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Hatching patterns in the silkworm, *Bombyx mori* L. under 'black-boxing' system: A photoperiodic perspective

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Abstract
Conventional methods of silkworm (*Bombyx mori* L.) hatching include incubation, exposure to light dark cycles etc., were in practice. As a recent technology for silkworm egg hatching, use of black-boxing method coupled with incubation is advocated in the contemporary Indian sericulture industry to get economical hatching of over 95%, in a single day with quick hatching. In the present study, an attempt is made to compare the photoperiodic way and the black-boxing method of hatching for better understanding the scientific principles behind the black-boxing method. For the studies, the DFLs of CSR2 x CSR4 were introduced into natural solar day condition, LD 12:12, continuous dark (DD) and continuous light (LL) on the third day of oviposition and continued till the completion of hatching experiments. For studies on black-boxing, the DFLs were initially maintained under LD 12:12 up to pin-head stage and introduced into black-boxing (DD) at 06.00 h on the pin-head stage. The DFLs were watched for stray-hatching on the penultimate day of hatching and after confirming stray-hatching, the dark phase in black-boxing is disrupted, exposing the eggs to light on the ultimate day of hatching at 06.00 h.

Hatching, in CSR2 x CSR4 bivoltine hybrid occurred very close to dawn under natural solar day, LD 12:12 conditions. Further, light-on phase is taken by *Bombyx mori* as signal for hatching. Hatching occurred for two consecutive days, with stray hatching on the first day and maximum on the second day. Intervals between two hatching peaks were around 24 h, hence, circadian. When the eggs are driven under continuous conditions (DD/LL) the hatching occurred for 2 consecutive days. Under continuous dark (DD), the hatching occurred for two consecutive days with advancement in peak hatching implying a free-running nature of hatching rhythm following 'gating' phenomenon. Hatching on the first day was highest with narrow hatching activity period. Under LL too, hatching occurred for two consecutive days, with low hatching on the first day and maximum on the second day. Further, hatching rhythm delayed with broadened of hatching under LL, indicating a near damp-out expression. When the eggs are kept under 'black-boxing' condition, the hatching rhythm was distinct, occurring immediately after interruption of darkness of black-boxing. Hatching was restricted to a single day with narrow hatching activity, measuring 2 h of hatching duration only. Hatching occurred at or after lights on phase indicating that the hatching rhythm is diurnal and lights-on is taken straightly as a signal for hatching. Hatching durations are more under continuous conditions (DD/LL) and less in LD 12:12 and black-boxing conditions.

Keywords: *Bombyx mori* L., black-boxing, photoperiodic, hatching

Introduction

Under light-dark schedules of natural day (≤ or ≥ 24 h; Saunders, 1982, 2002) [15, 16], animals exhibit rhythmic activities (Solberger, 1965) [25]. *Bombyx mori* was reported as a 'short-day' dependent insect (Kogure, 1933; de Wilde, 1962; Danilevskii, 1965; Lees, 1968; Beck, 1980; Saunders, 1982) [9, 7, 5, 10, 2, 15], while Shimizu (1982) [17] showed that an artificial diet produced long-day characteristics for diapausing in the next generation. As in the other insects (Beck, 1980; Saunders, 1982, 2002) [2, 15, 16], hatching in *Bombyx mori* is regulated by a circadian oscillator (Ananta Narayana *et al*., 1978; Tanaka, 1966a, b, c; Sivarami Reddy *et al*., 1984; Sivarami Reddy and Sasira Babu, 1990, Sivarami Reddy *et al*., 1998) [1, 26, 27, 28, 18, 19, 20]. It was also implicit that the oscillator, controlling the ultimate timing of hatching, was probably a specific element in the central nervous system in *Pectinophora gossypeilla* (Minis and Pittendrigh, 1968) [11] which was not 'differentiated' until mid point of embryonic development or it was present at the outset, but was not coupled to light cycles (Saunders, 1982, 2002) [15, 16]. Fugo *et al*. (1985) [8] pointed that the rhythmicity in hatching can be initiated by pulses of light when these are applied after the mid-point of embryonic development.

