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PHOTOPERIODIC MODULATION OF CIRCADIAN PROTEIN RHYTHM IN THE SILK GLAND OF *BOMBYX MORI* DURING FOURTH INSTAR DEVELOPMENT

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ABSTRACT
Circadian changes in the silk gland protein profiles were assayed in the fourth instar larva of *Bombyx mori*, under 12 h light and 12 h dark cycle (LD), continuous light (LL) and continuous dark (DD) conditions. The free running time of the protein rhythm, projected in the form of phase response curves shows characteristic peaks (elevated points) and troughs (low points) that presumably represent translation and transcription phases of the silk gene expression. The curves were further analyzed, in terms of the mean number of peaks and troughs and the interval between them with a view to determine the total number of protein synthetic cycles and the mean time required for the completion of each cycle (peak time + trough time), during the 24h-free running time or *tau* of the rhythm. Under LD and LL conditions, the protein rhythm followed a 24h cycle with 8 rounds of protein synthetic phases, each one being repeated at an interval of ~2.9h. But under DD condition, the rhythm included 7 rounds of synthetic phases that are repeated every ~3.3h. Obviously, the light condition under LD and LL, maintains the normal 24h protein rhythm while the dark condition under DD delays it by 3h and 12m.

INTRODUCTION

The growth of silk gland in silkworm is of paramount importance to the sericultural industry as it is responsible for the synthesis of silk proteins, the basic raw materials of the silk cocoon (Inoue *et al.,* 2000; Yong Hou *et al*., 2007; Sutherland *et al*., 2010). Prominently, the silk gland grows during the fourth and fifth larval instars, the rate of which is modulated by environmental factors such as the light, diet, temperature and relative humidity (Shimizu, 1982). The morphological and anatomical changes in the silk gland are associated with concomitant changes in its biochemical constituents, including proteins (Morimoto *et al*., 1968; Tashiro *et al*., 1968; Firling, 1977). In view of its importance for silk production, the silk gland attracted the attention of several researchers, especially with regard to silk proteins it synthesizes, during the larval development (Ravikumar and Sarangi, 2004; Su Li Seong *et al*., 2005; Yaginuma and Ushizima, 2005; Archana *et al*., 2006; Sarangi and Anitha, 2007; Sehnal and Sutherland, 2008). Further, the role of different regions of the silk gland in the synthesis of the core silk protein, fibroin and the adhesive silk protein, sericin has been highlighted (Prudhomme *et al*., 1985; Michaille *et al*., 1989) .

One interesting aspect of the insect chronobiology is the unraveling the mysteries of the circadian clock mechanism that opened-up new vistas for the exploration of its biochemical manifestations. A pioneering report on *Drosophila* (Konopka and Benzer,1971) , that lead to the identification of the first circadian clock gene *period* (per) which triggered further probes on similar lines. A large number of circadian clock genes such as the *period* (*per*), *timeless* (*tim*), *double time (dbt)* and the clock proteins were identified in silk moths and in other insects and specific functions were assigned to them (Naidoo *et al*., 1999; Goto and Denlinger, 2002; Froy *et al*., 2003; Hall, 2003; Sharma, 2003; Satyanarayana *et al*., 2004; Sehadova *et al*., 2004; Iwai *et al*., 2006) . Concerted efforts were also made to localize the endogenous pace makers or circadian clocks or oscillators in a variety of insects (Glossop and Hardin, 2002; Sehadova *et al*., 2004; Reppert, 2006). Some recent investigations have significantly contributed to the identification, isolation, cloning and determination of the expression patterns of silk genes (Michaille *et al*., 1989; Kimura *et al*., 1985; Obara and Suzuki , 1988; Ishikawa and Suzuki, 1985; Gizelak, 1995) and the genes of various other proteins (Yong Hou *et al*., 2007). The studies were further extended to determine the pattern of silk gene regulation through transcriptional and hormonal factors (Kodrik and Sehnal, 1994; Durand *et al*., 1992; Fukuta *et al*., 1993)

The studies largely contributed to our understanding of the transcriptional and translational profiles of insect clock genes that needs to be analyzed in terms of biochemical and physiological perspectives. No significant effort has since been made to elucidate the biochemical basis of circadian clock mechanism in *Bombyx mori*. Investigations on these lines could provide vital information related to the timing of silk gene expression and its modulation by the altered

photoperiodic conditions. The present investigation aims at making a preliminary attempt in this direction.

