Resetting of the circannual rhythm of the varied carpet beetle, *Anthrenus verbasci*, by low-temperature pulses

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Abstract. The varied carpet beetle, *Anthrenus verbasci*, has a circannual pupation rhythm and pupates in spring in the wild. The change in photoperiod has been shown to act as a predominant zeitgeber for this rhythm. However, it is unclear whether the change in ambient temperature acts as a zeitgeber. In the present study, we examined the effects of low-temperature pulses on this circannual rhythm by exposing larvae kept under constant short-day conditions (LD 12:12 h) at 20°C to a lower temperature of 15°C, 10°C, or 5°C for 8 or 12 weeks at various phases. Larval development and pupation were suppressed during exposure to low temperature, and pupation was induced in sufficiently grown larvae within two months of return to 20°C. These results were attributed to the suppression and stimulation of pupation not relevant to the circannual rhythm, i.e. masking of the circannual rhythm by temperature. Furthermore, long-term observations demonstrated the existence of phase-dependent phase shifts of circannual rhythm caused by low-temperature pulses. Circannual phase response curves to low temperature were constructed on the basis of the phase shifts obtained. A low-temperature pulse as a winter signal can reset the circannual rhythm of *A. verbasci*. It is probable that temperature plays a role as well as photoperiod for entrainment of this circannual rhythm to a natural year.

Key words. Circannual rhyrhm, diapause termination, larval diapause, low temperature, masking, phase response curve, phase shift, pupation, zeitgeber.

Introduction

Most organisms exhibit a specific behaviour, developmental event, or physiological change at a certain time of the day or in a certain season of the year. These are induced not only by exogenous factors but also by endogenous biological clocks and therefore can be observed with approximately daily or annual periodicity under constant conditions. These rhythms are called circadian rhythms and circannual rhythms, respectively. The period of these endogenous rhythms is almost fully temperature-compensated at various constant temperatures. Because the period of these rhythms is slightly different from that of the corresponding geophysical cycles, time or seasonal cues (zeitgebers) from the environment are needed to entrain the rhythm to a natural cycle (Pittendrigh, 1981a,b; Gwinner, 1986; Johnson *et al*., 2004). In circadian rhythms, both light and temperature are important zeitgebers for entrainment to a daily cycle. The phase of a circadian clock, which underlies the circadian rhythm, is shifted by applying a single light or temperature pulse in constant darkness and temperature. The magnitude of a phase shift depends on the strength of a pulse (intensity or amplitude, or exposure duration), and the direction of a phase shift (phase advance or phase delay) depends on the phase when a pulse is given. Because a pulse affects the phase of an endogenous clock, the phase shift induced is also observed in subsequent cycles. Phase response curves (PRCs) are obtained by plotting the magnitude of phase advances and delays as a function of the phase of a pulse. PRCs can also be used to detect oscillatory behaviour in biological clocks (Pittendrigh, 1981b; Rensing & Ruoff, 2002; Saunders, 2002; Johnson *et al*., 2004).

The existence of circannual rhythms has been confirmed under seasonally constant conditions in various seasonal traits of diverse species, e.g. hibernation, migration, germination, growth, moult, and reproduction (Gwinner, 1986; Goldman *et al*., 2004; Helm & Stevenson, 2014), but the physiological studies on circannual rhythms are much fewer than those on circadian rhythms. Although photoperiod is a predominant zeitgeber in many circannual rhythms, it is still almost unknown whether other environmental factors act as zeitgebers (Gwinner, 1986; Paul *et al*., 2008; Helm *et al*., 2013). The probability that temperature acts as a zeitgeber has been examined in the circannual rhythms of several species (see Gwinner, 1986; Andjus *et al*., 2000). In the golden-mantled ground squirrel, *Callospermophilus lateralis*, and thirteen-lined ground squirrel, *Ictidomys tridecemlineatus*, reared at 21°C, a temperature pulse of 6°C or lower applied at the spring phase induces a phase delay in the body weight rhythm, which is observed even in the subsequent years

(Mrosovsky, 1980, 1990; Joy & Mrosovsky, 1985). A 6-week high-temperature pulse (30°C against 4°C) at the mid-winter phase and a 20-week low-temperature pulse (6°C against 21°C) at the autumn phase, however, advance the timing of peak body weight and reproductive maturation of *C. lateralis* only in the year of exposure, and there is no evidence that rhythms are phase-advanced in subsequent years (Barnes & York, 1990; Mrosovsky, 1990). These two pulses probably mask the rhythms without affecting the phase of the underlying timing mechanism and, therefore, phase advances are not observed in subsequent cycles (Körtner & Geiser, 2000; Helm *et al*., 2013). The phenomenon of a single temperature pulse causing a phase advance of a circannual rhythm is still not observed in any species.

Insects are poikilotherms and therefore more susceptible to ambient temperature than homeotherms. Temperature, especially low temperature during winter, strongly influences development and seasonality in insects. Low temperatures not only cause the direct suppression of development but also prompt the induction and termination of winter diapause (Danilevskii, 1965; Danks, 1987; Hodek & Hodková, 1988). We focused on the relationship between ambient temperature and the pupation timing controlled by the circannual rhythm in the varied carpet beetle, *Anthrenus verbasci* (Miyazaki *et al*., 2012, 2014). *A. verbasci* shows a substantial difference in the larval growth rate between individuals and has a life-cycle duration from one to several years depending on the degree of development, because pupation is averted at least until the next year when larval growth is not sufficient for pupation (Blake, 1958; Miyazaki *et al*., 2009). A circannual pupation rhythm of this species displays a periodicity of approximately 40 weeks under constant conditions, and the temperature compensation in the period length has been confirmed in the range between 17.5°C and 25°C (Blake 1958, 1959; Nisimura & Numata, 2001, 2003). A photoperiodic change clearly induces a phase shift of this circannual rhythm (Nisimura & Numata, 2001; Miyazaki *et al*., 2005). When *A. verbasci* larvae were exposed to natural annual changes in photoperiod in Japan at a constant 20°C, pupation occurred in late winter in the first and second years (Nisimura & Numata, 2003; Miyazaki *et al*., 2006). However, when larvae were exposed to natural fluctuations in light and temperature, pupation was observed each year in mid-spring rather than in late winter (Nisimura & Numata, 2003; Miyazaki *et al*., 2009). It is likely that low temperatures during winter and early spring suppress pupation until mid-spring, and that this suppression also contributes to the synchrony of pupation under natural conditions (Blake, 1960; Nisimura & Numata, 2003).

Low temperatures not only directly influence the development of *A. verbasci* but also affect the physiological mechanism related to stimulation and suppression of pupation.

