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ORIGINAL ARTICLE

How long do diapause pupae of *Antheraea pernyi* (Lepidoptera: Saturniidae) store photoperiodic information?

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Key words. Silkmoth, pupal diapause termination, photoperiod

Abstract. Pupal diapause in the Chinese oak silkmoth (*Antheraea pernyi*) is maintained under short-day (SD) photoperiods but is terminated when pupae are exposed to long-day (LD) photoperiods for a specific number of days. This process suggests that pupae can count or retain memory of the number of LD days experienced. In this study, we investigated how long diapause pupae retain photoperiodic information acquired during LD exposure. Diapause pupae were first reared under SD conditions and then exposed to LD for 8 days – an insufficient duration to terminate diapause. Following this, the pupae were placed back under SD for 7, 14, or 21 days, and subsequently returned to LD to induce adult eclosion. Using the final transfer to LD as a reference point, we found that pupae exposed to SD for 7 or 14 days reached adulthood significantly earlier than control pupae that had not previously experienced LD. However, no significant difference in eclosion timing was observed between the 21-day SD group and the control group. These results suggest that photoperiodic information acquired during the 8-day LD exposure gradually decayed under SD conditions and was lost between 14 and 21 days after the transfer from LD to SD. Complete erasure of photoperiodic information under SD appeared to require approximately twice the duration of the initial LD exposure.

INTRODUCTION

Many insect species enter a hormonally mediated developmental arrest, known as diapause, to survive adverse environmental conditions such as cold winters (Tauber et al., 1986; Denlinger, 2022). Photoperiod is a major environmental cue that regulates the induction, maintenance, and termination of diapause (Saunders, 2002). Diapause can be programmed by exposing insects to either short-day (SD) or long-day (LD) photoperiods for a defined number of days. Insects are believed to count or retain a memory of the number of days of a given photoperiod they have experienced (Saunders, 1981). However, the mechanisms by which insects store this photoperiodic information remain largely unknown (Denlinger, 2022). Although *Pyrrhocoris apterus* has been shown to retain photoperiodic information under continuous darkness (Hodková, 2015), relatively few studies have investigated how long such information is preserved for diapause regulation.

The Chinese oak silkmoth, *Antheraea pernyi* (Guérin-Méneville), is a large moth domesticated in China for sericulture (Liu et al., 2010). Its presumed wild ancestor, *Antheraea roylei* (Moore), is a tropical and subtropical species native to central and southern China (Peigler, 2012). *A. pernyi* exhibits a unique life cycle that may have been fixed through artificial selection (Peigler, 2012). Interestingly, it displays a reversed geographical pattern: southern populations are univoltine, while northern populations are multivoltine (Peigler, 2012; Li et al., 2017). Diapause occurs during the pupal stage and is maintained under SD conditions in southern China but can be terminated by LD in northern populations. Notably, northern bivoltine larvae develop without entering diapause when reared under LD conditions.

The photoperiodic regulation of diapause in *A. pernyi* has been extensively studied (Tanaka, 1950; Williams & Adkisson, 1964; Williams, 1969). Long-day conditions with daylengths of 15 to 18 h are sufficient to terminate pupal diapause (Williams & Adkisson, 1964), and approximately 10 consecutive days of LD are required to initiate diapause termination (Wang et al., 2015). Brain transplantation experiments have demonstrated that the brain functions as the regulatory center of photoperiodic diapause (Bowen et al., 1984; Hasegawa & Shimizu, 1987). The release of prothoracicotropic hormone (PTTH) from the brain triggers the termination of diapause and initiates adult development (Denlinger et al., 2005).

In the present study, we investigated how long *A. pernyi* diapause pupae retain photoperiodic information acquired during LD exposure, which acts as a stimulus for diapause termination. Diapause pupae were initially reared under SD conditions and then exposed to LD for 8 days – an insufficient duration to terminate diapause. Subsequently, they were returned to SD for 7, 14, or 21 days and then transferred back to LD to induce adult development. We examined how the duration of SD exposure following the 8-day LD stimulus affected the timing of adult eclosion.

MATERIAL AND METHODS

Animals

Diapause pupae of a univoltine strain of *Antheraea pernyi* were obtained from the Faculty of Textile Science and Technology, Shinshu University (Ueda City, Japan; https://shigen.nig.ac.jp/wildmoth/). This strain originated from populations introduced to Japan in 1877 from Shandong Province, northern China, which included both univoltine and bivoltine strains (personal communication with the breeder).