MATERIALS AND METHODS

The present investigation was carried out on the PM x CSR_2 hybrid variety of *Bombyx mori,* reared under standard environmental conditions of 28° C, 85 % relative humidity (Krishnaswami, 1986). After hatching, the worms were fed with M_5 variety of mulberry leaves, five times per day at 6AM, 10 AM, 2 PM, 6PM and 10 PM, under normal 12hrs light and 12h dark conditions. After the third moult, the larvae were divided into three batches and reared separately under three different photoperiodic conditions, viz., 12hrs light and 12hrs dark cycle (LD), continuous light (LL) and continuous dark (DD). However, normal feeding pattern was continued throughout the fourth and fifth instar larval stages under the three photoperiodic conditions. Circadian rhythmicity in the protein profiles of the silk gland was analyzed on the third day of fourth instar larval stage. The silk glands, isolated every hour by dissecting the silkworm larvae in ice-cold Silkworm Ringer (Yamaoka *et al*., 1971), starting from 8 AM on day-3 through 8 AM on day-4 (*i.e.* for 25 hrs), were used for the assay of proteins.

Hour-to-hour changes in protein profiles (both total and soluble) of the silk gland were estimated (Lowry *et al*., 1951) in 1% homogenates of the tissue, prepared in ice-cold distilled water. The amount of proteins present in the tissue samples was computed using a standard prepared from bovine serum albumin, and the values were expressed as mg /g wet weight of the tissue. The structural protein content was obtained by subtracting the values of soluble proteins from those of total proteins. The experiment lasted for two consecutive days encompassing 12: 12 hrs of light and dark cycle (LD) for the first batch, continuous light (LL) for the second batch and continuous dark (DD) for the third batch. The first batch of the larvae reared under LD was treated as the control while those reared under LL and DD were treated as the experimental samples.

RESULTS

The data pertaining to circadian changes in the levels of total, soluble and structural proteins of the silk gland, assayed on hour-to-hour basis during the free running time of the rhythm, under LD, LL and DD conditions, are presented in Fig. 1 and in Tables 1, 2 and 3. The protein rhythm recorded during the period of 24hrs is designated the *free running period* or *tau,* which is depicted in the form of peaks (elevated points) and troughs (low points or depressions) in the phase response curves presented in Fig. 1. Further, the light period of the experiment is designated the photo phase and the dark period as the scoto phase.

Circadian changes in total proteins: Under LD, the total protein content of the silk gland showed 8 peaks, (elevated points) and 8 troughs (low points) during the 24hrs-free running period of the rhythm or *tau* (Fig. 1a). The first peak occurred at 8hrs on first day with a total protein value of ~64 mg / g wet wt. of tissue. The second peak occurred at 10hrs (~67 mg), third one at 13hrs (~57 mg), fourth one at 15hrs (~57 mg), fifth one at 18hrs (~ 54 mg), sixth one at 20hrs (~65 mg), seventh one at 1hrs (~77 mg) and the eighth peak at 5hrs (102 mg). Similarly, the first trough in the protein levels was observed at 9hrs (~56 mg), second one at 12hrs (~54 mg), third one at 14hrs (~52 mg), fourth one at 16hrs (~50 mg), fifth one at 19hrs (~34 mg), sixth one at 23hrs (~ 23 mg), seventh one at 2hrs (~55 mg) and the eighth one, next day at 8hrs (~50 mg). Out of 8 troughs, first four appeared during the first photo phase (8hrs to 18hrs on day -1).