Kuwana (1951) showed that when mature larvae of *A. verbasci* reared at 25°C were exposed to low temperature of 5–20°C for 1–5 months, the pupation rate within one month after the exposure prominently depended on the temperature and duration of the exposure. In addition, there remains a possibility that temperature as well as photoperiod effectively acts as a zeitgeber and resets the phase of the circannual rhythm of *A. verbasci*. Pupation of *A. verbasci* occurred in mid-spring under constant darkness and natural temperatures (Kuwana, 1951; Blake, 1960). These results indicate that the natural seasonal change in temperature can sufficiently synchronize pupation of *A. verbasci* to an appropriate season even without light. However, a temperature change from 25°C to 20°C had little or no effect on the phase of the circannual rhythm of *A. verbasci* under a constant photoperiod (Nisimura & Numata, 2003). In the present study, we examined effects of low-temperature pulses on the pupation timing and circannual rhythm in *A. verbasci*, by exposing at various phases the larvae kept under constant short days at 20°C to a lower temperature of 15°C, 10°C, or 5°C for 8 or 12 weeks, which corresponds to winter conditions in temperate zones. The centre of our interest is whether low temperature can shift the phase of this circannual pupation rhythm. However, the timing of pupation just after a low-temperature pulse likely does not reflect the phase shift of circannual rhythm, because a temperature pulse can affect the larval development and the physiological mechanism linked to stimulation and suppression of pupation without affecting the phase of circannual rhythm, i.e. masking by temperature (Mrosovsky, 1990; Körtner & Geiser, 2000). Therefore, we particularly noted the timing of pupation later than pupation just after a low-temperature pulse, for example the timing of pupation in the second cycle when a pulse was applied in the first cycle, for excluding the effects of masking and for clarifying phase shifts of this circannual rhythm.

Materials and methods

Insects

Adults of *A. verbasci* were collected in Minoh City, Osaka, Japan (34.8°N, 135.5°E), in April and May 2010. They were maintained on a diet of diluted honey under conditions of 16 h light and 8 h darkness (LD 16:8 h) at 25 ± 1°C. Pieces of wool were provided as oviposition substrates, and every day or every other day, the wool on which eggs had been laid was transferred to plastic boxes (61 mm × 43 mm × 17 mm), which were maintained

under the same conditions. Hatching of larvae was examined every day or every other day, and the number of larvae in a box was restricted to 50 or fewer. Within a week after hatching, the plastic boxes were transferred to LD 12:12 h at 20 ± 1°C. The boxes were placed in airtight containers containing a saturated solution of $NaNO_2$ to maintain the relative humidity at approximately 66%. The photoperiod was produced by 10-W fluorescent lamps (FL10WB; Hitachi Lighting Ltd., Tokyo, Japan) and timers, and the light intensity in the photophase was approximately 1.9 $W \cdot m^{-2}$. Dried bonito powder was provided as larval food and thereafter supplied *ad libitum*. A small amount of dried yeast (Asahi Food and Healthcare, Tokyo, Japan) was also supplied as a source of vitamins. Pupation was recorded each week for 130 weeks, although pupae were removed every day or every other day to prevent cannibalism.

Exposure to a lower temperature

Larvae kept at 20°C under LD 12:12 h were exposed to a lower temperature of 15°C, 10°C, or 5°C for 8 or 12 weeks (8- or 12-week pulse) beginning 8, 12, 16, 20, 24, 32, 40, or 48 weeks after hatching, or were kept continuously at 20°C. In order to acclimatize to new temperatures, the set temperature was changed by 1°C 2, 4, 6, 8, and 10 h after the onset of the scotophase. Because the temperature changed 5°C during a scotophase, for example, three days were required in the case of a transfer from 20°C to 5°C or vice versa. A pulse duration for 8 or 12 weeks to a lower temperature included the period spent for acclimatization. Pupation was also recorded during the exposure.

Results

Effects of low-temperature pulses at various phases

When larvae were kept continuously at 20°C under LD 12:12 h with no low-temperature pulses, as a control for experiments in which a low-temperature pulse was applied, their pupation showed a periodic pattern with a circannual period shorter than 1 year, as described in previous studies (Fig. 1A; Nisimura & Numata, 2001, 2003; Miyazaki *et al*., 2005, 2007, 2009; Matsuno *et al*., 2013). From two pupation groups that were separated by an interval without pupation, we defined ‘the first pupation group’ and ‘the second pupation group’. In the first pupation group, 468 larvae pupated 20–38 weeks after hatching, and their

median larval duration was 25 weeks. In the second pupation group, 97 larvae pupated 46–85 weeks after hatching, and the median larval duration was 62 weeks. The interval between medians for the first and second groups was 37 weeks.

Most larvae were immature 8 weeks after hatching. Because exposure to low temperature at 8 weeks after hatching directly suppressed larval growth in a temperature- and duration-dependent manner, the proportion of insects that pupated in the first circannual cycle was lower than that in the control (Fig. 1). When an 8-week or 12-week pulse of 15°C was applied 8 weeks after hatching, pupation in the first group was advanced by 1–2 weeks and that in the second group by 3–4 weeks over that in control insects (Fig. 1B, C). Thus, a pulse of 15°C given 8 weeks after hatching was effective to advance pupation not only in the first but also in the second circannual cycle. Although there was a possibility that the advance of pupation in the first cycle was attributed to masking of the circannual rhythm by temperature, the advance in the second cycle was definitely produced by the phase advance of this circannual rhythm. Therefore, we suggest that the change of temperature acted as a zeitgeber and advanced a phase of a circannual clock of *A. verbasci*. Exposure to 10°C over the same time strikingly reduced the proportion of the first pupation group and increased that of the second group. When an 8-week pulse of 10°C was applied, pupation in the first group was delayed by 1 week, but pupation in the second group was advanced by 8 weeks. After the second group, 13 larvae pupated with the median of 90 weeks. These insects likely pupated as the third group because the difference between median values of this group and the second group was 36 weeks and close to a circannual period of 37 weeks in the control insects (Fig. 1D). A 12-week pulse of 10°C advanced pupation by 1 week in the first group and by 5 weeks in second group (Fig. 1E). Exposure to 5°C obscured the pupation rhythm. When an 8-week pulse of 5°C was applied, pupation still showed a bimodal pattern, and two pupation groups were recognized. To calculate the median for each group, we defined the border of the two pupation groups as 53–54 weeks after hatching, during which no larvae pupated. The median larval durations for the first and second groups were 32 and 69 weeks, respectively, the interval between them being 37 weeks (Fig. 1F). A 12-week pulse of 5°C produced less synchronous pupation than an 8-week pulse. Larvae pupated almost every week over 29–96 weeks after hatching and the median in these insects was 54 weeks (Fig. 1G).

