F)** depict actograms with rhythms split into two components: leading (thick arrow) and lagging (thin arrow) ones. For other explanations see legend of Fig. 1.

(UOLX). Similar to intact crickets, all UOLX crickets showed a nocturnal rhythm under LD that free-ran in the ensuing LL, while unlike in intact crickets, no rhythm splitting was observed (Table 1); the UOLX crickets consistently showed a single nocturnal component throughout the recording period. The free-running period changed as days went by under LL, as exemplified in Fig. 1D and E. The average free-running period for the initial 10 days was 25.29 ± 0.88 h ($n$ = 18) and gradually shortened to be close to 24 h with an average of 24.04 ± 0.92 h ($n$ = 5) during 41–50 days of LL (ANOVA, $F_{4,47}$ = 3.9126, $P$ = 0.0080) (Fig. 2). Between the intact and UOLX crickets, there was no significant difference in the free-running period (factorial ANOVA, $F_{1,122}$ = 3.0904, $P$ = 0.0813) or in the slope of the change in free-running period (ANCOVA, $F_{1,128}$ = 1.9148, $P$ = 0.1688).

### Locomotor rhythms of ds*DsRed2*-, *Pdp1*$^{RNAi}$-, and *tim*$^{RNAi}$-treated crickets under LL

We have previously shown that delay shifts of the circadian clock caused by delayed light-off are associated with an increase in *Pdp1* mRNA levels, followed by the upregulation of *Clk* and *tim* (Kutaragi et al., 2016). In addition, we recently found that RNAi of *tim* often prevented reentrainment of LD cycles delayed by 6 h, and that the delay shifts were associated with TIM degradation (Moriyama et al., 2022). Since in LL, the clock normally slows its movement during the early subjective night, which is closely related to phase delay (Pittendrigh, 1981), these facts imply the possibility that *tim* and *Pdp1* are involved in lengthening the free-running period under LL. Thus, we investigated the effects of *tim* and *Pdp1* RNAi on locomotor rhythms under LL conditions. We used ds*DsRed2* treatment as the control. Similar to intact crickets, control crickets treated with ds*DsRed2* initially showed free-running nocturnal locomotor rhythms with a period longer than 24 h, with some showing rhythm splitting (Fig. 3A, B, Table 1). Again, the leading peak was weaker than the lagging peak in the cricket shown in Fig. 3A. However, for the cricket shown in Fig. 3B, the lagging peak was quickly weakened because it entered the subjective day of the other clock soon after the split. The free-running period gradually became significantly shorter (ANOVA, $F_{4,60}$ = 2.7656, $P$ = 0.0354) (Fig. 4). *Pdp1*$^{RNAi}$-treated crickets showed locomotor rhythms running with a period slightly longer than 24 h for at least the first several days, and the period soon became significantly shorter, often shorter than 24 h (ANOVA, $F_{3,25}$ = 3.2499, $P$ = 0.0386) (Figs. 3C, D; 4). Crickets treated with *tim*$^{RNAi}$ exhibited free-running rhythms with periods shorter than 24 h, except during the first 10 days of LL (Fig. 3E, F). The period was significantly shorter than that of crickets treated with ds*DsRed2*, and became still shorter during long-term LL exposure (ANOVA, $F_{5,40}$ = 2.6819, $P$ = 0.0350) (Fig. 4). Both *Pdp1*$^{RNAi}$-treated and *tim*$^{RNAi}$-treated crickets often showed rhythm splitting similar to that of intact crickets (Fig. 3D, F, Table 1), and the leading peak was always weaker than the lagging peak. Factorial ANOVA revealed that the free-running periods of *Pdp1*$^{RNAi}$-treated and *tim*$^{RNAi}$-treated crickets were significantly shorter than those of ds*DsRed2*-treated control crickets ($F_{2,117}$ = 21.7468, $P$ < 0.001). There was no significant difference in the slopes of the regression lines between ds*DsRed2*-treated control and *Pdp1*$^{RNAi}$-treated or *tim*$^{RNAi}$-treated crickets (ANCOVA, $F_{2,134}$ = 0.2248, $P$ = 0.7990).

