On the implications of temperature and humidity on egg hatching in the commercial silkworm, *Bombyx mori* L.

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Abstract: In the mulberry silkworm, *Bombyx mori* L., the first developmental marker event, the egg hatching is the most important one for the beginning of successful commercial silkworm cocoon crop. Many external factors such as photoperiod, temperature, humidity etc., directly affect hatching in silkworm. Two popular silkworm hybrids, PM x CSR2 (multivoltine x bivoltine) and CSR2 x CSR4 (bivoltine x bivoltine) were selected for hatching studies. Three photoperiodic conditions, LD 12 : 12, DD and LL were adopted. In combination, three temperature (25, 30 and 35 °C) and three relative humidity conditions (RH, 60, 70 and 80%) were considered for the study. In total, data on hatching patterns, hatching percentage and other related aspects of 2 silkworm hybrids x 3 photoperiodic x 3 temperature x 3 humidity conditions x 5 replications (= 270 individual batches) were recorded and analyzed. Hatching was very distinct, confining to a single day, with PM x CSR2 under LD 12 : 12 condition. Peak hatching occurred at or immediately after 'lights-on', taking it as the synchronizing signal. Eggs of CSR2 x CSR4 hatched for two consecutive days, with stray hatching on day-1 and maximum hatching on day-2, indicating circadian control over hatching. Driven under continuous conditions (DD/LL), hatching occurred for 2 days. Hatching was low on the day-1 and high on day-2 under both DD and LL conditions. Further, hatching distribution broadened with decrease in amplitude, indicating 'damp-out' or 'near damp-out' situation, especially with CSR2 x CSR4 under LL. Thus, *B. mori* hatching rhythm was circadian, diurnal, taking lights-on as synchronizing signal and followed the 'gating' phenomenon.

Keeping ambiguity of presenting 54 chronograms of hatching (for 2 hybrids x 3 photoperiodic conditions x 3 temperature x 3 humidity conditions), data on hatching patterns were computed for circular variables like mean vector (mean ϕ), length of mean vector (r) and angular standard deviation (s) using circular statistics. Mean vector indicated that hatching rhythmic patterns are not much affected by temperature and humidity conditions. However, r and s values varied significantly, indicating that height of peak hatching decreased and broadening of hatching increased under high temperature and low humidity conditions. Hatching percentage was more affected by high temperature and low humidity, decreasing hatching magnitude to below economical hatching. Similarly, high temperature and low humidity implicated in increased number of dead eggs. High temperature resulted in scorching and low humidity in desiccation of silkworm eggs. Results are discussed in terms of contemporary economic hatching in silkworm.

Keywords: Silkworm, *Bombyx mori* L., hatching, photoperiod, temperature, humidity.

1. INTRODUCTION

Many aspects of insect overt phenomena are clock controlled. These clocks get cues from external sources or environment like photoperiod, temperature and humidity. Photoperiod has been referred to as the strongest environmental factor affecting the overt phenomena than temperature and humidity. Among various overt phenomena, egg hatching is the most extensively studied as influenced by photoperiod. Egg hatching is a profoundly important event in the life history of

lepidopterans. In the silkworm, *Antheraea pernyi*, a behavioral programme is initiated in the early morning hours on the day of hatching whereby the pharate larva begins to gnaw its way out of the egg (Riddiford and Johnson, 1971; Sauman and Reppert, 1998). Sivarami Reddy and Sasira Babu, 1990; Sivarami Reddy *et al*., 1984; 1993a & b) reported egg hatching and various other phenomenon showing cyclical fluctuations in *B. mori*. Egg hatching is the earliest recognized behavior under circadian control in the silkworm (Sauman and Reppert, 1998). Similarly, implications of high temperature and low humidity on hatching were reported by Lakshminarayana Reddy (2001). An extensive account on the effects of high temperature and low humidity on pupation patterns in *B. mori* was published by Lakshminarayana Reddy *et al*. (2002). The present investigation describes the comprehensive effects of photoperiod in association with temperature (25, 30 and 35 °C) and humidity (RH, 60, 70 and 80%) on egg hatching patterns, as also hatching (%) and number of dead eggs in two popular commercial silkworm hybrids (PM x CSR2 and CSR2 x CSR4).

2. MATERIALS AND METHODS

Two popular silkworm hybrids; PM x CSR2 (multivoltine x bivoltine) and CSR2 x CSR4 (bivoltine x bivoltine) of the mulberry silkworm, *Bombyx mori* (L) were employed as experimental animals for the present study. Disease free layings (DFLs, each DFL is a group of 400 to 500 silkworm eggs laid by a single silk moth on a single day on specific egg laying sheet of paper) of these silkworm hybrids were procured, on the third day of oviposition, from the Silkworm Seed Production Centre (SSPC), National Silkworm Seed Organization (NSSO), Madanapalli, Chittoor District, Andhra Pradesh, India. The DFLs were transported to the Department of Sericulture, Sri Krishnadevaraya University, Anantapuramu during evening cool hours and immediately spread onto the pre disinfected plastic rearing trays (Nilkamal, India). The first developmental marker event in the life cycle of the silkworm, the hatching was studied. Five DFLs each of PM x CSR2 and CSR2 x CSR4 were kept primarily separately under three photoperiodic condition; natural solar day (LD 12 : 12), continuous dark (DD) and continuous light (LL). Three temperature (20, 25 and 30 °C) and three humidity (RH) conditions (60, 70 and 80%) were enrolled to study the implications on hatching. Hatching experiments were conducted in an environmental chamber (Kolarstat). Silkworm hatch from their eggs, generally, after 10 days of oviposition. The light yellowish coloured silkworm eggs turn into blue colour on or immediately after the eighth day (blue egg stage, Narasimhanna, 1987; Datta, 1992) of oviposition; the silkworm larvae hatch from the eggs two days from this specific stage (Datta, 1992). Rhythmic patterns in hatching of silkworm eggs was studied under the specified photoperiodic schedules (LD 12 : 12, DD and LL). The 24 h natural solar day was divided into 12 h dark part (scotophase) and 12 h light part (photophase). The photophase was initiated from 06.00 h and lasted 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 bulb, as light source for illuminating the experimental silkworm eggs during photophase of the day was arranged above the rearing tray, its height from the rearing tray was so monitored that the light intensity at the surface where the experimental silkworm eggs are exposed did not exceed 50 lux (Sivarami Reddy, 1993). For illuminating the eggs throughout the day (LL, 24 h), under constant conditions (around 50 lux), a 60 W bulb as light source was arranged. For the experimental silkworm eggs under continuous dark (DD) did not been provided with light. However, a dim red light source (below 0.1 lux, Sivarami Reddy, 1993) was utilized for efficient counting of silkworm larvae that hatched out from time to time. Added to the three primary photoperiodic conditions, three temperature (20, 25 and 30 °C) and three relative humidity (RH; 60, 70 and 80%) conditions were employed. In total, 2 silkworm hybrids x 3 photoperiodic conditions x 3 temperature conditions x 3 RH conditions with 5 replications each (2 x 3 x 3 x 3 x 5 = 270) were maintained. Precise number of silkworm larvae that hatched-out from silkworm eggs was counted, at one hour interval and recorded. The larvae, thus counted were regularly and gently transferred into separate rearing trays with a feather brush to avoid mixing.

