The Effect of Photoregime on the Calling Behavior of the Rice Leaf Folder Moth, *Cnaphalocrocis medinalis* (Lepidoptera: Crambidae)

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Abstract

The rice leaf folder, *Cnaphalocrocis medinalis*, showed a circadian rhythm in its calling behavior at 25 ± 1 °C. Female moths showed calling behavior most frequently 5–7 h into the scotophase under LD 15:9. Female moths at age 4 (days), after entraining at LD 15:9, showed a free-running rhythm for at least two more cycles in continuous darkness. The rhythm was damped out in constant light. The temporal pattern in the calling behavior was affected by different LD cycles (LD 15:9, 12:12, and 9:15), indicating that the rhythm in calling behavior was entrained by the LD cycles. By changing one photoregime condition to another after calling had been initiated, it was shown that *C. medinalis* females use the light-off signal to phase-set the circadian rhythm of calling behavior. Our results suggest that the rhythm in calling behavior is a circadian rhythm entrained by LD cycles.

Introduction

In many moths, the temporal synchronization of mating behaviors between females and males is mediated by sex pheromones, and the circadian rhythm of calling in females may be entrained by photoregime[2,4,10]. Furthermore, changes in the LD cycle may influence the amplitude and the phase of the calling rhythm[3,5,18,27], as do changes in other environmental factors such as temperature[2,4,5,7,27].

The calling activity of *C. medinalis* showed a distinct diel periodicity under LD 15:9[14]. Moths showed high activity 5–7 h into the scotophase. Calling was most active between age 3 and age 7 (days). However, our previous study did not show whether this is a circadian rhythm or how it is affected by LD cycles. The present set of experiments was designed to examine 1) the phase relationship between the calling behavior time and LD cycles, and 2) the mode of phase shift. We report here that the rhythm in calling behavior is a circadian rhythm that is entrained by LD cycles.

Materials and methods

1. Insects

The insects used were supplied from a stock culture and were originally collected in Osaka Prefecture, Japan, in 1985. They were supplied by Sumitomo Chemical Company (Tokyo, Japan) and have been successively

reared on an artificial diet (Silkmate 2 (S), Nihon Nosan Kogyo, Japan) at 25 ± 1 °C under LD 15:9. Pupae were sexed and separately kept in plastic-screen cages (30 x 30 x 30 cm). After emergence, female and male adults were kept separate and provided with 5% sucrose solution as food. A dim red light was used for visual observations during the dark period. The moths were designated as age 0 (days) upon adult emergence. The subsequent ages are expressed in days after emergence. The number of light cycles was counted immediately following the first light-on. The experiments were conducted in 2004.

2. Observation of calling behavior

Thirty virgin females were used in each experiment. They were confined individually in a transparent plastic cup (11 cm dia. x 10 cm ht.) and provided with 5% sucrose solution impregnated in a piece of cotton wool. The calling females exhibit a calling posture by extruding the pheromone gland while bending the abdomen dorsally. This calling posture is similar to that of other crambid moths, e.g., *Chilo suppressalis*[23], *Dichocrocis punctiferalis*[16], *Glyphodes pyloalis*[20], and *Diaphania indica*[15]. The number of females that performed the calling posture was recorded at 1-h intervals during the observation period.

(1) Experiment 1. Calling behavior under continuous darkness (DD) and under constant light (LL)

Circadian rhythms free-run under constant conditions. To examine the presence of a free-running rhythm in calling behavior, virgin females reared under LD 15:9 were transferred to continuous dark or constant light at the beginning or end of the fourth scotophase, respectively. Transferred moths were observed for 60 h at 1-h intervals. Non-transferred moths were used as a control and observed for 60 h at 1-h intervals.

(2) Experiment 2. Calling behavior under different LD cycles

To examine whether calling behavior is affected by different LD cycles, the effect of different dark durations of 24 hours on calling time was examined under LD 15:9, LD 12:12, and LD 9:15. Females were entrained from the final larval instar. They were observed at 1-h intervals from 1 h before light-off to 1 h after the next light-on for seven successive days, from age 0 to age 7.