### Locomotor rhythms under DD

To investigate whether the spontaneous change in the free-running period occurs in constant darkness (DD), we recorded the locomotor activity of 11 ds*DsRed2*-treated adult male crickets under long-term DD conditions. Figure 5A shows a representative actogram. The crickets were nocturnally active, and the nocturnal component free-ran in the ensuing DD. The free-running period was quite stable: the periods for the initial 10 days (days 1–10) and last 10 days (days 31–40) were 23.49 ± 0.20 (SD) h ($n$ = 11) and 23.30 ± 0.32 h ($n$ = 7), with no significant change found through ANOVA ($F_{3,30}$ = 0.8996, $P$ = 0.4529) (Fig. 5B).

### Changes in *opLW* expression during LL

The responses of the cricket circadian clock to light are dependent on the light information perceived in the compound eye (Tomioka and Chiba, 1984; Tomioka et al., 1990; Komada et al., 2015). In *Drosophila*, the structure and physiology of the compound eye deteriorate during long-term exposure to light (Lee and Montell, 2004). Thus, we examined the possibility that long-term changes in the free-running period and the shorter period in *tim*$^{RNAi}$-treated crickets were associated with a reduction in the circadian photoreceptor molecule opLW.

*opLW* mRNA levels in the compound eye were measured using qPCR in untreated and *tim*$^{RNAi}$-treated crickets. In untreated crickets, a slight increase was observed in *opLW* mRNA levels during 30 days of LL exposure (ANOVA, Tukey's test, $P$ < 0.01) (Fig. 6), suggesting that the shortening of the free-running period under prolonged LL is not related to the opLW reduction in the compound eye. In *tim*$^{RNAi}$-treated crick-

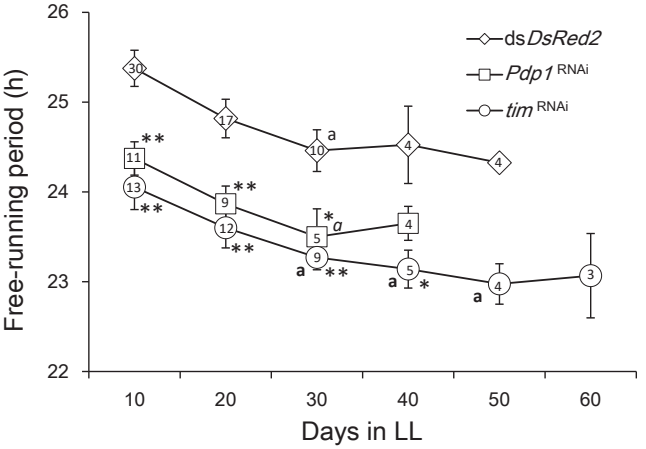


**Fig. 4.** Changes in free-running period under long-term LL in cricket *Gryllus bimaculatus,* treated with ds*DsRed2*, *Pdp1*$^{RNAi}$, or *tim*$^{RNAi}$. Abscissa indicates the period of every 10 days. Values indicate average at each 10-day period and vertical bars indicate SEM. Number in each symbol indicates number of crickets used. Lowercase letters a, *a*, and **a** indicate significant differences from the first 10 day period (Dunnett's test, $P$ < 0.05). Asterisks indicate that the value differs significantly from that of ds*DsRed2*-treated control crickets (factorial ANOVA followed by Tukey's test, *, $P$ < 0.05; **, $P$ < 0.01). The period in *Pdp1*$^{RNAi}$-treated and *tim*$^{RNAi}$-treated crickets was shorter than that in ds*DsRed2*-treated controls.