Under LL, 8 peaks and 8 troughs occurred in the levels of total proteins (Fig. 1a) during the *tau*. Peaks were observed at 8hrs (~67 mg), 10hrs (~50 mg), 13hrs (~80 mg), 15hrs (~59 mg), 23hrs (~61 mg), 1hrs (~51 mg), 4hrs (~65 mg) and the last one next day at 7hrs (~41 mg). Similarly, troughs appeared at 9hrs (~27 mg), 11hrs (~46 mg), 14hrs (~51 mg), 20hrs (~36 mg), 00hrs (~46 mg), 2hrs (~41 mg), at 6hrs (36 mg) and the eighth one, next day at 8hrs (~38 mg). Under DD, the total protein content levels recorded 6 peaks and 6 troughs during the 24hrs-free running period of the rhythm (Fig.1A). Peaks were observed at 11hrs (~98 mg), 17hrs (~94 mg), 20hrs (~ 63 mg), 23hrs (~75 mg), 4hrs (52 mg) and the sixth one, next day at 8hrs (~45 mg). Troughs under DD condition appeared at 8hrs (~55 mg), 14hrs (~ 46 mg), 18hrs (~34 mg), 22hrs (~37 mg), 2hrs (~24 mg) and the sixth one, next day at 7hrs (31mg).

Circadian changes in soluble proteins: Under LD, the soluble protein content of the silk gland showed 8 peaks and 8 troughs during the 24h-free running period of the rhythm (Fig. 1B). The first peak appeared at 10hrs (~51 mg) and the second one at 13hrs (~48 mg), the third one at 21hrs (~27 mg), the fourth one at 23hrs (~51 mg), the fifth one at 1hrs (~55 mg), the sixth one at 3hrs (~53 mg), the seventh one at 5hrs (~56 mg) and the eight one at 8hrs (~37 mg). Of the eight troughs, the first one made its appearance at 8hrs (~35 mg), the second one at 12hrs (~29 mg), the third one at 20hrs (~21 mg), the fourth one at 22hrs (~25 mg), the fifth one at 00hrs (~33 mg), sixth one at 2hrs (~39 mg), the seventh one at 4hrs (~51 mg) and eight one, next day at 6 hrs (~26 mg).

Under LL, 9 peaks and 8 troughs were recorded in the levels of soluble proteins of the silk gland (Fig. 1B). Peaks occurred at 8hrs (~ 49 mg), 10hrs (~35 mg), 12hrs (~44 mg), 5hrs (~38 mg), 17hrs (~34 mg), 22hrs (~35 mg), 1hrs (~30 mg), 3hrs (~35 mg) and the ninth one, next day at 8hrs (~27 mg). Likewise, troughs were observed at 9hrs (~14 mg), 11hrs (~32 mg), 14hrs (~16 mg), 16hrs (~33 mg), 21hrs (~22 mg), 23hrs (~26 mg), 2hrs (~25 mg) and the eight one, next day at 6hrs (~19 mg). Under DD, the levels of soluble proteins recorded 7 peaks and 7 troughs during the 24hrs-free running period of the rhythm (Fig. 1b). The peaks appeared at 9hrs (~40 mg), 11hrs (~71 mg), 17hrs (~50 mg), 20hrs (~39 mg), 23hrs (~43 mg), 4hrs (~44 mg) and the seventh one, next day at 8hrs (~24 mg). Troughs under this condition were observed at 8hrs (~21 mg), 10hrs (~35 mg), 14hrs (~ 30 mg), 18 hrs (~21 mg), 22 hrs (~31 mg), 2hrs (~5 mg) and the seventh one, next day at 7hrs (19 mg).

Circadian changes in structural proteins: Under LD, the structural proteins of the silk gland showed 7 peaks and 7 troughs during the 24hrs-free running period of the rhythm (Fig.1c). The first peak appeared at 8hrs (~29 mg), the second