Low-temperature pulses produced higher proportions of the first pupation group when given 12 weeks after hatching than 8 weeks after hatching (Fig. 2). An 8-week pulse of 15°C advanced pupation in the first group by 1 week, but delayed it in the second group by 3.5 weeks (Fig. 2B). A 12-week pulse of 15°C delayed pupation in the first group by 1 week and

that in the second group by 3 weeks (Fig. 2C). When an 8-week pulse of 10°C was applied, pupation in the first group was advanced by 1 week, whereas no shift of the pupation time was observed in the second group (Fig. 2D). A 12-week pulse of 10°C delayed pupation in the first group by 1 week and that in the second group by 4.5 weeks (Fig. 2E). When an 8-week pulse of 5°C was applied, pupation was delayed by 1 week in the first group and by 4 weeks in the second group (Fig. 2F). A 12-week pulse of 5°C delayed pupation in the first group by 3 weeks and that in the second group by 1 week (Fig. 2G). Effects of pulses of 15°C and 10°C were different between 8-week and 12-week exposures in the first pupation group—a 1-week advance in 8-week exposure and a 1-week delay in 12-week exposure. This is probably attributable to 4-week low-temperature exposure in the latter period of 12-week pulses directly suppressing the developmental process for pupation. In contrast, pulses of 5°C caused a delay in the first pupation group in both 8-week and 12-week exposures. The first pupation peak after exposure to lower temperature was sharper than that in control insects. This is probably attributable to the enhanced synchrony of pupation released from suppression of the pupation processes under low temperatures. On the shift of the pupation time in the second group, we observed no consistent effects of the temperature and duration of pulses applied 12 weeks after hatching (Fig. 2B–G).

Low-temperature pulses given 16 weeks after hatching induced a delay of pupation in both the first and second groups (Fig. 3). The range of delay in the first group was 1–5 weeks and the magnitude depended on the temperature and duration of the pulse, which can be attributed to the direct suppression of the pupation process by low temperatures, i.e. masking by low temperatures. In the second group, however, this dependence was obscure. The magnitude of delay in the second group was 2–3 weeks in the 8-week and 12-week exposures of 15°C and 10°C (Fig. 3B–E) and 3.5 and 5 weeks in the 8-week and 12-week exposures of 5°C, respectively (Fig. 3F, G). Thus, in the treatment at this phase, there was little or no dependence of the magnitude of circannual phase shift on the temperature and duration of the pulse.

In the same manner, low-temperature pulses given 20 weeks after hatching delayed pupation in both groups. These pulses were almost entirely superimposed on the first pupation group in the control, and therefore induced a larger delay of pupation in the first group (Fig. 4). The range of delay in the first group was 3–9 weeks (Fig. 4B–G). The inhibitory effect of exposure to 15°C on pupation was much weaker than that of exposure to 10°C or 5°C, and pupation was observed in many individuals during exposure to 15°C. Because exposure to 10°C or 5°C remarkably inhibited the metamorphosis, the median larval

duration in the first group shifted to 1–3 weeks after the end of exposure. In the magnitude of delay in the second group, dependence on the temperature and duration of a pulse was slightly observed, but the range of magnitude was 2–5 weeks (Fig. 4B–G), which were the same values as that in the case of the application 16 weeks after hatching (Fig. 3B–G).

When an 8-week pulse of 10°C or 5°C was applied 24 weeks after hatching, which corresponds to the pupation peak in the first group in the control, the pupation pattern in the first group was changed to a concave shape by the direct suppression of pupation. Pupation in the second group was delayed by 3–4 weeks (Fig. 5B, C). When an 8-week pulse of 10°C or 5°C was applied 32, 40, and 48 weeks after hatching, the proportion of pupation just after a pulse increased as the time of pulse application was late, because relatively small larvae that had not pupated as the first group grew gradually during 30–50 weeks after hatching and only in sufficiently grown larvae pupation was effectively stimulated by exposure to low temperature and subsequent return to 20°C (Fig. 5D–I). When an 8-week pulse of 10°C or 5°C was applied 32 weeks after hatching, a portion of larvae pupated within 5 weeks after the end of exposure. Most of the residual larvae pupated around 31 weeks after the end of exposure. We regarded this as a 9-week delay in the second group (Fig. 5D, E). When an 8-week pulse of 10°C or 5°C was applied 40 weeks after hatching, many larvae pupated within 8 weeks after the end of exposure. A portion of residual larvae pupated around 32 weeks after the end of exposure. We regarded this as 19-week and 17-week delays in the second group by the 10°C and 5°C pulses, respectively, although the number of individuals was only six in the 10°C treatment and pupation was dispersed over 28 weeks in the 5°C treatment (Fig. 5F, G). When an 8-week pulse of 10°C or 5°C was applied 48 weeks after hatching, most larvae pupated within 6 weeks after the end of exposure. Subsequently, four larvae in the 10°C treatment and eleven larvae in the 5°C treatment pupated 65–85 weeks after hatching, although they did not form a group with a clear phase shift (Fig. 5H, I).

Circannual PRCs to 8-week low-temperature pulses

We constructed circannual PRCs to 8-week low-temperature pulses of 10°C and 5°C (Fig. 6), according to the procedure of the construction of circannual PRCs to long-day pulses in *A. verbasci* (Miyazaki *et al*., 2005). The phase shift in the pupation group was plotted as a function of the phase at which an 8-week low-temperature pulse was started. The period in the circannual rhythm under a constant temperature of 20°C (37 weeks) was shown in terms of angle degrees (0–360°). The initial phase under LD 12:12 h at 20°C was regarded as 180°.

The 0–180° was regarded as the ‘subjective summer’ and the 180–360° as the ‘subjective winter’. The timing of pupation just after a low-temperature pulse may not reflect the phase shift of the circannual rhythm because of masking and, therefore, we excluded individuals that pupated within 10 weeks after the end of low-temperature pulses to assess the phase shift of circannual rhythm. A 19-week delay (−184.9°) induced by an 8-week pulse of 10°C applied 40 weeks after hatching (209.2°) was plotted as an 18-week advance (175.1°). In addition, the results when a pulse was applied 48 weeks after hatching were not included in these PRCs because they had no pupation group with a clear phase shift.

These curves indicate the existence of phase-dependent phase shifts of the circannual rhythm to low temperature. The PRC to 10°C pulses has large phase shifts and a distinct break point at the transition between delays and advances in the beginning of the subjective winter (Fig. 6A). Therefore, we categorize this curve as Type 0 (Winfree, 1970; Pittendrigh, 1981b; Johnson *et al*., 2004), although the largest phase shift is based on the results with only six individuals (Fig. 5F). The PRC to 5°C pulses resembles that to 10°C pulses in some phases. However, a difference in responsiveness to a pulse applied 8 weeks after hatching (257.8°) results in different shapes (Figs. 1D, F and 6A, B).