(3) Experiment 3. Calling behavior following a shift in light-off or -on

To examine whether calling behavior is affected by shifts in light-off or -on, the effect on calling behavior of shifting the light-off or -on timing of the day of the experiment was examined. Females used were reared under LD 12:12 until age 3. At age 4, the timing of either light-off or -on was shifted by ± 3 h to produce advanced or delayed on/off timing. They were then observed at 1-h

intervals from 1 h before light-off to 1 h after the next light-on.

3. Statistical analysis

In Experiment 1, to examine the presence of free-running rhythm in calling behavior, the effect of DD, LL, and LD on calling frequency were analyzed using a generalized linear mixed model (GLMM) with a binomial error structure and logit link function, in which the individual was incorporated as a random effect. The explanatory variables were condition treatment, time (hour), and the interaction between condition treatment and the time. Both condition treatment and time were regarded as the fixed effect, although condition treatment was the categorical variable and time was the continuous variable. The generalized linear mixed model analysis was conducted using the statistical program R version 2.11.0 (for Windows; R Development Core Team 2010)[19]. We used the "lmer" packages to conduct GLMM fitting. In Experiment 1, the total number of females that showed calling behavior was compared between the duration corresponding to the expected scotophase of DD and the scotophase of LD 15:9 in each age using the likelihood-ratio chi-square test employing contingency table analysis. In Experiment 2 and 3, data for the mean onset time of calling were fitted to the Weibull distribution and analyzed by means of parametric survival analysis to evaluate each LD condition treatment and light-off or -on signal treatment. Pairwise comparisons of the mean onset time of calling were determined using the log-rank test of the Kaplan-Meier method with Holm adjustment. The parametric survival analysis and the likelihood-ratio chi-square test were performed using JMP soft-ware, version 5.0.1 J (for Windows; SAS Institute Inc., Cary, NC, USA 2004).

Results

1. Experiment 1. Calling behavior under continuous darkness and under constant light

Model parameters for effects of DD and LL on calling frequency as estimated from a generalized linear mixed model are shown in Table 1. A significant difference was detected between LD 15:9 and LL. However, no significant difference was found between LD 15:9 and DD (Table 1). Under LD 15:9, calling gradually increased 2 h after light-off to reach a maximum at 5–6 h, and then gradually decreased (Fig. 1). In DD, calling showed a rhythm similar to that observed under LD 15:9, namely, a free-running rhythm (Fig. 1, Table 1). Under LD 15:9, at age 4, 5, and 6, female moths showed high activity at 16 h, 15 h, and 16 h, respectively, while, under DD, at age 4,

Table 1. Model parameters for effects of constant conditions on calling frequency as estimated from a generalized linear mixed model (errors=binomial, link=logit)

