Effects of photoregime on the diel rhythmicity of male responses to sex pheromones in *Glyphodes perspectalis* (Lepidoptera: Crambidae)

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Abstract

We examined the effect of the photoregime on the timing of male responsiveness to sex pheromones in the box tree pyralid, *Glyphodes perspectalis*. Circadian oscillation was observed in the male responsiveness of *G. perspectalis*, evidenced by the rhythmic expression of male responsiveness in the duration corresponding to the expected scotophase of the continuous light or continuous dark conditions, when males reared under 16L8D were transferred to continuous light or continuous dark conditions. When male responsiveness to the sex pheromone was measured throughout the scotophase under three different photoregimes, it was shown that the time to reach the maximal response after lights-off was rather constant (approximately 2 h), regardless of the duration of the scotophase in three cases of 6, 8 and 10 h. The maximal response in the three cases appeared to be maintained until the end of the scotophase. Furthermore, in an experiment involving ±2-h shifts of lights-on or -off from the usual 16L8D, male responsiveness peaked within 2 h after lights-off, and persisted throughout the remaining dark period. These results suggest that, at least under the present experimental conditions, the diel rhythmicity of male responsiveness based on the endogenous circadian rhythm is coordinated by the light-dark regime, and lights-off cues are critical for the timing and expression of the response rhythm leading to increased responsiveness.

Key words: *Glyphodes perspectalis*; sex pheromone; photoregime; responsiveness; circadian rhythm

INTRODUCTION

The box tree pyralid, *Glyphodes perspectalis* (Lepidoptera: Crambidae), is a pest of *Buxus* plants, originally distributed in Asian countries such as Japan, Korea, China, and India (Inoue, 1982; Zhou et al., 2005). Recently, due to introduction of the species, damage to *Buxus* plants by *G. perspectalis* has been reported in European countries such as Germany, the Netherlands, and Switzerland (EPPO, 2008; Krüger, 2008; Rennwald, 2008). Mature larvae feed on many leaves of *Buxus* plants species (e.g., *B. microphylla*, *B. microphylla* var. *insularis*, *B. sempervirens*, and *B. sinica*), resulting in withering and death (Uezumi, 1975, 2003; Osaka Plant Protection Association, 2005; Krüger, 2008).

Recently, for the purpose of pest control using pheromones, the sex pheromone components of *G. perspectalis* were identified as (Z)-11-hexadecenal (Z11-16:Ald) and (E)-11-hexadecenal (E11-16:Ald) at a ratio of 4 : 1 (Kawazu et al., 2007). The synthetic sex pheromone may not only be an effective monitoring tool for the timing of insecticide application, but also as a possible control agent, such as mating disruption. Fundamental research will be indispensable to develop applications using synthetic pheromone; however, biological research concerning sex pheromones, such as the periodicity of male responsiveness to sex pheromones or the release of female sex pheromones has not been clarified sufficiently.
In many moth species, sex pheromone-mediated mating behavior occurs within specific time windows during the scotophase under a diel light-dark cycle. Studies on the female release of and the male response to sex pheromones have shown that such behaviors are based on a circadian rhythm, modulated by various environmental factors, such as photoregime and temperature, to express diel periodicity (e.g., Baker and Cardé, 1979; Castro-villo and Cardé, 1979; Kamimura and Tatsuki, 1994; Linn et al., 1996; Rosén et al., 2003). The adaptive significance of circadian-based regulation of mating behavior relates to the added efficiency in the coordinated exchange between the sender and receiver, and the occurrence of mating during optimal environmental conditions (Cardé et al., 1975). There have been many reports on the circadian rhythm of female calling behavior (e.g., Baker and Cardé, 1979; Castrovillo and Cardé, 1979; Delisle and McNeil, 1987; Itagaki and Conner, 1988; Kamimura and Tatsuki, 1994; Del Socorro and Gregg, 1997; Webster and Yin, 1997). On the other hand, there are few reports on circadian rhythm in the male responsiveness to sex pheromones (Baker and Cardé, 1979; Castrovillo and Cardé, 1979; Linn et al., 1996; Rosén et al., 2003; Silvegren et al., 2005). Even less information is available on the modulation factors of the male responsiveness to sex pheromones. In the cabbage looper moth *Trichoplusia ni*, studies on male responsiveness to sex pheromones suggested that the time to reach maximal response levels was relatively constant (2–3 h) after lights-off, regardless of the duration of the scotophase and the timing of lights-on or -off, and circadian oscillation is involved in male responsiveness (Linn et al., 1996). For the rice leaffolder moth *Cnaphalocrocis medinalis*, only lights-on and -off cues are critical for the timing of the increase and decrease in male responsiveness to sex pheromones (Linn et al., 1996). For the box tree pyralid *G. perspectalis*, studies on male responsiveness to sex pheromones in the above ratio was prepared at 125 μg/ml. GC analyses showed that these compounds contain <0.1% of the corresponding geometrical isomers.

