Mem. Fac. Sci. Kyoto Univ. (Ser. Biol.), 12: 135–156, Oct., 1987

Circadian Clock Controlling the Eclosion Rhythm of the Silkworm, *Bombyx mori*: Its Characteristics and Dynamics

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(Received June 19, 1987)

Abstract The eclosion of the silkworm, *Bombyx mori,* occurred at peculiar times of entraining photoperiodic regimens with a stereotyped eclosion behavior. A transition from LL (light condition) to DD (dark condition) generated a circadian eclosion rhythm (DD-oscillation) with τ_{DD} (24.1 hr) from CT 12 or close to it. Stepwise transition from DD to LL or release into a prolonged light condition after entrainment under LD cycles generated an overt LL-oscillation with a short free-running period τ_{LL} (17.8 hr). Decrease of light intensity given in LL lengthened τ_{LL}. Single light-pulse exposed in DD produced a circadian rhythm with τ_{DD} and the medians of the first peak were roughly parallel to DD-onset. Single dark-pulse in LL generated eclosion rhythm particular to the duration of the dark-pulse applied. The phase response curve of the DD-oscillation showed Type 0 and there was an abrupt change from phase-delay to phase-advance at around CT 18 hr. The off-signal of the light-pulse applied at the first part of the subjective night caused a phase-delay and the on-signal of the light-pulse at the second part caused a phase-advance. A continuous-dynamics model of the circadian system was proposed to interprete the characteristic eclosion patterns in the silkworm.

INTRODUCTION

Many aspects of developmental event of insect occur once in the life-cycle and the timing is controlled by on-going circadian oscillator which is detected only by a rhythm of mixed-aged populations. The properties of the oscillator underlying the circadian rhythm have been investigated extensively in adult eclosion of *Drosophila* spp., and the studies of the eclosion rhythm have contributed much to current concepts of biological clocks (Pittendrigh, 1960, 1981).

Physiological aspects of insect eclosion have been studied primarily using the silkworm moths which show species-specific eclosion rhythms (Truman and Riddiford, 1970). Circadian clock controlling the eclosion rhythm of lepidoptera is located in brain and the eclosion behavior is mediated by a peptide hormone (Truman, 1971a; Taghert *et al.*, 1980). The hormone is produced by neurosecretory cells and released from the neurohemal organs of the brain (Copenhaver and Truman, 1986). It was purified from pupal heads of the silkworm *Bombyx mori* and the chemical properties of the isolated hormone were reported (Nagasawa *et al.*, 1985).

The domestic silkworm, *Bombyx mori,* differs from wild lepidopteran species in structure, physiology and ethological characteristics (Tazima, 1978). Generally speaking, domestication of animals brings about changes in reception of environments, endocrinological system and activity of parts of central nervous system (Immelmann, 1980).

Therefore it would be worthwhile to compare the circadian system of this unique species with other insects whose rhythms were well documented. We have reported that *Bombyx mori* shows a characteristic bimodal eclosion rhythm under certain photoperiodic regimens, and that one is controlled by an endogenous circadian rhythm and the other is raised up by exogenous light-effect mediated *via* compound eyes (Shimizu and Matsui, 1983). However, systematic study to propose a reliable scheme elucidating the dynamics of the eclosion rhythm has not been performed. The present paper deals with characteristics and dynamics of the circadian system that times adult eclosion in *Bombyx mori.*

MATERIALS AND METHODS

The silkworm (*Bombyx mori*) were provided from the Attached school-farm of Kyoto Institute of Technology and Sericulture. The silkworm larvae were fed on mulberry leaves and pupae were removed from their cocoons 2 days after pupation. Developmentally heterogenous pupae were used to see the persisting rhythms. They were kept at $25 \pm 0.5^{\circ}C$ under various light-dark cycles, LL or DD condition. Illumination was provided by 20-W fluorescent tubes (Toshiba Co. Ltd., Tokyo) at an intensity of 300 lux (4.5 μ Einstein m^{-2} sec^{-1}), unless otherwise stated. The moths emerged after 10 or 11 days after pupation. The number of adult moths which emerged from their skin was counted every hour on days of eclosion. During the scotophase emerging moths were observed under a dim red light.

The abdominal movements of developing moths were recorded according to the method described by Truman (1971a) with slight modification. Pupae were removed from cocoons 2 days after pupation and a thread was adhered to the apex of the abdomen of each. The thread from animals was attached to a small tip writting on a revolving smocked drum.

RESULTS

Recordings of pre-eclosion and eclosion behavior

Figure. 1 shows typical records of abdominal movements of *Bombyx mori* during the last portion of the adult development. Vibration of abdominal tip with very low amplitude was recorded from 24 hr early before the eclosion. Preparatory behavior that *Manduca sexta* shows few hours early prior to the adult emergency (Truman, 1984) was not observed in any individual of the silkworm. The pre-eclosion behavior consisted of an initial period of abdominal rotations (about 25 min in duration), and it was followed by an approximately equal period of quiesence. This was then followed by an abrupt eclosion behavior which consisted of peristatic abdominal contraction, and eventually moths escaped from the pupal cuticle within 3 min (Fig. 1).

The silkworm moths showed same pattern of the sequential stereotyped behaviors in scotophase (Fig. 1A), photophase (Fig. 1B) and light-on emergency (Fig. 1C). Difference in the eclosion behavior between male and femala moth was not observed. These kymograph-patterns of *Bombyx mori* resemble closely that of *Hyalophora cecropia* moth (Truman, 1971 a). An injection of saline extracts of pharate moth heads into the pharate animals released same stereotyped series of the eclosion behavior (data not shown).

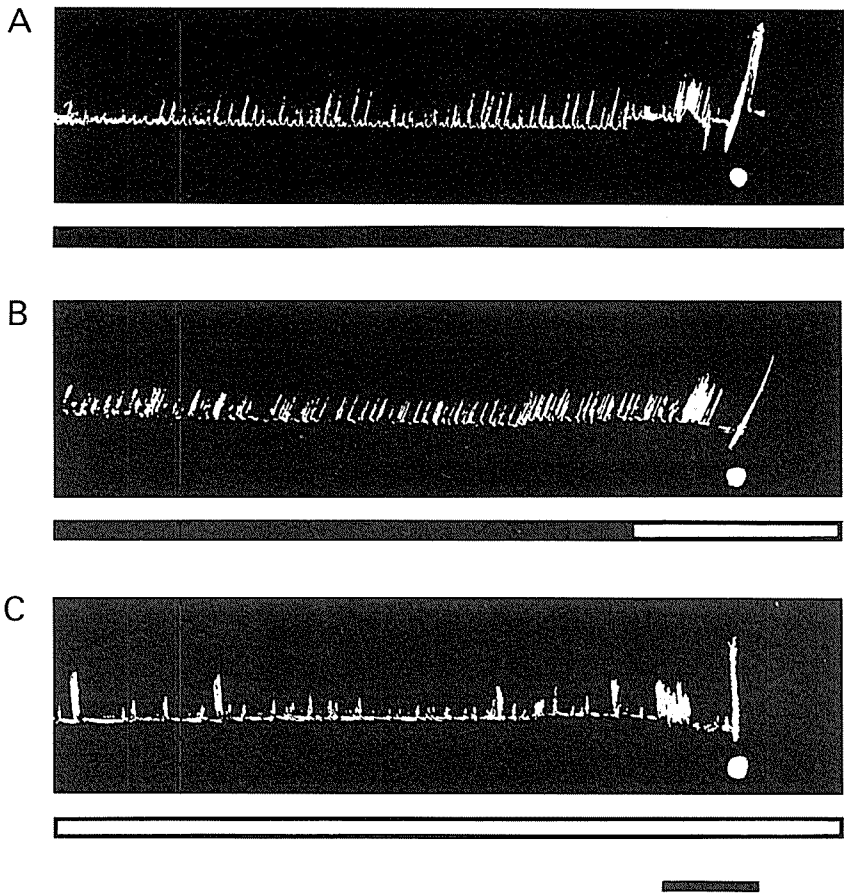

Fig. 1. Tracing of typical kymograph records showing the pre-eclosion and eclosion behavior of *Bombyx mori*. (A) moth emerged in scotophase of LD 8:16. (B) moth emerged after light-on under LD 16:8. (C) moth emerged in the late stage of photophase under LD 20:4. White dots identify the onset of the eclosion behavior. Closed and clear bars underneath each graph represent the scotophase and the photophase, respectively. Scale bar indicates 1 hr.

The eclosion of Bombyx mori under various entraining photoperiods

The silkworm pupae were removed from their cocoons 2 days after pupation and kept at constant temperature in light-dark regimens, which ranged from LD 4:20 to LD 23:1. In all regimens, the moths underwent the eclosion 8 or 9 days after the exposure of light-dark cycles. The cumulative data for each photoperiod is summarized in Figure 2.