	Parameter estimate	S.E.	d.f	Z value	P value
(Intercept)	−1.833696	0.106080	60	−17.286	<0.0001
DD treatment	−0.135065	0.102029	1	−1.324	0.186
LL treatment	−1.563630	0.154986	1	−10.089	<0.0001
time	−0.003243	0.002717	60	−1.194	0.233
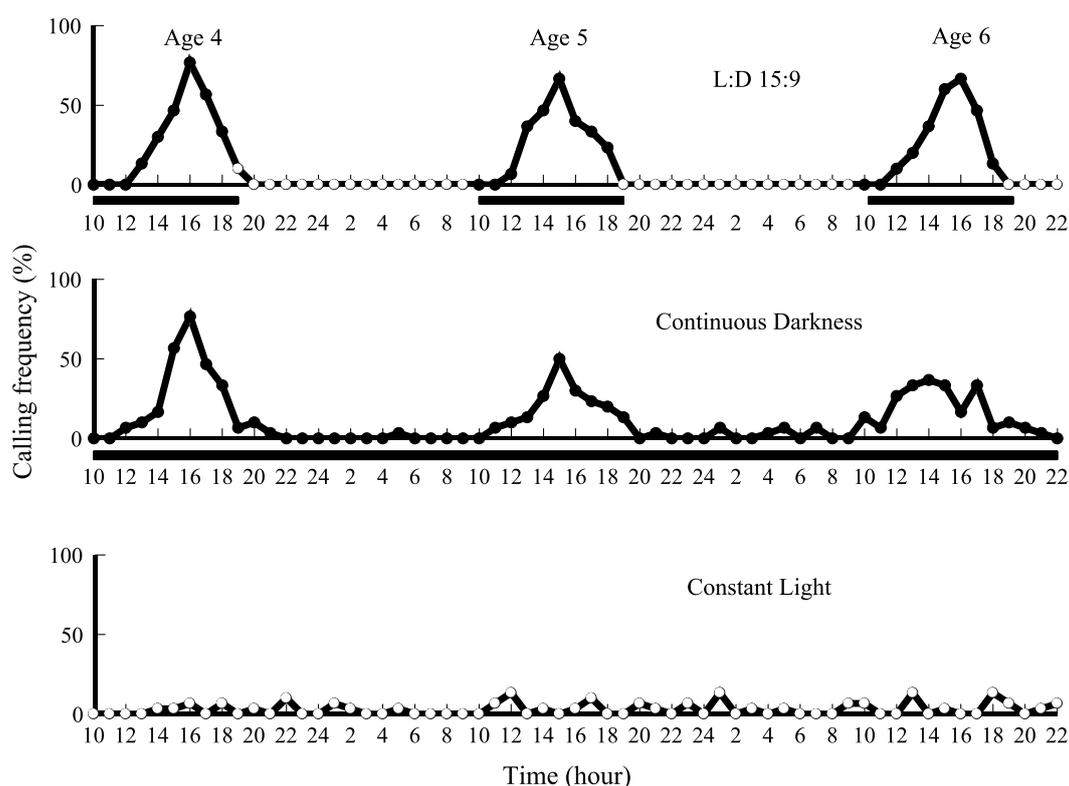
Fig. 1. Effect of continuous darkness and constant light on the temporal patterns of calling behavior in *C. medinalis* virgin females aged 4 to 6 under an LD 15:9 photoregime, continuous darkness (DD), and constant light (LL)
In DD and LL, females had been maintained under LD 15:9 until age 3, and were transferred at 10:00 h at age 4. Solid bars under the abscissa indicate the scotophase. Open and solid circles indicate the bioassays that were conducted under light-off and -on conditions, respectively. $N = 30$ for each group. Vertical axis (calling frequency) indicates the proportion of females that performed calling behavior.

5, and 6, female moths showed high activity at 16 h, 15 h, and 14 h, respectively. The peak of calling frequency at age 6 under DD appeared to be indistinct when compared to that of the same age under LD15:9. However, the total number of females that showed calling behavior in the duration corresponding to the expected scotophase of DD was not significantly higher than that in the scotophase of LD 15:9 in each age (age 4: $\chi^2 = 0.083$, $df = 1$, $P = 0.7736$; age 5: $\chi^2 = 2.860$, $df = 1$, $P = 0.0908$; age 6: $\chi^2 = 3.224$, $df = 1$, $P = 0.0726$). Under LL, calling behavior was sporadic (Fig. 1). Unlike in the control and the DD group, the

rhythmicity in calling activity was changed and no periodicity in calling activity was detected.

