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A phase response curve for circannual rhythm in the varied carpet beetle *Anthrenus verbasci*

Abstract We know that entrainment, a stable phase relationship with an environmental cycle, must be established for a biological clock to function properly. Phase response curves (PRCs), which are plots of phase shifts that result as a function of the phase of a stimulus, have been created to examine the mode of entrainment. In circadian rhythms, single-light pulse PRCs have been obtained by giving a light pulse to various phases of a free-running rhythm under continuous darkness. This successfully explains the entrainment to light-dark cycles. Some organisms show circannual rhythms. In some of these, changes in photoperiod entrain the circannual rhythms. However, no single-pulse PRCs have been created. Here we show the PRC to a long-day pulse superimposed for 4 weeks over constant short days in the circannual pupation rhythm in the varied carpet beetle *Anthrenus verbasci*. Because the shape of that PRC closely resembles that of the Type 0 PRC with large phase shifts in circadian rhythms, we suggest that an oscillator having a common feature in the phase response with the circadian clock, produces a circannual rhythm.

Keywords *Anthrenus verbasci* · Biological clock · Circannual rhythm · Entrainment · Phase response curve

Abbreviations LD: Light/dark · PRC: Phase response curve

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Introduction

The study of biological rhythms and the awareness of their existence became widespread during the twentieth century (Dunlap et al. 2004). We know that most biological rhythms have an endogenous period that is close to a period of certain astronomical cycles and that they entrain to an external cycle under natural conditions. These rhythms are designated by the prefix "circa." Five circarhythms related to external cycles exist, and they are: circatidal, circadian, circasemilunar, circalunar and circannual (Saunders 2002; Dunlap et al. 2004). Studies show that a self-sustaining oscillator generates the circadian rhythm, and we know both the anatomical location and the cellular and molecular bases of the oscillator (Saunders 2002; Dunlap et al. 2004). However, it is still unclear whether other circarhythms are generated by self-sustaining oscillators with the period of the apparent rhythms (Neumann 1981; Gwinner 1986; Morgan 1991; Dunlap et al. 2004).

We know that entrainment, a stable phase relationship with an environmental cycle, must be established for a biological clock to function properly (Pittendrigh 1981; Johnson et al. 2003). Phase response curves (PRCs), which are plots of phase shifts that result as a function of the phase of a stimulus, have been created to examine the mode of entrainment (Pittendrigh and Minis 1964; Johnson 1999; Saunders 2002). In circadian rhythms, PRCs are determined by six different protocols (Aschoff 1965). The protocol most commonly used is the one in which single light pulses systematically perturb one full circadian cycle of an organism that is free-running in continuous darkness. In this protocol, a single light pulse advances the phase, delays the phase, or has little or no effect. The outcome is determined by the phase of the circadian rhythm in which the pulse was given. In single-pulse PRCs in circadian rhythm, a light pulse in early subjective night generally delays the phase whereas a light pulse in late subjective night advances the phase. Under

natural conditions, therefore, light advances the rhythm in the morning or delays it in the evening, so that entrainment can be established. Thus, the PRCs produced with single light pulses not only successfully explain the mechanism of entrainment by light-dark cycles, but also can be used as probes which detect the phase, the period, and the amplitude of circadian oscillators (Pittendrigh and Minis 1964; Pittendrigh 1981; Johnson 1999; Saunders 2002).

Circannual rhythms were first clearly described in the late 1950s and ultimately detected in various animals and plants (Pengelley and Fisher 1957; Blake 1959; Mrosovsky 1978; Gwinner 1986; Dunlap et al. 2004). However, very few details of the physiological basis of circannual rhythms exist (Gwinner 1986; Dunlap et al. 2004). The only reliable example of a circannual rhythm in insects is shown when the circannual rhythm of the varied carpet beetle, *Anthrenus verbasci*, restricts pupation in the spring (Blake 1959; Nisimura and Numata 2001, 2003). Adults of this species are found around May in central Japan, and larvae pupate in April under natural photoperiod and temperature (Kiritani 1958; Nisimura and Numata 2003). When larvae are reared under 12 h of light and 12 h of darkness (LD 12:12) at various constant temperatures, the insect shows a periodical pattern in pupation at about 40-week intervals. This population rhythm shows a self-sustaining feature, temperature compensation, and entrainment to a zeitgeber, and therefore the rhythm satisfies all the criteria for real endogenous circarhythms (Nisimura and Numata 2001). The predominant zeitgeber is the change in photoperiod, and this rhythm with a period considerably shorter than a year must be entrained to an exact year, responding to change in photoperiod under natural conditions (Nisimura and Numata 2001, 2003). Some vertebrates also respond to changes in photoperiod for circannual rhythms (Goss 1969; Gwinner 1977, 1986; Dunlap et al. 2004), although no single-pulse PRCs have been created in the circannual rhythms of any organisms.