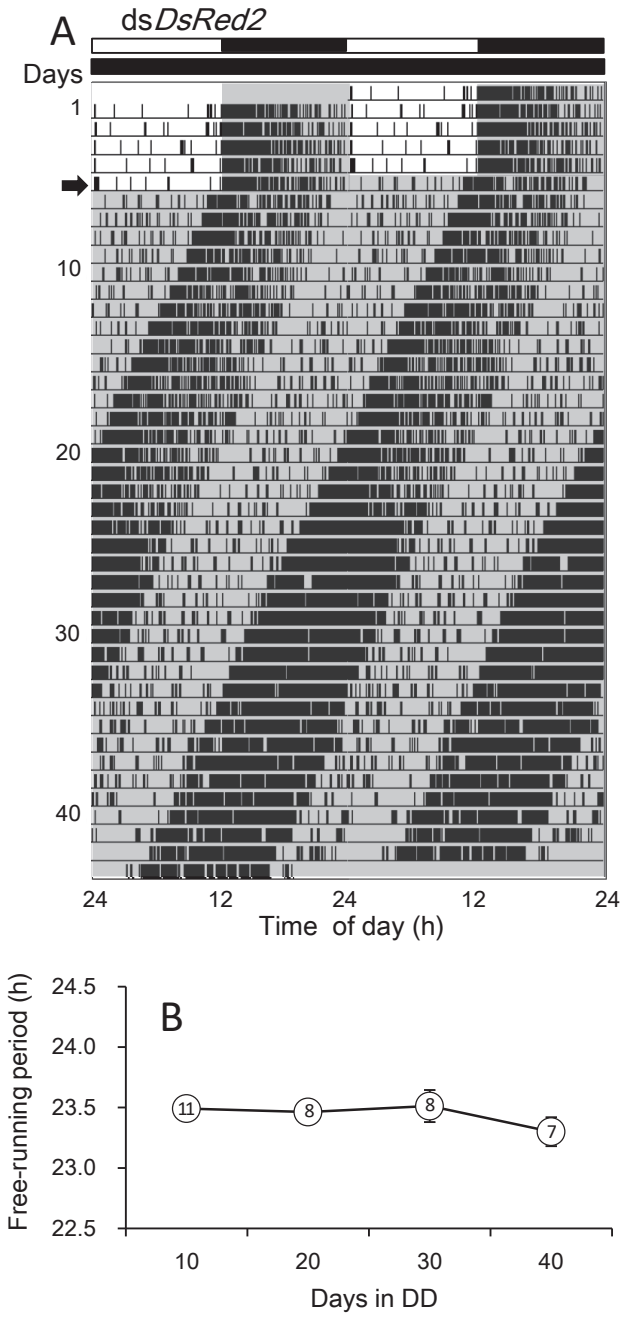


**Fig. 5.** Free-running rhythm of ds*DsRed2*-treated adult male crickets *Gryllus bimaculatus* in long-term constant darkness. **(A)** A representative double-plot actogram of a cricket which was kept under LD for the first 5 days and then transferred to DD. Arrow indicates the day of transfer to DD. **(B)** Average free-running periods with SEM for every 10-day period. Number in each symbol indicates number of crickets used. For further explanations see Figs. 1 and 2.

ets, a significant increase in *opLW* mRNA levels was found from days 10 to 20 in LL, and the level was not significantly different from that of untreated crickets (ANOVA, Tukey's test, $P > 0.05$) (Fig. 6). Thus, the shorter free-running period in *tim*$^{RNAi}$-treated crickets may not be attributable to reduced sensitivity to light.