**Materials and Methods**

**Insects.** *G. perspectalis* were collected from the box tree, *B. microphylla*, at RIKEN (Saitama Pref., Japan) in 2005 and have been successively reared on box tree leaves. The insects were kept at 25°C under 16L8D conditions with lights-off at 10:00 h and lights-on at 18:00 h, under a light intensity of 1,500 lx during the photophase and 5 lx during the scotophase. In rearing and all experimental conditions, a dimmed red lamp (5 lx) and fluorescent lamp (1,500 lx) were used during the scotophase and photophase, respectively. After emergence, males and females were separated and provided with 5% honey solution as food. The insects used in the present study were reared under the same conditions as above, unless otherwise described. Preliminary observations showed that all moths emerged during the photophase. The moths were designated as age 0 during the photophase in which they emerged. The subsequent ages were started at the time of each incidence of lights-on.

**Pheromone.** Synthetic aldehyde compounds of the sex pheromone components (Z11-16:Ald and E11-16:Ald) were obtained from Pherobank ( Wageningen, The Netherlands). A hexane solution of the two synthetic pheromone components in the above ratio was prepared at 125 μg/ml. GC analyses showed that these compounds contain <0.1% of the corresponding geometrical isomers.

**Bioassay.** A laboratory bioassay was conducted with a screen cage (32×22×30 cm) under the same conditions as described above except for the photoregime. Ten virgin males were released in the cage. To investigate the temporal patterns of male responsiveness, three experiments were conducted involving shifts of scotophase length and either lights-on or -off timing from the usual photoregime (16L8D). The details of those photoregime conditions are described in the results of each experiment. A filter paper strip (2×4 cm) impregnated with the pheromone was placed in the center of the cage. Males were observed for their response to the pheromone for 6 h after lights-off. The behavior of males was scored as 1,0, and 0 upon detection of male orientation, flying, and no response, respectively.
with 1 μl hexane solution containing the synthetic pheromone blend was suspended in the cage from the center of the top screen so that it was about 5 cm below the top. The blend ratio of compounds Z11-16:Ald and E11-16:Ald was 4:1, and the amount of Z11-16:Ald was 100 ng. In a preliminary experiment, we confirmed that with the dosage used here, disruption due to contamination of the screen cage did not occur. The dosage was selected so as to more clearly demonstrate the window of male responsiveness, because it was the lowest dosage to obtain the maximum response level in a preliminary experiment. Immediately after the introduction of filter paper containing the synthetic pheromone blend into the cage, the number of contacts by males with claspers extruded to the treated filter paper for 5 min was counted. Each bioassay was replicated 3–10 times. For statistical analyses, differences among mean numbers of contacts of males were tested for significance by Tukey-Kramer’s test.

RESULTS

Experiment 1. Effect of continuous light or darkness on male responses to pheromones

To examine the existence of a free-running rhythm in male responses to the pheromone source, virgin males reared under 16L8D were transferred to continuous light or dark conditions. Males reared under 16L8D were transferred to continuous light or continuous dark conditions at the beginning or end of the 3rd scotophase, respectively. Male responses to the pheromone source were examined at 2-h intervals for 32 h under continuous light and for 48 h under continuous darkness. Moths of the same age under 16L8D were used as a control and observed for 56 h at 2-h intervals.