It is apparent that the 'black-boxing' technique has been appropriately adopted in recent silkworm rearing technology as advocated by the Central Sericultural Research and Training Institute, Central Silk Board, Mysore (Datta, 1992) [6]. However, the scientific explanation is not available. Certain issues like why the silkworm eggs under incubation are to be black-boxed or introduced into continuous dark (DD) condition at pin-head stage, why the darkness of black-boxing should be disturbed with light at 06.00 h on the ultimate day of hatching, what are the results on hatching and the procedure of black-boxing is of mere academic or of industrial applicable etc., are not explained. Therefore, an attempt is made to explain the credible principles behind the 'black-boxing' technology, comparing the phenomenon with hatching patterns under natural day, LD 12:12, continuous dark, DD, continuous light, LL and 'black boxing', using popular bivoltine silkworm (*Bombyx mori*) hybrid, CSR2 x CSR4 in the contemporary Indian sericulture.

Materials and Methods

The popular bivoltine hybrid, CSR2 x CSR4 was used for the studies on rhythmicity in hatching process of the silkworm, *Bombyx mori* L. Disease free layings (DFLs, commonly called) of CSR2 x CSR4 were procured, on the third day of oviposition, from the Silkworm Seed Production Centre (SSPC), National Silkworm Seed Organization (NSSO), Bangalore, Karnataka, India. The DFLs were transported to the laboratory (Department of Sericulture, Sri Krishnadevaraya University, Anantapur) during evening cool hours, spread into the pre disinfected rearing trays. In total 20 DFLs were procured @ 5 DFLs each for four experimental conditions, LD 12:12, DD, LL and black boxing. DFLs were introduced into experimental conditions immediately with 5 + 5 DFLs into LD 12:12 and 5 DFLs each into DD and LL conditions. For natural day, LD 12:12, the solar day (24 h) was divided into two equal parts; 12 h light phase (photophase) and 12 h dark phase (scotophase). The photophase was initiated from 06.00 h and lasted for 12 h at 18.00 h local time. Similarly, the scotophase was imposed from 18.00 h and continued up to 06.00 h local time. A 60 W florescent bulb, as light source for illuminating the experimental DFLs during photophase of rearing period was arranged above the rearing tray, its height from the surface of experimental silkworm DFLs was so monitored that the light intensity at the surface measured 50 lux. For LL conditions, continuous light of 50 lux is provided while for DD condition, the entire period was complete darkness. 5 DFLs kept separately under LD 12:12 conditions were introduced into DD (darkness) at 06.00 h on the day of pin-head stage initiation for hatching studies under black-boxing. The DFLs under LD 12:12, DD, LL and black-boxing were carefully observed for stray hatching on the penultimate day of hatching. On the ultimate day of hatching, darkness in the black-boxing experiment, was interrupted by exposing the DFLs to light (50 lux) at 06.00 h local time and continued till completion of hatching. For observations in the dark, under LD 12:12, DD and black-boxing experiment, a dim red tight source was used, as the embryos are reported to be insensitive to dim red light. All the experimental batches were provided with a room temperature of 25±1 ^{o}C and relative humidity (RH) of 80±5%. Precise number of larvae that hatched out from eggs was counted. The counting was done at one hour interval and recorded. The counted larvae were gently transferred into a separate tray with a feather brush for further studies, if needed. Data on hatching thus recorded were represented as distribution diagrams (hourly histograms, resolved for 24 h, $\omega = 360^{o}$). The experiments on hatching were repeated for 5 times. Macroscopic data recorded were treated statistically (ANOVA). All the values, below 5% (< 0.05) are designated as significant and those below 1% (< 0.01) level as highly significant.

Results

I. Hatching under normal LD cycle, LD 12:12 conditions

In the present study, egg hatching in CSR2 x CSR4 was predominantly diurnal (Fig. 1.). Hatching was observed during early hours of the light part of natural LD cycle (LD 12:12). Notably, the hybrid recorded egg hatching for only one day, with stray (negligible) hatching on the earlier day which was not considered. Hatching has been completed in a single day. The observed patterns in hatching are diurnal as the activity occurred during the day time. In addition, hatching occurred immediately after 'lights-on' and should be described that the hybrid is taking 'lights-on' as a signal for hatching. Hatching occurred in the early hours (06.00 to 09.00 h).