The eclosion pattern was charactristic of the particular photoperiod. In photoperiodic regimen of LD 4:20, LD 8:16 and LD 12:12, the silkworm showed bimodal eclosion rhythm with one peak in scotophase, and with one at light-on. Male and female moths emerged in both fractions without any significant deviation. The mean phase-angle of the scotopeak from light-off was 14.2 hr for LD 4:20, 12.3 hr for LD 8:16 and 10.5 hr for LD 12:12. Then it was not fixed relative to the timing of light-off.

In LD 16:8 only one peak just after light-on occurred, and in photoperiodic

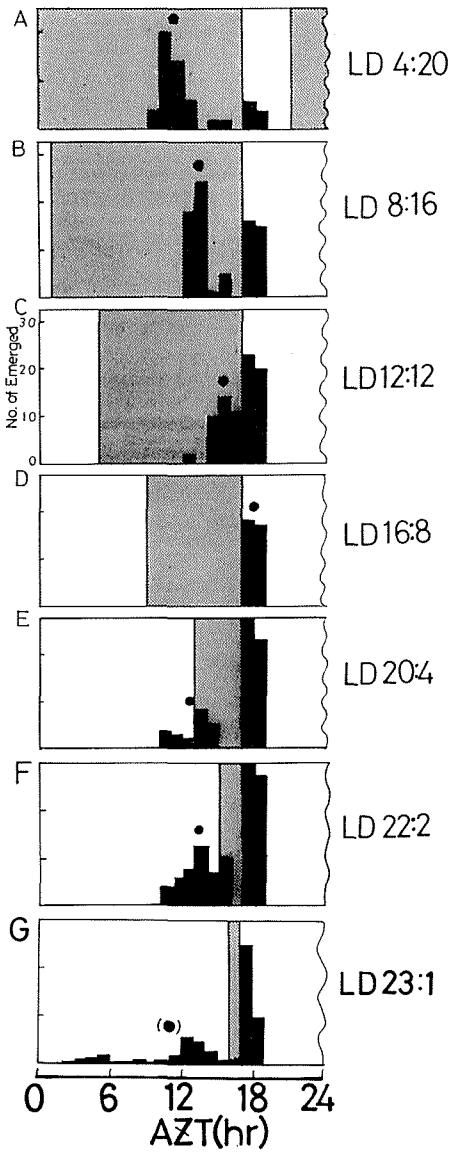

Fig. 2. The eclosion of *Bombyx mori* under various photoperiodic regimens of 24 hr light-dark cycles. Shaded zones represent the scotophase. The filled cycles show the median of each eclosion peak.

regimens (LD 20:4, LD 22:2) with a long photophase the light-on peak and a mass eclosion just before light-off were observed. In a regimen with a very short scotophase (LD 23:1), the gate was wide in the photophase.

We have demonstrated that the eclosion at light-on is evoked by an exogenous light effect which is mediated *via* compound eyes of pharate moths (Shimizu and Matsui, 1983). The light-on emergency occurred by a light-pulse in DD or by a dark-pulse in LL, and it masked the endogenous eclosion rhythm.

Generations of free-running rhythm by LL/DD and DD/LL transition

A group of the pupae that had been kept in LL were transferred to DD condition.

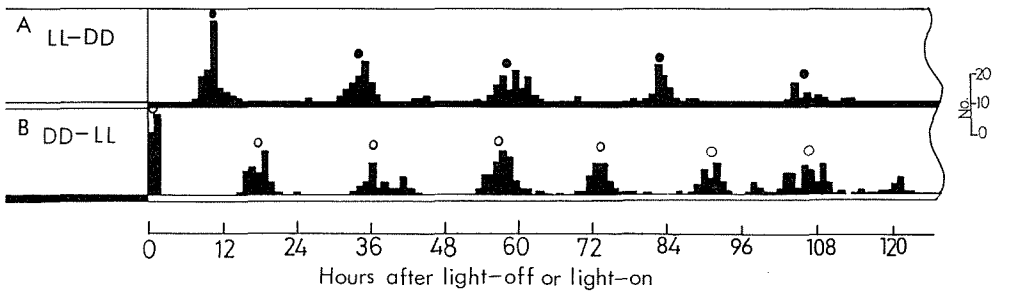

Fig. 3. Generations of eclosion rhythm by LL/DD (A) and DD/LL (B) transition. Pupae that had been kept in LL (300 lux) were transferred to DD-condition at time 0 (A). Pupae that had been kept in DD were transferred to LL-condition of 300 lux at time 0 (B). Closed circles and open circles denote the median of each eclosion peak.

Figure. 3A shows that clear circadian rhythm was initiated by the LL/DD transition with a free-running period τ_{DD} of 24.1 hr. Hereafter we call this circadian oscillation in continuous darkness DD-oscillation. The interval between DD-onset and the first peak was 10.5 hr (n=6). This suggests that phase of the circadian oscillator at LL/DD transition is just equivalent to the onset of 12 hr-scotophase in steady state entrainment to LD 12:12; DD oscillation begins from CT 12 or close to it. Thus the median of the first eclosion peak is assigned to CT 22.5 (τ_{DD} is taken as 24.0 hr).

Transfer from DD to LL of a high intensity (300 lux) produced a well-synchronizing eclosion rhythm (LL-oscillation) with a short τ_{LL} of 17.8 hr. In contrast with other insects reported (for example, *Drosophila pseudoobscura*) the eclosion rhythm of *Bombyx mori* persisted several times in LL with high rhythmicity and a rather narrow gate even under a high intensity of light (Fig. 3B).

The transition of DD/LL produced a first peak about 1 hr after light-on and the interval between LL-onset and the second peak was 18.8 hr (n=3). Therefore medians of each eclosion peak were observed to occur at intervals of modulo$\times\tau_{LL}+1$ hr. If we suppose that the specific phase for a release of the signal dictating eclosion behavior is about 1 hr before the eclosion itself, it occurs at interval of modulo$\times\tau_{LL}$. Hence, on this supposition LL-oscillation begins just from the specific release phase by DD/LL transition.

The phase relationship between DD and LL-oscillation, and entrainment mechanism these are subjects of the present study on the eclosion rhythm of the silkworm.

Characterization of the LL-oscillation

To know the property of LL-oscillation, further experiments were carried out. A transfer from DD to LL of a relatively high intensity (300 lux) generated a persisting rhythm in LL as shown above (Fig. 4A). However, a transfer from DD to LL of a low intensity (3 lux) did not produce any synchronizing rhythm (Fig. 4B). An exogenous light-on eclosion peak was observed by the transfer to such a dim light-condition, but the eclosion became virtually arrhythmic. Thus a high intensity of light is required to produce and/or maintain the LL-oscillation.

Dark pulses were imposed in LL after DD/LL transfer as shown in Figure. 4. The off-transition of dark-pulse was aligned at a same time (just 8 hr after the light-off), but

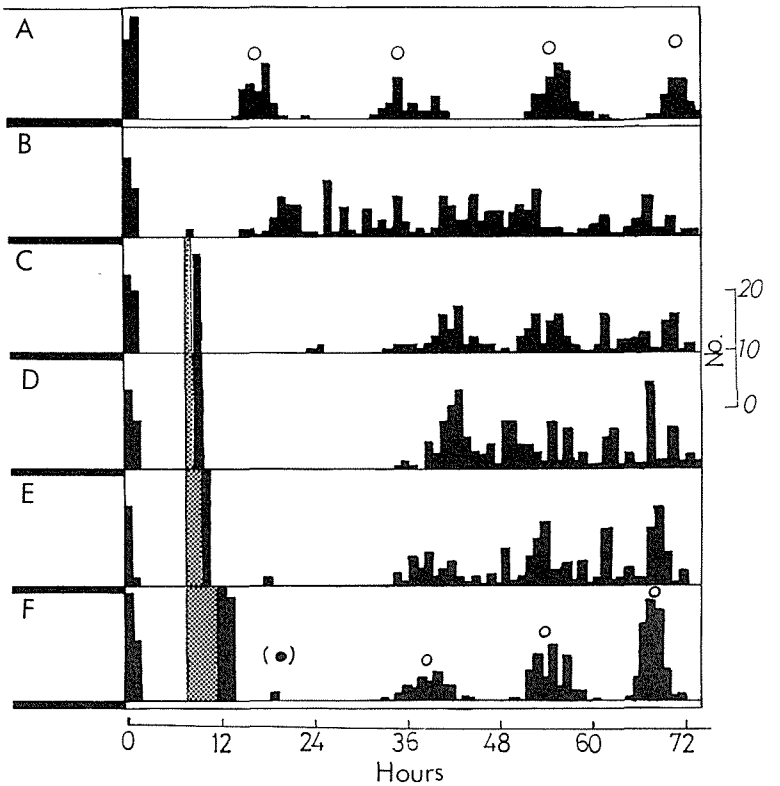

Fig. 4. Effect of low light-intensity and dark-pulse on the LL-oscillation which had been initiated by DD/LL transition. Pupae which had been reared in DD were transferred to LL of 300 lux (A, C-F) and 3 lux (B). Dark-pulses of varying the duration were inserted in LL-condition as shown in the figure. The light-off timing of dark-pulse was aligned just at 8 hr after the onset of LL. The dark-duration was 0.5 hr (C), 1 hr (D), 2 hr (E) and 4 hr (F), respectively.

the duration of darkness was varied from 0.5 to 4 hr. Compared to the unpertabated control (Fig. 4A), dark-pulses of 0.5–2 hr scatterred the emergency pattern over a wide range and resulted in vitual arrythmicity (Fig. 4C, D, E). However, 4-hr-duration pulse produced a new clear mode of persisting rhythm (Fig. 4F).