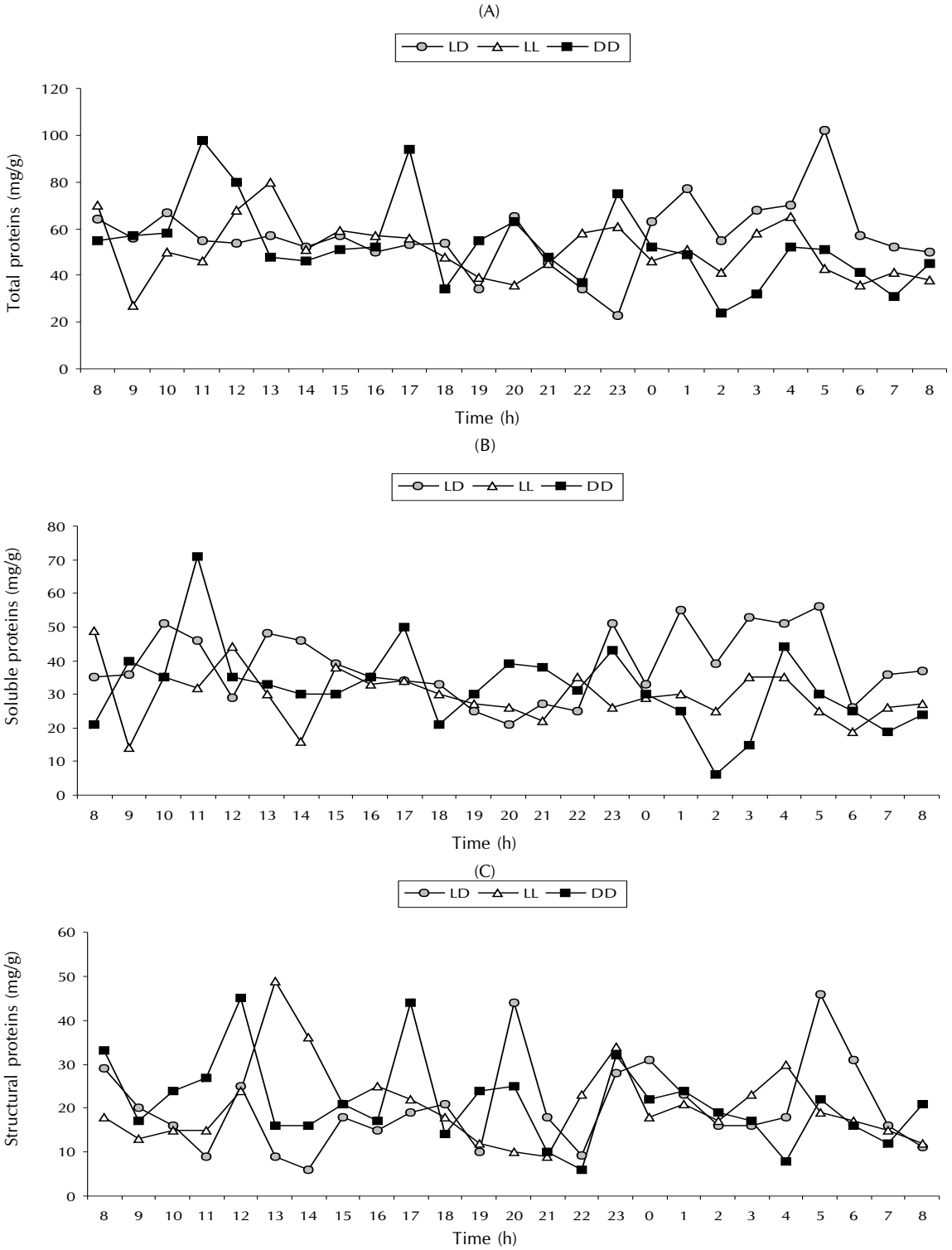

Figure 1: Phase response curves (PRC) of the circadian protein rhythms in respect of total (A), soluble (B) and structural (C) proteins in the silk gland of *Bombyx mori, under* 12hrs light: 12hrs dark cycle (LD); continuous light (LL) and continuous dark (DD) conditions. Each phase represents the hourly assay of protein levels, starting from 8hrs on day-5 to 8hrs on day-6 during fourth instar development. Each value, expressed as mg protein/g wet weight of tissue represents the mean ± S.D of four separate observations (P values: < 0.001)

one at 12hrs (~25 mg), the third one at 15hrs (18 mg), the fourth one at 18hrs (~21 mg), the fifth one at 20hrs (~44 mg), sixth one at 0hrs (~31 mg) and the seventh one at 5hrs (~46 mg). Of the seven troughs, the first one made its appearance at 11hrs (~9 mg), second one at 14hrs (~6 mg), third one at 16hrs (15 mg), fourth one at 19hrs (~9 mg), the fifth one at 22hrs (~9 mg), sixth one at 3hrs (~16 mg) and the seventh one, next day at 8hrs (~11 mg).