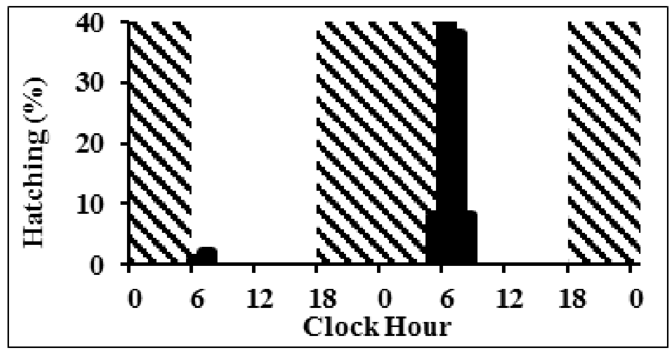

Fig 1: Distribution of hatching (%) in CSR2 x CSR4 of *Bombyx mori* L. under LD 12:12 conditions. Note stray hatching on the first day and the rest on the second day. Also note that the hatching occurred just after 'lights-on' phase of the LD cycle. Cross-hatched area in the histogram indicates the dark phase imposed and the open area, the light phase of the day.

II. Hatching under continuous dark (DD) conditions

The activity of hatching in the CSR2 x CSR4 under continuous dark (DD) condition is presented in Fig. 2. In the case of DD, hatching occurred for two consecutive days. However, hatching on the first day was more compared to that occurred on the second day. It is interesting to note that hatching rhythm advanced, hatching initiated before 04.00 h local time and concluded at 06.00 h. Similar patterns were recorded for hatching on the second day also. Interval between these two hatching rhythmic components is around 24 h, hence its occurrence is circadian. Further, as the hatching did not complete in a single day, and continued to occur on the next day, revealed 'gating' phenomenon.

III. Hatching under continuous light (LL) conditions

Under LL conditions, hatching in the bivoltine silkworm hybrid, CSR2 x CSR4 occurred after 06.00 h local time (Fig. 3). However, the peak of hatching is seen occurring at or after 10.00 h local time. Thus, hatching delayed from that observed for LD 12:12 conditions (Fig. 1). Also, hatching activity broadened, taking more than 6 h width while that for LD 12:12 and DD was just 3-4 h. Other characters of the hatching rhythm are comparable to that observed under LD 12:12 and DD. Thus, hatching occurred for two consecutive days, the intervals between two consecutive peaks is around 24 h and hence, circadian. Further, the hatching followed gating phenomenon. Broadening of hatching activity indicate the 'damping-out' condition.

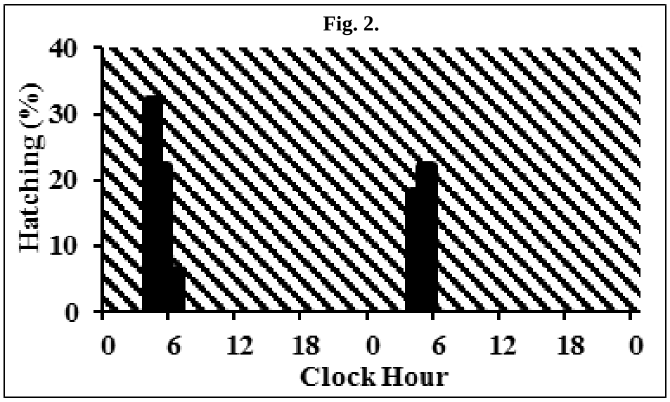

Fig 2: Distribution of hatching (%) in CSR2 x CSR4 of *Bombyx mori* L. under continuous dark (DD) condition. Note hatching for two consecutive days (gates) with more hatching on the first day. Also, hatching durations were narrowed. The hatching occurred before 06.00 h of the local time and therefore advanced with free running rhythmicity. Hatching activity (peak) is very sharp.