Under 16L8D, the responsiveness of males showed diel periodicity, which was first observed at the onset of the scotophase and increased to peak 2 h after lights-off, being maintained at the same level until 2 h before the next lights-on (Fig. 1A). Under continuous darkness, male responsiveness showed a rhythm similar to that observed under 16L8D (Fig. 1B), namely, a free-running drift.

Fig. 1. Effect of continuous light and darkness on male responses to sex pheromones: 16L8D photoregime (A), continuous darkness (B) and continuous light (C). Solid bars under the abscissa indicate the scotophase. The abscissa indicate the time of day. Means with the same letter in each figure are not significantly different at p<0.05 by Tukey-Kramer's test. Values marked with * indicate significantly lower values at 12, 14, and 16 h at age 4 under continuous darkness and at 12, 14, and 16 h at age 2 and 3 under continuous light compared with values at the same times in the control according to Tukey-Kramer’s test (p< 0.05). Each bioassay was replicated 3 times. Open and solid circles indicate the bioassays that were conducted under lights-on and -off conditions, respectively.
Male responsiveness at 12, 14 and 16 h at age 3 under continuous darkness was not significantly different from that of the control group at the same period under 16L8D; however, male responsiveness at 12, 14 and 16 h at age 4 under continuous darkness was significantly lower than that of the control group at the same period under 16L8D. The total amount of male responsiveness per 24 h at age 3 and 4 under continuous darkness was not significantly different from those of the control group in the same periods under 16L8D. Under continuous light, male responsiveness showed a rhythm similar to that observed under 16L8D (Fig. 1C), namely, a free-running rhythm. Male responsiveness at 12, 14 and 16 h at age 2 and 3 under continuous light was significantly lower than that of the control group in the same period under 16L8D. The total amount of male responsiveness per 24 h at age 3 under continuous light was significantly lower than in the control group at the same age under 16L8D.

**Experiment 2. Effect of different photoregimes on the temporal pattern of male responses to pheromones**

To examine whether male responses to sex pheromones are affected by different light-dark durations, the effect of photoregimes of different dark durations of 24 hours on the temporal patterns of male responses to the pheromone source was examined using the following photoregimes: 18L6D, 16L8D, and 14L10D. The males used had been conditioned to these photoregimes from the final larval stage. At age 3, bioassays were conducted at 2-h intervals from the beginning of lights-off until 2 h before the next lights-on and immediately after lights-on. Thus, the first and final bioassays were under the photophase in each test.

The temporal patterns of male responses to the pheromone source under the three different photoregimes are shown in Fig. 2. Under 18L6D, no response was observed under the photophase at 2 h before lights-off (Fig. 2A). The responsiveness of males was first observed at the onset of scotophase and then markedly increased and peaked at 2 h after lights-off. At the onset of the next lights-on, a weak response was observed. Under the usual photoregime (16L8D), the responsiveness of males was first observed at the onset of scotophase and then markedly increased to peak at 2 h after lights-off, being maintained at the same maximal level until 2 h before the next lights-on (Fig. 2B). Under 14L10D, the time dependence of the responsiveness showed a similar pattern to that under 18L6D and 16L8D (Fig. 2C); however, although the decrease was not significantly different from the maximal level, a tendency toward a decrease of the male response at 2 h before the end of the scotophase was observed in the data. Regardless of the scotophase length, the responsiveness of males was first observed at the onset of the scotophase and then markedly increased to peak at 2 h after lights-off. A similar maximum level was shown until 2 h before the end of the scotophase. The longer the duration of the scotophase, the longer the duration of the maximal response level. A weak male response to sex pheromone was observed under the lights-on condition at the onset of the next lights-on for all photoregimes.

