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IMPACT OF PHOTOPERIOD ON CIRCADIAN PROTEIN AND PROTEASE RHYTHMS IN THE DIGESTIVE SYSTEM OF SILKWORM, BOMBYX *MORI*

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ABSTRACT
Photoperiod-induced clock-shifting in circadian protein and protease rhythms were studied in the larval digestive system of *Bombyx mori* under 12h light-dark cycle (LD), continuous light (LL) and continuous dark (DD) conditions. The hourly changes, reflected as peaks and troughs in the phase response curves of protein and protease rhythms, were interpreted as protein synthetic cycles in gut wall and protein releasing cycles in gut content. In the gut wall, the protein rhythm showed 6 synthetic cycles of 4h duration each under LD and 7 cycles of 3.4h duration each under LL and DD. As a result, the 24h-free running period of LD rhythm is advanced to 20.4h under LL and DD. The gut wall protease rhythm maintained 6 synthetic cycles of 4h duration each under LD and DD and 7 cycles of 3.4h duration each under LL. Consequently, the 24h protease rhythm of LD and DD is advanced to 20.4h under LL. The gut content protein rhythm showed 6 releasing cycles of 4h duration each under LD, 7 cycles of 3.4h duration each under LL and 5 cycles of 4.8h duration each under DD, resulting in rescheduling of the 24h LD rhythm to 20.4h under LL and 28.8h under DD. However, the gut content protease rhythm maintained 7 releasing cycles, each with duration of 3.4h uniformly under LD, LL and DD. The analysis of mean peak values of proteins and protease levels reveal that dark cues are necessary for their synthesis and both dark and light cues are essential for their release.

INTRODUCTION

The Silkworm digestive system, with its distinct regions of foregut, midgut and hindgut together with glandular components constitutes the first site wherein the mulberry leaf is subjected to chemical treatment for its ultimate conversion to silk, the exquisite fiber of sericulture industry (Cermenati *et al*., 2007). The digestive juice is a rich source of digestive enzymes, ions, vitamins and many more biochemical constituents that emanate from glandular cells of the gut wall and from the diet (Santos *et al*., 1984; Terra and Ferreria, 2005; Xia *et al*., 2007). In view of its importance in digestion and absorption of mulberry leaves, the biochemical assays of the gut and its contents have emerged as potential areas of research in silkworm biochemistry. Largely, the biochemical studies focused on the ontogenic variations in proteins, lipids, amino acids and nucleic acids during the life cycle of *Bombyx mori* (Yamashita and Hasegawa, 1974; Horie *et al*., 1982; Konno *et al*., 1996; Leonardi *et al*., 2001; Ponnuvel *et al*., 2003; Ravikumar and Sarangi, 2004). Protein-uptake from the mulberry diet and its digestion by proteases in the gut is of special interest, as the amino acids so generated, provide raw materials for the silk protein synthesis in the silk gland. Hence, the profiles of these two biochemical constituents (proteins and proteases) have been extensively studied with reference to larval development and metamorphosis (Yamashita and Hasegawa, 1974; Engelmann and Geraerts, 1980; Horie *et al*., 1982; Sarangi and Anitha, 2007). Despite the availability of voluminous data on ontogenic protein changes (Yamashita and Hasegawa, 1974; Horie *et al*., 1982; Konno *et al*., 1996; Leonardi *et al*., 2001; Ponnuvel *et al*., 2003; Ravikumar and Sarangi, 2004), the circadian protein changes in the silkworm gut have not been taken-up so far. Nevertheless, the circadian studies on silkworm clock genes and their expression pattern and products such as TIM, PER, CRY, DBT (Naidoo *et al*., 1999; Froy *et al* 2003; Hall, 2003; Sharma, 2003; Satyanarayana *et al*., 2004; Sehadova *et al*., 2004; Iwai *et al*., 2006) and those on proteins and amino acid profiles of the silk gland, fat body, haemolymph and segmental muscle (Sailaja and Sivaprasad, 2010, a, b., Sailaja *et al*., 2011; Sivaprasad and Sailaja, 2011) have immensely contributed to the understanding of the silkworm chronobiology. Additionally, such studies on the protein profiles of the gut would facilitate setting appropriate dietary timings for the silkworm. The current investigation attempts to analyze circadian rhythmic changes in the profiles of total proteins vis-a-vis the protease activity in the digestive system of *B. mori* under altered photoperiodic conditions.

MATERIALS AND METHODS

The pure Mysore x CSR_2 hybrid variety of the Silkworm *Bombyx mori,* reared under standard environmental conditions of 28ºC, 85% relative humidity (Krishnaswami, 1986), was taken as the test species for the present study. After hatching, the

worms were feed with M_5 variety of mulberry leaves, five times per day at 6 AM, 10 AM, 2 PM, 6 PM and 10 PM, under normal 12h light and 12h dark conditions. After third moult, the larvae were divided into three batches and reared separately under three different photoperiodic conditions viz., 12h light and 12h dark cycle (LD), continuous light (LL) and continuous dark (DD), but fed uniformly five times a day as usual. Circadian rhythmicity in the levels of proteins and protease activity of the silkworm gut was analyzed for a period of 25h spawning between day 5 and day 6 of fifth instar development. The gut wall tissue was isolated every hour by mid dorsally dissecting the silkworm larvae in ice cold silkworm Ringer (Yamaoka *et al*., 1971) starting from 6 AM on day 5 through 6 AM on day 6 (*i.e.* for 25h) at the same time the digestive juice was extracted through hypodermic syringe by inserting it into the lumen of the gut. The digestive juice so collected was kept in a test tube under ice cold conditions till the mulberry leaf pieces are settled at the bottom. Later, the supernatant was decanted and used for the assay.