Data on hatching thus recorded were represented as histograms (distribution diagrams, hourly histograms, resolved for 24 h, ω = 360º) and peak hour of hatching was identified. Using the values on peak hour, the unit (individual) vector of each replication was calculated (resolving a day or 24 hrs into a complete cycle, w = 360°). From these values, the circular variables like mean vector (mean □), length of mean vector (r) and angular standard deviation (s) corresponding to hatching was computed through circular statistics (Chassé and Théron, 1988). The data on these values (mean □, r and s) were then analyzed statistically using the test of Mardia- Watson-Wheeler (Mardia, 1972) to test the significance of differences, if any. Further, the data on pupation rate (%) and number of dead eggs were also recorded and analyzed statistically (ANOVA, 2 - way classification with 5 observations per cell).

3. RESULTS

Hatching patterns under natural day conditions (LD 12 : 12, temperature of 25 °C and RH of 80%): Silkworm egg hatching under natural day condition (LD 12 : 12) with temperature of 25 °C and relative humidity of 80% for PM x CSR2 is resolved as chronogram and presented in Figure 1. As seen from the figure, hatching in PM x CSR2 initiated at 06.00 h in the morning and completed by 09.00 h. The peak of hatching was seen at 08.00 h, thus hatching occurring in the morning and phase-locked to dawn. Hatching was very sharp and seen only on a single day.
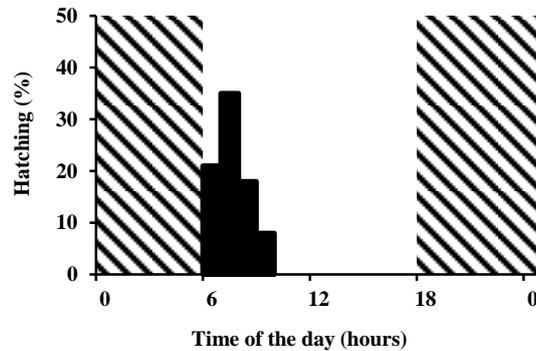

Figure 1: Distribution of hatching (%) in PM x CSR2 of *Bombyx mori* L. under LD 12 : 12 conditions with temperature of 25 °C and RH of 80%. Note entire hatching on a single day. Also note that the hatching occurred just after 'lights-on' phase of the LD cycle. Cross-hatched area in the histogram indicates the dark phase imposed and the open area, the light phase of the day.

Similarly, hatching of CSR2 x CSR4 silkworm hybrid under natural solar day condition (LD 12 : 12) conditions with temperature of 25 °C and relative humidity of 80% are presented as chronograms in Figure 2.
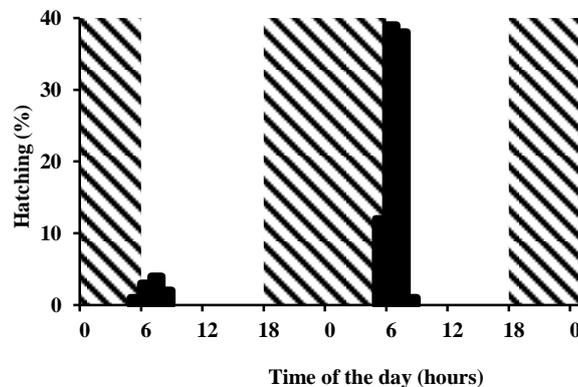

Figure 2: Distribution of hatching (%) in CSR2 x CSR4 of *Bombyx mori* L. under LD 12:12 conditions with temperature of 25 °C and RH of 80%. Note stray hatching on day-1 and maximum hatching on day-2 (gating-phenomenon). Also note that the hatching occurred around 'lights-on' phase of the LD cycle. Cross-hatched area in the histogram indicates the dark phase imposed and the open area, the light phase of the day.

In the case of CSR2 x CSR4, hatching under natural day condition (LD 12 : 12) with temperature of 25 °C and relative humidity of 80% occurred for two consecutive days with stray hatching on day-1 and maximum hatching on day-2. The interval between two peaks of hatching was ≈ 24 h and hence circadian rhythmicity expression. Hatching occurred at or just after lights-on (dawn) phase and hence diurnal with phase-locked to dawn. The peak expression of hatching in CSR2 x CSR4 was sharp with distinct expression.

Hatching patterns under Continuous dark conditions (DD, temperature of 25 °C and RH of 80%): Egg hatching in multivoltine x bivoltine silkworm hybrid, PM x CSR2 is presented in Figure 3.
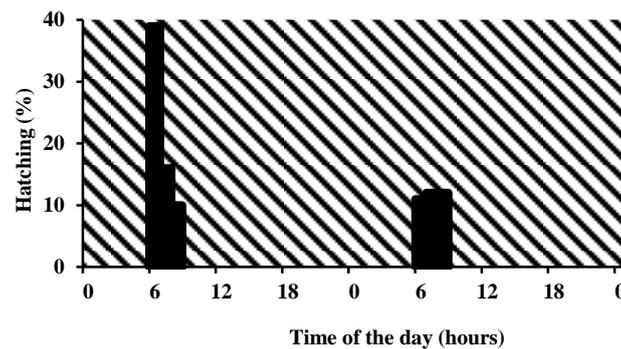
Figure 3: Distribution of hatching (%) in PM x CSR2 of *Bombyx mori* L. under DD conditions with temperature of 25 °C and RH of 80%. Note hatching on two consecutive days with more hatching on day-1 and less hatching on day-2. Also note that the hatching occurred just at/after 06.00 h of the LD cycle on either day, peaks occurring 24 h apart from each other, indicating a circadian hatching system. Cross-hatched area in the histogram indicates the dark phase (DD) imposed.

Hatching occurred for two consecutive days in PM x CSR2, with more hatching on day-1 and less hatching on day-2. Hatching occurred just at/after 06.00 h of the LD cycle on either day. Duration between peaks hatching was ≈ 24 h, indicating circadian control. Hatching rhythmicity on both days was very sharp.

