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# Phase Responses in the Circannual Rhythm of the Varied Carpet Beetle, *Anthrenus verbasci*, Under Naturally Changing Day Length

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In the varied carpet beetle, *Anthrenus verbasci*, we examined the effects on the circannual pupation rhythm of a short-day or long-day pulse under naturally changing day length at a constant 20°C. A short-day pulse for 4 weeks caused a prominent phase delay or advance under constant long days, but had little or no effect on the phase under naturally changing day length between 4 August and 24 November. A long-day pulse for 4 weeks given under naturally changing day length caused a phase shift in the first pupation group, as under constant short days. A long-day pulse given on 4 August, 1 September, or 29 September caused a phase delay, and a pulse given on 27 October or 24 November caused a phase advance. Pupation was least synchronous just before the transition from delaying to advancing. However, the magnitude of phase delays was much smaller under natural day length than under short days. In the second pupation group, larvae pupated at the same time as in the control experiment without a long-day pulse, and this result can be attributed to entrainment to the geophysical year by long days in spring and summer.

**Key words:** *Anthrenus verbasci*, biological clock, circannual rhythm, entrainment, phase response curve

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## INTRODUCTION

Circannual rhythms have been reported in various animals and plants. Under constant conditions without seasonal information, these rhythms persist with an endogenous period close to a year and entrain to an annual cycle under natural conditions (Gwinner, 1986; Goldman *et al.*, 2004). The varied carpet beetle, *Anthrenus verbasci* (L.) (Insecta, Coleoptera, Dermestidae), shows a circannual pupation rhythm (Blake, 1959; Nisimura and Numata, 2001). Under natural conditions, larval development of this species takes one or more years, and fully grown larvae pupate in spring. Under short-day conditions, the rhythm persists with a period of about 40 weeks, although under long-day conditions pupation is less synchronous. The zeitgeber to control this circannual rhythm is the change in photoperiod (Nisimura and Numata, 2001).

Phase response curves (PRCs) are plots of phase shifts that result as a function of the phase of a stimulus, and they were devised to examine the mode of entrainment (Johnson, 1999; Saunders, 2002). Relevant to the circannual rhythm in *A. verbasci*, Miyazaki *et al.* (2005) determined a PRC to a long-day pulse superimposed for 4 weeks over constant short-day conditions. The PRC reflects the

nature of the oscillation producing the circannual rhythm. A long-day pulse causes a phase delay or a phase advance, depending on the phase at which the pulse is given, similarly to a light pulse under continuous darkness in circadian rhythms. The circannual PRC resulting from these phase shifts closely resembles a circadian PRC. Miyazaki *et al.* (2005) thus assumed that the circannual rhythm is generated by a circannual oscillator having features in common with the circadian clock, including the phase response.

Nisimura and Numata (2003) showed that the circannual pupation rhythm in *A. verbasci* entrains to an exact year under naturally changing day length at a constant temperature, and they explained this entrainment as a phase delay induced by a decrease in day length in autumn. However, temporal changes in the phase of the circannual oscillator under naturally changing day length remained unclear. If we compare the phase shift caused by a photoperiodic stimulus under naturally changing photoperiod with the PRC resulting from a photoperiodic stimulus under constant photoperiodic conditions, we can describe the temporal change in phase under naturally changing day length in terms of the phase in the PRC.

Under naturally changing day length, phase shifts depend on the season at which a photoperiodic stimulus is given in the circannual rhythm of the willow warbler *Phylloscopus trochilus*, the European starling *Sturnus vulgaris*, and the rainbow trout *Oncorhynchus mykiss* (Gwinner, 1971, 1973; Randall *et al.*, 1998). In these species, however, PRCs resulting from the same stimulus under constant

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photoperiodic conditions have not been obtained; therefore, we cannot describe temporal phase changes in terms of the phase of PRCs.