In the present study, we examined the phase response of the circannual pupation rhythm in *A. verbasci* by exposing larvae kept under LD 12:12 to LD 16:8 for 4 weeks (long-day pulse) at various phases of the rhythm. LD 12:12 and LD 16:8 are regarded as short-day and long-day conditions, respectively, because the critical daylength is between 13 and 14 h (Nisimura and Numata 2003). Then we constructed a single-pulse PRC.

Materials and methods

Adults of *A. verbasci* were collected in Osaka City, Japan (34.7°N, 135.5°E), in April and May of 2002. They were kept under LD 16:8 at 25 ± 1°C, and their progeny larvae were transferred to LD 12:12 at 20 ± 1°C within a week after hatching. The 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^{-2}. A relative humidity of about 66% was maintained with a saturated solution of $NaNO_2$. Dried yeast (Asahi Food and Healthcare, Tokyo) and dried bonito powder were provided as larval food (Nisimura and Numata 2001).

The larvae were kept continuously under LD 12:12 at 20°C, or exposed to LD 16:8 at 20°C for 4 weeks. This was described in terms of a "long-day pulse" of the following number of weeks after hatching: 0, 4, 8, 12, 16, 20, 24, 28, 32, 36, 40, 44, or 48. The pupation of all larvae was recorded each week.

Results

In the control insects kept continuously under short-day conditions, the pupation showed a periodic pattern with a period shorter than 1 year as described in previous studies (Nisimura and Numata 2001, 2002, 2003). In the first and second pupation groups, the median larval duration was 24 and 61 weeks, respectively, and the interval was 37 weeks (Fig. 1a). When larvae were exposed to long-day conditions for the first 4 weeks, pupation was delayed by 4–5 weeks in the first and second groups (Fig. 1b). When a long-day pulse at 4 weeks after hatching was received, pupation of both groups was delayed by 9–9.5 weeks (Fig. 1c). When a long-day pulse was given at 8 weeks after hatching, pupation produced a result that was much less synchronous with the above three conditions. However, the median larval duration of the first group was delayed by 13 weeks, compared to the control insects (Fig. 1d). When a long-day pulse was given at 12 weeks after hatching, however, pupation in the first group was advanced by 3 weeks, although we could not define the second group (Fig. 1e). The delivery of a long-day pulse at 16 weeks after hatching was found to advance pupation by 2–3 weeks in both groups (Fig. 1f). When a long-day pulse was given at 20 weeks after hatching (the beginning of pupation in the first group in the control experiment), the pulse had no effect on pupation in the first group. However, the pulse advanced pupation in the second group by 3 weeks (Fig. 1g). A long-day pulse at 24 weeks after hatching advanced pupation in the second group by 4 weeks (Fig. 1h). When a long-day pulse was given at 28 weeks after hatching, pupation of the second group was slightly advanced (Fig. 1i). When a long-day pulse was given at 32 weeks after hatching, pupation of the second group was slightly delayed (Fig. 1j). A long-day pulse at 36 or 40 weeks after hatching delayed pupation in the second group (Fig. 1k, l). Also, the amplitude of the delay was similar to that when a long-day pulse was given 36 weeks earlier (Fig. 1b, c). A long-day pulse at 44 or 48 weeks after hatching split the second group into two groups as follows: in one group, pupation was advanced; in the other, pupation was delayed (Fig. 1m, n). Whether a long-day