Discussion

Although it has been demonstrated that *A. verbasci* uses the change of photoperiod as a zeitgeber for entrainment of the circannual pupation rhythm (Nisimura & Numata, 2001, 2003; Miyazaki *et al*., 2005, 2006), there remained the possibility that the change of temperature also acts as a zeitgeber for this rhythm. Nisimura & Numata (2003) concluded that a decrease of temperature does not play a significant role as a zeitgeber, because a temperature decrease from 25°C to 20°C had little or no effect on the phase of this circannual rhythm under LD 12:12 h. However, the present results indicate that effects of winter temperature, which is lower than 10°C for 3 or 4 months in Osaka, are not negligible for synchronization of this circannual rhythm and pupation timing to a natural annual cycle.

Although the circannual rhythm of *A. verbasci* has been recorded at constant 15°C or higher, at the temperatures lower than 15°C the progress of the circannual rhythm has been unknown (Blake, 1958; Nisimura & Numata, 2001). Therefore, we should consider the possibility that exposure to low temperature merely stops or slows the oscillation of a circannual clock, as observed in circadian clocks (e.g. Bünning, 1959; Roberts, 1962; see

Bünning, 1973). However, as a 12-week pulse of 5°C applied 12 weeks after hatching had little effect on the phase of the second group (Fig. 2G), we exclude this possibility, excepting effects of pulses of 5°C applied 8 weeks after hatching (Fig. 1F, G, see below).

Exposure to low temperature and temperature increase are effective factors for termination of larval diapause and the synchronization of pupation in many insects including some coleopterans with long larval diapause (Danilevskii, 1965; Danks, 1987; Hodek & Hodková, 1988; Shintani & Ishikawa, 1997; Higaki, 2005; Terao *et al*., 2012). In addition, it has been reported that pupation time is advanced by a 4-month exposure to 15°C or 20°C in *A. verbasci* reared at 25°C and by an 8-week treatment of 15°C in the black carpet beetle, *Attagenus unicolor*, reared at 28°C (Kuwana, 1951; Baker, 1982, 1983). A circannual clock for pupation of *A. verbasci* probably controls a downstream physiological mechanism in common with larval diapause in other species (Blake, 1958; Miyazaki *et al*., 2014). It is likely that exposure to low temperature for 8 weeks or more and return to 20°C can stimulate pupation in sufficiently grown larvae of *A. verbasci* without involving the circannual rhythm, similar to diapause larvae of other insects. Masking by temperature has been shown also in the circannual rhythm of *C. lateralis* (Barnes & York, 1990; Mrosovsky, 1990). Temperature masks circadian rhythms controlling insect metamorphosis also. In the circadian eclosion rhythm of the onion fly, *Delia antiqua*, bursts of eclosion appear immediately after a temperature rise (Watari, 2002). Therefore, the pupation just after a low-temperature pulse likely does not reflect the phase of the circannual rhythm in *A. verbasci*. By this reasoning, it is difficult to distinguish a phase shift of circannual pupation rhythm from induction of pupation just after an exposure to low temperature and a subsequent temperature increase. However, we detected the phase shift of circannual rhythm by long-term observations.

A phase advance induced by a single high or low temperature pulse has failed to be distinctly observed in circannual rhythms (Barnes & York, 1990; Mrosovsky, 1990). However, the present study demonstrated for the first time a clear circannual phase advance caused by a temperature pulse, by observation of pupation timing in the next cycle also. A pulse of 15°C or 10°C applied 8 weeks after hatching was obviously effective for the phase advance in the second cycle (Fig. 1B–E). The magnitude of the advance was larger in the exposure to 10°C than that to 15°C. This indicates that phase-advance responses depend on temperature or amplitude of the temperature change. In exposure to 10°C, 8-week and 12-week pulses advanced pupation of the second group by 8 and 5 weeks, respectively. Both pulses shifted the median of pupation to 37–38 weeks after the end of exposure (Fig. 1D, E). It is probable that a pulse of 10°C over 8 weeks or more can set the pupation phase to 37–38 weeks after

the end of exposure. We assume that temperature increase from 10°C to 20°C acts as a seasonal signal of 'the end of winter' or 'the beginning of spring' and conforms the pupation phase of the next cycles to approximately 37 weeks (a circannual year for this rhythm) after a temperature increase.

The end of 8-week pulses starting 12 weeks after hatching is the same time as that of 12-week pulses starting 8 weeks after hatching. However, the former pulses of 15°C and 10°C did not cause a clear phase advance in the second cycle in contrast to the latter pulses of 15°C and 10°C (Figs. 1C, E and 2B, D). Therefore, it is probable that the phase of onset of exposure is more important for determination of phase shift than the end of exposure. Moreover, low-temperature pulses applied in the range of 12–24 weeks after hatching produced only a 0–5-week delay in the second group regardless of the degree of low temperature, the exposure duration, and the phase of pulse onset (Figs. 2–4 and 5B, C). Thus, it seems that a low-temperature pulse in this range of circannual phases shows a relatively weak effect on phase shift, as a light pulse applied in the subjective day of circadian rhythm does (Pittendrigh, 1981b; Johnson *et al*., 2004).

When an 8-week pulse of 10°C or 5°C was applied 32 or 40 weeks after hatching, pupation occurred 31–33 weeks after the end of exposure (Fig. 5D–G). Under these regimes, a temperature increase from 10°C or 5°C to 20°C probably acted as a signal of the end of winter and set the pupation phase to approximately 32 weeks after the end of exposure, so that a large phase delay in the second group was observed, although the phase relationship between the end of exposure and the median pupation time differed by approximately 6 weeks from the relationship when an 8-week pulse of 10°C was applied 8 weeks after hatching (Fig. 1D).

A pulse applied 32, 40, or 48 weeks after hatching also caused pupation in sufficiently grown larvae within two months after exposure (Fig. 5D–I). As described above, this can be attributed to masking by temperature. However, we do not exclude another possibility relating to phase shifts: for insects kept under constant photoperiod and temperature, the individual variations of the circannual phase in the second cycle are larger than those in the first cycle, and these variations probably produce differences in the phase at which a pulse is given. Therefore, both advances and delays might have been produced by a pulse given apparently in the same phase (Miyazaki *et al*., 2005). Unfortunately, it is difficult to distinguish the phase advance of circannual pupation rhythm and induction of pupation by masking by temperature based on pupation in the second cycle only.