In the present study, we focused our attention on the nature of the oscillation producing the circannual rhythm in *A. verbasci* under naturally changing day length. We first examined the effect of a short-day pulse superimposed for 4 weeks over constant long-day conditions. We then observed the phase responses to long-day and short-day pulses for 4 weeks given under naturally changing day length from August to November. The goal was to describe temporal changes in phase under naturally changing day length in terms of the phases of PRCs.

## MATERIALS AND METHODS

Adults of *A. verbasci* were collected in Osaka City, Japan (34.7°N, 135.5°E), in April and May in 2002 and 2003. The adults and their eggs were kept under 16 h light and 8 h darkness (LD 16:8) at 25±1°C, and larvae within a week after hatching were used for experiments at 20±1°C and about 66% relative humidity. Dried bonito powder and dried yeast were provided as larval food, as described by Nisimura and Numata (2001). A constant photoperiod was produced by white fluorescent lamps (NEC Lighting, Tokyo) and timers (Omron, Kyoto), and the light intensity in the photophase was about 0.9 Wm<sup>-2</sup>. For rearing under naturally changing day length at a constant temperature, an incubator was placed beside a window facing north in the laboratory. The transparent side of the

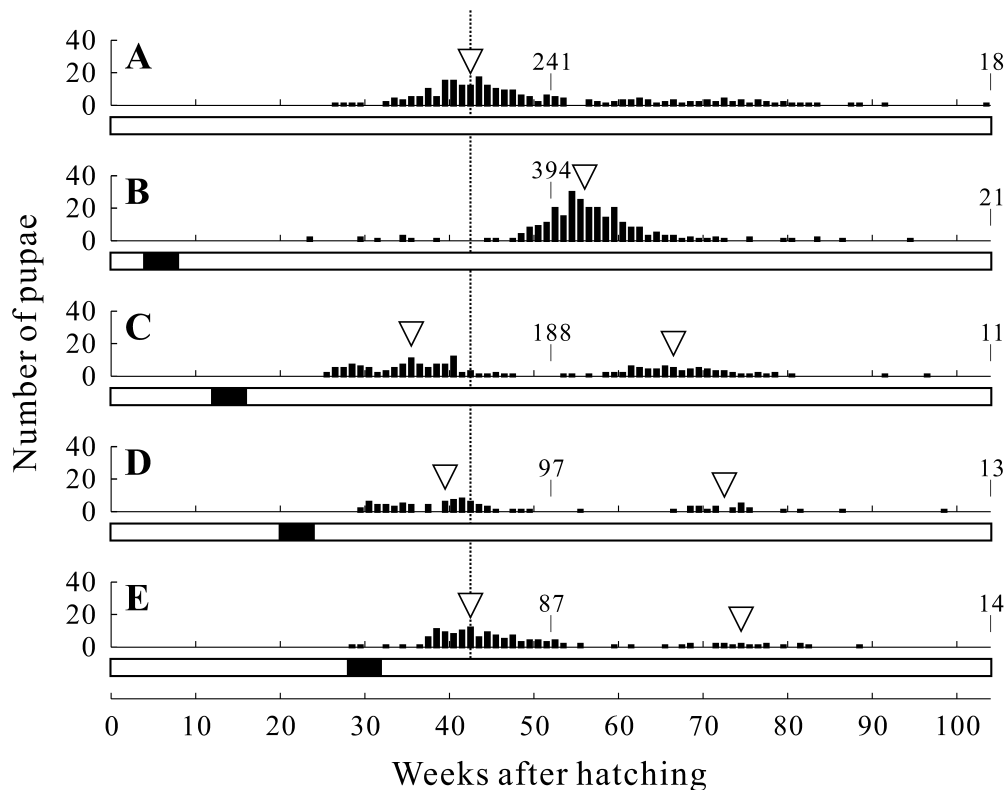
incubator faced the window, and only natural sunlight was permitted to enter it. At midday on sunny days, the light intensity inside the incubator was 5–22 Wm<sup>-2</sup>.