**Experiment 3. Effect of shifts in lights-on or -off timing on temporal patterns of male responses to pheromones**

To examine whether male responsiveness to sex pheromones is affected by shifts in lights-on or -off
Timing, temporal patterns of male responses to the pheromone source were examined. The males tested were subjected to a 16L8D photoregime until age 2. At age 3, the timing of lights-on or -off was shifted by ±2 h to generate advanced or delayed on/off timing and a different scotophase length. Bioassays were conducted only on the day (age 3) when the timing of lights-on or off was shifted and were conducted at 2-h intervals from the beginning of lights-off until 2 h before the next lights-on and immediately after lights-on. Thus, the first and final bioassays were under the photophase in each test.

Temporal patterns of male responses to the pheromone source under the conditions of shifted lights-on or -off are shown in Fig. 3. Under the usual photoregime (16L8D), the time dependence of the responsiveness showed a similar pattern to that in Experiments 1 and 2 (Fig. 3A). When the timing of lights-off was shifted by ±2 h, the responsiveness of males was first observed at the onset of the scotophase and increased to peak at 2 h after lights-off, being maintained at the same level until 2 h before the next lights-on (Fig. 3B, C). When the timing of lights-on was shifted by ±2 h, the change was not shown in the timing of the increase of male responsiveness and the high level of male responsiveness in the scotophase (Fig. 3D, E). The responsiveness of males was entrained to shifts in the timing of lights-on or off (photoregime shifts) immediately. Regardless of either shifted on/off timing or the length of the scotophase, the responsiveness of males was first observed at the onset of the scotophase and increased to peak at 2 h after lights-off, being maintained at the same level until 2 h before the next lights-on. In this experiment, the weak male response to sex pheromone was observed at the onset of the next lights-on in each test.

DISCUSSION

When *G. perspectalis* moths were shifted to continuous light or continuous dark from the 16L8D photoregime, the responsiveness of males showed a similar rhythm to those that remained under the 16L8D photoregime (Fig. 1). This result suggests that diel periodicity in male responsiveness to pheromones is regulated on a circadian basis. Circadian rhythms of male responsiveness were reported for other moth species, such as *Agrotis segetum* (Rosén et al., 2003), *Grapholitia molesta* (Baker and Cardé, 1979), *Laspeyresia pomonella* (Castrovillo and Cardé, 1979), *Spodoptera littoralis* (Silvegren et al., 2005), and *Trichoplusia ni* (Linn et al., 1996). The present results show that during the anticipated scotophase at age 4 in continuous dark, the mean number of contacts per replicate was significantly reduced (Fig. 1B). However, the total amount of male responsiveness per 24 h at age 3 and 4 under continuous darkness was not significantly different from that of the control group at the same period under 16L8D. Under constant conditions, circadian oscillation in the free-running period of each individual difference resulted in the broadening and reduction of the peak of response rhythm. The reduction of the peak of response
rhythm under continuous darkness may be due to desynchronisation in the absence of external zeitgebers. The results suggest that the existence of a light-dark photoregime entrain the diel periodicity of male responsiveness. When moths were transferred from 16L8D to continuous light, the mean number of contacts per replicates was slightly reduced, keeping the duration of the response unchanged (Fig. 1C). The expression of male responsiveness appears to be suppressed by continuous light. On the other hand, the results of male responsiveness under light-dark regimes showed that *G. perspectalis* has the general nocturnal habits of animals. Such a weaker male response to pheromones under continuous light is also observed in other nocturnal moths, such as *Trichoplusia ni* (Linn et al., 1996), including calling behavior in *Dioryctria abietella* (Fatzinger, 1973) and *Helicoverpa assulta* (Kamimura and Tatsuki, 1994). These results indicate that *G. perspectalis* has the character of a typical nocturnal circadian rhythm and in accordance with a well-known circadian rule. A similar tendency of activity was reported in other dark-active animals, such as *Lycosa tarentula* (Ortega-Escobar, 2002), *Leucophaea maderae*, *Bystroria fumigata* and *Periplaneta americana* (Roberts, 1960).