Fig. 1 The effect of a long-day pulse on the pupation rhythm under short-day conditions in *Anthrenus verbasci*. Larvae were kept under light/dark (LD) 12:12 (*filled bars*), and exposed to LD 16:8 (*empty bars*) for 4 weeks at various times (temperature 20°C). *Numerals with vertical lines* show the numbers of insects remaining as larvae after 52 or 104 weeks. Each *triangle* indicates the median for each pupation group. *Vertically dotted lines* cross through the median for each pupation group under constant short-day conditions
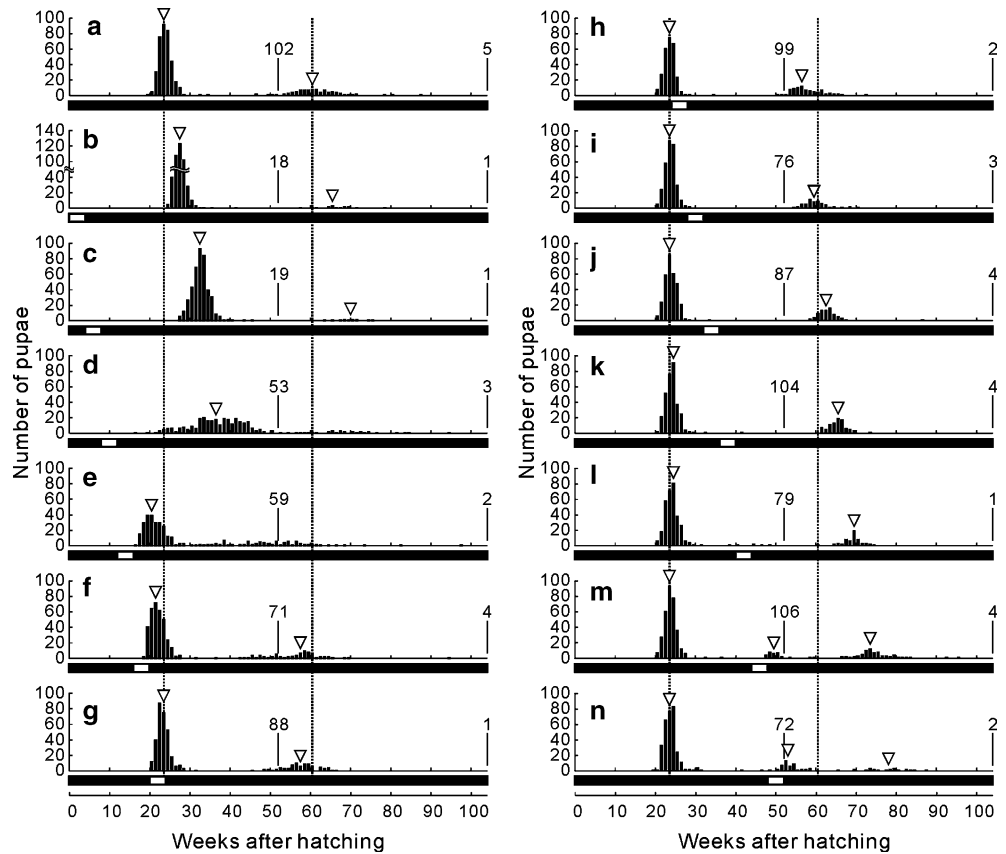
pulse advanced or delayed the phase of the circannual rhythm in *A. verbasci* depended upon the phase in which the pulse was given.

From these results, we constructed a PRC for the circannual rhythm by plotting the phase shift as a function of the phase at which a long-day pulse was started (Fig. 2a). In this curve, the period in the circannual rhythm under constant short-day conditions (37 weeks) was shown in terms of angle degrees (0–360°). In circadian rhythms, the phase after a 12-h photophase is regarded as the beginning of the subjective night, i.e. 180°. In the circannual rhythm of *A. verbasci*, in the same way, the phase after exposing to long-day conditions for 26 weeks (half a year) can be regarded as the beginning of the subjective winter, i.e. 180°. When larvae were transferred to short-day conditions after exposure to long-day conditions for 26 weeks, the next pupation peak was 23 weeks after the transfer (Nisimura and Numata 2001, Fig. 4e). In the present study, the period from the beginning of the control experiment to the first pupation peak was 24 weeks, similar to the above value (Fig. 1a). In circadian rhythms, moreover, a light pulse given at 180° generally delays the phase by the duration of the pulse (e.g. Saunders 1978), and a 4-week long-day pulse at the beginning of the experiment delayed the phase by 4–5 weeks in the circannual rhythm of *A. verbasci* (Fig. 1b). Therefore, the initial phase was regarded as 180°.