In the first experiment, larvae reared under LD 16:8 were exposed to LD 12:12 for 4 weeks beginning 4, 12, 20, or 28 weeks after hatching, or kept continuously under LD 16:8. In the second experiment, larvae from 26 May or 2 June 2003 reared under naturally changing day length were exposed to LD 16:8 or LD 12:12 for 4 weeks beginning on 4 August, 1 September, 29 September, 27 October, or 24 November 2003, or kept continuously under naturally changing day length. The number of larvae was not counted at the beginning of the experiments, but at least 125 individuals were used for each experimental series. The pupation of all larvae was recorded each week.

## RESULTS

### Phase response under long-day conditions

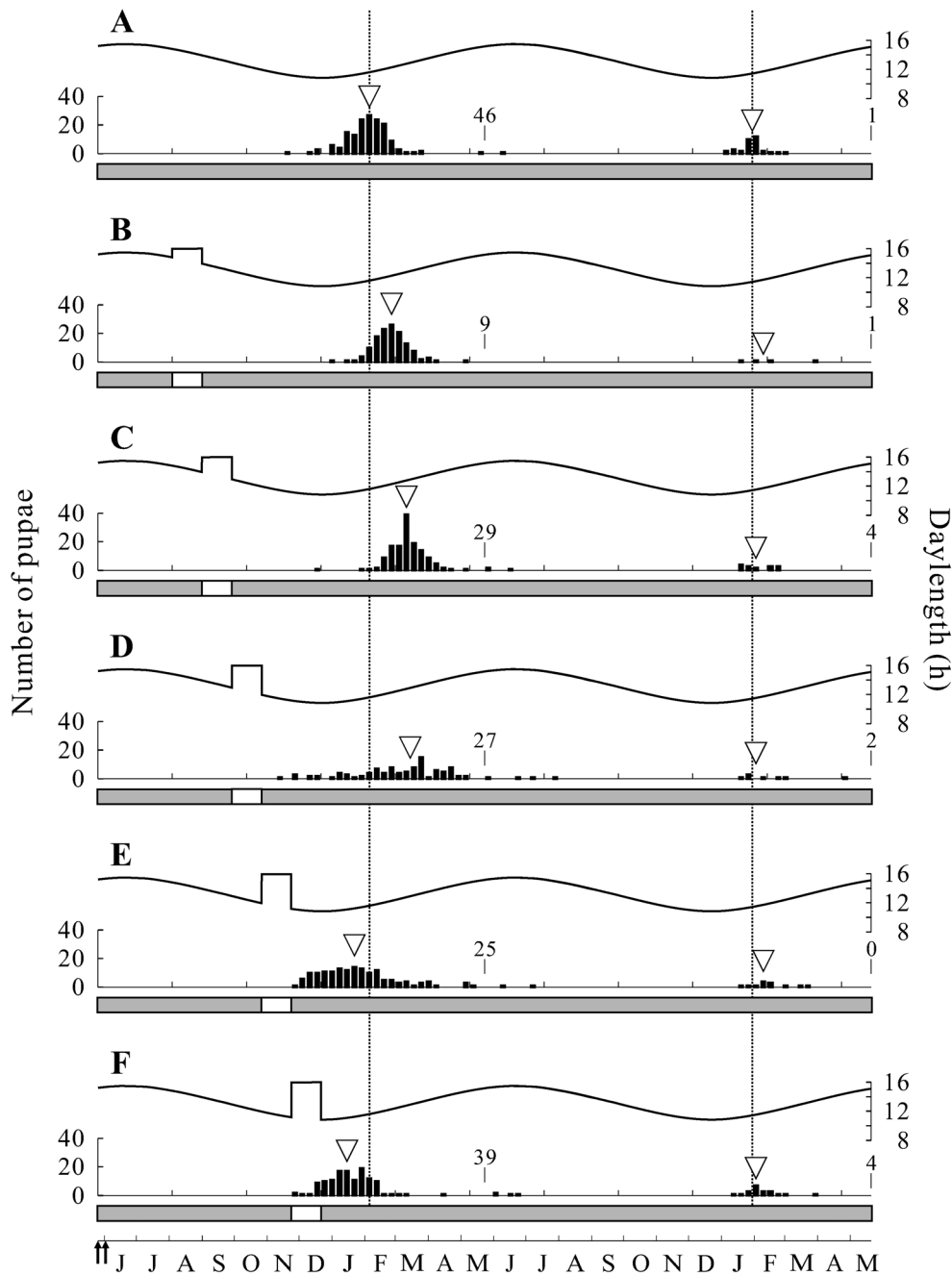
In the control insects kept continuously under long-day conditions, many individuals pupated before 55 weeks after hatching, and their median larval duration was 43 weeks (Fig. 1A), which was close to that of the first pupation group under the same conditions in a previous study (Nisimura and Numata, 2001). However, we could not define the second group because of less synchrony of pupation (Fig. 1A). When a short-day pulse was given at 4 weeks after hatching, the median in pupation was delayed by 13.5 weeks (Fig. 1B). When a short-day pulse was given at 12, 20, or 28