Hatching in CSR2 x CSR4 silkworm hybrid of *B mori* under continuous dark conditions (DD) with temperature of 25 °C and RH of 80% are presented in Figure 4. As observed for PM x CSR2, hatching in CSR2 x CSR4 was also appeared for two consecutive days with more hatching on day-1 and less on day-2. Hatching occurred just before lights-on phase on either day, peeks being 24 h apart from each other. Therefore, hatching in CSR2 x CSR4 is circadian and phase locked to lights-on. Expression of hatching was free-running (Figure 4).
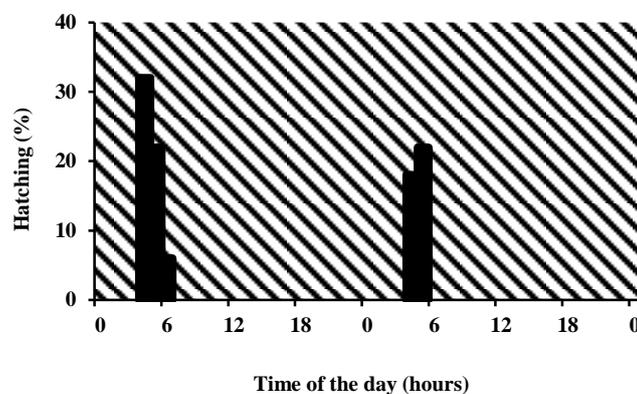

Figure 4: Distribution of hatching (%) in CSR2 x CSR4 of *Bombyx mori* L. under DD conditions with temperature of 25 °C and RH of 80%. Note hatching on two consecutive days with more hatching on day-1 and less hatching on day-2. Also note that the hatching occurred just before 'lights-on' phase of the LD cycle on either days, peaks occurring 24 h apart from each other, indicating a circadian and free-running hatching system. Cross-hatched area in the histogram indicates the dark phase (DD) imposed.

Hatching patterns under Continuous light conditions (LL, temperature of 25 °C and RH of 80%):

Hatching patterns of PM x CSR2 under continuous light (LL) with temperature of 25 °C and RH of 80% are presented in Figure 5. Hatching in PM x CSR2 (multivoltine x bivoltine silkworm) hybrid of *B mori* under continuous light conditions (LL) with temperature of 25 °C and RH of 80% are presented in Figure 5. Hatching appeared for two consecutive days with more hatching on day-2 and less on day-1. Hatching occurred just after lights-of phase on either day, peeks being 24 h apart from each other. Therefore, hatching in PM x CSR2 is circadian, phase locked to lights-on phase. As the hatching is occurring at the same time on both days, it is expressing the free-running periodicity (Figure 5).
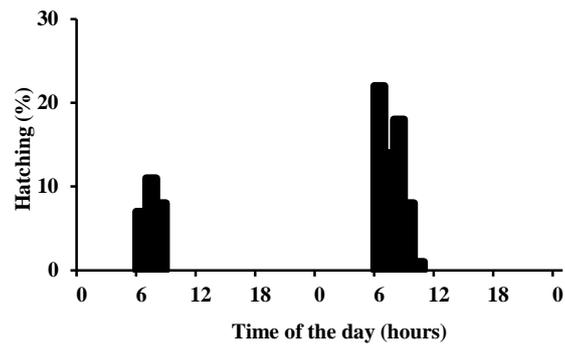
Figure 5: Distribution of hatching (%) in PM x CSR2 of *Bombyx mori* L. under LL conditions with temperature of 25 °C and RH of 80%. Note hatching on two consecutive days with more hatching on day-2 and less on day-1. Also note hatching occurred just after 'lights-on' phase of the LD cycle on either day, peaks occurring 24 h apart from each other, indicating a circadian and free-running hatching system.

Similarly, hatching in CSR2 x CSR4 of *Bombyx* silkworm eggs occurred for two consecutive days. Hatching in CSR2 x CSR4 bivoltine x bivoltine silkworm hybrid of *B mori* under continuous light conditions (LL) with temperature of 25 °C and RH of 80% are presented in Figure 6. Hatching in CSR2 x CSR4 appeared for two consecutive days, with more hatching on day-2 and less on day-1. Hatching occurred after 06.00 h on either day, peeks being 24 h apart from each other. Therefore, hatching in CSR2 x CSR4 is circadian. However, hatching peak delayed to occur at mid-part of the day indicating free running nature (Figure 6).
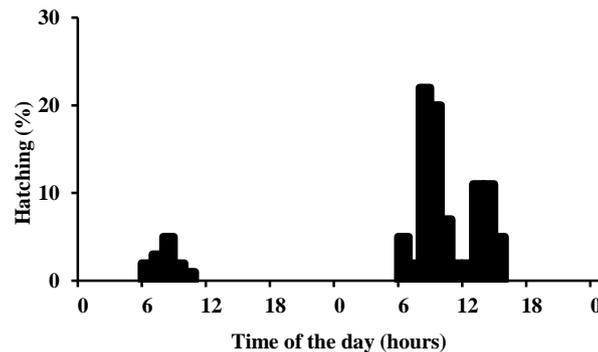

Figure 6: Distribution of hatching (%) in CSR2 x CSR4 of *Bombyx mori* L. under LL conditions with temperature of 25 °C and RH of 80%. Note hatching on two consecutive days with more hatching on day-2 and less hatching on day-1. Also, note that the hatching occurred after 06.00 h with more delayed towards mid-part of the LD cycle on either day, peaks occurring 24 h apart from each other, indicating a circadian and free-running hatching system.

Hatching patterns under natural day (LD 12 : 12) conditions and low temperature (25 °C) and low humidity (RH of 60%):

Hatching in PM x CSR2 under natural day (LD 12 : 12) conditions and low temperature (25 °C) and low humidity (RH of 60%) are presented as histograms in Figure 7.

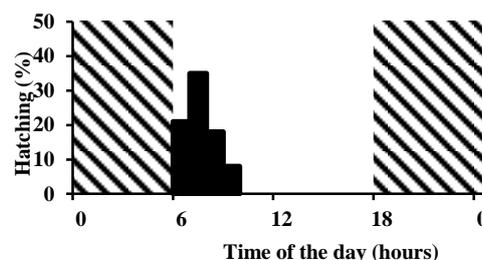**Figure 7: Distribution of hatching (%) in PM x CSR2 of *Bombyx mori* L. under LD 12 : 12 conditions with low temperature of 25 °C and low humidity, RH of 60%. Note entire hatching on a single day. Also note that the hatching occurred just after 'lights-on' phase of the LD cycle. Cross-hatched area in the histogram indicates the dark phase imposed and the open area, the light phase of the day.**

Page | 30

The patterns in hatching in PM x CSR2 under low temperature and low humidity was similar to that observed for conditions of LD 12 : 12, temperature of 25 °C and humidity of 80% (Figure 1). Perusal of the chronogram (Figure 7), it is clear that the hatching rhythmic characters were similar, hatching occurring immediately after lights-on phase (06.00 h) with peak expression at 07.00 h and slightly broadening in hatching histogram. Further, the phase is locked to lights-on signal with diurnal expression. Added, entire hatching occurred on a single day.