Except for pulses applied 8 weeks after hatching, differences in the degree of low

temperature and the exposure duration among pulses produced unremarkable differences in the effects on the circannual phase. In contrast, differences in the phase of pulse onset affected the direction and magnitude of phase shift. A PRC to 8-week pulses of 10°C reflects the phase-dependent phase shifts to low-temperature stimuli (Fig. 6A). This circannual PRC is categorized as Type 0 (Winfree, 1970; Pittendrigh, 1981b; Johnson *et al*., 2004). We previously constructed a circannual PRC by exposing *A. verbasci* larvae kept under LD 12:12 h to long-day conditions of LD 16:8 h for 4 weeks at various circannual phases at a constant 20°C. The shape of this PRC is also Type 0 (Miyazaki *et al*., 2005). PRCs enable us to explain the mode of entrainment of biological rhythms to the environmental cycle (Pittendrigh, 1981b; Saunders, 2002; Johnson *et al*., 2004). Thus, it is probable that temperature plays a role as well as photoperiod for entrainment of circannual rhythm of *A. verbasci* to a natural year. Especially, temperature increase seems to act as an effective seasonal cue. We also previously constructed a Type 1 PRC, which has small phase shifts and a continuous transition between delays and advances (Winfree, 1970; Pittendrigh, 1981b; Johnson *et al*., 2004), for this circannual rhythm by exposing larvae to LD 16:8 h for 2 weeks at various phases at 20°C (Miyazaki *et al*., 2007). It is probable that a Type 1 PRC to low-temperature pulses is obtained by exposure to 10°C for durations shorter than 8 weeks.

When a low-temperature pulse was applied 8 weeks after hatching, a pulse of 5°C had different effects from pulses of 15°C and 10°C. An 8-week pulse of 5°C delayed pupation but pulses of 15°C and 10°C advanced it (Fig. 1B, D, F). This resulted in the difference in the shape between PRCs to pulses of 10°C and to pulses of 5°C (Fig. 6). A PRC to pulses of 5°C displayed only phase delay responses (Fig. 6B). Such PRCs have also been reported in disturbance of insect circadian rhythms by 4-min pulses of 40°C and 120° (~8-h) pulses of 1°C (Maier, 1973; Wiedenmann, 1977). Moreover, the present study showed that a 12-week pulse of 5°C applied 8 weeks after hatching produced the least synchronous pupation of *A. verbasci* (Fig. 1G). Although the cause for these differences of influence between 10°C and 5°C is still unclear, a possible explanation is on the immature developmental stage 8 weeks after hatching. Exposure to 5 °C in the immature stage likely has a stronger suppressive effect on the physiological mechanism than that in the later stages. This effect may have induced the cessation or deceleration of the oscillation of a circannual clock and the delay of the pupation phase. Another possible explanation is that the difference in influences between a pulse of 5°C and a pulse of 10°C or 15°C depends on the phase of circannual rhythm of *A. verbasci*. According to the limit cycle model for biological rhythms, state variables of a clock oscillate in phase space around a trajectory of the limit cycle (Lakin-Thomas, 1995; Winfree,

2000; Johnson *et al*., 2004). If a pulse of 5°C applied 8 weeks after hatching can drive state variables of a circannual oscillator to the region close to a phaseless point (singularity) in the phase space or can scatter the phases of individual oscillators of the multioscillator system, arrhythmicity or unpredictable phase shifts would be induced. Such phenomena have been reported in many biological rhythms (Winfree, 1970, 2000; Lakin-Thomas, 1995; Johnson *et al*., 2004). In *A. verbasci* kept under LD 12:12 h at 20°C, asynchronous pupation was evoked by a 4-week pulse of LD 16:8 h applied 8–10 weeks after hatching (Miyazaki *et al*., 2007). It should be examined in a future study whether a 4-week pulse of LD 16:8 h and a 12-week pulse of 5°C applied 8 weeks after hatching have a similar effect on the circannual pupation rhythm of *A. verbasci*.

The present study showed that low temperature resets the phase of a circannual clock of *A. verbasci* in a phase-dependent manner, as reported in studies on circadian clocks (Rensing & Ruoff, 2002; Saunders, 2002). This suggests that temperature plays a role as a zeitgeber, as well as photoperiod, for entrainment of circannual rhythm in *A. verbasci* to a natural year and that *A. verbasci* can predict seasons without photoperiodic information, as suggested by Blake (1960). *A. verbasci* is known as a household and museum pest that feeds on woollen goods, dried animal products, and zoological specimens (Griswold, 1941; Hinton, 1945). In environments where photic information is lacking, temperature is probably crucial to synchronize the timing of pupation and reproduction among individuals. The present study, however, did not show actual entrainment of a circannual period of approximately 37 weeks to a natural annual period of 52 weeks by temperature changes. Further experiments are required to explicitly test the temperature entrainment of this circannual rhythm. To extend the interval between two pupation peaks to one year, summer and autumn signals are needed between the two peaks to induce a large phase delay. Because this is achieved by long days in photoperiodic entrainment (Nisimura & Numata, 2001; Miyazaki *et al*., 2006), high temperature also likely plays a significant role as summer and autumn signals in temperature entrainment. Moreover, temperature may provide more actual entrainment in a sinusoidal manner than in a square-wave manner (Yoshii *et al*., 2009; Miyazaki *et al*., 2011). To clarify the temperature entrainment of this rhythm to an exact year, it is necessary to examine effects of high-temperature pulses and effects of continuously changing temperatures over a year.

Pupation of *A. verbasci* occurs in February under natural photoperiod at 20°C but in April under natural photoperiod and temperature in Osaka (Nisimura & Numata, 2003). Nisimura & Numata (2003) explained that the pupation time of *A. verbasci* depends on the circannual rhythm entrained to natural photoperiod, and low temperatures in winter inhibit

the downstream pupation processes. However, the present results pointed out a possibility that, irrespective of the phase of the circannual clock, sufficiently grown larvae pupate in spring by temperature masking as in many other insects with larval diapause (Danilevskii, 1965; Danks, 1987; Hodek & Hodková, 1988). Although we discuss mostly the role of temperature as a zeitgeber, to understand the life cycle in *A. verbasci* under natural conditions, we should integrate the role of the circannual rhythm, and the effect of temperature without intervention of the circannual rhythm.