**Fig. 1.** Effects of a short-day pulse on the pupation rhythm under long-day conditions in *Anthrenus verbasci*. Larvae were kept under LD 16:8 (empty bars) and exposed to LD 12:12 (filled portions of bars) for 4 weeks at various times after hatching (temperature 20°C). Panel A represents a control experiment with no LD 12:12 pulse; panels B–E represent separate experiments with an LD 12:12 pulse at different times after hatching. Each numeral above a vertical line indicates the number of insects remaining as larvae, which differs from the total number of insects that subsequently pupated because of mortality. A triangle indicates the median of each pupation group. The vertical dotted line crosses through the median of the first pupation group in the control experiment.

weeks after hatching, a clearly periodic pattern of pupation was observed (Fig. 1C-E). Delivery of a short-day pulse at 12 weeks after hatching advanced pupation in the first group by 7 weeks (Fig. 1C). A short-day pulse at 20 weeks after hatching advanced pupation in the first group by 3 weeks

(Fig. 1D). A short-day pulse at 28 weeks after hatching did not shift pupation in the first group (Fig. 1E). Under these three conditions, the interval between the medians of larval duration in the first and second groups was 31–33 weeks.

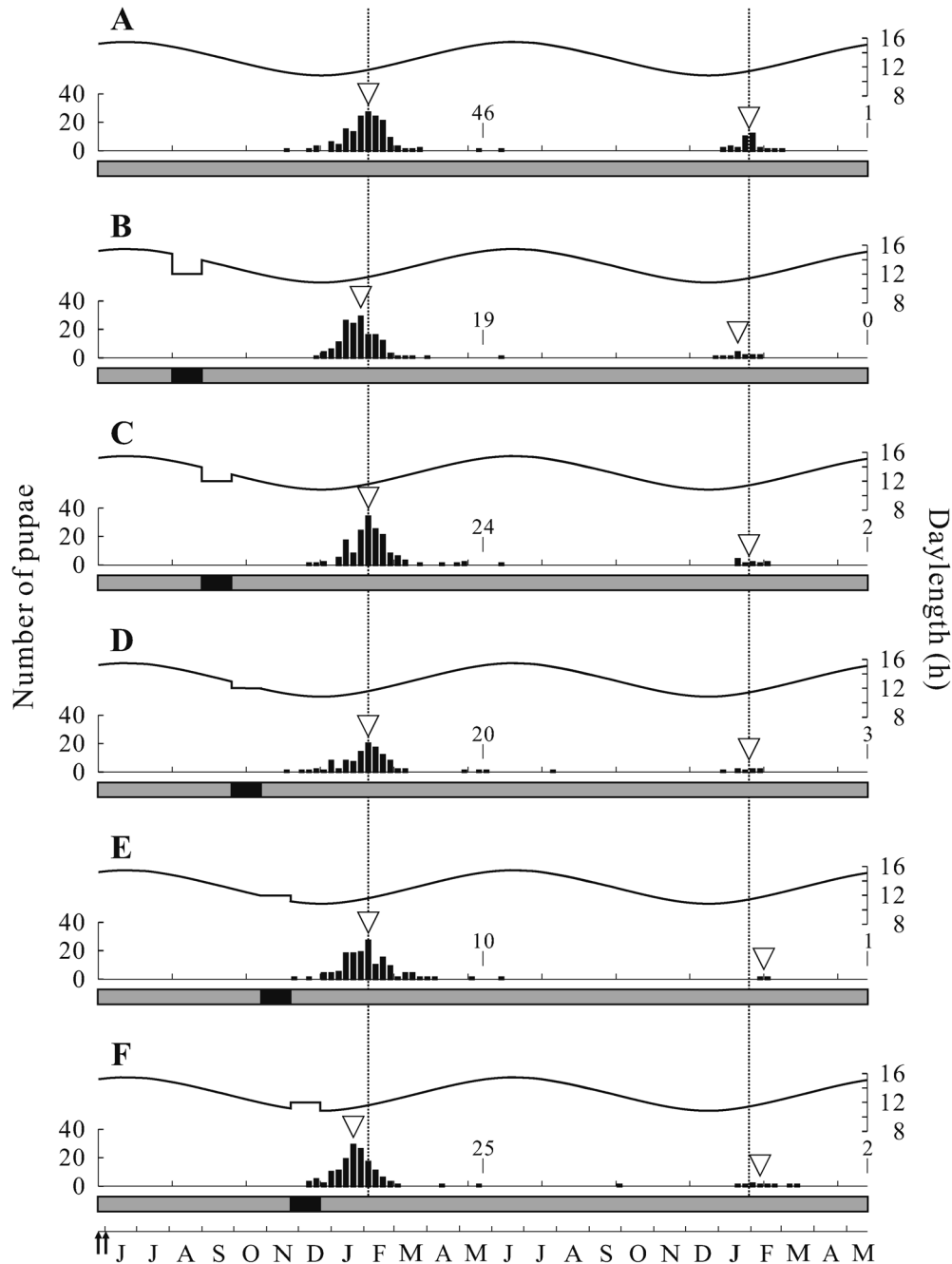