Then a systematic experiment varying the light-intensity of LL was carried out to reveal the intenisty effect on the LL-oscillation driven. Some different batchs of pupae which had been reared in DD were exposed to light of 300 lux for 2 hr. Then two batches were transferred to light condition of 3000 lux (Fig. 5A) and 3 lux (Fig. 5C), respectively. Another one group was reared under 300 lux successively (Fig. 5B) and the other one was returned to DD condition (Fig. 5D). As shown in Figure. 5, the intervals between eclosion peaks under 3 lux were significantly longer than those under 3000 and 300 lux. Thus an increase of light-intensity shortened the free-running period of LL-oscillation. This strongly suggests that the DD-and LL-oscillator are not driven by separate pacemakers but by a single pacemaker of which motion is controlled by the environmental light-intensity.

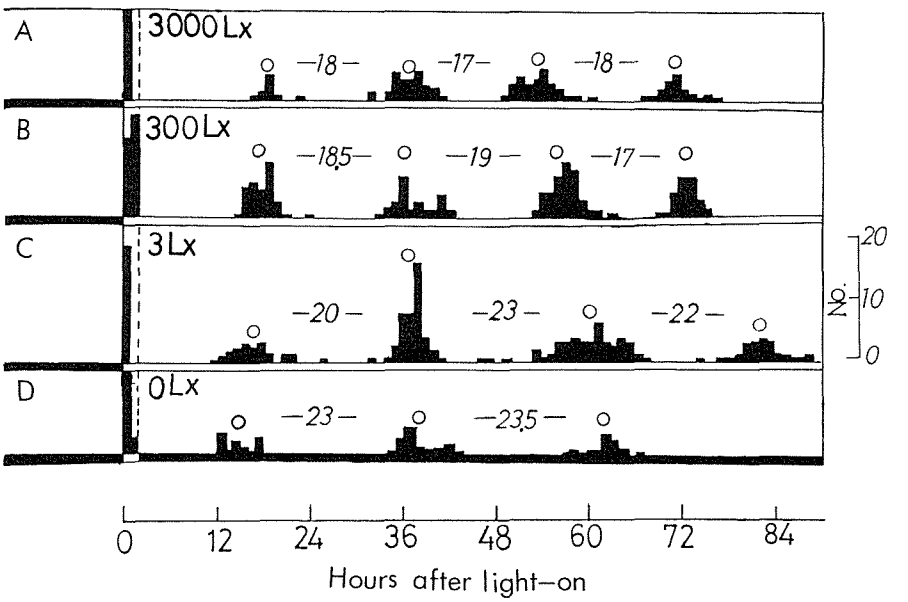

Fig. 5. Effect of light-intensity on the free-run rhythm in LL. Separate populations of pupae which had been kept in DD were exposed to light of 300 lux for 2 hr, and then one batch was transferred to a light condition of 3000 lux (A) and the other was transferred to 3 lux (C). One group (B) was reared under 300 lux successively and the other (D) was retured to DD (0 lux) condition. Numbers in each panel represent the interval between peak medians.

Generations of eclosion rhythm by single light-pulse in DD

Insect rhythm can be intitiated by exposure of single nonrecurrent light-pulse in DD. To see the effect of light-pulse on eclosion of *Bombyx mori,* single light-pulses varying duration were exposed to pupae which had been kept in DD condition (Fig. 6). Since the light-pulse contains both light-on and light-off signal, the light-on transition initiated the LL-rhythm at first. Therefore in light-pulses with a long duration (20 or 24 hr), the eclosion peaks which were governed by LL-oscillator fell in the light fraction just before the light-off. In the light-pulse of 16 hr-duration, a minor peak corresponding these eclosion peak "survived" in darkness just after the light-off. Figure. 6 shows clearly that light-pulses of various durations produce a circadian rhythm in DD with τ_{DD}.

Medians of first eclosion peak shown after the onset of DD are roughly parallel to DD onset. The average of interval between DD onset and the first peak-median is 11.9 hr (from 11.3 to 13.5 hr). The transition LL/DD produced the first peak 10.5 hr later after the onset of DD (Fig. 3A). These facts suggested that about 1.5 hr was required to reset or convert the on-goinig LL-oscillation to DD-oscillation.

Generation of eclosion rhythm by single dark-pulse in LL

When single dark-pulse was interposed in LL condition, short dark-duration (0.5-2 hr) did not generate any synchronizing eclosion rhythm in LL (Fig. 7). However, dark-pulse of 4 hr and longer than 4 hr generated a clear rhythmicity. The mean phase angle of the first peak following light-on peak was not fixed relative to the timing of light-on. The dark-pulse with 4 and 8 hr duration produced an eclosion peak about 22 hr

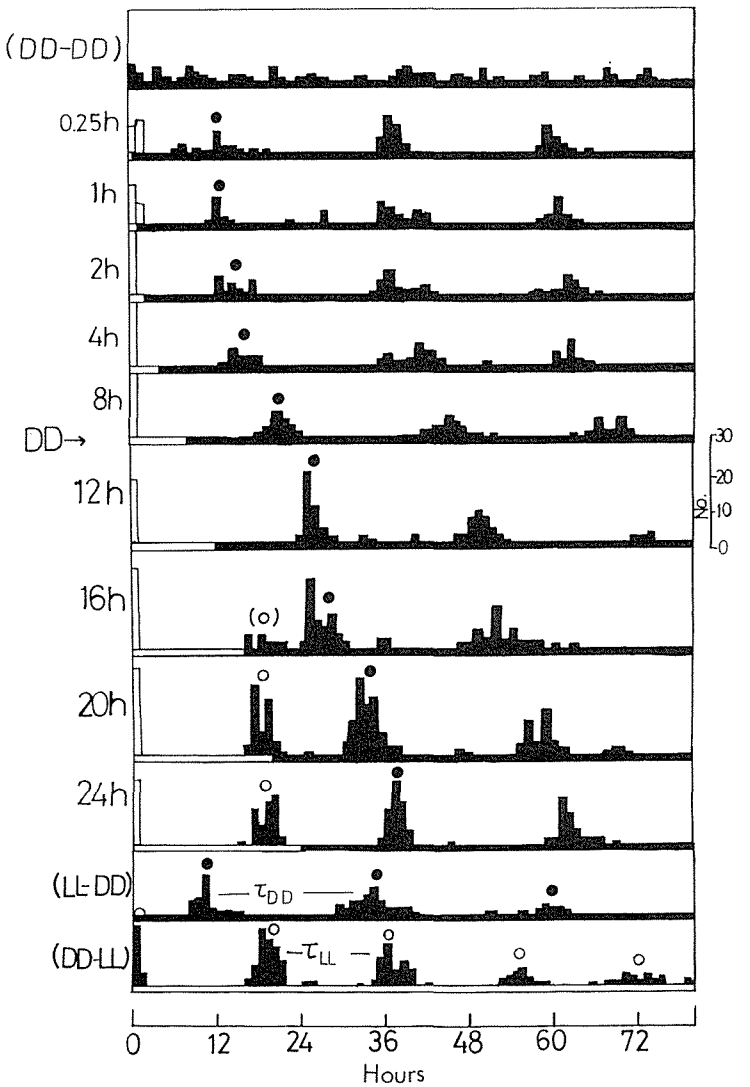

Fig. 6. Generations of eclosion rhythm by single light-pulse varying the duration from 15 min to 24 hr. The top panel shows DD-control. Closed circles indicate the median of each eclosion peak of DD-oscillation, and open circles indicate that of LL-oscillation. (LL-DD)and (DD-LL) panel show LL/DD and DD/LL transition experiment, respectively. Closed and clear bars underneath each panel represent light and dark condition, respectively.

after light-on. In dark-pulses with a long duration (12-20 hr), the first eclosion gate (gate I) which was controlled by DD-oscillation occurred in the dark period, and the first eclosion peak occurred at approximately same time of Gate II, irrespective of the difference of the light-on timing. In all cases eclosion peaks occurred with a short free-running period τ_{LL} in LL.