Larvae were reared under natural field conditions on oak trees (*Quercus acutissima*) until cocoon formation, which typically occurred from mid-June to late July, when daylength ranged from 14.1 to 14.7 h. Harvested cocoons were kept indoors at approximately 22°C and maintained under natural photoperiod conditions. In early October, when the daylength was approximately 11.8 h, the cocoons were transported to our laboratory. Upon arrival, pupae were stored at 25°C under a 12L : 12D cycle (lights on 10:00–22:00; short-day [SD] photoperiod) for 4–8 weeks. They were then exposed to either a 16L : 8D cycle (lights on 10:00–02:00; long-day [LD] photoperiod) or remained under the same SD conditions, according to the experimental design. Throughout the experiments, pupae were maintained at 25°C. Both male and female individuals were included. Illumination was provided by white fluorescent lamps with an intensity of 100–200 lux at the level of the insects.

The duration from the start of LD exposure to adult emergence varied across years, with an average range of 34 to 45 days (data not shown). As the larval rearing schedule was nearly the same each year, this variation was likely due to environmental factors other than photoperiod, such as temperature or host plant quality. To minimize inter-lot variability, analyses were conducted within each experimental cohort using individuals from the same lot.

To assess the effects of photoperiodic exposure on diapause status, we recorded the number of moths that emerged each day. Diapause termination was quantified as the cumulative percentage of emerged adults per group, with group sizes ranging from 23 to 68 individuals.

Statistical analysis

The incidence of diapause termination was compared among experimental groups using Kaplan-Meier survival analysis followed by log-rank tests. Differences were considered statistically significant at $p < 0.05$.

RESULTS AND DISCUSSION

Long-day photoperiod functions as a diapause-terminating stimulus

We initially confirmed whether our experimental animals responded appropriately to photoperiods in terms of diapause regulation. We made three groups (Fig. 1A): A (SD→LD), B (SD→8dLD→SD) and C (SD→SD).

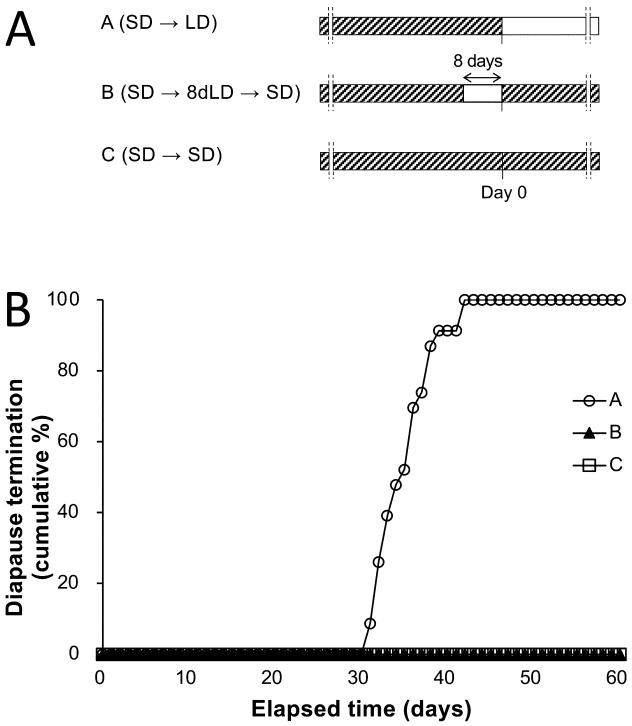

Fig. 1. Time course of pupal diapause termination in *Antheraea pernyi*. (A) Photoperiodic regimes of groups A (SD→LD), B (SD→8dLD→SD) and C (SD→SD). Group A (SD→LD) was transferred from SD to LD photoperiod on day 0. Group B (SD→8dLD→SD) was exposed to LD for 8 days until day 0, then returned to SD. Group C (SD→SD) remained under SD through the study. White and shaded bars indicate LD and SD photoperiods, respectively. Vertical parallel dotted lines across bars indicate long periods of successive identical photoperiods. (B) Time course of diapause termination in all groups. Emerged adults were counted daily, and incidence of diapause termination is expressed as ratios (%) of cumulative numbers of emerged adults per group of 23–25 individuals. LD – long-day; SD – short-day.

Group A (SD→LD) was transferred from SD to LD on day 0. Group B (SD→8dLD→SD) was exposed to LD for 8 days until day 0, then returned to SD. Group C (SD→SD) remained under SD throughout the study. Only Group A pupae became adults (Fig. 1B), which was in line with previous findings that pupal diapause is maintained under SD, but terminated after pupae are transferred from SD to LD (Williams & Adkisson 1964; Williams 1969). However, we found here that diapause was not terminated when pupae were returned to SD after exposure to LD for 8 days (Fig. 1B). Although the LD photoperiod functioned as a stimulus to terminate pupal diapause, 8 days under LD was insufficient to achieve this. Our results were consistent with the previous finding that 5 days is insufficient, whereas 11 days terminates pupal diapause in *A. pernyi* (Wang et al., 2015). Therefore, we considered that our pupae were validated for experimentation.