Hatching in CSR2 x CSR4 under natural day (LD 12 : 12) conditions with low temperature (25 °C) and low humidity (RH of 60%) are presented as histograms in Figure 8. Hatching patterns were similar to those observed for CSR2 x CSR4 under LD 12 : 12 conditions with 25 °C temperature and RH of 80%. Thus, hatching occurred after lights-on phase for two consecutive days, with low or stray hatching on day-1 and high hatching on day-2. The peak hatching occurred at or after 06.00 h with broadening of hatching chronograms. Thus, the hatching rhythm is diurnal, phase locked to lights-on, circadian and broadening of hatching activity.
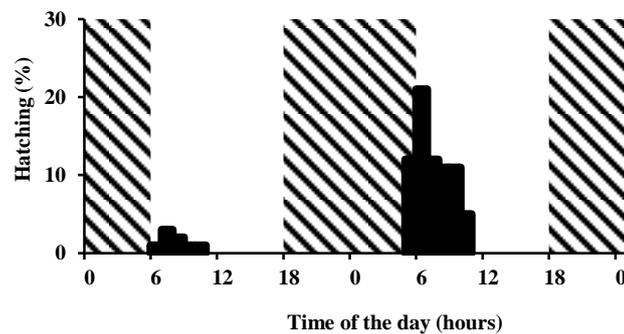

Figure 8: Distribution of hatching (%) in CSR2 x CSR4 of *Bombyx mori* L. under LD 12 : 12 conditions with temperature of 25 °C and low humidity, RH of 60%. Note entire hatching on a two consecutive days. Also note that the hatching occurred just after 'lights-on' phase of the LD cycle. Cross-hatched area in the histogram indicates the dark phase imposed and the open area, the light phase of the day.

Hatching patterns under natural day (LD 12 : 12) conditions with low temperature (25 °C) and medium humidity (RH of 70%):

Hatching patterns of PM x CSR2 under natural day (LD 12 : 12) conditions with low temperature (25 °C) and medium humidity (RH of 70%): The hatching patterns of PM x CSR2 under natural day (LD 12 : 12) photoperiodic condition with temperature of 25 °C and medium humidity (RH of 70%) are presented in Figure 9.
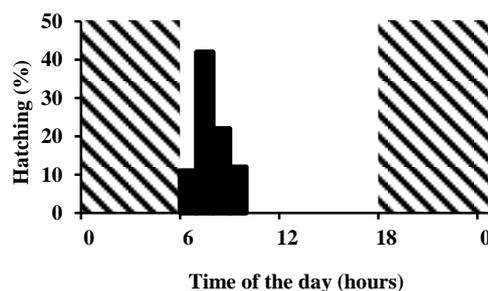

Figure 9: Distribution of hatching (%) in PM x CSR2 of *Bombyx mori* L. under LD 12 : 12 conditions with temperature of 25 °C and medium humidity, RH of 70%. Note entire hatching on a single day. Also note that the hatching occurred just after 'lights-on' phase of the LD cycle. Cross-hatched area in the histogram indicates the dark phase imposed and the open area, the light phase of the day.

The patterns in hatching in PM x CSR2 under LD 12 : 12 condition with temperature of 25 °C and medium humidity of 70% RH resembled that observed for conditions of LD 12 : 12, temperature of 25 °C and humidity of 80% (Figure 1). Perusal of the chronogram, it is clear that the hatching rhythmic characters were similar, hatching occurring immediately after lights-on phase (06.00 h) with peak expression at 07.00 h and slightly broadening in hatching histogram. Further, the phase is locked to lights-on signal with diurnal expression. Added, entire hatching occurred on a single day.

Hatching patterns of CSR2 x CSR2 under natural day (LD 12 : 12) conditions and low temperature (25 °C) and medium humidity (RH of 70%): The hatching patterns of CSR2 x CSR4 under natural day (LD 12 : 12) photoperiodic condition with temperature of 25 °C and medium humidity (RH of 70%) are presented in Figure 10.
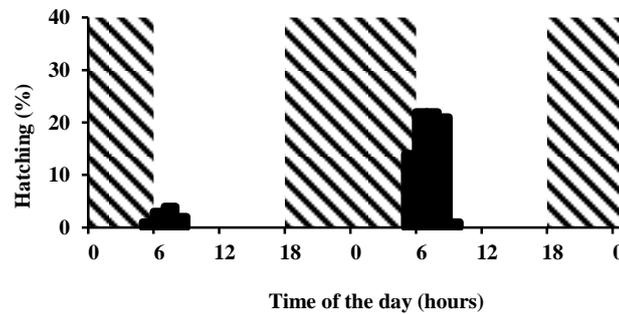

Figure 10: Distribution of hatching (%) in CSR2 x CSR4 of *Bombyx mori* L. under LD 12 : 12 conditions with low temperature of 25 °C and medium humidity, RH of 70%. Note hatching on two consecutive days. Also note that the hatching occurred just after 'lights-on' phase of the LD cycle. Cross-hatched area in the histogram indicates the dark phase imposed and the open area, the light phase of the day.

Hatching in CSR2 x CSR4 under natural day (LD 12 : 12) conditions and low temperature (25 °C) and low humidity (RH of 70%) are presented as histograms in Figure 10. Hatching patterns were similar to those observed for CSR2 x CSR4 under LD 12 : 12 conditions with 25 °C temperature and RH of 80%. Thus, hatching occurred after lights-on phase for two consecutive days, with low or stray hatching on day-1 and high hatching on day-2. The peak hatching occurred at or after 06.00 h with broadening of hatching chronograms. The hatching rhythm was diurnal, phase locked to lights-on, circadian and broadening of hatching activity.

Studies on the rhythm characteristics: Since the temperature and humidity with two silkworm hybrids resulted in huge amount of graphs (histograms), it is not only difficult for comparison but also leading to bewilderment. In such situations, resolving the data on hatching patterns for two silkworm hybrids (PM x CSR2 and CSR2 x CSR4) under three temperature conditions (25, 30 and 35 °C) with three humidity conditions (RH, 60, 70 and 80%) into three rhythmic characteristics, mean vector (mean □), length of mean vector (r) and angular standard deviation (s) calculated through circular statistics were felt appropriate. The rhythmic characteristics of this developmental marker event (hatching), the circular variables computed through circular statistics like mean vector (mean □), length of mean vector (r) and angular standard deviation (s) of multivoltine x bivoltine silkworm hybrid, PM x CSR2 are given in Table 1. Similarly, the rhythmic characteristics for hatching of bivoltine x bivoltine silkworm hybrid, CSR2 x CSR4 are given in Table 2. The data presented in Table 1 and 2 clearly indicate that the hatching occurred at or around 06.00 h, the onset of photophase, as the mean vector scattered to occur at or around 90°. The advancement in mean □□ from 90° in LD 12 : 12 condition into dark phase has been observed for DD conditions, both for PM x CSR2 and CSR2 x CSR4, indicating that the peak expression in hatching is advancing into dark phase of the day under continuous dark (DD). On the other hand the mean □ under LL conditions, delayed into light part of the day, from 90° for LD 12 : 12, indicating that the peak hatching expression occurred away from 06.00 h to somewhere near 12.00 (180°). Both the cases of DD and LL clearly indicated the free running rhythmicity of hatching. However, these variations are not significant. The delay in hatching rhythm has been more intense under low humidity coupled with high temperature (Table 1 and 2).