These results can be interpreted by both phase-shifting property of DD-oscillator and by a continuous-dynamics scheme of DD- and LL-oscillation. The continuous-dynamics scheme which we propose here is as follows:when DD-oscillator is released

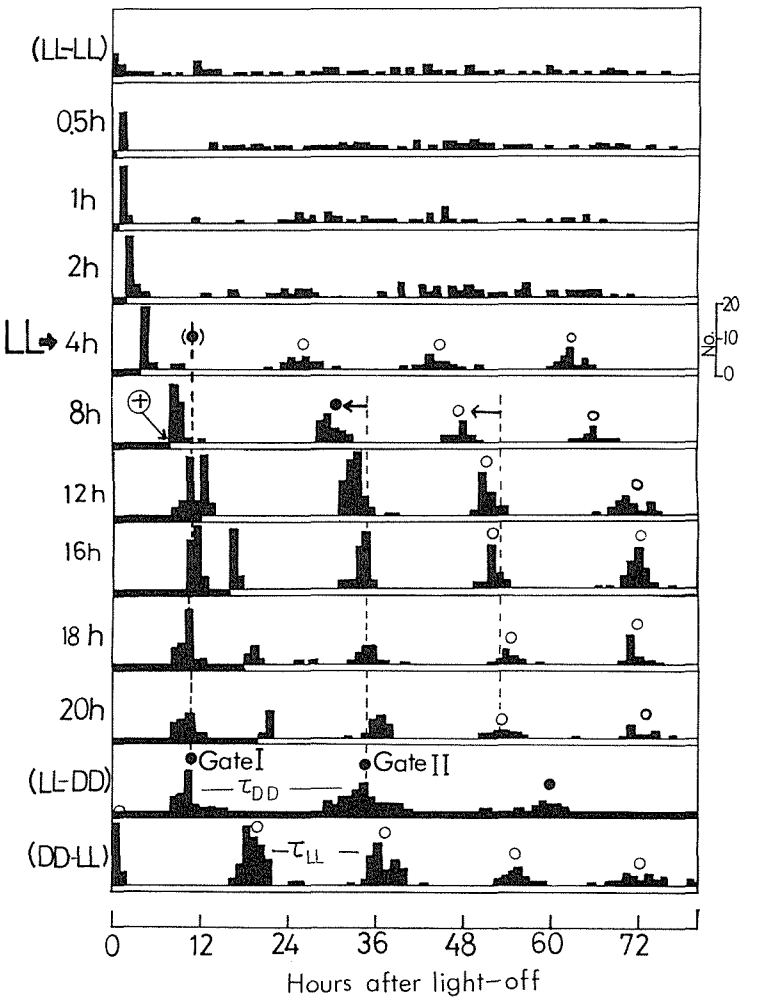

Fig. 7. Generations of eclosion rhythm by single dark-pulse interposed in LL. The top panel shows the LL-control. (LL-DD) and (DD-LL) panel show LL/DD and DD/LL transition, respectively. Closed circles indicate the median of each eclosion peak controlled by DD-oscillation, and open circles indicate that by LL-oscillation. Arrows in the panel of 8 hr-dark experiment show the phase advance of eclosion gate by the stepwise light-on transition (mark ⊕ in the panel). Gate I and gate II indicate the first and second gate of DD-oscillation in continuous darkness. See the text for details.

into LL, it moves to the next predicted phase point for the release of information dictating eclosion (e.g., Gate II in Fig. 7) without any change of the pacemaker-velocity, and there the oscillator is converted into LL-oscillator or replaced by it. This interpretation will be examined further in another experiments.

In the 4 hr dark-pulse experiment, the DD-oscillation that was initiated by LL/DD transfer underwent without any influence by the transfer itself, because the light-on signal fell on CT 16 where the DD-oscillator is insensitive to light-on signals (Fig, 9). Then the oscillation proceeds to the next gate (Gate I), and was converted to LL-oscillation which free-run with τ_{LL} successively. In this case the eclosion peak which

should be observed at the phase corresponding to Gate I was "absorbed" to light-on peak.

In the case of 8 hr dark-pulse, the light-on signal (indicated by a mark⊕ in Fig. 5) advanced the phase of the DD-oscillator, and so the next eclosion peak corresponding to Gate II advanced about 3 hr (the advance is indicated by arrows). In long dark-pulses (16-20 hr) the light-on signal of dark-pulse fell on the insensitive phase of DD-oscillator, and the first eclosion peak occurred at approximately same time of Gate II.

Phase-response curve by light-pulses applied to DD-oscillation

Several separate populations of pupae were entrained by prior exposure to LD 8:16 and released into DD-free run. With an aim of obtaining a phase-response curve, one population served as control, while each of others reveived a 2 hr light-pulse at different circadian time (Fig. 8). Our preliminary results showed light-pulse of 15 min duration produced the same response of phase-shift as 2 hr light-pulse, and so we applied 2 hr light-pluse to remove emerging moths from incubation chamber, which eclosed by exogenous light-on effect during the period.

The results showed that pulses applied between CT 10.2 (DD-onset) and CT 18.2 produced a phase delay, whereas those given between CT 18.2 and CT 24 produced a phase advance. In the rest of circadian time light-pulse had little or no effect on the phase of the resultant eclosion. Thus the switch of the phase-shift from delay to advance occurred at around CT 18.2. This phase-response curve belongs to Type 0 (Winfree, 1970).

Phase shifts of the eclosion with light-pulses varying duration applied at various circadian time

It has been demonstrated in *D. pseudoobscura* that "off" signal offered during the first half of the subjective night caused phase-delay and "on" signal offered during the second half caused phase-advance shift (Chandrashekaran *et al.*, 1973). The experiments here were designed to test whether the nature of phase-shift of *Bombyx* circadian rhythm is fitted to such a feature of *Drosophila,* or not.

As shown in Figure. 9, different populations of pupae which had been reared under LD 8:16 regimen experienced the "on" transition of the pulse given at different hour between the first part of subjective night, but experienced "off" transition at the same time (CT 18.2). In one group (Fig. 9E) the last photophase of LD 8:16 was prolonged to CT 18.2. The results showed that pulses applied split the eclosion peak into three fractions, that is light-on peak, non-shifted peak and phae-delay peak. The delay-shifts were roughly of the same magnitude regardless of the length of the pulses applied, when the "off" transition was given at the same time.

In other experiments (Fig. 9G, H, I), on-transition of pulses was aligned at the same circadian time (CT 18.2) and "off" transition occurred at differnet times. In one group of pupae (Fig. 9J), they were kept in continous light condition after the stepwise on-transition. The results showed also that the advance of phase-shift is the same magnitude regardless of the difference of the light-off timing. These observations demonstrated that the DD-oscillator of *Bombyx mori* responses selectively either to "off" and "on" signal of light pulse offered during the subjective night.

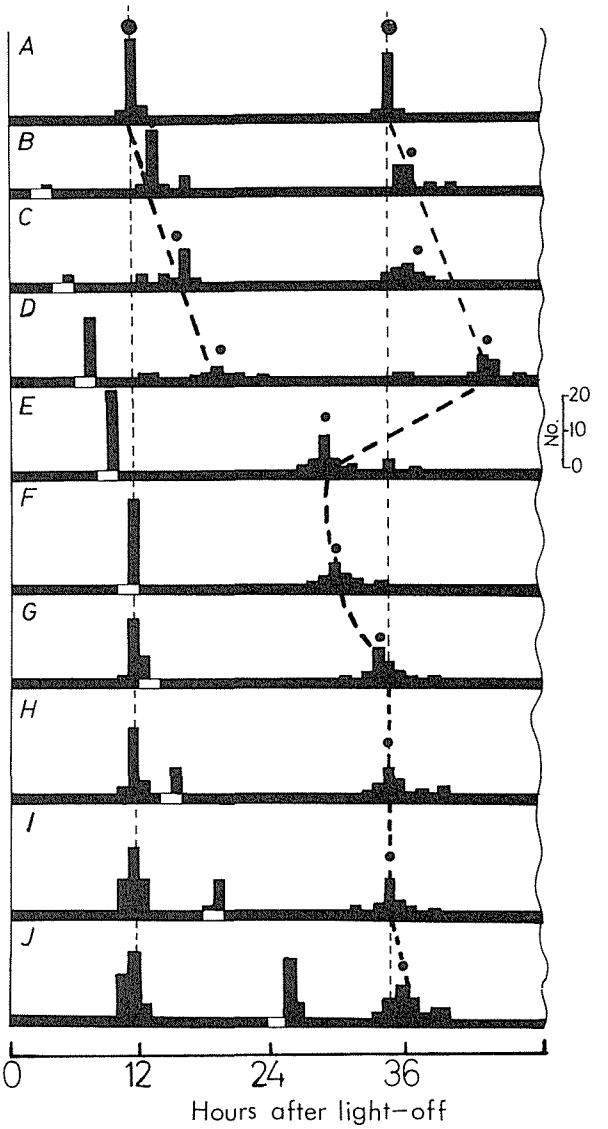

Fig. 8. Phase-shifts of circadian rhythm by applications of light pulses (300 lux, 2 hr) at various circadian times after entrainment under LD 8:16. One population of pupae which had been entrained under LD 8:16 was kept in DD from the normal onset of the scotoperiod without light pulse (A). Dashed line connects the median point of each peak.