**Fig. 2.** Effects of a long-day pulse on the pupation rhythm under naturally changing day length at a constant temperature (20°C) in *Anthrenus verbasci*. Larvae were kept under naturally changing day length (grey bars) and exposed to LD 16:8 (empty portion of bars) for 4 weeks at various times after hatching. Panel A represents a control experiment with no LD 16:8 pulse; panels B-F represent separate experiments with an LD 16:8 pulse at different times after hatching. Arrows at bottom left show the beginning of the experiments. Each numeral above a vertical line indicates the number of insects remaining as larvae, which differs from the total number of insects that subsequently pupated because of mortality. A triangle indicates the median of each pupation group. A vertical dotted line crosses through the median of each pupation group in the control experiment. The solid curve in each panel indicates the natural day length including 1 h of twilight at Osaka, Japan (35°N) for most of the experiment, and the artificial day length produced by white fluorescent lamps during the LD 16:8 pulse.

### Effect of long-day pulses under naturally changing day length

Larvae kept continuously under naturally changing day length at 20°C showed a periodic pattern in pupation at an interval of 51.5 weeks, and their rhythm entrained to an exact year. In the first pupation group, most individuals

pupated in January and February 2004, and the median date of pupation was in early February (Fig. 2A). When a long-day pulse was given on 4 August, pupation in the first group was delayed by 3 weeks (Fig. 2B). When a long-day pulse was given on 1 September, pupation in the first group was delayed by 5 weeks (Fig. 2C). Although a long-day