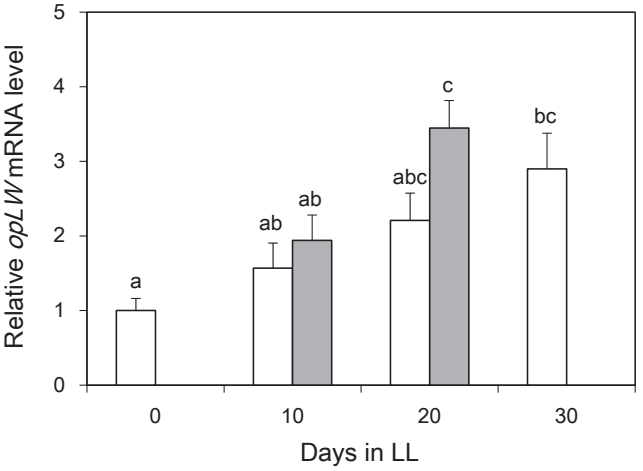


**Fig. 6.** *opLW* mRNA expression under LL in the compound eye of untreated crickets (open columns) and *tim*$^{RNAi}$-treated crickets (*Gryllus bimaculatus*) (gray columns). Values are the average of five–eight samples relative to *rpl18a* mRNA levels normalized by the value for untreated crickets kept under LD (Day 0). Different lower case letters indicate that the values are significantly different (ANOVA followed by Tukey's test, $P < 0.05$). *opLW* expression increased slightly during 30 days of LL in untreated adult male crickets and between day 10 to 20 in *tim*$^{RNAi}$-treated crickets.

## DISCUSSION

### *tim*$^{RNAi}$ shortened free-running period under LL

The present study demonstrated that *tim*$^{RNAi}$ treatment significantly shortened the free-running period under prolonged LL. This shortening effect was consistent with the effect observed under DD (Danbara et al., 2010), suggesting that *tim* was involved in the control mechanism of the free-running period. A similar period shortening was found in *tim* knockout linden bugs *Pyrrhocoris apterus* (Kotwica-Rolinska et al., 2022), suggesting that *tim* may share common functions in the clock machinery in *G. bimaculatus* and *P. apterus*. The shortening of the free-running period cannot be attributable to photoreceptor dysfunction because *tim*$^{RNAi}$-treated crickets were robustly synchronized with the light cycle (Fig. 3E, F).

Studies have shown that *tim*$^{RNAi}$ ablates daily rhythms of expression of *tim*, but not *cry2*, which maintains rhythmic expression with a slight change in its peak phase (Danbara et al., 2010; Tokuoka et al., 2017). Thus, the locomotor rhythms in *tim*$^{RNAi}$-treated crickets should be attributable to *cry2* oscillations that may operate with a shorter period. *tim* most likely works with *per* to form a *per*/*tim*-oscillatory loop that may couple to the *cry2* loop to determine the period of the entire clock system. The shorter free-running period in *tim*$^{RNAi}$-treated crickets under LL is probably caused by a lack of phase responsiveness during the early subjective night. In LL the clock normally slows its movement down, which is closely related to phase delay, during the early subjective night (Pittendrigh, 1981). This hypothesis is consistent with our recent finding that *tim*$^{RNAi}$ disrupted delay shifts of the clock caused by a phase delay of the LD cycle (Moriyama et al., 2022).

*Pdp1*$^{RNAi}$ also had period shortening effects. This may

occur through a reduction in *tim* expression because, in the *Drosophila* clock, PDP1 enhances the transactivation of *Clk*, the product protein CLK transactivating *tim* (Cyran et al., 2003). Actually we have recently shown that *Pdp1*$^{RNAi}$ reduces TIM levels (Moriyama et al., 2022). However, our previous results showed that *Pdp1*$^{RNAi}$ upregulated *Clk* expression under LD conditions and had little effect on free-running periods under DD (Narasaki-Funo et al., 2020). The role of *Pdp1* in the clock oscillatory mechanism may differ under different lighting conditions.

**Spontaneous changes in free-running period under LL**

The present study showed that the free-running period of locomotor rhythms gradually became shorter during prolonged LL in *G. bimaculatus*. The rhythm initially ran with a period longer than 24 h, as previously reported by Tomioka and Chiba (1982), whereas the period became shorter to almost equivalent to that under DD at 31–40 days (Figs. 2, 5). In addition, the period shortening under LL appeared to be independent of *Pdp1* and *tim*, as there were no significant differences in the slopes of the regression lines of period change among ds*DsRed2*-, *Pdp1*$^{RNAi}$-, and *tim*$^{RNAi}$-treated crickets. There are several possible explanations for this spontaneous change in the free-running period.