Table 1: Interval between peaks (elevated points) of protein levels in the silk gland of the fourth instar larva of *Bombyx mori* during the free running period of the rhythm under 12 hrs light / dark cycle (LD), continuous light (LL) and continuous dark (DD) conditions

Protein type	Photo-period	No. of peaks	Interval between peaks in hours 1-2	2-3	3-4	4-5	5-6	6-7	7-8	8-9	9-10	Mean interval in hrs
Total proteins	LD	8	2	3	2	3	2	5	4	-	-	2.6
	LL	8	2	3	2	8	2	3	3	-	-	2.9
	DD	6	6	3	3	5	4	-	-	-	-	3.5
Soluble proteins	LD	8	3	8	2	2	2	2	3	-	-	2.8
	LL	9	2	2	3	2	5	3	2	5	-	2.7
	DD	7	2	6	3	3	5	4	-	-	-	3.3
Structural proteins	LD	7	4	3	3	2	4	5	-	-	-	3.0
	LL	7	2	3	3	7	2	3	-	-	-	2.9
	DD	8	4	3	2	3	3	6	3	-	-	3.0

Source: Figure 1

Table 2: Interval between troughs (low points) of protein levels in the silk gland of the fourth instar larva of *Bombyx mori* during the free running period of the rhythm under 12 hrs light / dark cycle (LD), continuous light (LL) and continuous dark (DD) conditions

Protein type	Photo-period	No. of troughs	Interval between troughs in hrs 1-2	2-3	3-4	4-5	5-6	6-7	7-8	8-9	9-10	Mean interval in hrs
Total Proteins	LD	8	3	2	2	3	4	3	6	-	-	2.9
	LL	8	2	3	6	4	2	4	2	-	-	3.0
	DD	6	6	4	4	4	5	-	-	-	-	3.8
Soluble proteins	LD	8	4	8	2	2	2	2	2	-	-	2.8
	LL	8	2	3	2	5	2	3	4	-	-	2.6
	DD	7	2	4	4	4	4	3	-	-	-	3.0
Structural Proteins	LD	7	3	2	3	3	5	5	-	-	-	3.0
	LL	7	2	4	6	3	2	6	-	-	-	3.3
	DD	8	5	2	2	4	2	4	3	-	-	2.8

Source: Figure 1

Table 3: Analysis of the phase response curves of the protein rhythm in the silk gland of the fourth instar larva of *Bombyx mori*, in terms of number of peaks and troughs and the interval between them, under 12hrs light / dark cycle (LD), continuous light (LL) and continuous dark (DD) conditions

Parameter	Photoperiodic condition LD	LL	DD
Average number of peaks	~ 8(8 + 8 + 7 =23 / 3 = 7.7)	8(8 + 9 + 7 =24 / 3 = 8)	7(6 + 7 + 8 = 21 / 3 = 7)
Average number of troughs	~ 8(8 +8 + 7 =23 / 3 = 7.7)	~8(8 + 8 + 7 =23 / 3 = 7.7)	7(6 + 7 + 8 = 21 / 3= 7)
Mean interval between peaks	2.8h(2.6 + 2.8 + 3.0= 8.4 / 3 = 2.8)	~2.8h(2.9 + 2.7 + 2.9 = 8.5 / 3 = 2.83)	~3.3h(3.5+3.3 + 3.0 = 9.8 / 3 = 3.26)
Mean interval between troughs	2.9h(2.9 + 2.8 + 3.0= 8.7 / 3 = 2.9)	~3 h(3 + 2.6 + 3.3 = 8.9 / 3 = 2.96)	3.2h(3.8 + 3.0 + 2.8 = 9.6 / 3 = 3.2)
Combined mean interval of peaks and troughs	~2.9h(2.8 + 2.9 = 5.7 / 2 = 2.85	2.9h(2.8 + 3 = 5.8 / 2 = 2.9)	~3.3h(3.3 + 3.2 = 6.5 / 2 =3.25)