**Fig. 3.** Effects of a short-day pulse on the pupation rhythm under naturally changing day length at a constant temperature (20°C) in *Anthrenus verbasci*. Larvae were kept under naturally changing day length (grey bars) and exposed to LD 12:12 (filled portion of bars) for 4 weeks at various times after hatching. Panel A represents a control experiment with no LD 12:12 pulse; panels B-F represent separate experiments with an LD 12:12 pulse at different times after hatching. Arrows at bottom left show the beginning of the experiments. Each numeral above a vertical line indicates the number of insects remaining as larvae, which differs from the total number of insects that subsequently pupated because of mortality. A triangle indicates the median of each pupation group. A vertical dotted line crosses through the median of each pupation group in the control experiment. The solid curve in each panel indicates the natural day length including 1 h of twilight at Osaka, Japan (35°N) for most of the experiment, and the artificial day length produced by white fluorescent lamps during the LD 12:12 pulse.

pulse given on 29 September produced much less synchronous pupation than the above three conditions, the median date of pupation in the first group was delayed by 5.5 weeks, compared to the control insects (Fig. 2D). When a long-day pulse was given on 27 October, however, pupation in the first group was advanced by 2 weeks (Fig. 2E). When a long-day pulse was given on 24 November, pupation in the first group was advanced by 3 weeks (Fig. 2F). Thus, the phase shift in the circannual rhythm caused by a long-day pulse depended on the season in which the pulse was given under naturally changing day length. However, the median date of pupation in the second group changed by 1.5 weeks or less compared with the control insects, and most insects in the second group pupated in January and February 2005.

#### Effect of short-day pulses under naturally changing day length

When a short-day pulse was given on 4 August, pupation in the first group advanced slightly (Fig. 3B). A short-day pulse given on 1 September, 29 September, or 27 October did not change the median date of pupation in the first group (Fig. 3C-E). When a short-day pulse was given on 24 November, pupation in the first group advanced by 2 weeks (Fig. 3F). The effect of a short-day pulse on the first pupation group was less prominent than that of a long-day pulse. The median date of pupation in the second group changed by 2 weeks or less compared with the control insects, and most insects in the second group pupated in January and February 2005, as with the long-day pulses.

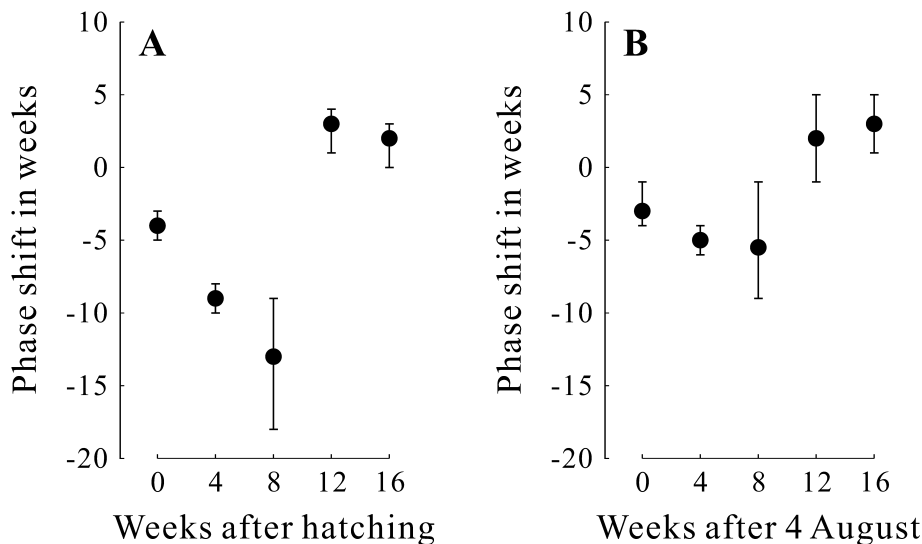
### DISCUSSION

In our previous study, we obtained a circannual PRC for *A. verbasci* by superimposing a long-day pulse for 4 weeks over constant short-day conditions, which closely resembles the Type 0 PRC in circadian rhythms (Miyazaki *et al.*, 2005). In the circannual PRC, we showed the period of the rhythm