Properties of DD-oscillator entrained under LD 8:16 regimen

To know the dynamics of DD-oscillator entrained by LD 8:16, the photoperiodic condition of the LD cycle was manipulated on one day of eclosion (Fig. 10). Fig. 10C showed the control where pupae were kept in LD 8:16 successively. Continuous light condition (Fig. 10A) and continuous darkness (Fig. 10G) from the normal time of the onset of photophase did not shift the eclosion gate in scotophase. This indicates the continuous light illuminated between CT 2 and CT 22 did not influence the velocity of

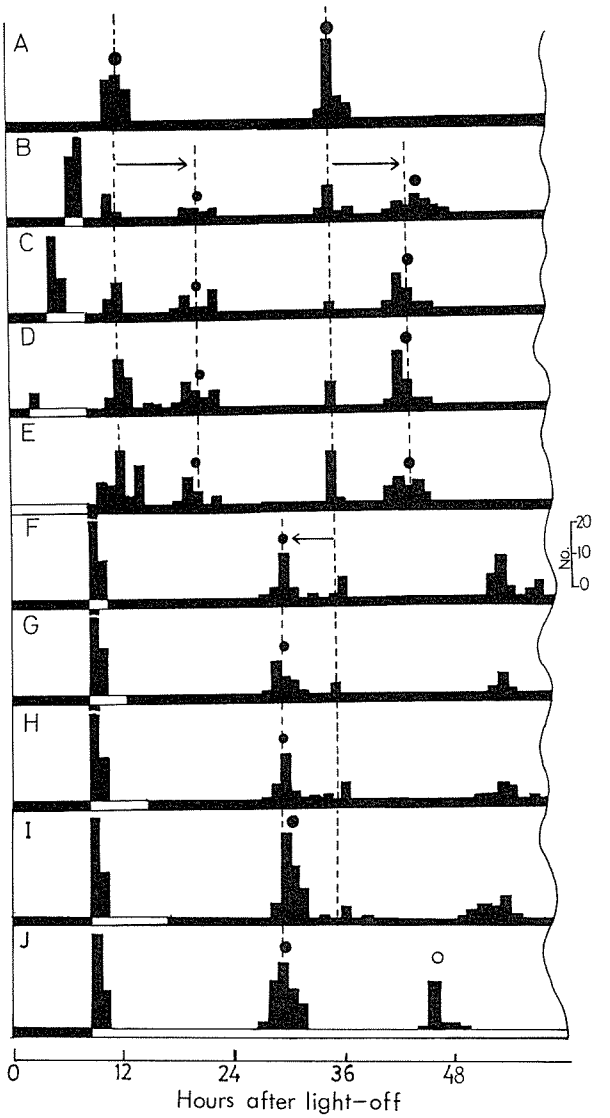

Fig. 9. Phase shifts of circadian eclosion rhythm with light pulses (300 lux) varing the duration. Closed circles denote the median of each eclosion peak. Arrows indicate the phase shifts of the eclosion peak.

the DD-oscillator. Two hours-advance of beginning the photophase caused a slight advance of the eclosion as predicted by the phase-resposne (Fig. 10B). The manipulation of 8L-photophase (Fig. 10D, E) did not affect the eclosion phase. These results suggest that under this photoperiodic regimen the entrainment of *Bombyx* eclosion rhythm is not parametric one which involves a continuous modulation of the circadian pacemaker's velocity, bur non-parametric one which involves instataneous phase shift of the pacemaker (Pittendrigh, 1981; Pittendrigh *et al.*, 1984).

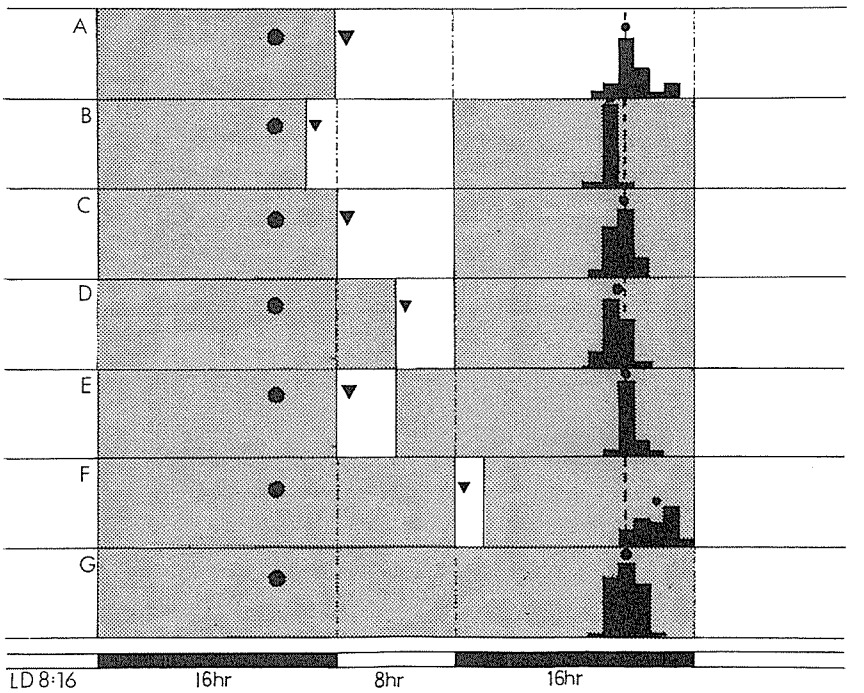

Fig. 10. Effect of manipulations of photoperiodic condition on the entrained eclosion pattern. The pupae were kept under LD 8:16 regimen from the early stage of pupal-adult development, and at 9 day after pupation the light-dark condition was manipulated as shown in this figure. Closed circles represent the median of scotophase eclosion and closed triangles represent that of light-on eclosion.

Eclosion rhythm in LL after an entrainment under LD 8:16

Pupae which had been raised under LD 8:16 showed eclosion rhythm, of which peak occurred in late scotophase (Fig. 11A). One group of pupae was transferred to LL 2 hr before the normal light-on time (Fig. 11B). Other groups were transferred at normal light-on (Fig. 11C) and 2hr intervals (Fig. 11D, E, F), respectively. As control one group was transferred to DD from the normal onset-time of the scotophase (Fig. 11G).

In all cases the first eclosion peak following light-on one occurred at approximately same time of the eclosion gate in LL (dashed line arround closed circles), regardless of the difference of the transfer-timing. The eclosion rhythm persisted at least 4 cycles with τ_{LL} (Fig. 9H: this photoperiodic manipulation was same as Fig. 11C). These results can be interpreted by the continuous-dynamics scheme proposed above: the on-going circadian oscillator moves in light-condition to the next gate with an hour-glass behavior and is converted to the LL-oscillator.

Effect of dark-pulse on entrained eclosion rhythm in prolonged light condition

Pupae which had been entrained in LD 8:16 were maintained in LL from the normal onset of photophase, and dark pulses of 5 hr duration were inserted in the continuous light condition as shown in Figure 12 at various times. Under the prolonged light-condition the first gated peak was found 22 hr after the onset of light, and at least three

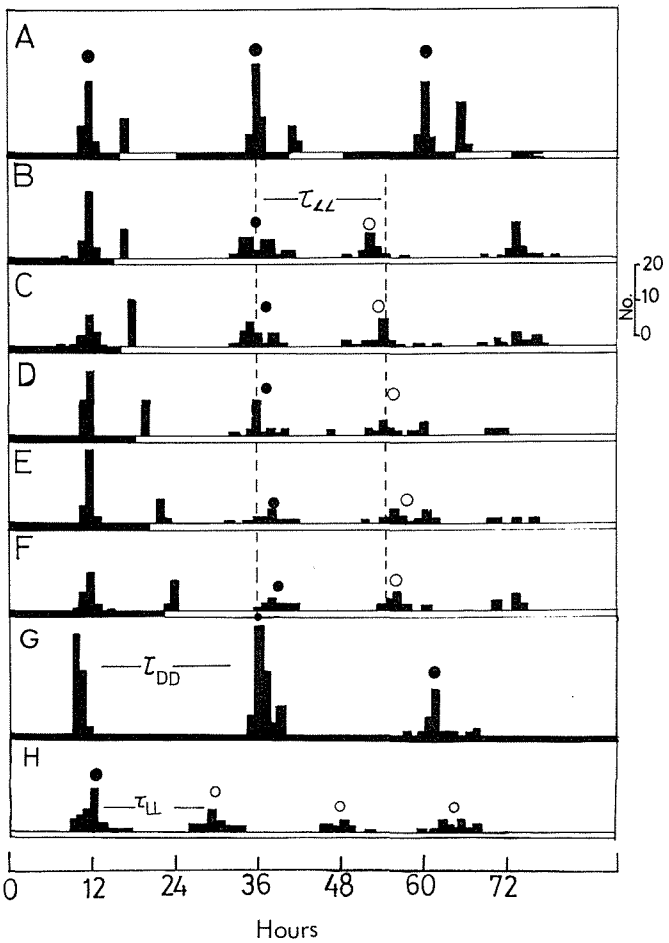

Fig. 11. Eclosion rhythms in LL or DD after entrainment under LD 8:16 regimen. (A) rearing under LD 8:16. (B) transfer from LD 8:16 to LL 2 hr before the normal onset of light-on. (C) (H) transfer to LL from normal onset of light-on. (D) (E) (F) transfer to LL at 2 hr intervals after the normal onset of light-on. (G) transfer to DD condition from the normal onset of light-off. Closed circles indicate the median of each eclosion peak controlled by DD-oscillation, and open circles indicate that of each peak controlled by LL-oscillation.

consecutive cycles occurred (Fig. 12A). As predicted by the phase-response curve a phase-delay was observed when the off-signal of dark-pulse fell during the subjective day or the first part of the subjective night (Fig. 12D, E, F). If not so, (Fig. 12B, C), no phase shift was observed.