The length of the mean vector (r) and their angular variance (s) also differed greatly. As the imposed temperature varied, these values also varied. The least value of the length of mean vector (r) was observed for PM x CSR2 hatching (0.66) and for CSR2 x CSR4 (0.64) under LL and imposed high temperature and low RH of 35°C and 70% (Table 1 and 2), indicating that the hatching magnitude at high temperature and low humidity considerably reduced. Similarly, highest value for s was observed for CSR2 x CSR4 (21.44°) under the same temperature and humidity conditions (Table 2). The highest value of r and least values of s were recorded for two silkworm hybrids under imposed temperature and humidity conditions with LL. It is generally observed that the length of mean vector and the angular variance are inversely related. Thus, the mean □ did not vary much compared to all photoperiodic, temperature and humidity conditions, but the r and s values significantly varied.

Table 1: Rhythmic characteristics (circular variables, mean □, r and s) of hatching in PM, CSR2 of the silkworm, *Bombyx mori* L. under LD 12:12, DD and LL with different temperature conditions in combination of different humidity conditions. (n=5).

Photoperiodic condition	Circular variables	RH at 25 °C			RH at 30 °C			RH at 35 °C		
		60%	70%	80%	60%	70%	80%	60%	70%	80
LD 12:12	Mean φ	90°	90°	90°	105°	105°	105°	120°	120°	105°
	r	0.91	0.92	0.98	0.99	0.96	0.89	0.99	0.99	0.99
	s	5.66°	4.89°	4.99°	5.99°	6.66°	6.43°	6.11°	6.41°	5.88°
DD	Mean φ	60°	60°	60°	75°	75°	75°	75°	90°	75°
	r	0.96	0.99	0.89	0.92	0.96	0.91	0.92	0.96	0.94
	s	8.22°	8.78°	10.12°	8.91°	14.44°	12.64°	18.88°	17.99°	21.22°
LL	Mean φ	120°	150°	135°	180°	180°	150°	150°	150°	120°
	r	0.88	0.86	0.85	0.81	0.79	0.76	0.69	0.71	0.66
	s	17.22°	14.99°	17.22°	18.42°	24.22°	26.33°	28.44°	27.66°	31.66°

Table 2: Rhythmic characteristics (circular variables, mean □, r and s) of hatching in CSR2 x CSR4 of the silkworm, *Bombyx mori* L. under LD 12:12, DD and LL with different temperature conditions in combination of different humidity conditions. (n=5).

Photoperiodic condition	Circular variables	RH at 25 °C			RH at 30 °C			RH at 35 °C		
		60%	70%	80%	60%	70%	80%	60%	70%	80
LD 12:12	Mean φ	105°	90°	90°	105°	105°	105°	120°	120°	105°
	r	0.99	0.99	0.99	0.99	0.98	0.98	0.96	0.96	0.96
	s	5.99°	5.96°	5.99°	0.59°	7.66°	8.23°	7.99°	8.14°	9.66°
DD	Mean φ	90°	75°	75°	75°	75°	90°	90°	75°	90°
	r	0.99	0.87	0.92	0.96	0.94	0.96	0.92	0.92	0.94
	s	15.66°	14.82°	15.54°	17.22°	22.15°	20.46°	21.44°	23.14°	26.16°
LL	Mean φ	90°	75°	75°	75°	75°	90°	90°	75°	90°
	r	0.99	0.87	0.92	0.96	0.94	0.96	0.92	0.92	0.64
	s	15.66°	14.82°	15.54°	17.22°	22.15°	20.46°	21.44°	23.14°	26.16°

Hatching percentage: The hatching in silkworm is the crucial first step for the beginning of commercial silkworm rearing. Amount of eggs hatched (hatching percentage) decides the entire success of the cocoon crop. Hatching percentages for the two silkworm hybrids (PM x CSR2 and CSR2 x CSR4) under designated photoperiodic conditions (LD 12 : 12, DD and LL) exposed to selected temperature (25, 30 and 35 °C) in combination with selected relative humidity (RH, 60, 70 and 80%) conditions were calculated from recorded data and presented in Table 3 for PM x CSR2 and in Table 4 for CSR2 x CSR4. From the data, it is clear that hatching percentage affected by all the imposed conditions; photoperiod, temperature and humidity. Precisely, hatching was high with temperature of 25 and 30 °C in combination with RH of 80% under LD 12 : 12 and DD conditions. On the other hand, hatching was low with LL and with high temperature combined with low humidity conditions. Highest hatching (%) was observed for PM x CSR2 exposed to 25 °C at RH of 80% (Table 3) and for CSR2 x CSR4 at 25 °C combined with RH of 80% (Table 4). The lowest hatching was recorded for both the silkworm hybrids exposed to 30 °C and 60% RH (Table 3 and 4). Differences in hatching (%) recorded were highly significant ($p < 0.01$).

Table 3: Hatching percentages for PM x CSR2 under photoperiodic conditions (LD 12 : 12, DD and LL) exposed to selected temperature (25, 30 and 35 °C) in combination with selected relative humidity (RH, 60, 70 and 80%). Values are mean of five replications (n = 5).

Photoperiodic conditions	Description	RH at 25 °C			RH at 30 °C			RH at 35 °C		
		60%	70%	80%	60%	70%	80%	60%	70%	80
LD 12:12	Average	91.400	93.200	96.800	90.200	88.600	92.000	84.600	85.800	82.000
	SD (±)	1.342	2.280	1.789	3.271	2.074	1.581	4.336	2.864	4.848
DD	Average	92.600	96.000	98.200	92.200	94.400	95.000	91.200	90.200	86.000
	SD (±)	1.673	1.414	0.837	2.775	2.510	2.828	2.168	3.421	3.391
LL	Average	91.000	87.800	95.000	90.200	87.000	90.200	89.800	86.600	86.600
	SD (±)	3.674	2.775	2.550	2.950	3.808	1.304	3.564	3.975	4.722

Table 4: Hatching percentages for CSR2 x CSR4 under photoperiodic conditions (LD 12 : 12, DD and LL) exposed to selected temperature (25, 30 and 35 °C) in combination with selected relative humidity (RH, 60, 70 and 80%). Values are mean of five replications (n = 5).