2. Experiment 2. Calling behavior under different LD cycles

Under the three LD cycles tested, no calling was observed at age 0. Calling behavior was seldom observed at ages 1 and 2, but was most actively performed after age 3 (Fig. 2). There was a considerable variability in temporal patterns of calling behavior among different LD cycles. In each age, there were significant differences in the
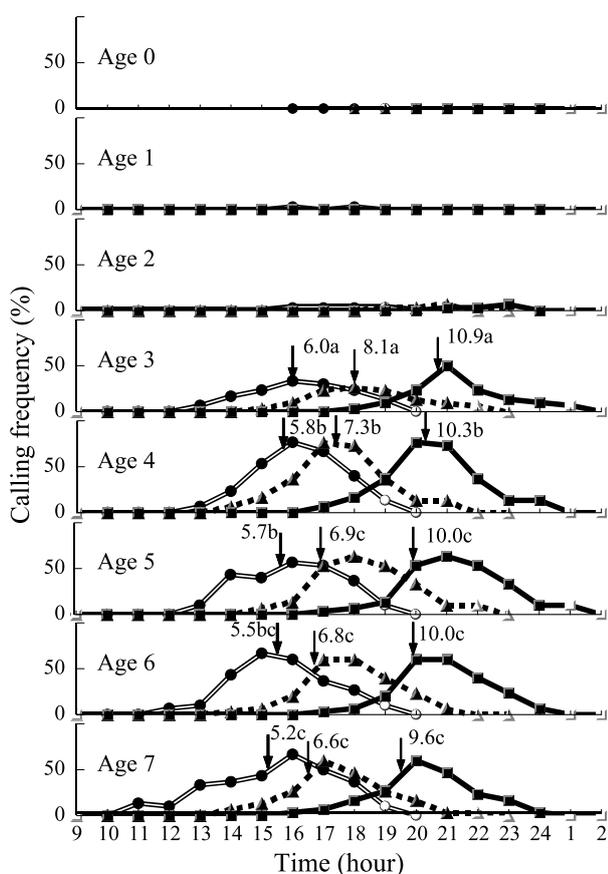
Fig. 2. **Effect of different LD cycles on the daily calling patterns of *C. medinalis* virgin females for seven successive days after emergence; under LD 15:9, LD 12:12, and LD 9:15**

Numbers near the arrows indicate the mean onset time of calling; values followed by the same letters in each LD cycle are not significantly different according to parametric survival analysis.

In all LD cycles, the light-off was conducted at 10:00 h. Open and solid symbols indicate the bioassays that were conducted under light-off and -on conditions, respectively. $N = 30$ for each group.

■ L:D 9:15, ▲ L:D 12:12, ● L:D 15:9.

mean onset time of calling among the three LD cycles (age 3: $\chi^2 = 75.0215$, $df = 2$, $P < 0.0001$; age 4: $\chi^2 = 130.0824$, $df = 2$, $P < 0.0001$; age 5: $\chi^2 = 108.9857$, $df = 2$, $P < 0.0001$; age 6: $\chi^2 = 137.8844$, $df = 2$, $P < 0.0001$; age 7: $\chi^2 = 105.4198$, $df = 2$, $P < 0.0001$). On the whole, the mean onset time of calling was significantly later when the duration of the scotophase was long. Under LD 15:9, at age 4, most calling activity was first observed (6.7%) at 3 h after light-off, then gradually increased, reaching a maximum (76.7%) at 6 h, and then gradually decreased. Under LD 12:12, at age 4, calling activity was first observed (6.7%) at 4 h after light-off, and gradually increased to reach a maximum (73.3%–76.7%) at 7–8 h, and

then gradually decreased. Under LD 9:15, at age 4, moths showed high activity (73.3%–76.7%) 10–11 h into the scotophase. Few females exhibited the calling posture under light-on conditions in any LD cycles. At age 3 to age 7, the tendency to fluctuate showed a similar pattern to that at age 4 in each LD cycle. The mean onset time of calling differed significantly among age 3 to age 7 in each LD cycle (LD 15:9: $\chi^2 = 38.5672$, $df = 4$, $P < 0.0001$; LD 12:12: $\chi^2 = 55.7960$, $df = 4$, $P < 0.0001$; LD 9:15: $\chi^2 = 38.4122$, $df = 4$, $P < 0.0001$). The mean onset time of calling shifted significantly earlier with ageing under all LD cycles. The mean onset time of calling significantly advanced from 6.0 h at age 3 to 5.2 h at age 7 under LD 15:9. The mean onset time of calling significantly advanced from 8.1 h at age 3 to 6.6 h at age 7 under LD 12:12. The mean onset time of calling significantly advanced from 10.9 h at age 3 to 9.6 h at age 7 under LD 9:15.