The effect of dark-pulse applied just after the first eclosion peak needs more complicated explanation (Fig. 12G). In this case LL-oscillator which operated from the preceding eclosion gate was converted to DD-oscillator and the DD-oscillator moved to the next eclosion gate (indicated by a closed circle in parenthesis); again DD-oscillator was converted to LL-oscillator by continuous illumination.

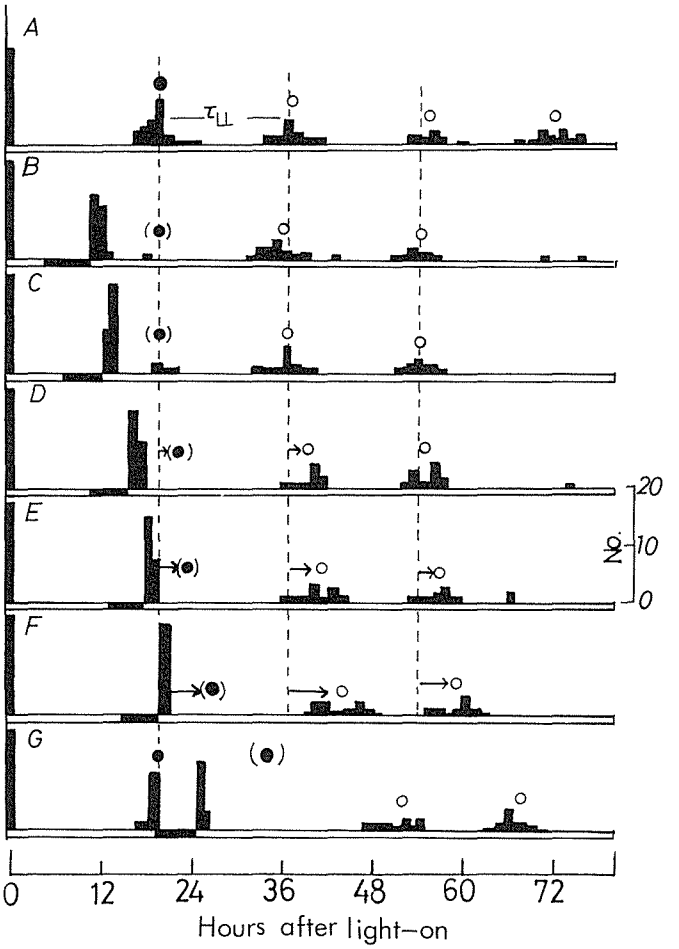

Fig. 12. Effect of dark-pulse on the eclosion rhythm in LL after entrainment under LD 16:8. Pupae which had been reared under LD 8:16 were kept in LL from the normal onset of photophase (A). Closed and open circles represent the median of eclosion peaks which were governed by DD and LL-oscillation, respectively. Closed circles in parenthesis represent the presumed eclosion peak which had been absorbed into the light-on peak. Arrows indicate the phase-delay shift caused by the dark-pulses.

Eclosion rhythm in DD and LL after entrainment under LD 16:8

To know the entrain mechanism in LD 16:8, the pupae which had been reared under LD 16:8 were transferred into DD from the normal onset of scotophase (Fig. 13A) or into LL from the normal onset of photophase (Fig. 13B), respectively. The photoperiodic regimen produced single peak at about 60 min after the light-on (Fig. 2D). The circadian free-running gates in DD were found at almost same times as the entrained light-on eclosion. This shows that the eclosion peak shown under LD 16:8 was a "mixture" of endogenous gated-eclosion and exogenous eclosion which is evoked by a starlet light-on effect.

Release into LL produced a peak about 20 hr later after light-on followed by

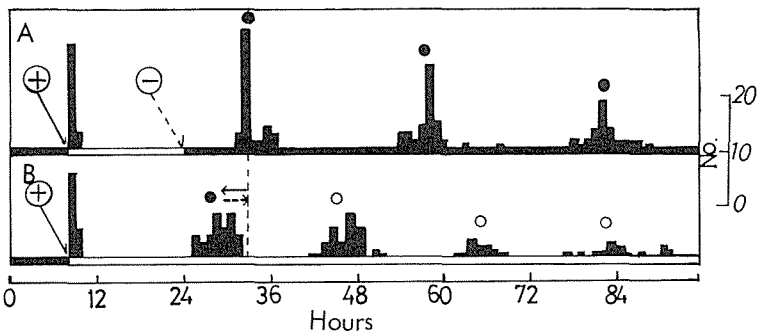

Fig. 13. Eclosion rhythms in LL and DD after entrainment under LD 16:8. (A) transfer to DD from the light-off time. (B) transfer to LL from the light-on time. Solid and dashed arrow represent a phase-advance and phase-delay, respectively. The light-on signal (mark ⊕) acts as a phase-advancer and the light-off signa l (mark ⊖) acts as a phase-delayer.

LL-oscillation with τ_{LL} (Fig. 13B). These observations can be interpreted both by the phase-response property of DD-oscillator and by the continous-dynamics between DD and LL oscillator. That is, the light-on signal (mark ⊕ in Fig. 13) and light-off signal (mark ⊖) of the photoperiodic regimen act as phase-advancer and phase-delayer, respectively. The magnitude of the advance and the delay was same, and so the phase-shift did not result. In LL the entrained DD-oscillator whose phase was advanced by the light-on signal moved to the eclosion gate (closed circle in Fig. 13B) and then it was converted to LL-oscillator.

Eclosion rhythm in DD and LL after an entrainment under LD 22:2

To reveal the characteristics of the oscillation in photoperiodic regimen with a extreme long photophase, the pupae which had been entrained under LD 22:2 were transferred into DD or LL condition (Fig. 14). In DD condition, the first peak occurred between 11 and 12 hr after the onset of the light-off, and a circadian rhythm persisted with τ_{DD} (Fig. 14A). This eclosion pattern was same as the experiment of 16 hr-light

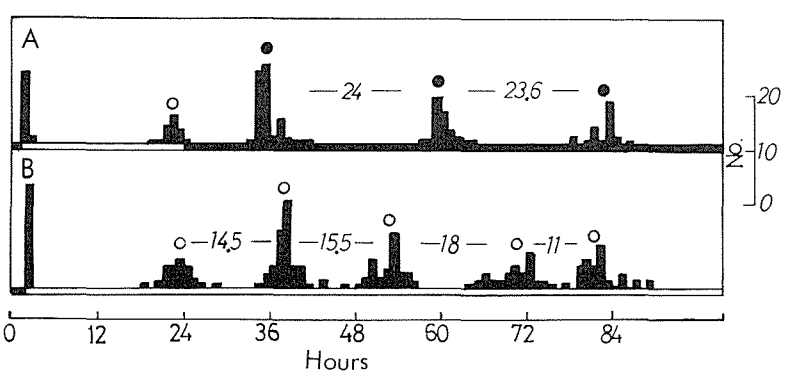

Fig. 14. Ecloiosn rhythms in LL and DD after entrainment under LD 22:2. (A) transfer to DD from the light-off time. (B) transfer to LL from the light-on time. Numbers in each panel represent the intervals between peak medians.

duration in Figure. 6. In LL-condition, eclosion rhythm persisted with τ_{LL} and a rather broad gate (Fig. 14B). These results showed that the eclosion peak ocurred in the photo-phase of LD 22:2 was governed by the LL-oscillator.

DISSCUSSION

The silkworm, *Bombyx mori,* shows a stereotyped eclosion behavior which resemble that of *Hyalophora cecropia* (Fig. 1). Adult eclosion of of silkmoths is triggered by a neurosecretory hormone which is released from neurohemal organs of brain (brain-corpora allata complex), and which acts directly on the central nervous system to elicit the eclosion behavior (Truman, 1978). In *Bombyx mori* the release takes place about 40 min before the eclosion (Fugo *et al.,* 1984). Copenhaven *et al.* (1986) reported that in *Manduca sexta* the secreation of the eclosion hormone from corpora cardiac-corpora allata complex was directly correlated with an increase in the electric activity of group Ia neurosecretory cells. Thus the electric activity of the neuron cells, rather than eclosion *per se,* seems to be a preceding event controlled by the central clock system. In *Manduca sexta* a sudden increase in the tonic firing of several units within nervi corporis cardiaci-1+2 from the neurosecretory cells occurred approximately 2 to 3 hr before the eclosion. In the case of *Bombyx mori* the timing of such synaptic input is presumed to take place about 60 min before the eclosion.