under constant short-day conditions in terms of angle degrees (0–360°), and considered 0–180° as “subjective summer” and 180–360° as “subjective winter”. The phase shift changed from delayed to advanced in the middle of subjective winter. Therefore, a long-day pulse caused a phase delay as an “autumn signal” and a phase advance as a “spring signal”. In the present study, a long-day pulse caused phase shifts in the first pupation group under naturally changing day length at a constant 20°C. This phase response resembles that obtained by giving a long-day pulse under constant short-day conditions, both in the direction of the phase shift and the degree of synchronization (Fig. 4A, B).

The phase response between August and November was similar to that in subjective winter under constant short-day conditions, because the phase shift caused by a long-day pulse changed from delayed to advanced in October. A long-day pulse given just before the transition from delaying to advancing caused the least synchronous pupation. Therefore, the circannual oscillator changes its phase from August to November under naturally changing day length, as occurs in subjective winter under constant short-day conditions. In the second pupation group, however, larvae pupated at the same time as in the control experiment without a long-day pulse, regardless of the phase response of the first group. This result can be attributed to entrainment to the geophysical year by long days in spring and summer.

Nisimura and Numata (2001) reported that *A. verbasci* showed a periodicity of 32 weeks in pupation under long-day conditions of LD 16:8, although a smaller proportion of larvae pupated less synchronously than under short-day conditions of LD 12:12 at 20°C. In the present study, however, the timing of pupation under constant long-day conditions of LD 16:8 did not show a clear periodic pattern. We interpret this discrepancy to indicate that the circannual rhythm is not prominent under long-day conditions because these condi-



**Fig. 4.** Comparison of phase responses in the pupation of *Anthrenus verbasci* under constant short-day conditions and under naturally changing day length. Closed circles represent the phase shift (shown as median/interquartile) in the first pupation group, induced by a long-day pulse given at various times after hatching. **(A)** Phase response to a 4-week long-day pulse under a constant short-day condition (temperature 20°C) (redrawn from Miyazaki *et al.*, 2005). **(B)** Phase response to a 4-week long-day pulse under naturally changing day length (temperature 20°C).

tions have an inhibitory effect on pupation (Nisimura and Numata, 2001), and sometimes a periodic pattern was not detected. In other animals, the obvious expression of circannual rhythms is restricted within a narrow range of photoperiodic conditions (Gwinner, 1986). In *Phylloscopus trochilus*, for example, clear circannual rhythms were observed under short-day conditions of LD 12:12, but the rhythms disappeared under long-day conditions of LD 18:6 (Gwinner, 1971).

Despite the lower level of synchrony of pupation in *A. verbasci* under constant long-day conditions in the present study, a short-day pulse superimposed for 4 weeks over constant long-day conditions caused a prominent phase delay or advance, similarly to a long-day pulse under short-day conditions (Miyazaki *et al.*, 2005). When a short-day pulse was given at 12, 20, or 28 weeks after hatching, the interval between the first and second pupation groups was about 32 weeks, corresponding to the interval under constant long-day conditions observed by Nisimura and Numata (2001). Therefore, we consider a period of about 32 weeks to be intrinsic for this rhythm under long-day conditions, which is shorter than the period of about 37 weeks under short-day conditions (Nisimura and Numata, 2001; Miyazaki *et al.*, 2005). In *Oncorhynchus mykiss*, the circannual period is also significantly shorter under long-day conditions of LD 18:6 than under short-day conditions of LD 6:18 (Duston and Bromage, 1986).