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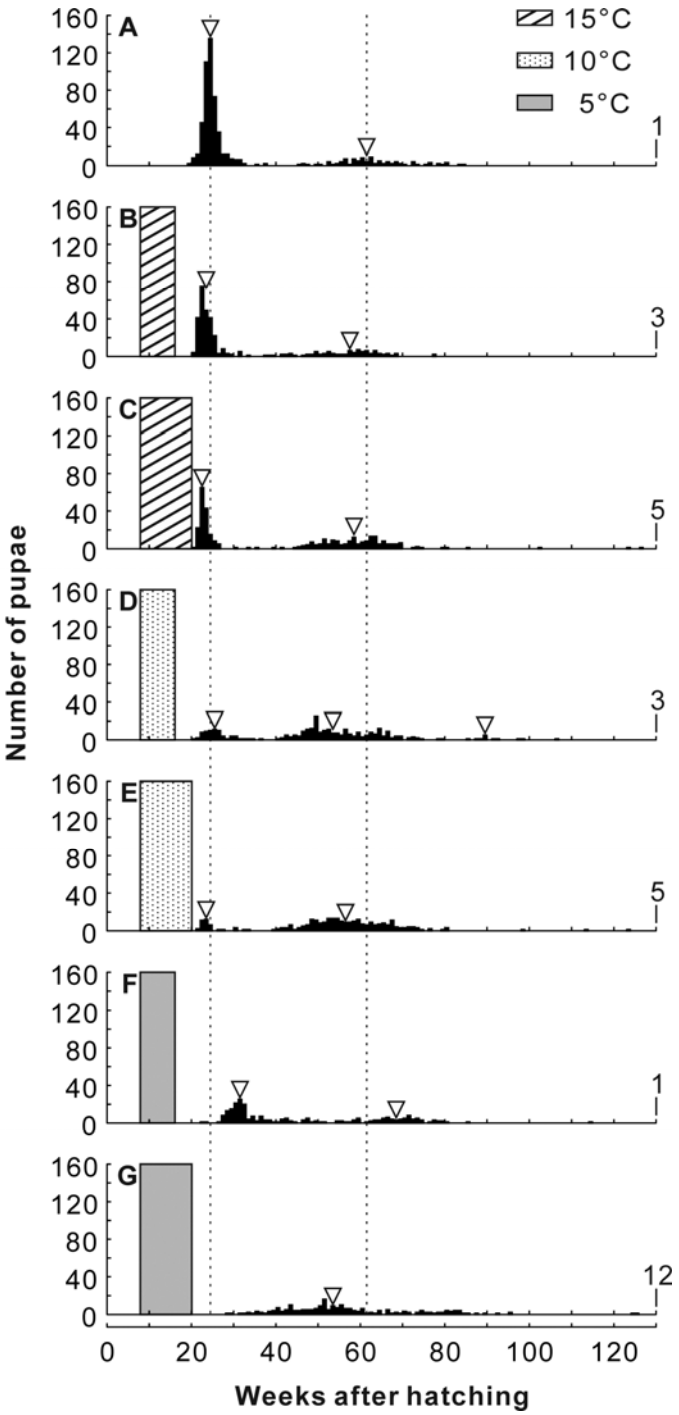

Fig. 1

Fig. 1. Effects of low-temperature pulses given 8 weeks after hatching on the circannual pupation rhythm under LD 12:12 h at 20°C in *Anthrenus verbasci*. The pulse duration is 8 weeks (B, D, F) or 12 weeks (C, E, G). Numerals with vertical lines indicate the numbers of insects remaining as larvae after 130 weeks. The triangle indicates the median of each pupation group. The vertical dotted lines pass through the median of each group under a constant temperature of 20°C.

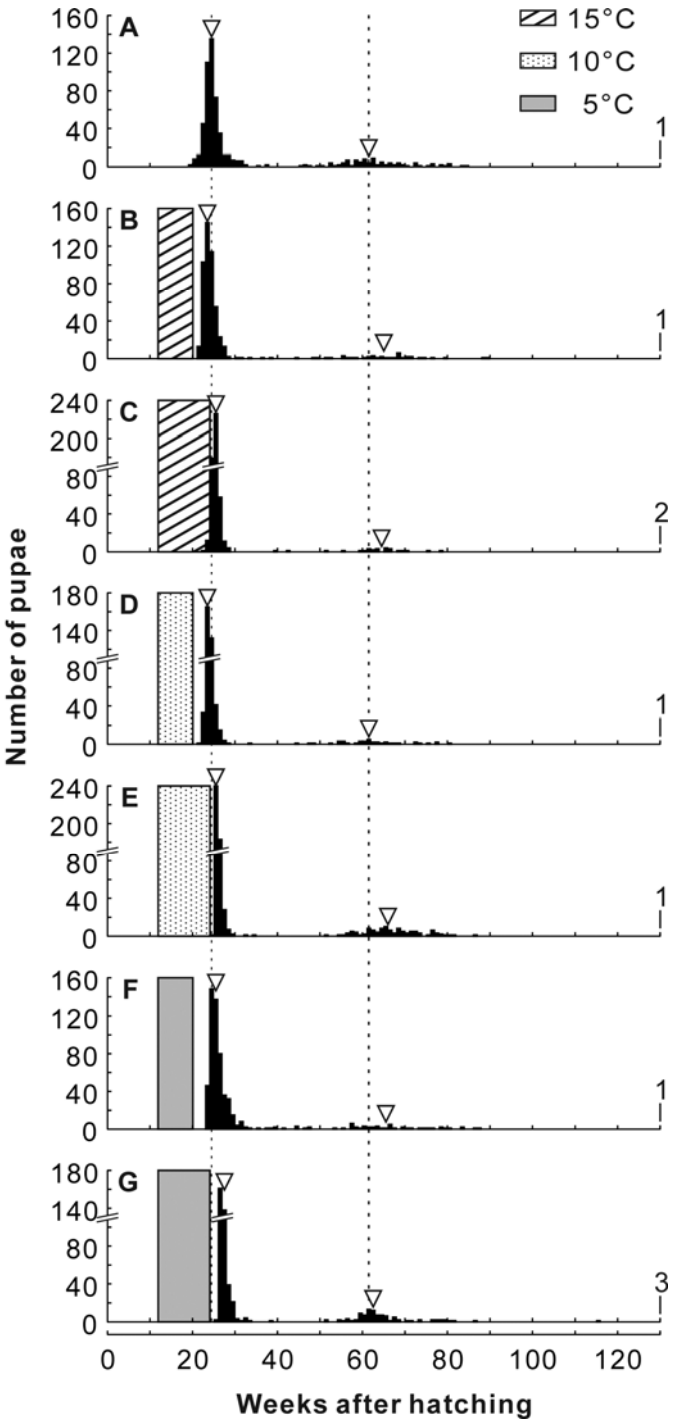

Fig. 2

Fig. 2. Effects of low-temperature pulses given 12 weeks after hatching on the circannual pupation rhythm under LD 12:12 h at 20°C in *Anthrenus verbasci*. The pulse duration is 8 weeks (B, D, F) or 12 weeks (C, E, G). Numerals with vertical lines indicate the numbers of insects remaining as larvae after 130 weeks. The triangle indicates the median of each pupation group. The vertical dotted lines pass through the median of each group under a constant temperature of 20°C.