One of the characteristic feature in *Bombyx* eclosion rhythm is the light-on peak which is evoked by an exogenous light effect. We repoted that the eclosion clock of the silkworm is entrained by an extraocular photoreceptor, but the exogenous light-on eclosion is mediated *via* compound eyes of pharate moths (Shimizu *et al.,* 1981; Shimizu and Matsui, 1983). According to this exogenous eclosion the diel rhythm of the silkworm shows a bimodal pattern under some photoperiodic regimens. The exogenous (masking) light-on effect is evoked at any circadian time, and sweeps the next predicted eclosion peak which is governed by an endogenous circadian system. Little is known about the physiological mechanism which evokes such an exogenous light-on offect. Vigorous shaking of pharate moths and steep change of temperature did not evoke any such abrupt eclosion. On the other hand an exposure of pharate moths which had been kept in DD-condition to a dim light (0.1 lux) brought about the light-on emergency. The adaptive significance of the light-on emergency might be a compensatory event to scavenge the "dull" pharate moths that failed to eclose at the eclosion gate in spite of their complete development, since under natural photoperiodic regimens the silkworm emerged just before the dawn (Fig. 2). Truman (1972) reported also in *Hyalophora cecropia* eclosion that the light-on signal perceived by their compound eyes caused an eclosion behavior and thus partially masked the rhythm pattern.

In entrained steady state the rhythm takes a definite phase-relationship to the driving light-dark cycles (Fig. 2). With some photoperiods less than 16 hr, one eclosion peak lay before dawn, and light-on peak occurred at the onset of the photophase. The mean phase-angle of the scotopeak from light-off was not fixed relative to the timing of light-off. Manipulations of LD 8:16 regimen (Fig. 10) and free-run kinetics after an entrainment under the photoperiod (Fig. 11) revealed that the scotopeak was governed by the endogenous DD-oscillator in a mode of non-parametric entrainment mechanism. Under LD 16:8 the eclosion peak becomes unimodal, and only one peak is observed just

after light-on. As stated above, this eclosion peak under this photoperiodic regimen is a "mixture" of endogenous and exogenous eclosion. Whereas under LD 20:4 and LD 22:2 one eclosion peak occurred just before the light-off (dusk). Another entrainment mechanism which differs from that underlying the eclosion rhythm in shorter photoperiods is presumed to operate in these long photoperiods. Free-running rhythm in DD and LL after entrainment under LD 22:2 (Fig. 14) showed that the eclosion peak shown in the late photophase is governed by LL-oscillator whose period is shortened to about 15 hr. However, single exposure of 2 hr-dark pulse in LL did not produce any synchronizing rhythm (Fig. 7) and it pertubated the LL-oscillation initiated by DD/LL transition (Fig. 4E). Therefore the speculation about a goverment by an on-going LL-oscillator under LD 22:2 seems to be conflict with those observations. At the present time no reliable explanation for this discripancy can be offered. Recurrent dark pulses having even a short duration might establish a firm LL-oscillation.

Single stepwise transfer from LL to DD generates a clear circadian rhythmicity (DD-oscillation) with beginning from CT 12 (or close to it) as reported in other insect species (Fig. 3). The dark-pulse experiments in LL (Fig. 7) show that at least 4 hr of darkness is required to establish the persisting LL-rhythm. In this system the preceding establishment of DD-oscillation seems to be required for that of LL-oscillation. This indicates that 4 hr darkness is required for the establishment of the overt DD-oscillation. Therefor, DD-oscillator may be at a phase equivalent to CT 16, not to CT 12 at the transfer itself:after a 4 hr-dark-reaction the pacemaker begins to operate from CT 16. Another alternative explanation is that during the first 4 hr of darkness there is no phase-setting and the oscillator is initiated from CT 16 4 hr after LL/DD transition. Same augement has been given in the circadian oscillator of *Drosophila* to interpret the anomous results of light-pulse experiments applied during the first 4 hr of darkness following the LL/DD transition (Saunders, 1982).

The DD-oscillation of *Bombyx mori* governs the eclosion rhythm under diel light-dark cycles (LD 4:20 to LD 16:8) by non-parametric entrainment mechanism as mentioned above. The phase-response curve (Fig. 8) of the silkworm showed Type 0 (Winfree, 1970), and at around CT 18 there was an abrupt change from phase-delay to phase-advance. The maximum magnitude (6 hr) of the phase-advance was smaller than that (9.6 hr) of phase-delay. In the present study we did not examine whether steady state phase-shift of advance will reach after several transient cycles, or not. Our phase-shift experiments (Fig. 8 and 9) separated the effect of both "on" and "off" signal of light-pulse or of complete photoperiod as described by Chandrashekaran *et al.* (1973). The light-off signal in the early part of the subjective night acted as a new dusk and caused a phase-delay. On the other hand, the light-on signal in the later half of the night acted as a new dawn and caused a phase-advance.

Stepwise transfer from DD to LL or release into a prolonged light condition after entrainment under LD cycles produces an overt persisting LL-rhythm with a short free-running period (Fig. 3B). In the stepwise transfer-experiment the gate width of eclosion in late populations becomes broder than those in early ones. The enlargement of the gate width might be due to an undifferentiation of the pacemaker or to a failure of the perception of the light-on signal in late groups. A transfer from DD to LL of high intensity (300 lux) generated a persisting LL-rhythm, but that to LL of low intensity (3 lux) did not produce any synchronizing rhythm (Fig. 4B). The rhythm, however,

persisted clearly in dim-LL (3 lux), when the pupae were transferred from DD to a high-LL (300 lux) for first 2 hr and then kept under the dim-LL (Fig. 5C). This observation indicates that a generation of LL-rhythm needs a steep gradient in a transfer from DD to LL. The period of LL-rhythm was dependent upon the light-intensity given in LL, and the increase of intensity shortened τ_{LL} from 23-24 hr (0 lux) to 17-18 hr (300 lux). This continuity of the free-running period suggests an identity of the pacemaker governing the DD- and LL-oscillation. Another characteristic of LL-oscillation is a rapid break-down of the rhythm by an exposure to darkness of a short duration (Fig. 4). In other words, the LL-oscillator stops rapidlly in darkness. An insertion of a long dark period (4 hr) reconstructed a new mode of LL-rhythm.

The effect of LL on the population rhythm in insects differs from species to species reported. We demonstrated here *Bombyx mori* eclosion rhythm persists several times in LL even under a high-intensity. Pflüger and Neumann (1971) reported that a population of *Clunio marinus* (from St. Jean-de-Lutz) released from LD 12:12 into LL showed a free-running rhythm of pupal eclosion. Saunders (1979) described that continuous light of a high intensity also failed to stop the eclosion in *Sarcophaga argyrostoma,* although it might broaden the eclosion peaks. Rensing and Hardeland (1967) observed that rhythm of puparium-formation in *Drosophila victoria* persisted at least 3 cycles. Mizoguchi and Ishizaki (1982) reported that after entrainment under LD 12:12 gate-width of the gut purge rhythm in *Samia cynthia* growed steadily in LL and finally rhythmicity was lost entirely on day 5 in LL. Takeda (1983) reported that LL-rhythm of eclosion in *Diatraea grandiosella* gradually became desynchronized and broken down at the third cycles. By Truman using *Anthereae pernyi* it was reported that the adult eclosion in LL became arrhythmic after one transient cycle. In eclosion rhythm of *D. pseudoobscura* some investigations about the effect of LL showed a fairly rapid damping out to eventual arrhythmicity, the number of persisting cycles depending upon the intensity (Englemann, 1966; Winfree, 1974; Pittendrigh, 1981).

Thus *Bombyx mori* and *D. pseudoobscura* are contrastive insect species showing LL-rhythm and LL-arrhythmia, respectively. It is worthwhile to compare the charactristics of the eclosion rhythm in *Bombyx mori* with those of the well-documented *D. pseudoobscura.* Contrary to the high rhythmicity of *Bombyx* eclosion in LL, high intensity of LL causes arrhythmia in *Drosophila,* and transfer to DD reinitiates a rhythm whose phase projects to CT 12 at the beginning of the dark free-run. *Drosophila* rhythm, however, persists at low intensities with τ_{LL} of a longer free-running period than in DD, and a transfer of the persisting LL-rhythm under low intensities to DD resets the phase of the pacemaker to CT 12, no matter when the step into darkness occurred (Pittendrigh, 1981). These evidence imply that although the overt rhythm of *Drosophila* eclosion may be damped out in LL, constant light of high intensity does not suppress the motion of the underlying pacemaker, and that it is reset to CT 12 or close to it, when transferred to DD (Saunders, 1982). Some observations obtained here show that when LL-rhythm of *Bombyx* is released into DD condition newly-established DD-rhythm is initiated from CT 12 (or close to it). Thus the fundamental feature of dynamics of LL-oscillation seems to be identical in both species, though high intensities shorten τ_{LL} in *Bombyx* and lenghthen in *Drosophila.*

Bombyx eclosion rhythm was initiated by an exposure of single light-pulse in DD (Fig. 6). Since the light pulse contains both light-on and light-off signal, the light-on

transition initiated the LL-oscillation at first. The intervals between DD-onset and the median of the first peak were distributed from 11.3 to 13.5 hr, and the mean of the medians was 11.9 ± 0.4 (S. D.) hr. The interval between DD-onset and the first peak which occurred after LL/DD transition was 10.5 hr (Fig. 3). This value was significantly shorter than those observed in light-pulse experiments (Fig. 6). This implies that the process of stopping LL-oscillator and the construction of DD-oscillator needs first 1.5 hr of the darkness.