In the circannual rhythm of *A. verbasci*, the PRC to a long-day pulse closely resembles light-pulse PRCs in circadian rhythms (Miyazaki *et al.*, 2005). In the same way, we can suppose that a short-day pulse acts like a dark pulse under continuous light in circadian rhythms. Although dark-pulse PRCs have not been examined as extensively as light-pulse PRCs in circadian rhythms, some have been mirror images of light-pulse PRCs. That is, dark pulses cause phase-dependent phase shifts in a direction opposite to that of phase shifts caused by light pulses at equivalent circadian phases. (e.g., Subbaraj and Chandrashekar, 1978; Boulos and Rusak, 1982; Johnson *et al.*, 1989; also see Johnson (1999)).

However, the PRC to a short-day pulse does not seem to be a mirror image of the PRC to a long-day pulse in the circannual rhythm of *A. verbasci*. In this rhythm, Miyazaki *et al.* (2005) regarded the initial phase under constant short-day conditions as the beginning of subjective winter. Under constant long-day conditions, we assume that the initial phase is around the onset of subjective summer, because the phase is different by about half a cycle from that under constant short-day conditions (Fig. 1 of Miyazaki *et al.* (2005)). A short-day pulse given at 12 weeks after hatching, which is in late subjective summer, caused a phase advance to the phase 24 weeks before pupation. The phase at the onset of the short-day pulse corresponds to the beginning of subjective winter and, therefore, the pulse was considered to be an "autumn signal" (see Miyazaki *et al.* (2005)). A short-day pulse given at 20 weeks after hatching also caused a phase advance as an "autumn signal". When a short-day pulse was given at 4 weeks after hatching in early subjective summer, however, the pulse probably acted as a "spring signal" and resulted in a prominent phase delay. Moreover, a short-day pulse given at 28 weeks after hatch-

ing, which is in subjective winter, did not shift the phase.

A hypothesis of alternate half-cycles of subjective summer and winter under long-day conditions facilitates interpretation of these phase shifts. According to this hypothesis, a short-day pulse causes a phase delay in early subjective summer and a phase advance in late subjective summer, and has little or no effect in subjective winter. Therefore, the subjective summer in the PRC to a short-day pulse probably corresponds to the subjective winter in the PRC to a long-day pulse, and vice versa. Because we examined the phase shift by a short-day pulse only in four phases, further investigations are necessary to know the exact relationship among these PRCs.

Even though a short-day pulse of LD 12:12 caused a phase shift under long-day conditions of LD 16:8, it had little or no effect on phase responses under naturally changing day length at a constant 20°C. One possible explanation for this apparent contradiction is that, because the difference between 12 h and natural day length when a pulse was given was much less than 4 h, the effect of photoperiodic stimuli was less prominent. Nevertheless, a short-day pulse on 24 November advanced pupation in the first group by 2 weeks. When the short-day pulse was given, the day length including 1 h twilight was shorter than 12 h. A pulse of slightly longer day length probably produced a similar effect to that of a long-day pulse.

In this study, the circannual rhythm in *A. verbasci* showed a phase response under naturally changing day length similar to that under constant short-day conditions between 4 August and 24 November, when the day length was monotonically decreasing at a rate of about 2 minutes per day, from 14 h 48 min to 11 h 9 min including 1 h of twilight. However, the insect did not recognize these changing day length as being identical to constant short-day conditions. The magnitude of the phase delay caused by a long-day pulse was much smaller under naturally changing day length than under short-day conditions (Fig. 4A, B).

This difference can be attributed to the amplitude of photoperiodic changes, because when a long-day pulse delayed pupation in the first group, the natural day length including 1 h twilight was longer than 12 h and, therefore, differed from 16 h by less than 4 h. However, the difference in the magnitude of phase delay is probably also due to modulation of the rhythm by decreasing day length. In the latter case, the circannual rhythm of *A. verbasci* entrained to an exact year, not only by phase shifts induced by a decrease or increase in day length crossing the critical value, as in discrete (nonparametric) entrainment of the circadian rhythm, but also by continuous modulation of the phase by the seasonal fluctuation of natural day length, as in continuous (parametric) entrainment of the circadian rhythm (Johnson *et al.*, 2003).

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