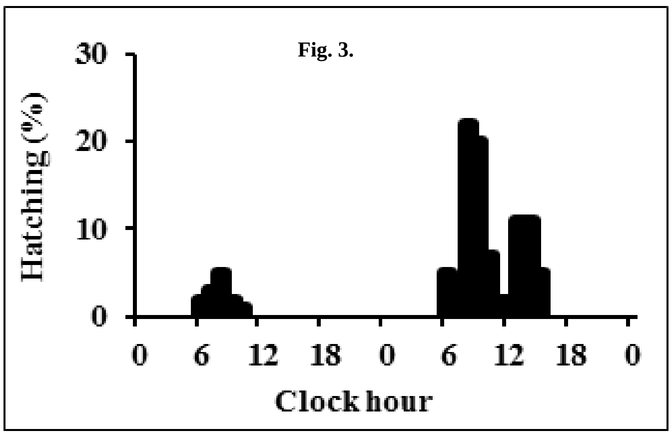

Fig 3: Distribution of hatching (%) in CSR2 x CSR4 of *Bombyx mori* L. under continuous light (LL) condition. Note hatching occurring for two consecutive days, with more hatching on the second day. Also note that the hatching occurred after 06.00 h of local time indicating a delay. Hatching activity broadened.

IV. Hatching under 'black boxing' condition

Results on 'black-boxing' technique on hatching patterns in the bivoltine silkworm hybrid, CSR2 x CSR4 are depicted in Fig. 4. Hatching invariably confined to a single day only. Also, the hatching magnitude, at peak hour, was more in the hybrid, indicating that the black-boxing is more effective in inducing single day hatching, with high magnitude. Hatching occurred, as observed in all the other experimental conditions, at or after lights on phase, again indicating that the hatching rhythm is diurnal and 'lights-on' is taken as a signal for hatching.

V. Hatching durations under different photoperiodic conditions

Hatching durations of commercial silkworm is the most important one for the sericulture farmer. Therefore, hatching durations were computed in the bivoltine hybrid, CSR2 x CSR4 which are the extaracted value from the mcroscopic data recorded for hatching patterns under all the imposed photoperiodic conditions. Hatching duration, from the initiation point of hatching to its completion (Fig. 5.) showed interesting results. Hatching under LD 12:12 and DD conditions are comparatively low when compared to that under DD and LL. The differences in hatching durations under LD 12:12, DD and LL are significant (< 0.05). These durations (LD 12:12, DD and LL) when compared to black-boxing condition (Fig. 5.) indicates that the hatching duration is very narrow in the bivoltine hybrid, CSR2 x CSR4, recording only 2 h. This hatching duration is highly significant (< 0.01).

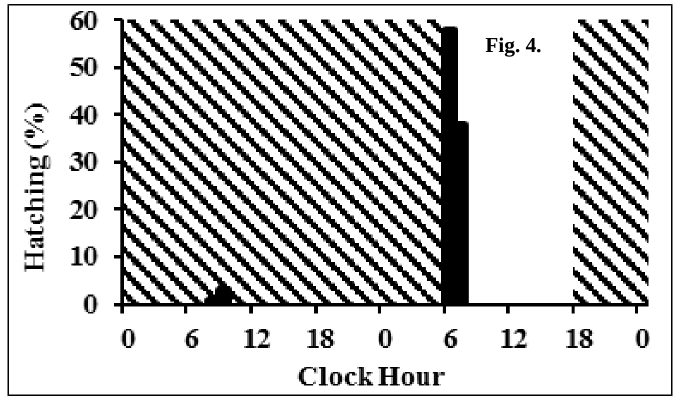

Fig 4: Distribution of hatching (%) in CSR2 x CSR4 of *Bombyx mori* L. under 'black-boxing' condition. Note stray hatching on day-one under dark condition. Also note that the hatching occurred just after 'lights-on' phase, terminating dark in black-boxing condition. The rhythm peak is very sharp. Cross-hatched area in the histogram indicates the dark phase imposed and the open area, the light phase.