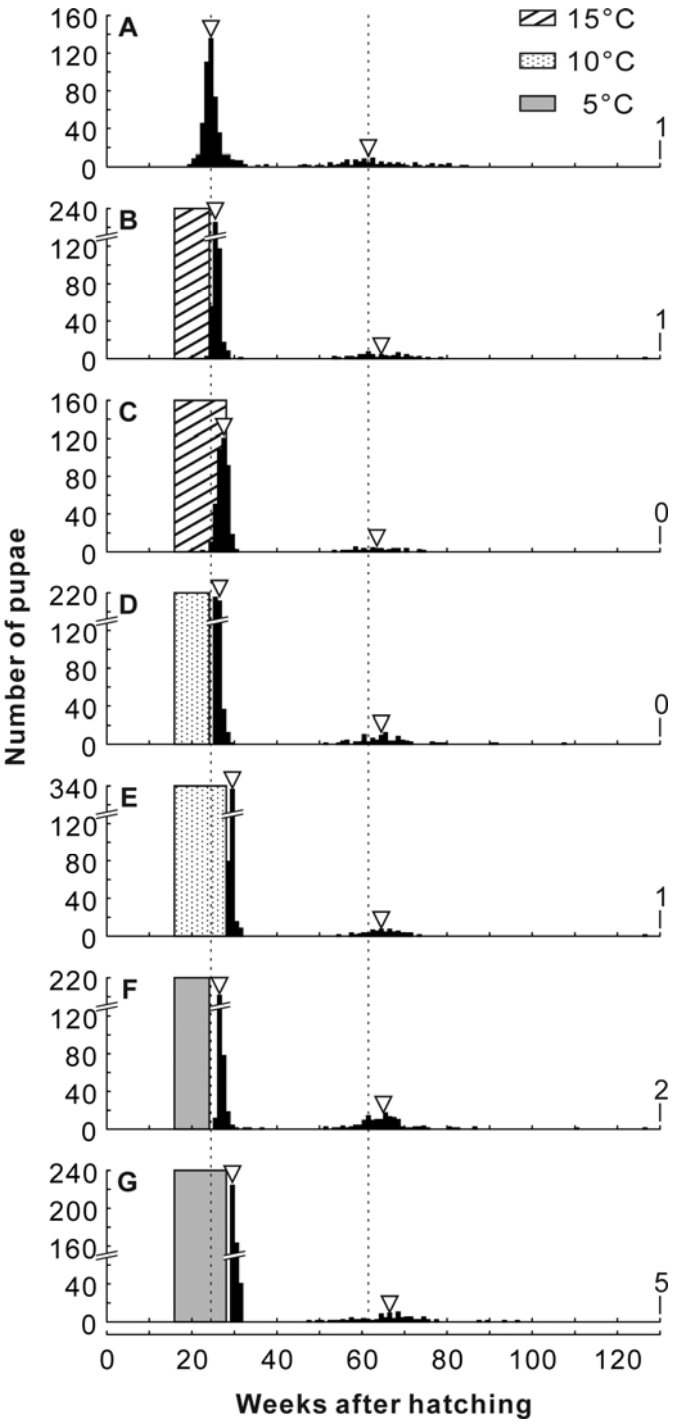

Fig. 3

Fig. 3. Effects of low-temperature pulses given 16 weeks after hatching on the circannual pupation rhythm under LD 12:12 h at 20°C in *Anthrenus verbasci*. The pulse duration is 8 weeks (B, D, F) or 12 weeks (C, E, G). Numerals with vertical lines indicate the numbers of insects remaining as larvae after 130 weeks. The triangle indicates the median of each pupation group. The vertical dotted lines pass through the median of each group under a constant temperature of 20°C.

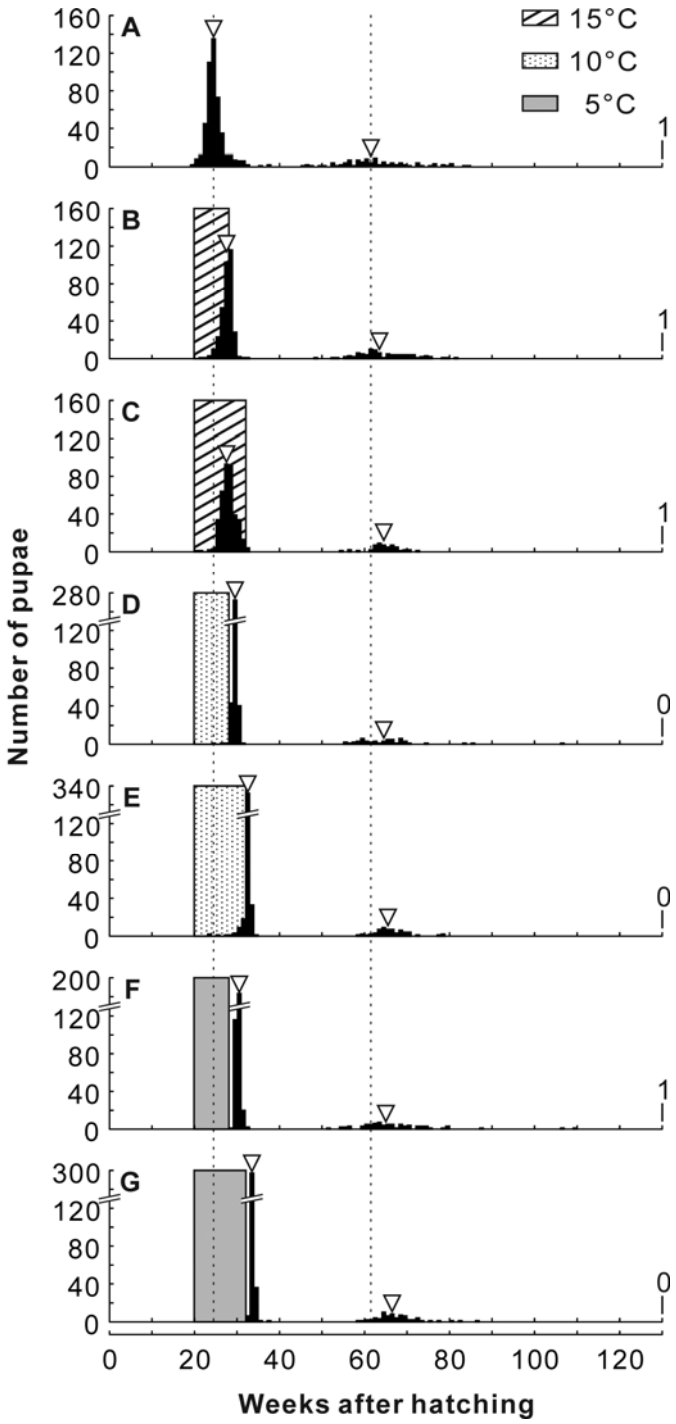

Fig. 4

Fig. 4. Effects of low-temperature pulses given 20 weeks after hatching on the circannual pupation rhythm under LD 12:12 h at 20°C in *Anthrenus verbasci*. The pulse duration is 8 weeks (B, D, F) or 12 weeks (C, E, G). Numerals with vertical lines indicate the numbers of insects remaining as larvae after 130 weeks. The triangle indicates the median of each pupation group. The vertical dotted lines pass through the median of each group under a constant temperature of 20°C.