Photoperiodic conditions	Description	RH at 25 °C			RH at 30 °C			RH at 35 °C		
		60%	70%	80%	60%	70%	80%	60%	70%	80
LD 12:12	Average	91.400	93.200	96.800	85.600	88.600	91.400	78.600	85.800	82.000
	SD (±)	1.342	2.280	1.789	3.050	2.074	2.408	4.393	2.864	4.848
DD	Average	92.400	95.200	97.600	96.800	85.400	87.000	88.000	87.000	82.400
	SD (±)	2.702	2.588	1.140	1.789	3.912	3.271	1.000	6.042	3.286
LL	Average	87.200	89.200	90.800	82.600	85.600	87.000	80.200	81.800	81.200
	SD (±)	3.114	2.387	2.387	4.615	4.775	1.643	2.387	5.357	4.712

Data were further averaged for all temperature (25, 30 and 35 °C) and relative humidity (60, 70 and 80%), irrespective of photoperiodic conditions exposed. Thus, computed data on hatching response to temperature for PM x CSR2 are presented in Figure 11 and hatching response to humidity with PM x CSR2 in Figure 12. It can be seen that hatching is indirectly related to increase in temperature with equation of $y = -4.833x + 98.48$ and $R^2 = 0.997$. The differences in hatching against temperature are thus statistically highly significant ($p < 0.01$) as also evidenced by R^2 value.
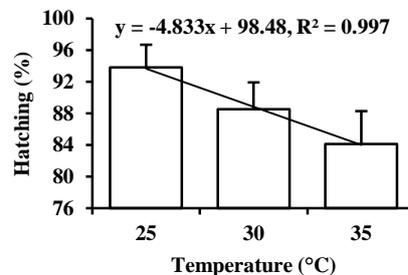

Figure 11: Response of hatching (%) in silkworm (*Bombyx mori* L) hybrid, PM x CSR2 eggs to exposed temperature conditions irrespective of photoperiod and humidity (n =15).

Data on response of hatching to humidity in PM x CSR2 irrespective of photoperiod and temperature are presented in Figure 12. Perusal of data revealed that hatching directly responded to enrolled humidity conditions. Thus, hatching was low (below 90%) with low humidity (60% RH) and increased to over 90% with high RH of 80%. The curve equation was $y = 1.566x + 86.8$ with $R^2 = 0.858$. The equation also denotes that hatching is directly related to humidity conditions.
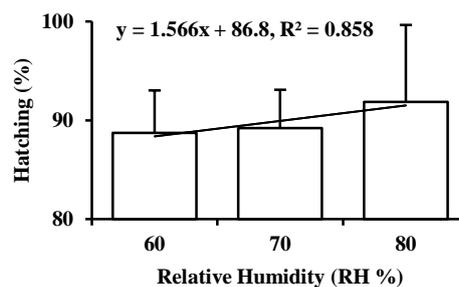

Figure 12: Response of hatching (%) in silkworm (*Bombyx mori* L) hybrid, PM x CSR2 eggs to exposed humidity conditions irrespective of photoperiod and temperature (n =15).

Similarly, the computed data on hatching response to temperature in CSR2 x CSR4 irrespective of photoperiodic and humidity conditions are presented in Figure 13. The curve equation was $y = -5.833x + 99.82$ with $R^2 = 0.996$, thus indicating negative correlation of hatching to temperature conditions. At low temperature of 25 °C, hatching in CSR2 x CSR4 was very economical, with hatching of around 95% (Figure 13). Hatching was uneconomical (around 80%) with increased temperature of 35 °C. The differences in hatching (%) responding to exposed temperatures are statistically highly significant ($p < 0.01$) as also supported by R^2 value (0.996). Thus, temperature inversely affected hatching in CSR2 x CSR4 and the relation is negative to temperature.
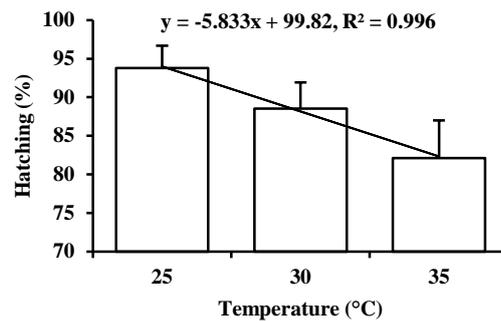
Figure 13: Response of hatching (%) in silkworm (*Bombyx mori* L) hybrid, CSR2 x CSR4 eggs to exposed temperature conditions irrespective of photoperiod and humidity (n =15).

Computed data on hatching response to humidity in CSR2 x CSR4 irrespective of photoperiodic and temperature conditions are presented in Figure 14. The curve equation was $y = 2.433x + 83.28$ with R^2 value of 0.878, thus indicating direct relation of hatching to humidity conditions. At low RH of 60%, hatching in CSR2 x CSR4 was non-economical, recording less than 85% hatching (Figure 14). Hatching was economical (over 90%) with increased humidity of 80%. The differences in hatching (%) in responding to exposed humidity are statistically highly significant (*p < 0.01*) as also supported by R^2 value (0.878). Thus, humidity directly affected hatching in CSR2 x CSR4 and the relation is a direct one to humidity.
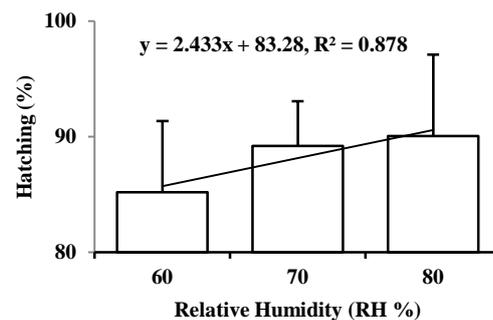

Figure 14: Response of hatching (%) in silkworm (*Bombyx mori* L) hybrid, CSR2 x CSR4 eggs to exposed humidity conditions irrespective of photoperiod and temperature (n =15).

Number of dead eggs: While counting eggs for accounting hatching percentage in DFLs of two silkworm hybrids, PM x CSR2 and CSR2 x CSR4, certain number of dead eggs were found that were not present at the beginning of hatching experiments. These eggs were counted and recorded for each hybrid under three photoperiodic combination (LD 12 : 12, DD and LL) exposed to three temperature (25, 30 and 35 °C) in combination with three relative humidity conditions (RH, 60, 70 and 80%). Such data are presented in Table 5. For PM x CSR2 and in Table 6 for CSR2 x CSR4.

Table 5: Number of dead eggs observed in DFLs of PM x CSR2 under photoperiodic conditions (LD 12 : 12, DD and LL) exposed to selected temperature (25, 30 and 35 °C) in combination with selected relative humidity (RH, 60, 70 and 80%). Values are mean of five replications (n = 5).

Photoperiodic conditions	Description	RH at 25 °C			RH at 30 °C			RH at 35 °C		
		60%	70%	80%	60%	70%	80%	60%	70%	80
LD 12:12	Average	0.200	0.400	0.000	1.000	0.400	0.800	5.600	1.800	1.400
	SD (±)	0.447	0.894	0.000	0.707	0.894	1.095	2.074	0.837	0.548
DD	Average	0.600	0.600	0.000	0.600	0.400	0.400	2.600	0.600	1.000
	SD (±)	1.342	0.548	0.000	0.548	0.548	0.548	1.949	0.894	0.000
LL	Average	0.800	0.600	0.400	0.600	0.400	0.600	8.000	2.800	0.800
	SD (±)	0.447	0.894	0.894	0.894	0.548	0.548	1.581	2.950	0.447

Table 6: Number of dead eggs observed in DFLs of CSR2 x CSR4 under photoperiodic conditions (LD 12 : 12, DD and LL) exposed to selected temperature (25, 30 and 35 °C) in combination with selected relative humidity (RH, 60, 70 and 80%). Values are mean of five replications (n = 5).