Photoperiodic information decreased under short-day photoperiod

We examined whether diapause pupae retained photoperiodic information that was accumulated during exposure to LD after transfer from LD to SD. We made three groups (Fig. 2A): D (SD→8dLD→7dSD→LD), E (SD→8dLD→LD)

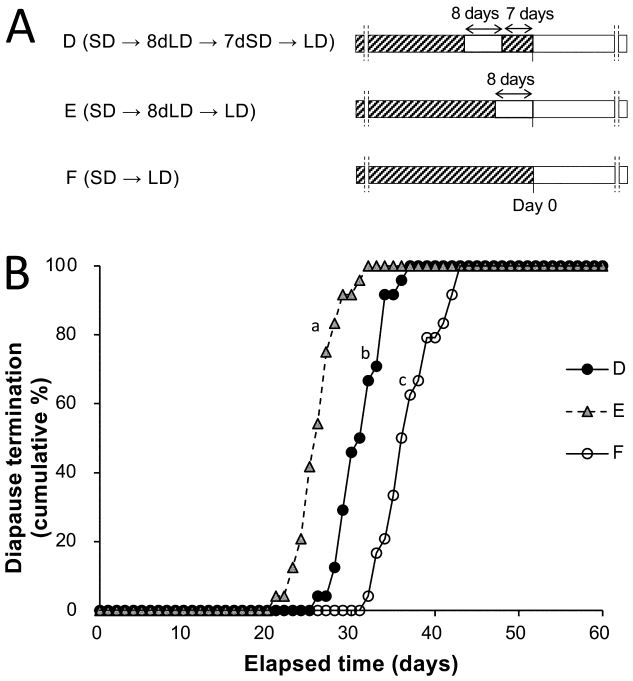

Fig. 2. Effects of exposure to LD photoperiod for 8 days followed by SD for 7 days on the timing of diapause termination. (A) Photoperiodic regimes of groups D (SD→8dLD→7dSD→LD), E (SD→8dLD→LD) and F (SD→LD). Group D (SD→8dLD→7dSD→LD) was exposed to LD for 8 days, followed by SD for 7 days then returned to LD on day 0 for adult eclosion. Group E (SD→8dLD→LD) was transferred from SD to LD 8 days before day 0 and remained there. Group F (SD→LD) was transferred from SD to LD on day 0 and remained there. White and shaded bars indicate LD and SD photoperiods, respectively. Vertical parallel dotted lines across bars indicate long periods of successive identical photoperiods. (B) Time course of diapause termination. Emerged adults were counted daily, and incidence of diapause termination is expressed as ratios (%) of cumulative numbers of emerged adults per group of 24 individuals. Superscript letters indicate significant differences among groups (Kaplan-Meier analysis with the log-rank tests, $P < 0.05$). LD – long-day; SD – short-day.