Source: Fig. 1

Under LL, 7 peaks and 7 troughs were recorded in the levels of structural proteins (Fig. 1C). Peaks occurred at 8hrs (~18 mg), 10hrs (~15 mg), 13hrs (~49 mg), 16hrs (~25 mg), 23hrs (~ 34 mg), 1hrs (~ 21 mg) and the seventh one next day at 4hrs (30 mg). Troughs were recorded at 9hrs (~13 mg), 11hrs (~15 mg), 15 hrs (~21 mg), 21 hrs (~9 mg), 00hrs (~18 mg), 2hrs (~17 mg) and seventh one next day at 8hrs (~12 mg). Under DD, the structural protein content of the silk gland recorded 8 peaks and 8 troughs during the 24hrs-free running period of the rhythm (Fig.1c). Peaks appeared at 8hrs (~33 mg), 12h (~ 45 mg), 15hrs (~21 mg), 17hrs (~44 mg), 20hrs (~25 mg), 23hrs (~32 mg), 5hrs (~22 mg) and the eighth one, next day at 8hrs (~21 mg). Troughs were observed at 9hrs (~17 mg), 14hrs (~16 mg), 16hrs (~17 mg), 18hrs (~14 mg), 22hrs (~6 mg), 00hrs (~22 mg), 4hrs (~8 mg) and the eighth one, next day at 7hrs (~12 mg). During the remaining period of the free running time minor falls and elevations were observed in all the three types of proteins, which are not statistically significant to be treated as peaks and troughs.

DISCUSSION

Circadian rhythms enable organisms to live in harmony with the rhythms of the nature by re-adjusting their physiological events to occur at an appropriate time of the day (Saunders, 2002). The current study clearly demonstrates the prevalence of circadian rhythmicity in the protein profiles of the silk gland in *Bombyx mori,* and this is reflected in the form of peaks and troughs in the phase response curves of the free running time or *tau* (Fig.1). The findings are in consistent with the earlier ones (Yong Hou *et al*., 2007; Sehadova *et al*., 2004; Iwai *et al*., 2006; Shimizu *et al*., 2001; Kyung *et al*., 2006), that tissue-specific endogenous pacemakers in *Bombyx mori* and other insects control the circadian rhythms. Light being the most dominant *zeitgeber* (time giver) of the circadian rhythm modulates the circadian clock mechanism in insects by a phenomenon called resetting or entrainment or clock shifting

during the larval development (Peschel *et al*., 2009). The clock-shifting mechanism allows the animal to continue its circadian rhythm, but sets the rhythm ahead or behind the normal free running time (Wallace *et al*., 1991). In *Bombyx mori*, the free running period of the protein rhythm is clock-shifted under the impact of altered photoperiodic conditions, viz., LD, LL and DD, that manifested in the form of variations in the average number of peaks and troughs and the mean interval between them (Fig. 1; Tables 1, 2 and 3). The mean number of peaks and troughs stood approximately at the same level, *i.e.* 8, both under LD, LL conditions and 7 under DD condition. Similarly, the combined mean interval of peaks + troughs stood at 2.9hrs for both LD and LL conditions and ~3.3hrs for DD condition.

The reasons for the appearance of peaks and troughs in protein levels are not clear. However, they signify two vital stages of gene expression, i.e., the translation and transcription phases respectively in the silk gland of *Bombyx mori* during the fourth instar development. Evidently, the peaks represent the phases of protein biosynthesis, while the troughs depict the active phases of transcription that alternate with each other in the silk gland at regular intervals. While, the mean interval between troughs is indicative of the timing of the translation process, during which the protein levels peak to heights, the intervals between peaks represent the timing of transcription during which the cells prepare for the next phase of translation. The combined mean interval between peaks and troughs is thus indicative of the time required for the protein synthetic cycle in the silk gland. Though, the rhythm of particular protein is not examined in the present study, it might relates to the silk proteins; fibroin and sericin, apart from a host of other proteins including metabolic enzymes, trans-membrane proteins, heat-shock proteins, immuno- proteins, proteases, tubulins (Inoue *et al*., 2000; Nirmala *et al*., 2001; Jin *et al*.,2004; Takasu *et al*.,2005; Kyung *et al*., 2006; Zhang *et al*., 2006; Yong Hou *et al*., 2007) , that are synthesized by the silk gland from time to time during larval development. More particularly the sericin-2 gene is known to be expressed actively during fourth instar development, and hence it is presumed that the sericin (floss protein) is the most abundantly synthesized silk protein during this instar, while the synthesis of fibroin (core silk protein) takes precedence during the fifth instar development (Ishikawa and Suzuki, 1985; Michaille *et al*., 1989; Kludkiewicz *et al*., 2009). The ups and downs in the levels of structural proteins indicate the consolidation of silk proteins, more significantly, of sericin-2 during peak phases (Inoue *et al*., 2000; Sehnal and Zurovec, 2004), that may involve processes like the gelation, adhesion and crystallization (Takasu *et al*., 2005). The trough phases, apart from indicating the timing of transcription, probably reflect the timing of protein denaturation and hydrolysis that could have been triggered by an intracellular proteolytic mechanism that maintains homeostasis in the levels of silk proteins during metamorphosis (Chen and You, 2004; Ciechanover, 2005).