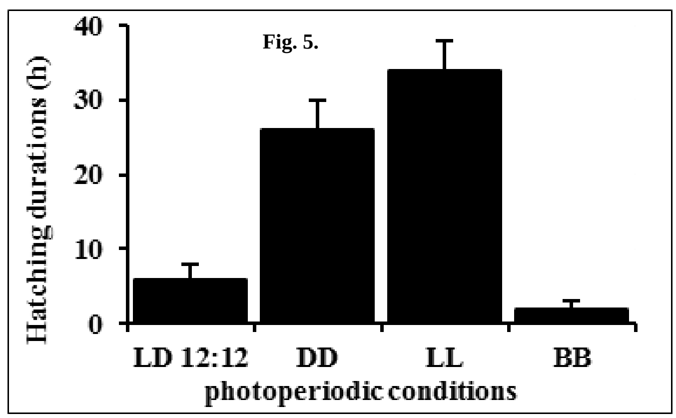

Fig 5: Hatching durations in the bivoltine silkworm hybrid, CSR2 x CSR4 of *Bombyx mori* L. under LD 12:12, DD, LL and under black-boxing (BB) conditions. Note hatching durations are very high under LL and DD conditions and are very low under black-boxing and LD 12:12 conditions. The values are mean of 5 replications (± SD). The differences in hatching durations between LD 12:12, DD and LL are significant (5%) while those compared to black-boxing conditions are highly significant (1%).

Discussions

Among different developmental marker events of insects, as influenced by photoperiods, the rhythmic pattern in the first and foremost developmental marker event, the hatching of eggs received considerably very good attention. In the silkworm, *Bombyx* mori also, there are several reports on the hatching patterns. The present study is the extension of such studies for the latest popular bivoltine hybrid, CSR2 x CSR4 of *B. mori*. Rhythmic patterns in eclosion, oviposition and hatching, as affected by photoperiods in silkworm were reported (Yamaoka and Hirao, 1975; Yamaoka *et al*., 1976; Anantha Narayana *et al*., 1978; Sivarami Reddy *et al*., 1984, 90, 93a and b; Sivarami Reddy and Sasira Babu, 1990, Sivarami Reddy *et al*., 1998) [29, 30, 1, 18, 20, 19, 22]. Anantha Narayana *et al*. (1978) [1] explained that the hatching peak in PM of *B. mori* to be very close to dawn under alternating cycles of light and dark, and they (Anantha Narayana *et al*., 1978) [1] linked the 'light-on' as the synchronizing signal. In the present study also, the hatching in CSR2 x CSR4 was observed at or immediately after lights-on under LD 12:12 condition and black-boxing condition as well. Therefore, the hatching rhythm is a diurnal one. Pupal eclosion rhythm in *D. pseudoobscura* (Pittendrigh, 1966) [12] suggest the existence of a self-sustained oscillator which partitions a mixed age population into daily active peaks, with certain hours of the day constituting 'forbidden zones' and certain others as allowed zones or 'gates', directed by the clock(s). Works of Skopik and Pittendrigh (1967) [24] and Pittendrigh and Skopik (1970) [14] indicate that if the animals in a mixed-age population are not at the 'correct' morphogenic state to utilize one particular allowed zone or gate, they are required to remain waiting till the next gate for utilization, the intervening hours constituting a 'forbidden zone'. The gates or forbidden zones reoccur with circadian frequency, as in the present study. The gating of certain developmental stages by such a mechanism is probably ubiquitous in insect population rhythms (Saunders, 1982, 2002) [15, 16] and therefore, *Bombyx* is not an exemption. Thus, hatching in CSR2 x CSR4 followed 'gating phenomenon' indicating 'mixed-age' population characteristics, as evident from occurring hatching in two consecutive days under DD (Fig. 2.) and LL conditions (Fig. 3). Further, occurrence of consecutive hatching peaks at or around 24 h intervals notably indicate that the hatching rhythm is a circadian one.

Early investigations on the effect of LL on the eclosion rhythm in *D. pseudoobscura* (Pittendrigh and Bruce, 1957; Chandrashekaran and Loher, 1969a, b) [13] showed fairly rapid damp-out to an eventual arrhythmicity under LL condition. In the present study, it is clear that the underlying driving oscillation in the hatching of *Bombyx mori* is under the control of circadian system as hatching followed gating pattern. Hatching peaks broadening was reported by Sivarami Reddy (1993) [20], Sivarami Reddy and Sasira Babu (1990) [19], Sivarami Reddy *et al*. (1998) [22] with photophase over 12 h as also under continuous light (LL) conditions though not expressing the complete damp-out situation of the rhythmicity. They (Sivarami Reddy, 1993; Sivarami Reddy and Sasira Babu 1990; Sivarami Reddy *et al*., 1998) [20, 19, 22] also reported that since the number of photoperiodic cycles experienced by the experimental silkworm embryos is limited (7-8 cycles, Sivarami Reddy and Sasira Babu, 1990) [19], complete damping-out or arrhythmicity may not be expected. Such near damp-out conditions were also seen with CSR2 x CSR4 under LL condition (Fig. 3).