In Experiment 2, male responses to sex pheromones are also affected by different light-dark regimes (Fig. 2). The results showed that the temporal patterns of male responsiveness were entrained by the photoregimes under which the insects had been held. There was a clear relationship between the lights-off signal and the time (2 h) to reach the maximal response, regardless of the scotophase length. Once the maximal response was reached at 2 h after lights-off, response levels were relatively constant until the end of the dark period under all three photoregimes tested. The window of male responsiveness was wide when the duration of the scotophase was long. Such a tendency of male responsiveness to sex pheromones was also reported in other moth species, e.g., *Agrotis ipsilon* (Gemeno and Haynes, 2000), *T. ni* (Linn et al., 1996), *Platyptilia carduidactyla* (Haynes and Birch, 1984), *C. medinalis* (Kawazu et al., 2003), and *Herpetogramma phaeopteralis* (Meagher et al., 2007). The wide window of male responsiveness will be maintained if the scotophase length increases with the season or the latitude where the males occur. Thus, it appears that the probability of males to locate females will rise if the duration of the scotophase increases with the season or habitat.

To clarify the overall mating behavior, it will also be necessary to investigate the effect of the photoregime on the calling behavior of female *G. perspectalis*.

Under 18L6D and 16L8D, no decrease of male responsiveness 2 h before the end of the scotophase was observed (Fig. 2A, B); however, the tendency toward a decrease 2 h before the end of the scotophase under 14L10D was observed, although the decrease was not significantly different from the maximal level in the data (Fig. 2C). This decrease in responsiveness appeared to be affected by the circadian rhythm (Fig. 2C). Such a decrease is also observed in male responsiveness under continuous darkness in the responsive period of approximately 8 hours, despite the difference in the experiment method. From these results, it seems that male responsiveness is based on the circadian rhythm, modulated by the photoregime, to express diel periodicity.

In Experiment 3, male responsiveness to sex pheromones was also affected by the lights-on or -off timing (Fig. 3). Thus, the temporal patterns of male responsiveness were similar to those in Experiment 2, regardless of the scotophase length and the timing of lights-on or -off. Only weak responsiveness was shown in the photophase even when it corresponded to the original scotophase. In contrast, the maximal response level was maintained in the scotophase even when it corresponded to the original photophase. The temporal patterns of male responsiveness were not only entrained by the photoregime, but were also affected by lights-on and -off signals. Furthermore, there are two possible explanations for the effects of lights-on and -off signals on male responsiveness i.e., 1) the Zeitgeber and 2) the masking effect. This remains to be proved in the future.

Experiments involving shifts in the time of lights-on or -off also supported the idea that the lights-off cue is critical for the expression and timing of the male response rhythm, the same as in other moth species, e.g., *C. medinalis* (Kawazu et al., 2003) and *T. ni* (Linn et al., 1996). With *G. perspectalis*, it is thought that male responsiveness is a function of the scotophase portion of the photoregime, and even though the lights-off cue is clearly important, both lights-on and -off cues are
critical for sustaining the response rhythm. The diel rhythmicity of male responsiveness based on the endogenous circadian rhythm is coordinated by the light-dark regime. *G. perspectalis* have an adaptive nature with flexibility in the circadian rhythm. It will be necessary to investigate whether the diel periodicity of male responsiveness based on the endogenous circadian rhythm can entrain non-24-hour periodicity and whether temperate compensation exists in the responsiveness of males.

Knowledge regarding the diel temporal activity pattern of *G. perspectalis* is important to understand intraspecific communication and its role in the temporal organization of a community. In *G. perspectalis*, the periodicity of male responsiveness is endogenously controlled, modified by exogenous factors. The rapid response to exogenous cues modifying the underlying circadian rhythm appears to allow *G. perspectalis* moths to function under different seasonal conditions. *G. perspectalis* may achieve optimal temporal synchrony with its mates mainly by its circadian rhythm. For many moth species, such diel temporal isolation is likely important as a reproductive isolating mechanism.

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