One possibility is that the change may be due to the aging of the circadian clock itself. However, this seems unlikely because the free-running period was very stable without any significant changes under long-term constant darkness (Fig. 5). Second, one could argue that the period change is caused by some interaction between two optic lobe clocks, as has been suggested in New Zealand weta (Lewis, 1994). However, the period shortening under prolonged LL was observed even after unilateral removal of the optic lobe (Figs. 1D, E; 2). Thus, the change in period should be attributable to a single circadian clock and its photic entrainment pathway.

Currently, it seems most likely that some component(s) involved in the photic entrainment pathway gradually changes owing to long-term LL exposure. Our results showed that the free-running period of intact crickets became even shorter than 24 h (Figs. 1, 2), which is typical for crickets kept in DD (Tomioka and Chiba, 1982). Although this result suggests that photoreception may weaken during long-term exposure to LL, qPCR results revealed that the expression level of *opLW*, the circadian photoreceptor gene (Komada et al., 2015), actually increased both in untreated and *tim*$^{RNAi}$-treated crickets (Fig. 6). Thus, circadian light perception may function even after exposure to LL for 40 days. These results on opsin content are in contrast to those found in *Drosophila*, in which long-term LL exposure leads to retinal damage and blindness, and retinal dysfunction in morphology and physiology is paralleled by a gradual decline in rhodopsin concentration (Lee and Montell, 2004).

Therefore, some component(s) of the entraining pathway downstream of the photoreceptor seemed to be affected by long-term LL exposure. The circadian clock may be one of them. Long-term changes in free-running period are often caused not only by LL but also entrainment to LDs with a period longer or shorter than 24 h. For example, in the cockroach *Leucophea maderae*, entrainment to LD13:13 lengthened the free-running period of locomotor rhythms, whereas that to LD11:11 shortened it under constant darkness afterwards (Page, 1982). The altered free-running period was maintained even after the optic lobe was transplanted to another individual (Page, 1982), suggesting that the molecular oscillatory mechanism of the clock may be altered and the alteration remains stable over a long period. Although the mechanism of long-term change in free-running period remains undefined, it is likely related to the period changes observed during long-term exposure to LL. Another important question is the mechanism of the gradual change in the free-running period due to long-term LL exposure. These issues need to be addressed at the molecular level in future studies.

**Rhythm splitting under LL**

In this study, some crickets simultaneously exhibited two rhythmic components that ran with different periods or maintained an out-of-phase relationship. Since no rhythm splitting was observed in the crickets with the optic lobe unilaterally removed (Fig. 1, Table 1), rhythm splitting was most likely caused by differential effects of LL on the two optic lobe circadian clocks. In long-term recordings under LL, the two rhythmic components showed similar gradual changes in free-running period (Fig. 1B). Similar changes in free-running periods were also observed in *tim*$^{RNAi}$- and *Pdp1*$^{RNAi}$-treated crickets (Fig. 3D, F). This may be due to the underlying two clocks having a common property of spontaneous changes in free-running periods, or to them determining their period through mutual interaction via neural pathways (Tomioka et al., 1991; Yukizane and Tomioka, 1995). It is also likely that the period changes caused by long-term LL in the two components were independent of *Pdp1* and *tim*. Future studies should focus on the differences in photoresponsiveness between the left and right clocks and the physiological significance of these differences for mutual coupling.

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## COMPETING INTERESTS

The authors have no competing interests to declare.

## AUTHOR CONTRIBUTIONS

YM and K Tomioka conceived and designed the study. YM, K Takeuchi, and K Tomioka performed molecular and behavioral experiments. K Takeuchi and K Tomioka analyzed the data. YM and K Tomioka wrote the original manuscript draft. All authors read and approved the final manuscript.

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