3. Experiment 3. Calling behavior following a shift in light-off or -on

Under LD 12:12, the time-dependence of calling behavior showed a similar pattern to that in Experiment 2 (Figs. 3 and 2). When light-off or -on was shifted by +3 h or –3 h, calling behavior was first observed at 4–5 h after light-off, and gradually increased to reach a maximum at 8 h. It then sharply decreased at the onset of the next light-on (Fig. 3). This sharp reduction may be due to the inhibitory effect of light-on. When light-off or -on was shifted by –3 h or +3 h, calling behavior was first observed at 5 h after light-off, and gradually increased to reach a maximum at 8 h, and then gradually decreased (Fig. 3). Irrespective of light-off or -on time, it was first observed at 4–5 h after light-off, and gradually increased to reach a maximum at 8 h. No significant difference in the period between mean onset time of calling and light-off was noted among the treatments ($\chi^2 = 4.4136$, $df = 4$, $P = 0.3529$). The period between mean onset time of calling and light-off was nearly constant at about 7.0 h after light-off in every case. In each test, calling behavior was seldom observed at the onset of the next light-on.

Discussion

The free-running rhythm of calling behavior in DD showed a similar phase relationship to that under LD 15:9 (Fig. 1). Circadian calling rhythms were reported for many species, such as *Trichoplusia ni*[21], *Dioryctria abietella*[6], *Laspeyresia pomenella*[2], *Grapholitha molesta*[1], *Pseudaletia unipuncta*[24], *Helicoverpa armigera*[17], *Manduca sexta*[8], *Helicoverpa assulta*[10], and *Lymantria dispar*[27]. The present results show that during the anticipated scotophase at age 6 in DD, the peak of calling frequency ap-
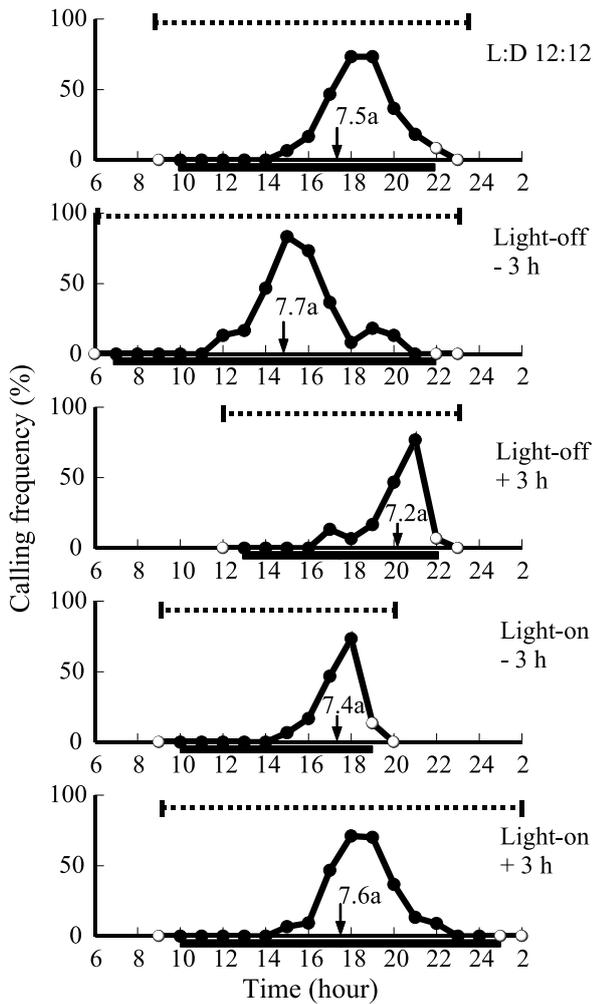
Fig. 3. Effect of shifts in the light-off or -on timing from LD 12:12 on the temporal patterns of calling behavior in *C. medinalis* virgin females