Discussion

In single-pulse PRCs in circadian rhythm, a light pulse in early subjective night generally acts as a "new dusk" and causes a phase to delay. In contrast, a light pulse in late subjective night acts as a "new dawn" and causes a phase to advance. Pulses applied in subjective day have little or no effect on the phase (Pittendrigh 1981; Johnson 1999; Saunders 2002). Figure 2b shows a typical example of this. In circannual rhythms, however, PRCs have been achieved only in the rainbow trout *Oncorhynchus mykiss* and *A. verbasci*. These circannual PRCs were constructed by one-step transitions from long-day to short-day conditions (Duston and Bromage 1988; Nisimura and Numata 2001). Spawning in *O. mykiss* and pupation in *A. verbasci* was delayed as the photoperiod was later changed. Accordingly, these PRCs monotonically decreased, and after perturbation, the rhythms reached a similar phase, such as late summer or autumn. In *O. mykiss*, a similar phase response was shown when 2 months of continuous light was introduced to naturally-changing daylengths. However, this curve does not satisfy the definition of the PRC because the rhythm entrained to seasonally-changing daylengths before perturbation. Spawning is delayed as continuous light is applied at a later time, with the post-perturbation phase always occurring near summer
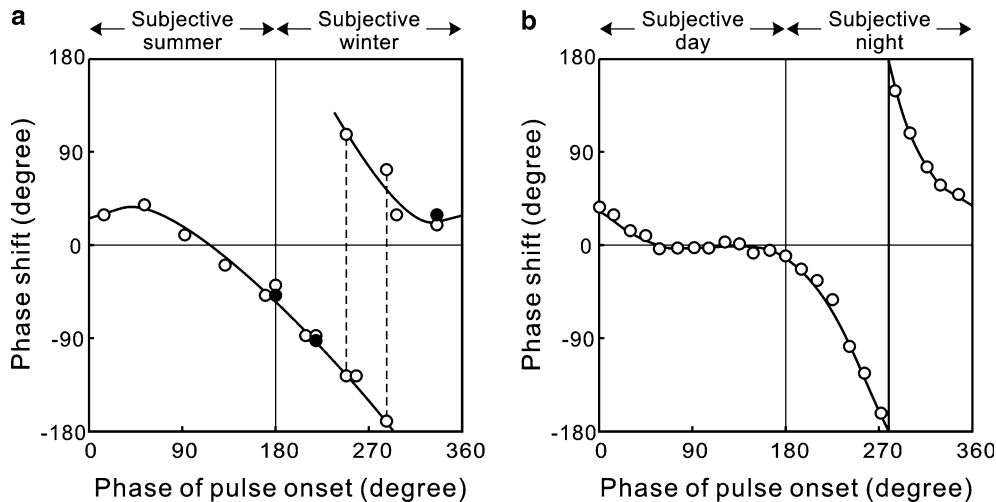
Fig. 2 Comparison of phase response curves for circannual and circadian rhythms. **a** Phase response curve to 4 week long-day pulses in the circannual pupation rhythm of *Anthrenus verbasci*. The period under constant short-day conditions (37 weeks) is shown in terms of angle degrees (0–360°), and the initial phase is regarded as 180° (see text for further explanation). *Open circles* represent the phase shifts in the first pupation group after perturbation (Fig. 1b–n). *Closed circles* represent the phase shifts in the second pupation group after perturbation (Fig. 1b, c, f). *Broken lines* show split in the phase response (Fig. 1m, n). **b** Type 0 phase response curve to 15 min light pulses in the circadian eclosion rhythm of *Drosophila pseudoobscura*. Modified from Pittendrigh and Minis (1964) Am Nat 98: 261–294 The University of Chicago Press, **c** 1964, The University of Chicago

solstice (Randall et al. 1998). If the post-perturbation phase is stable, the response curve does not reflect the oscillation of the rhythm before perturbation. This condition is shown also in circadian PRCs with stronger light pulses (Saunders 1978; Johnson 1999).