Notably, the hatching under black-boxing system did not express gating phenomenon, which is desired for economic hatching. Datta (1992) [6] explained that the development of embryos in silkworm eggs attain uniformity, avoiding mixed age characteristics under black-boxing system of hatching. Occurrence of hatching immediately after interrupting darkness under black-boxing system (lights on) invariably indicate that the hatching rhythm take 'lights-on' as signal of synchronization for hatching. The issue of expressing mixed-age characteristics in the population of *Bombyx mori* has been reported by Sivarami Reddy *et al*. (2011) [23]. It is apparent that the 'black-boxing' technique has been fittingly adopted in contemporary silkworm rearing technology as advanced by the Central Sericultural Research and Training Institute, Central Silk Board, Mysore (Datta, 1992) [6]. However, the scientific explanation is scanty. Certain issues like why the silkworm eggs under incubation are to be black-boxed or introduced into continuous dark (DD) at pin-head stage or blue egg stage are not explained. The results of the present study with black-boxing and the next coming discussions give more clear insight into the facts behind the black-boxing technique.

The basic principles of black-boxing can be traced from the reports of Minis and Pittendrigh (1968) [11] who demonstrated that the egg hatching rhythm in *P. gossypiella*, by systematic transfer of cultures from LL into DD every 5.5 h during embryonic development, could not be initiated until after the midpoint of embryogenesis. Saunders (1982, 2002) [15, 16] suggested that either the oscillator controlling the egg hatching was not differentiated until mid embryogenesis, or that it was present from the out-set, but was not coupled to the light-dark cycles. Saunders (1982, 2002) [15, 16] considered the second of these alternatives as a possibility, as a pinkish pigment in the eggs of *P. gossypiella* was noticed at about the time when rhythm initiation could be achieved. In *B. mori* too, Fugo *at al*., (1985) [8] reported that the rhythm of hatching can be initiated by pulses of light when these are administered after the mid-embryonic development which almost coincides with the stage of differentiation of the central nervous system. In 'black-boxing' experiment, in the present study also, oscillator controlling the egg hatching was noticed to be at pin-head stage and reflected in a distinct hatching rhythmicity. The hatching rhythm under black-boxing system was comparable to that under LD 12:12 the eggs are given a light pulse at 06.00 h on the ultimate day of hatching. The rhythm is obvious under all the photoperiodic conditions. However, the rhythm was nearly ambiguous in LL. Thus, the fact that the oscillator or clock, controlling the egg hatching is well recognized by embryos at pin-head stage in the present study with black-boxing system of hatching. Further, the hatching rhythm under DD and LL continued for 2 consecutive days, which is not an economical aspect in silkworm hatching particularly and silkworm rearing primarily. Further, hatching rhythmic patterns were broadened (damped-out) under LL which adds further complications to hatching phenomenon. The economic point in hatching durations is also clearly demonstrated when the hatching durations under all four test conditions (LD 12:12, DD, LL and black-boxing, Fig. 5) are compared. Thus the hatching durations are very less (2 ± 0.67 h) under black-boxing conditions (Fig. 5.), while these are around 6 h under LD 12:12 conditions and more than 24 h under the remaining two experimental conditions (DD and LL). Towards the extreme position, hatching durations under

LL were too high (34 h, Fig. 5).

Hence, the hatching experiments with three photoperiodic conditions, LD 12:12, DD and LL are considered more as of academic interest while that under black-boxing system as of industrial interest. Ultimately, it is concluded that i. oscillator controlling the egg hatching is noticed to be at pin-head stage (therefore, an appropriate point of time for initiating black-boxing), b. darkness in the black-boxing has to be interrupted by exposing the eggs under black-boxing at 06.00 h on the ultimate day of hatching and continued in light till completion of hatching, c. hatching is restricted to a single day and single peak, d. hatching under black-boxing system of technology did not extend to next day, e. hatching durations are economically low, recording only 2 hours with high and quick hatching

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