> Females had been reared under LD 12:12 until age 3. At age 4, the light-off signal was shifted by –3 h or + 3 h and the light-on signal was shifted by –3 h or + 3 h. In the control, the moths were maintained under LD 12:12 throughout. Solid bars under the abscissa indicate the scotophase. Numbers near the arrows indicate the mean onset time of calling; values followed by the same letters are not significantly different according to parametric survival analysis. Open and solid circles indicate the bioassays that were conducted under light-off and -on conditions, respectively. $N = 30$ for each group. The dotted line indicates the duration of observation.

running period by each individual resulted in a broadening and lowering of the peak of calling rhythm, possibly due to desynchronization in the absence of external cues. These results suggest that the existence of LD cycles entrain the diel periodicity of calling behavior in *C. medinalis*. The rhythm was considerably diminished in LL and became irregular (Fig. 1). The expression of calling activity thus appears to be suppressed by LL. This has also been seen in other moth species such as *Dioryctria abietella*[6], and *Helicoverpa assulta*[10].

In Experiment 2, the temporal pattern in the calling behavior was also affected by different LD cycles (Fig. 2). The results showed that the temporal pattern in calling behavior was entrained by the LD cycles under which the insects had been held (Fig. 2). In Experiment 2, the mean onset time of calling was significantly later when the duration of the scotophase was long, indicating that the circadian calling rhythm of *C. medinalis* is not constant under different LD cycles, but is entrained by each regular LD cycles. Consequently, the results of Experiments 1 and 2 demonstrate that calling behavior is based on the circadian rhythm, modified by the LD cycle, to express diel periodicity. The mean onset time of calling significantly shifted earlier with ageing under all three LD cycles tested. A similar trend has also been reported in some other species, e.g. *Chilo suppressalis*[12], *Glyphodes pyloalis*[20], *Manduca sexta*[8], and *Diaphania indica*[15]. It is thus suggested that sexually mature older moths are more competitive than their younger counterparts in that they call and attract males first[22]. Females aged less than 2 seldom adopted a calling posture throughout the dark period under the LD cycles examined. Long-distance migration occurs in many insect species while the adults are still reproductively immature[9]. The reproductively immature stage of *P. unipuncta* moths has been implicated in their seasonal migration[3,24,25]. *C. medinalis* is also a long-distance migrator[11,26], so the reproductively immature period, 2 days after emergence, may be linked to this migration.

In Experiment 3, the temporal patterns of calling behavior were not only entrained by LD cycles, but were also affected by light-off or -on. When light-off was shifted by ± 3 h, the initial phase of the temporal pattern of calling behavior shifted accordingly. However, irrespective of how on/off timing was shifted, the period between the mean onset time of calling and light-off was nearly constant at about 7.0 h after light-off (Fig. 3), suggesting that *C. medinalis* females use light-off to phase-set the circadian rhythm of their calling behavior. Only light-off and light-on cues are critical for the timing of the increase and decrease in male responsiveness to the sex pheromone in *C. medinalis*[13]. It is of interest that the

peared to be indistinct when compared to that of the same age under LD 15:9 (Fig. 1). However, the total number of females that showed calling behavior in the duration corresponding to the expected scotophase of DD was almost the same as that in the scotophase of LD 15:9 in each age. Under DD, the different circadian oscillation in the free-

female calling rhythm based on circadian oscillation is different from the male responsiveness to the sex pheromone that was affected by only light-off and light-on signals. We conclude that the calling rhythm is coordinated by circadian oscillation.

Acknowledgments

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