Photoperiodic conditions	Description	RH at 25 °C			RH at 30 °C			RH at 35 °C		
		60%	70%	80%	60%	70%	80%	60%	70%	80
LD 12:12	Average	0.600	1.000	0.000	0.600	0.200	0.600	7.600	0.600	2.200
	SD ($\pm$)	0.894	1.414	0.000	0.894	0.447	0.548	2.074	0.548	1.304
DD	Average	0.600	0.400	0.200	0.200	0.600	0.600	5.600	1.000	1.200
	SD ($\pm$)	0.894	0.548	0.447	0.447	1.342	0.548	2.510	1.225	0.837
LL	Average	0.400	0.800	0.200	0.800	0.400	1.200	10.800	0.800	1.800
	SD ($\pm$)	0.548	1.304	0.447	0.837	0.894	1.643	2.387	0.837	0.837

Interesting observation is that the number of dead eggs is more under high temperature and low humidity conditions and the same are almost nil under low temperature and high humidity conditions. Thus, number of dead eggs was very almost nil under LD 12 : 12, DD and LL with 25 °C of temperature and 80% RH for PM x CSR2. Surprisingly, number of dead eggs was 8 for PM x CSR2 under LL with 35 °C and 60% RH. The trend in number of dead eggs with CSR2 x CSR4 was similar to that of PM x CSR2, however, with increase in number of dead eggs for CSR2 x CSR4 (11) under LL condition with 35 °C temperature and 60% RH conditions.

Data were further averaged for all temperature (25, 30 and 35 °C) and relative humidity (60, 70 and 80%), irrespective of photoperiodic conditions exposed. Thus, computed data on number of dead eggs in response to temperature for PM x CSR2, irrespective to photoperiodic condition and humidity conditions are presented in Figure 15 and in response to humidity in Figure 16. It can be seen that number of dead eggs is directly related to increase in temperature with curve equation of $y = 1.366x - 1.444$ and $R^2 = 0.889$. The differences in number of dead eggs against temperature are statistically highly significant ($p < 0.01$) as also evidenced by R^2 value (0.889).
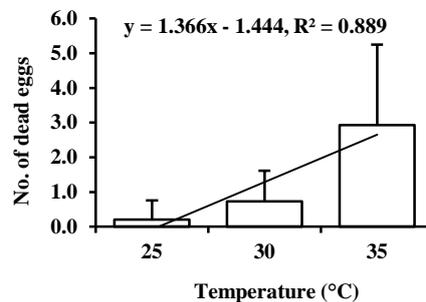

Figure 15: Response of number of dead eggs in DFLs of silkworm (*Bombyx mori* L) hybrid, PM x CSR2 to exposed temperature conditions irrespective of photoperiod and humidity (n =15).

Data on response of number of dead eggs in DFLs of PM x CSR2 to humidity, irrespective of photoperiod and temperature are presented in Figure 16. Perusal of data revealed that dead eggs directly responded to enrolled humidity conditions. Thus, number of dead eggs was high (around 2) with low humidity (60% RH) and decreased to over almost nil with high RH of 80%. The curve equation was $y = -0.766x + 2.822$ with $R^2 = 0.814$. The equation also denotes that number of dead eggs is inversely related to humidity conditions.
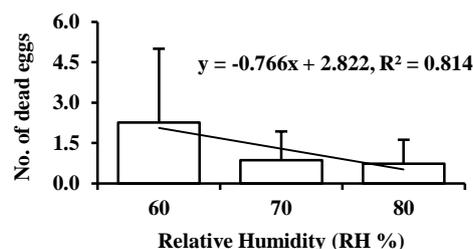

Figure 16: Response of number of dead eggs of DFLs in silkworm (*Bombyx mori* L) hybrid, PM x CSR2 exposed humidity conditions irrespective of photoperiod and temperature (n =15).

Similarly, the computed data on number of dead eggs in response to temperature in CSR2 x CSR4 irrespective of photoperiodic and humidity conditions are presented in Figure 17. The curve equation was y = 1.466x - 1.444 with R^2 = 0.733, thus indicating negative correlation of number of dead eggs to temperature conditions. At low temperature of 25 °C, number of dead eggs in CSR2 x CSR4 was very low (almost nil, Figure 17). Number of dead eggs was high ($\approx$ 4) with increased temperature of 35 °C. The differences in number of dead eggs in responding to exposed temperatures are statistically highly significant ($p < 0.01$) as also supported by R^2 value (0.733). Thus, temperature inversely affected number of dead eggs in CSR2 x CSR4 and the relation is negative to temperature.
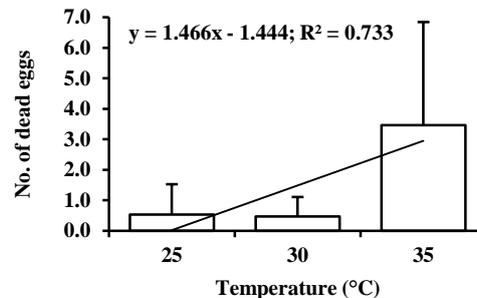

Figure 17: Response of number of dead eggs in DFLs of silkworm (*Bombyx mori* L) hybrid, CSR2 x CSR4 to exposed temperature conditions irrespective of photoperiod and humidity (n =15).

With regard to the number of dead eggs in the DFLs of CSR2 x CSR4 in response to humidity, (Figure 18), the response was an inverse relation, low number of dead eggs under high humidity conditions and high number of dead eggs under low humidity. The curve equation was y = -x + 3.488; R^2 = 0.627. Under low humidity condition (60% RH), number of dead eggs was high (around 3). The number of dead eggs decreased to as low as below 0.5 under high humidity condition (80% RH). Differences in the number of dead eggs against humidity conditions were statistically highly significant ($p < 0.001$). The R^2 value (R^2 = 0.627) also denotes the above significance.
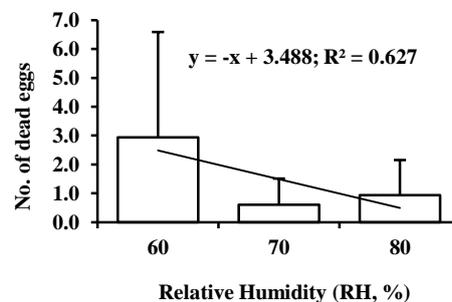

Figure 18: Response of number of dead eggs in DFLs of silkworm (*Bombyx mori* L) hybrid, CSR2 x CSR4 to exposed humidity conditions irrespective of photoperiod and temperature (n =15).

4. DISCUSSIONS

Egg hatching is primarily an important event in the life history of lepidopterans. Fully grown embryos in the eggs start piercing its egg shell to enter into a new word for continuation of its progeny. In the silkworm, *Antheraea pernyi*, a behavioral programme is initiated in the early morning hours on the day of hatching whereby the pharate larva begins to gnaw its way out of the egg (Riddiford and Johnson, 1971; Sauman and Reppert, 1998). It takes about 90 min of continuous chewing to make a hole in the chorion, large enough for the animal to escape. Proper initiation of this behavioral programme is critical because it frees the larva from the constraints of life in the egg to fulfilling its biological destiny in the outside world.