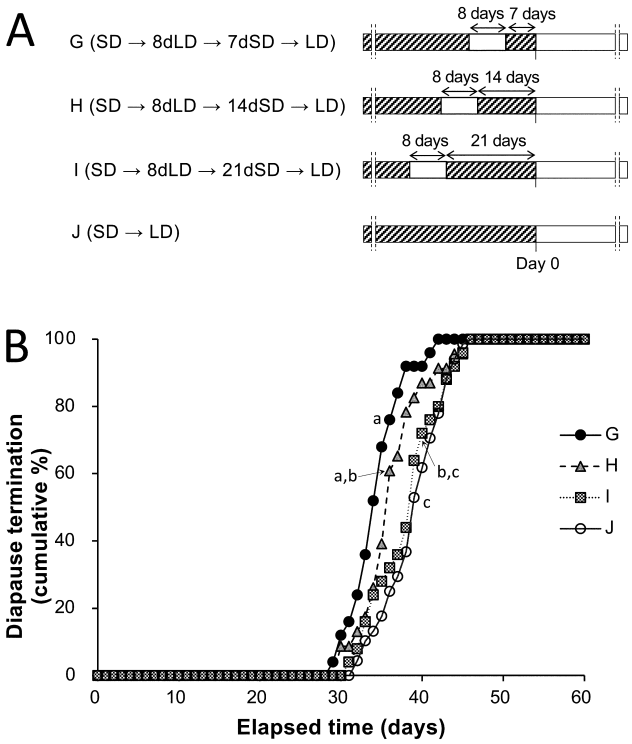

Fig. 3. Effects of exposure to LD photoperiod for 8 days followed by 7, 14 or 21 days of SD on timing of diapause termination. (A) Photoperiodic regimes of groups G (SD→8dLD→7dSD→LD), H (SD→8dLD→14dSD→LD), I (SD→8dLD→21dSD→LD) and J (SD→LD). Group G (SD→8dLD→7dSD→LD) was exposed to LD for 8 days, followed by SD for 7 days then returned to LD on day 0 for adult eclosion. Groups H (SD→8dLD→14dSD→LD) and I (SD→8dLD→21dSD→LD) differed from Group G only in terms of 14 and 21 days under SD, respectively, after exposure to LD for 8 days. Group J (SD→LD) without LD stimulation before day 0, was transferred from SD to LD on day 0 where they remained. White and shaded bars indicate LD and SD photoperiods, respectively. Vertical parallel dotted lines across bars indicate long periods of successive identical photoperiods. (B) Time course of diapause termination. Emerged adults were counted daily, and incidence of diapause termination is expressed as ratios (%) of cumulative numbers of emerged adults per group of 23–68 individuals. Superscript letters indicate significant differences among groups (Kaplan-Meier analysis with the log-rank tests, $P < 0.05$). LD – long-day; SD – short-day.

and F (SD→LD). Group D (SD→8dLD→7dSD→LD) was exposed to LD for 8 days, followed by SD for 7 days then returned to LD on day 0 for adult eclosion. Group E (SD→8dLD→LD) was transferred from SD to LD 8 days before day 0 and remained there. Group F (SD→LD) was transferred from SD to LD on day 0 and remained there. If all the photoperiodic information accumulated during exposure to LD for 8 days was stored after 7 days under SD, Group D would reach adulthood at the same time as Group E that was transferred to LD 8 days before day 0. On the other hand, if such photoperiodic information was completely lost after 7 days under SD, Group D would reach adulthood at the same time as Group F that was not exposed to LD before day 0. Our results showed that Group D reached adulthood significantly later than Group E and significantly earlier than Group F (Fig. 2B). These findings indicated that Group D pupae stored some photoperiodic information after 7 days under SD. This suggested that photoperiodic information accumulated under LD decreased after transfer to SD.

How long did pupae store photoperiodic information?

We investigated how long pupae stored photoperiodic information accumulated by exposure to LD after being transferred to SD. We examined the effects of exposure to LD for 8 days followed 7, 14 or 21 days of SD on the timing of eclosion. We made four groups (Fig. 3A): G (SD→8dLD→7dSD→LD), H (SD→8dLD→14dSD→LD),I(SD→8dLD→21dSD→LD) and J (SD→LD). Group G (SD→8dLD→7dSD→LD) was exposed to LD for 8 days, followed by SD for 7 days then returned to LD where they remained. The day of the final transfer from SD to LD was defined as day 0. Groups H (SD→8dLD→14dSD→LD) and I (SD→8dLD→21dSD→LD) differed from Group G only in that they were exposed to SD for 14 and 21 days, respectively, after exposure to LD for 8 days, and were otherwise

treated like Group G. Group J (SD→LD) was a control without LD stimulus before day 0 that was transferred from SD to LD on day 0 where they remained. Our results showed that Groups G and H (7 and 14 days under SD, respectively) reached adulthood significantly earlier than the Group J control (Fig. 3B). However, the timing of adult eclosion did not significantly differ between Groups I (21 days under SD) and Group J (control; Fig. 3B). Group G tended to reach adulthood earlier than Group H (Fig. 3B). These findings showed that a longer duration of SD after 8 days under LD damped the effects of the LD stimulus on diapause termination. Therefore, photoperiodic information accumulated during exposure to LD for 8 days gradually decreased under SD and eventually disappeared between 14–21 days after transfer from LD to SD.

Temporal rate of decay of photoperiodic information

Photoperiodic information regulating diapause may be acquired during exposure to SD, LD, or both, depending on the insect species (Saunders, 2002). This information can be erased by switching to the opposite photoperiod. However, the rate at which such information decays over time has not been well characterized. Our results show that in *A. pernyi* pupae, photoperiodic information gained through LD exposure gradually decays when transferred to SD before diapause termination occurs. Complete erasure of this information appears to require approximately twice the duration of the LD exposure. This ability to store and gradually lose photoperiodic information is likely essential for silkmoths to adapt accurately to seasonal photoperiods in natural environments.

This study is novel in investigating the temporal dynamics of photoperiodic information decay. Our findings may aid in identifying molecular candidates involved in photoperiodic memory within the silkmoth brain, as such molecules may exhibit temporal changes corresponding to the observed decay. In our experiments, pupae were maintained under SD conditions for at least four weeks before diapause termination assays began. However, the minimum duration of SD exposure required to enable effective diapause termination upon subsequent LD exposure remains unclear. Further elucidation of these temporal dynamics in relation to the molecular mechanisms of photoperiodic memory may provide new insights into the regulation of insect diapause.

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