Dark-pulse experiments in LL also give us an important information about the dynamics controlling the *Bombyx* eclosion rhythm. A long dark-pulse interposed in LL generated an overt LL-rhythmicity (Fig. 7). These eclosion patterns can be interpreted by a scheme of continuous-dynamics of DD and LL-oscillator as described: when the DD-oscillator is released in LL, it goes forward to the next critical phase-point where the clock signal to dictate the eclosion behavior (the eclosion itself is assigned to CT 22.5) is released and then LL-oscillation begins. The eclosion patterns shown in LL after entrainment under LD 8:16 (Fig. 11) and LD 16:8 (Fig. 13) can be explained also on this continuous-dynamics model.

Dynamics and phase relationship between DD-oscillation and LL-oscillation are summarized in Fig. 15. The oscillatory motion of both oscillators are represented as two separate closed cycles. Each oscillator is not driven by separate pacemakers, but by a physiologically identical pacemaker which can be represented by two limit cycles on a plane (Peterson and Jones, 1979). Transition of LL/DD generated an endogenous DD-oscillation with τ_{DD} (24 hr) from CT 12 or close to it (CT 16). The eclosion occurs at CT 22.5, and the clock signal dictating the eclosion is assigned to CT 21.5. DD-oscillation is separated into three fractions from a view of the property of the phase response:light-off sensitive (off-sen in the figure), light-on sensitive (on-sen) and photo-insensitive circadian phase. When the DD- oscillator is released into LL, it is converted

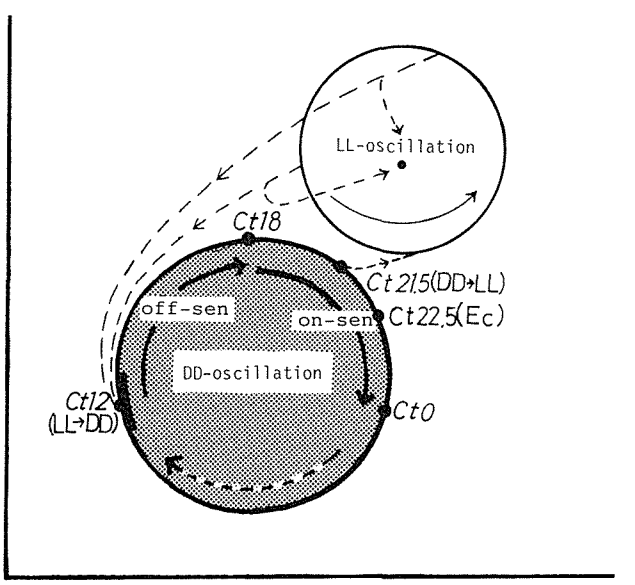

Fig. 15. A scheme elucidating the characteristics and dynamics of the eclosion rhythm in *Bombyx mori*. See the text for details.

to LL-oscillator from CT 21.5 without any transient phase. Transition of DD/LL produced LL-oscillation with a short τ_{LL} (17.8 hr), and when on-going LL-oscillator is exposed to darkness of a long period, it is converted to DD-oscillator from around CT 12 or close to it (dashed line converging to CT12 in Fig. 15). Increase of the light-intensity given in LL shortens the free-running period. In darkness of a short period the LL-oscillator is driven toward to its singularity before the construction of the DD-oscillator and it becomes arrhythmic (dashed line converging into the cycle center in Fig. 15). About 1.5 hr is required for the construction process. *Bombyx* eclosion rhythm can be interprted appropriately in terms of this continuous-dynamic model.

Acknowledgements

We would like to express our sincere gratitude to Professor. C. S. Pittendrigh for a critical reading of the manuscript and invaluable comments. We wish to thank Professor J. Aschoff for discussing our study of this paper and for many helpful comments. Thank is also due to Dr. S. Dann for his kind discussion and advice.

References

Chandrashekaran, M. K., A. Johnsson and W. Engelmann (1973) Possible "dawn" and "dusk" roles of light pulses shifting the phase of a circadian rhythm. *J. Comp. Physiol.* 82: 347–356.

Copenhaver, P. F. and J. W. Truman (1986) Control of neurosecretion in the moth *Manduca sexta*: physiological regulation of the eclosion hormone cells. *J. Comp. Physiol.* 158: 445–455.

Engelmann, W. (1966) Effect of light and dark pulses on the emergency rhythm of *Drosophila pseudoobscura*. *Experientia* 22: 606–608.

Fugo, H., Y. Iwata and M. Nakajima (1984) Eclosion hormone activity in haemolymph of eclosing silkworm, *Bombyx mori*. *J. Insect Physiol.* 30: 471–475.

Immelmann, K. (1980) Introduction to ethology. Plenum, New York.

Mizoguchi, A. and H. Ishizaki (1984) Circadian clock controlling gut-purge rhythm of the saturniid *Samia cynthia ricini*: its characterization and entrainment mechanism. *J. Comp. Physiol.* 155: 639–647.

Nagasawa, H., T. Kamito, T. Takahashi, A. Isogai and H. Fugo (1985) Eclosion hormone of the silkworm, *Bombyx mori*: purification and determination of the N-terminal amino acid sequence. *Insect Biochem.* 15: 573–578.

Peterson, E. L. and M. D. R. Jones (1979) Do circadian oscillators ever stop in constant light ? *Nature* 280: 677–679.

Pflüger, W. and D. Neumann (1971) Die Steuerung einer gezeitenparallelen Sclüpfrhythmick nach dem Sanduhr-Prinzip. *Oecologia* 7: 262–266.

Pittendrigh, C. S., V. G. Bruce and P. Kaus (1958) On the siginificance of transients in daily rhythms. *Proc. Nat. Acad. Sci.* 44: 965–973.

Pittendrigh, C. S. (1960) Circadian rhythm and the circadian organization of living system. *Cold Spring Harb. Sym. Quant. Biol.* 25: 159–184.

Pittendrigh, C. S. (1981) Circadian system: entrainment. In *Handbook of Behavioral Neurobiology, Vol 4, Biological rhythm* (ed. J. Ascoff), pp. 95–124. Plenum, New York.

Pittendrigh, C. S., J. E. Elliott and T. Takamura (1984) The circadian component in photoperiodic induction. In *Photoperiodic Regulation of Insect and Molluacan Hormones* (CIBA Foundation Symp. 104: ed. R. Porter and G. M. Collins), pp. 26–47. Pitman, London.

Saunders, D. S. (1979) The circadian eclosion rhythm in *Sarcophaga argyostoma*: delineation of the responsive period for entrainment. *Physiol. Ent.* 4: 263–274.

Saunders, D. S. (1982) *Insect Clocks* (2nd. ed.), Pergamon, Oxford.
Shimizu, I., S. Kitabatake and M. Kato (1981) Effect of carotenoid deficiency on photosensitivities in the silkworm, *Bombyx mori*. *J. Insect Physiol.* 27: 593–599.
Shimizu, I. and K. Matsui (1983) Photoreceptions in the eclosion of the silkworm, *Bombyx mori*. *Photo. and Photo.* 37: 409–413.
Taghert, P. H., J. W. Truman and S. E. Reynolds (1980) Physiology of pupal ecdysis in the Tobacco hornworm, *Manduca sexta*. *J. Exp. Biol.* 88: 339–349.
Takeda, M. (1983) Ontogeny of the circadian system governing ecdysial rhythms in a homometabolous insect, *Diatraea grandiosella*. *Physiol. Ento.* 8: 321–331.
Tazima, Y. (1978) The silkworm: an important laboratory tool. Kodansha, Tokyo.
Truman, J. W. and L. M. Riddiford (1970) Neuroendocrine control of ecdysis in silkmoths. *Science* 167: 1624–1626.
Truman, J. W. (1971a) Physiology of insect ecdysis I The eclosion behavior of saturniid moths and its hormonal release. *J. Exp. Biol.* 54: 805–814.
Truman, J. W. (1971b) Hour-glass behavior of the circadian controlling eclosion of the silkmoth *Anthearea pernyi*. *Proc. Nat. Acad. Sci.* 68: 595–599.
Truman, J. W. (1972) Physiology of insect rhythms. II. The silkworm brain as the location of the biological clock controlling eclosion. *J. Comp. Physiol.* 81: 99–114.
Truman, J. W. (1978) Hormonal release of stereotyped motor programma from the isolated nervous system of the *cecropia* silkmoth. *J. Exp. Biol.* 74: 151–173.
Truman, J. W. (1984) The preparatory behavior rhythm of the moth *Manduca sexta*: and ecdysteroid-triggered circadian rhythm that is independent of the brain. *J. Comp. Physiol.* 155: 521–528.
Winfree, A. T. (1970) An integrated view of the resetting of a circadian clock. *J. Theor. Biol.* 28: 327–374.

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