In this instance, we first show a PRC that reflects the nature of the oscillation producing the circannual rhythm by applying long-day pulses. In such a PRC, a long-day pulse carries two different meanings, depending on the phase at which the pulse is given. We propose that when a long-day pulse acts as an "autumn signal," it causes a phase delay. When a long-day pulse acts as a "spring signal," it causes a phase advance, in the same way as a light pulse acts as a "new dusk" or a "new dawn" in circadian rhythms. The circannual PRC to long-day pulses can explain the entrainment to the naturally-changing daylengths, as in nonparametric entrainment of the circadian rhythm that involves almost instantaneous phase shifts in response to short light pulses, although we cannot deny the possibility that the angular velocity in the circannual rhythm is continuously influenced by naturally-changing daylengths, as in parametric entrainment of the circadian rhythm. Circadian PRCs that are based on nonparametric entrainment may be used to calculate and predict all formal aspects of the phenomenon with considerable precision (Pittendrigh 1981; Saunders 2002). In the circannual rhythm of *A. verbasci*, the period under constant short-day conditions was about 37 weeks,

considerably shorter than a year. Under natural conditions, therefore, most important for entrainment appears the delay in the phase of the circannual rhythm by long-day conditions in early autumn. Because most individuals pupate in February under naturally-changing daylengths at constant 20°C (Nisimura and Numata 2003), long-day conditions in early spring seem to play no important role in the entrainment. Therefore, our discussion on the entrainment of the circannual rhythm under natural conditions depending on the PRC to one-step transitions from long-day to short-day conditions is still valid (Nisimura and Numata 2003).

This circannual PRC to long-day pulses closely resembles the Type 0 PRC of circadian rhythms with its large phase shifts and a break point in the middle of the subjective night (Winfree 1970; Johnson 1999) (Fig. 2a, b). In the circannual rhythm of *A. verbasci*, the long-day pulse near the break point caused a phase delay in some subjects, and caused a phase advance in others. There are large individual variations in the endogenous period of circannual rhythms (Gwinner 1986) (see Fig. 1a). These variations probably produced differences in the phase at which a long-day pulse was given. Therefore, both advances and delays were produced by a pulse given apparently in the same phase. We observed less synchronous pupation when a long-day pulse was given at 8 weeks after hatching, than when a pulse was given at some other point. This treatment resulted in a large phase delay in the first circannual cycle. It should also be noted that, in the circadian eclosion rhythms of flies *Drosophila pseudoobscura* and *Sarcophaga argyrostoma*, arrhythmicity is most marked when single light pulses commence at phases close to the maximum phase shift (Winfree 1970; Saunders 1978). Because of this, we recognize again the similarity between circannual and circadian rhythms in responses to single pulses of a zeitgeber. Moreover, even though only phase delays occur probably under natural conditions as discussed above, both the delays and advances are similar between circadian and circannual PRCs. This similarity in PRCs indicates

that there is a point common between the circadian and circannual mechanisms.

Although the mechanism that generates the endogenous circannual rhythm has not been clarified, three hypotheses have been proposed for the mechanism (Mrosovsky 1978; Gwinner 1986). (1) Circannual rhythms are derived from circadian rhythms through frequency demultiplication (Gwinner 1973). This hypothesis seems acceptable, because it is generally accepted that a circadian oscillator exists in most organisms (Dunlap et al. 2004), and no other oscillator is necessary for this hypothesis. However, the experimental results did not support this in all the animals examined including *A. verbasci* (Gwinner 1981; Carmichael and Zucker 1986; Nisimura and Numata 2002). (2) Circannual cycles are composed of a series of stages that take a relatively fixed amount of time to complete (Mrosovsky 1978). This hypothesis theoretically explains the circannual mechanism, although no direct experimental results have supported it in the past (Mrosovsky 1978). (3) Circannual rhythms are generated by a self-sustaining oscillator with a period of about 1 year analogous to the circadian oscillator. One defect of this hypothesis is that one of the major characteristics of circadian rhythms, temperature compensation, has rarely been shown in circannual rhythms of homeotherms (Pengelley and Asmundson 1969; Gwinner 1986). In *A. verbasci*, however, temperature compensation in the period of circannual rhythm was clearly shown with the advantage of a poikilotherm (Blake 1959; Nisimura and Numata 2001). Moreover, we show here that there is a common feature in phase responses between the circannual rhythm of this species and circadian rhythms in other organisms. Therefore, it is quite adequate to assume that, at least in *A. verbasci*, the circannual rhythm is generated by a circannual oscillator.

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