The timing of gene expression is subjected to alteration by the light cues as reflected in the present investigation. The protein synthetic phase repeats every 2h, 54m under LD and LL conditions and 3hrs, 8m under DD condition in tune with changes in the timing of gene expression. The transcriptional and translational events of the silk gene expression are apparently stimulated by the light but delayed by the dark condition. The free running time of the protein rhythm is thus, advanced under the influence of photoperiod and delayed under the scoto period. If, the interval between peaks and troughs is considered as the time taken for the completion of one round of transcription and translation, it is likely that each round could take about 2hrs and 54m both under LD and LL conditions and about 3hrs and 18m under DD condition. Clearly, under both LD and LL conditions, the protein rhythm of the silk gland includes 8 rounds of synthetic phases, each lasting for duration of about 2hrs, 54m. Similarly, under dark conditions the protein rhythm comprises 7 rounds of synthetic phases, each one repeating at an interval of about 3hrs and 18m.

The advances in investigations related to the genetical and molecular mechanism of circadian clock mechanism have not been substantiated by biochemical correlates. As such direct evidence for protein rhythms remains largely unknown, except for a few references related to circadian clock genes and their expression timings (Goto and Denlinger, 2002; Syrova *et al*., 2003; Grima *et al*., 2004; Hardin, 2004; Shafer *et al*., 2004; Stoleru *et al*., 2004). Though the frequency of circadian rhythms has not been correlated with reference to photoperiod, the latter has a profound effect on protein biosynthesis in insects. However, our findings are in agreement with the earlier observations made in crickets and certain other insects that more proteins are synthesized under altered conditions of photoperiod (Kenny and Saunders, 1991; Koga *et al*.,2005; Peschel *et al*., 2009). How the circadian behaviour of protein rhythm of tissues is controlled is not known; but it is likely that the juvenile hormone secreted by the corpora allata does so by modulating the peripheral oscillators of the silk gland (Koga *et al*., 2005) a point that needs elucidation. Further, the silk gland maintains the circadian rhythm more or less at a constant rate even under continuous dark condition (DD), probably by taking cues from the diet, which could act as the prime *zeitgeber* and quickly reset the clock in peripheral organ (Damiola *et al*., 2000; Kita *et al*., 2002; Stokkan *et al*., 2001). Since, the silkworms, reared under continuous dark condition were also fed 5 times a day, they carried through the rhythm more or less on the same lines as done by the worms grown under LD. It is likely that the silkworm might have at least two-oscillators in the silk gland, in which one responds to the light and the other to the dark, as observed in *Drosophila* (Forster, 2000). Accordingly, in the animals that are deprived of natural light cues, the circadian rhythm shifts from the standard 24hrs pattern. In the present case the shift resulted in the delay of each transcription- translation cycle by 24 minutes under DD condition, so that the circadian clock in the silk gland is shifted to a free running time of ~27hrs, 12m instead of normal 24hrs rhythm maintained under LD and LL conditions. Hopefully, our findings (Sailaja and Sivaprasad, 2010) on the protein rhythms could provide further conclusive proof for the phase-shifting influence of light on the protein rhythm in the silkworm.

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