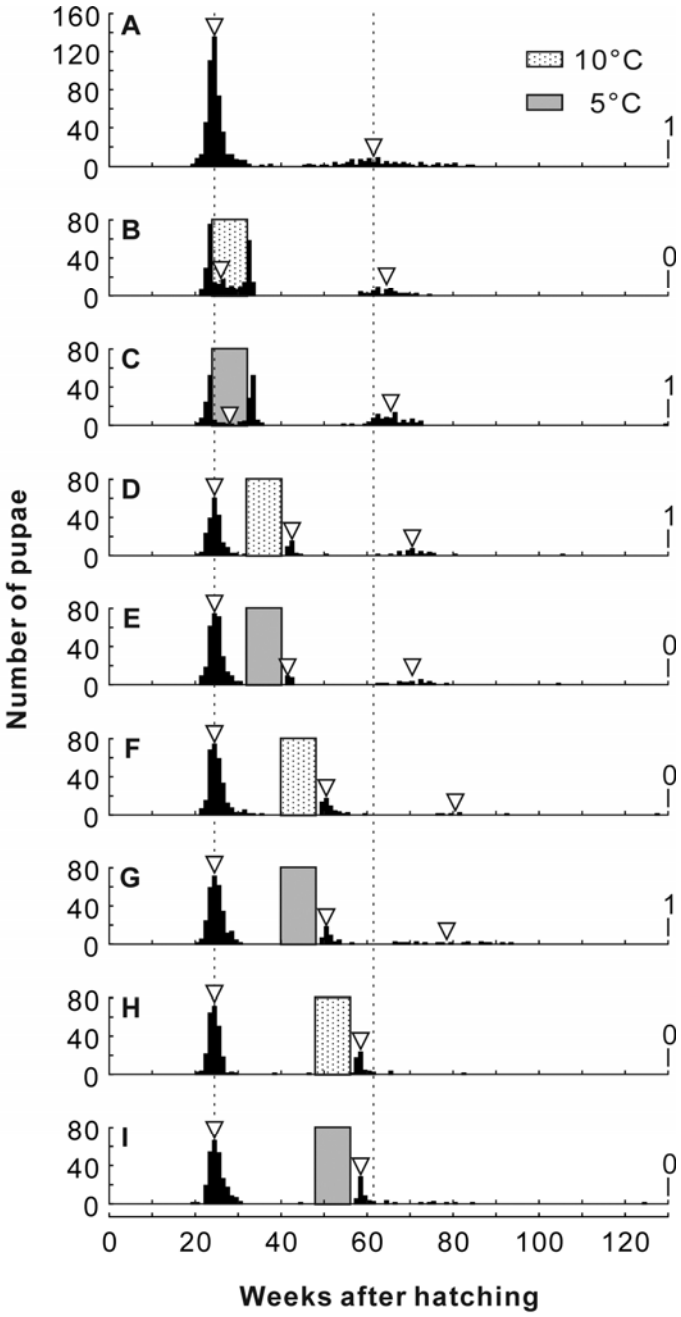

Fig. 5

Fig. 5. Effects of 8-week low-temperature pulses given 24 (B, C), 32 (D, E), 40 (F, G), or 48 (H, I) weeks after hatching on the circannual pupation rhythm under LD 12:12 h at 20°C in *Anthrenus verbasci*. Numerals with vertical lines indicate the numbers of insects remaining as larvae after 130 weeks. The triangle indicates the median of each pupation group. The vertical dotted lines pass through the median of each group under a constant temperature of 20°C.

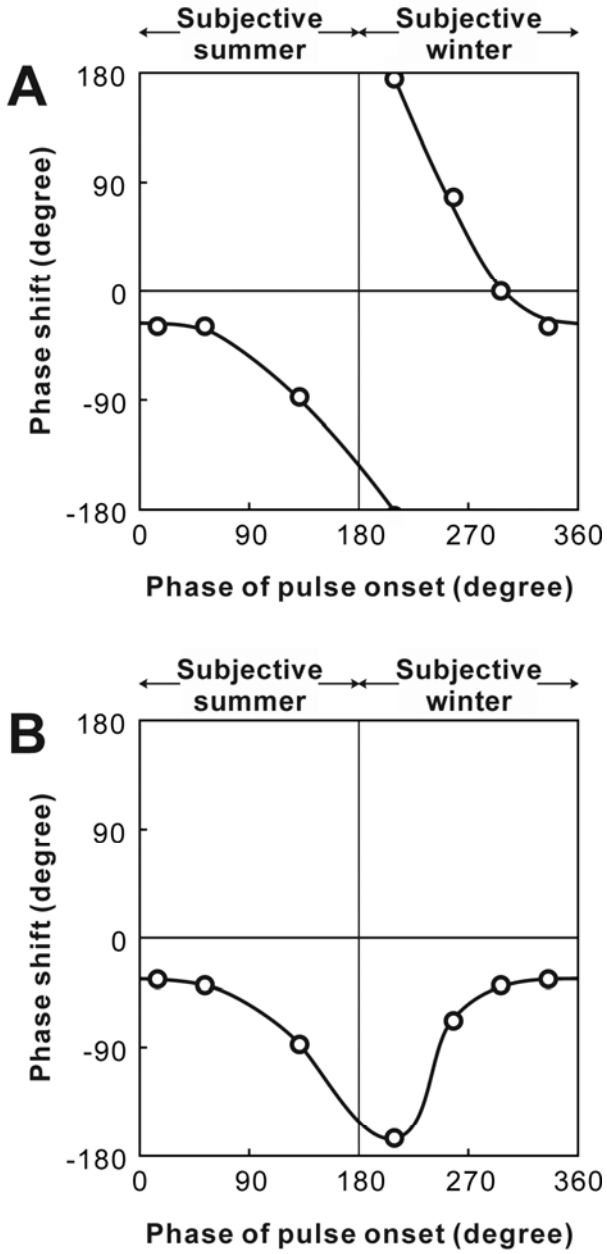

Fig. 6

Fig. 6. Circannual phase response curves to 8-week low-temperature pulses of 10°C (A) or 5°C (B) in *Anthrenus verbasci*. The circannual period under a constant temperature of 20°C (37 weeks) is shown in terms of angle degrees (0–360°), and the initial phase is regarded as 180° (see text for further explanation). The median pupation time in the control is 0° in phase shift. Phase advance and delay are shown with the positive and negative values in phase shift, respectively. Open circles represent the phase shifts in the pupation group more than 10 weeks after pulse exposure.