Egg hatching is the earliest recognized behavior under circadian control in the silkworm (Sauman and Reppert, 1998). It is well established that the daily rhythm in hatching in many insects is controlled by circadian system (Beck, 1980; Saunders, 1982, 2002). As in the other insects (Beck, 1980; Saunders, 1982), hatching in *Bombyx mori* is also 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). It was also assumed 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) 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). Fugo *et al*. (1985), based on their experimental results, further pointed that the rhythmicity in hatching can be initiated by pulses of light when these are applied after the mid-point of embryonic development.

Thus, the rhythm of hatching is very distinct when the *Bombyx mori* eggs are driven under LD 12 : 12 condition. It is also clear that under LD 12 : 12 conditions the hatching occurred very near or immediately after lights-on (Figure 1). Therefore, light-on is taken by *Bombyx* as signal for hatching. However, in CSR2 x CSR4, a bivoltine hybrid, hatching occurred for two days, with stray hatching on the first day and maximum hatching on the second day (Figure 2). The interval between these two hatching peaks is ≈ 24 h, hence, circadian. Hatching in PM x CSR2, however confined to a single day (Figure 1). When the eggs are driven under continuous conditions (DD/LL) the hatching occurred for 2 days. Under DD, hatching occurred for two days, with high hatching on day-1 and low hatching on day-2 (Figure 3) for PM x CSR2. For CSR2 x CSR4, the issue was different, that hatching was low on day-1 and maximum on day-2 (Figure 4). Under LL conditions, interestingly, the hatching rhythm was broadened with consequential peak decreasing with CSR2 x CSR4 (bivoltine hybrid) alone (Figure 6), while the same was distinct for PM x CSR2 (multivoltine x bivoltine hybrid, Figure 5). This implies that in *Bombyx mori,* hatching rhythm is self-sustained. Further, hatching rhythm followed a 'gating' phenomenon, the gates re-occurring nearly 24 h apart.

The implications of temperature and humidity on hatching (Figures 7, 8, 9 and 10) with both the hybrids (PM x CSR2 and CSR2 x CSR4) are clear at 26 °C with 60% RH for PM x CSR2 (Figure 7) and CSR2 x CSR4 (Figure 8) and at 25 °C with RH of 70% for PM x CSR2 (Figure 9) and for CSR2 x CSR4 (Figure 10) that temperature and humidity conditions too affect the rhythmicity in *B. mori*. For more clarifications on this aspect, hatching rhythmicity experiments were conducted under three photoperiodic conditions (LD 12 : 12, DD and LL) with three temperature (25, 30 and 35 °C) in combination with three humidity conditions (60, 70 and 80% RH) for two silkworm hybrids (PM x CSR2 and CSR2 x CSR4). To avoid ambiguity in verifying all chronograms, the data on hatching was analyzed for mean vector (mean ▢), length of mean vector (r) and angular standard deviation (s) through circular statistics (Chassé and Théron, 1988). Phenomenally, the mean ▢ did not significantly (statistically) deviated from appearing at lights-on phase (06.00 h) occurring at or around 06.00 h. Surprisingly, the length of mean vector (r) and angular standard deviation (s) varied significantly, indicating that the hatching rhythm broadened with reduced hatching peaks under high temperature and low humidity conditions, emphasizing a near arrhythmicity or damping-out of hatching. The situation is still grave under LL condition.

The observed rhythmic patterns in hatching, in the present study, under all temperature and humidity conditions suggest the circadian and endogenous nature of the rhythmicity. The endogenously free-running nature and circadian control over the phenomenon has been demonstrated in *Bombyx mori* (Sivarami Reddy *et al*., 1993a). Hatching in *B. mori* was reported as diurnal (Sivarami Reddy and Sasira Babu, 1990). Larval to larval ecdysis was reported to be instar dependent (Krishnaswami *et al*., 1973; Krishnaswami, 1986; Sivarami Reddy *et al*., 1990). A comparative analysis of marker events in the pupation patterns in the silkworm *Bombyx mori* under different temperature and humidity conditions through circular statistics (Chassé and Théron, 1988) served as good example of chronobiological markers in population rhythm. The non-significant variations in the mean ▢, indicate the homogeneity nature of the rhythm under temperature and humidity conditions studied.

Insects generally require an adequate level of environmental conditions in order to keep their physiological system working normal (Mellors *et al*., 1984; Bauer, 1976). Among the environmental conditions, photoperiod, temperature and relative humidity are the most important ones. Datta (1992) emphasized optimum environmental conditions of 28 °C and 80% humidity during silkworm egg incubation and hatching for economical hatching (more than 90%). Thus, economical hatching in PM x CSR2 and CSR2 and CSR4 are more justified. In PM x CSR2 higher hatching (%) compared to bivoltine x bivoltine hybrid, CSR2 x CSR4 was observed. The productive bivoltine breeds and hybrids are reported to be susceptible to high temperature and low humidity (Kato *et al*., 1979). However, in the present study, hatching seemed to be dependent on more acclimatized female partner of the hybrid (PM) in PM x CSR2. As supported by the observation in the present study, hatching in CSR2 x CSR4 is significantly (at 5% level) influenced by both temperature and humidity. Suresh Kumar and Yamamoto (1995) reported that the hybrids are more tolerant than the pure races. Bursell (1970) also viewed that tropical species are more resistant to temperature than temperate species. Thus, more hatching in PM x CSR2 is justified compared to less hatching in CSR2 c CSR4.

Further, the aspect of appearing dead eggs in experimental silkworm eggs under different photoperiodic conditions coupled with temperature and relative humidity was examined carefully. It is observed that the occurrence of dead eggs is mainly dependent on temperature and humidity rather than on photoperiodic conditions imposed. Thus, temperature has a direct or positively related to number of dead eggs in PM x CSR2 (Table 5 and Figure 15, collective effect under LD 12 : 12 condition only) while humidity effected inversely (Table 5 and Figure 16). Similar results are seen with CSR2 x CSR4 (Table 6 and Figure 17 and 18). The main inference is that temperature is affecting in a charring mode while humidity in desiccation way. Therefore, the insect eggs, in general and *Bombyx* eggs in particular prefer early hours of the day, as reported for hatching in *B. mori* (Sivarami Reddy and Sasira Babu, 1990) and for eclosion for Drosophila (Pittendrigh, 1966), thus insects follow the phenomenon of minimizing the risk of desiccation.

It is inferred that temperature and humidity, thought not directly affecting the expression of hatching patterns, implicated on the quantum of hatching and width of rhythmicity to a great extent. Further, the hatching (%) and dead egg appearance are also greatly connected to temperature and humidity conditions imposed.

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