Hour- to- hour changes in protein profiles of the gut wall and gut content were estimated by the method of Lowry *et al* (1951) in 1% homogenate of the gut wall tissue and 1:9 diluted gut content (digestive juice) in ice cold distilled water. The protein levels computed using standard bovine serum albumin, were expressed as mg/g wet weight of gut wall tissue or 1 mL of digestive juice. Likewise hour to hour changes in the protease activity was estimated by the method of Davis and Smith (1955) in 5% homogenate of the gut wall and 1:19 diluted digestive juice in ice cold distilled water. The enzyme activity was computed using an amino acid standard and expressed in μ moles of tyrosine/ mg protein/ hour. The whole experiment lasted for two consecutive days encompassing 12:12 h 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 larva reared under LD was treated as the control while those reared under LL and DD were treated as the experimental samples.

RESULTS

The circadian protein and protease activity rhythms of the gut wall and gut content under three photoperiodic conditions LD, LL and DD were projected in phase response curves (PRCs) and presented in Figs. 1 to 4. The PRCs were analyzed in terms the number of peaks (elevated points) and troughs (low points) and time intervals between them and shown in Tables 1 to 6.

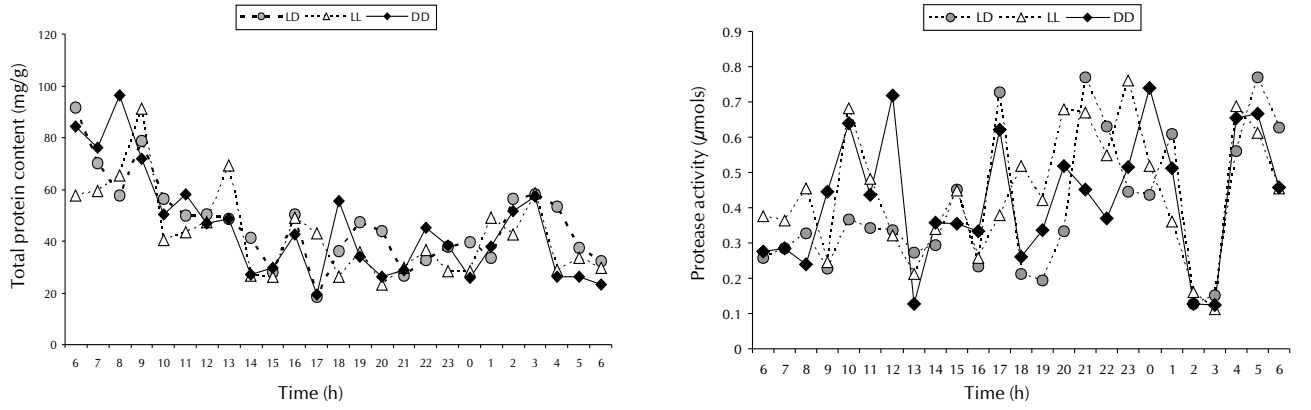

Figure 1: Phase response curves (PRCs) of the 24 h circadian protein (A) and protease (B) rhythms (from 6AM on day 5 to 6AM on day 6) in the Gut wall of fifth instar larva of *Bombyx mori*, under12 h light: 12 h dark cycle (LD), continuous light (LL) and continuous dark (DD) conditions. (p values: < 0.001)

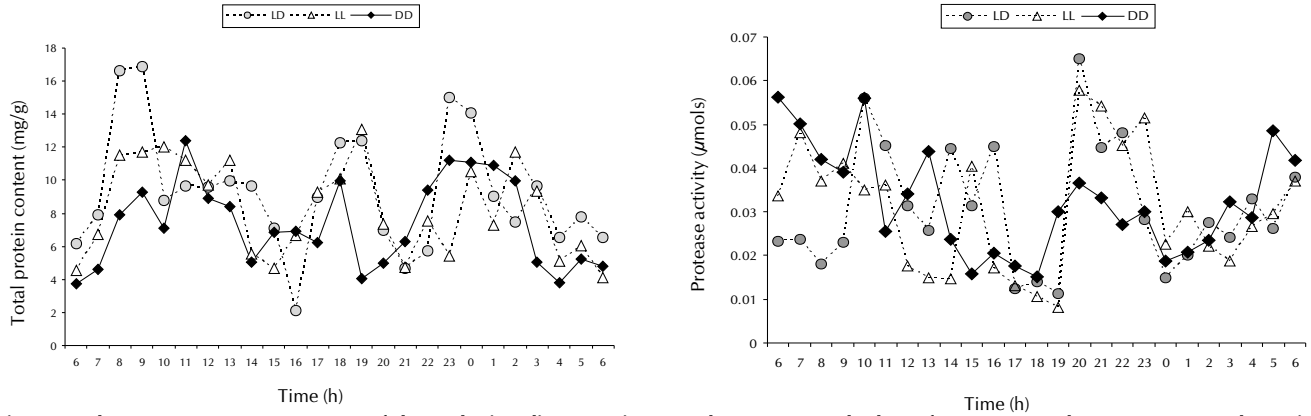

Figure 2: Phase response curves (PRCs) of the 24 h circadian protein (A) and protease (B) rhythms (from 6AM on day 5 to 6 AM on day 6) in the gut content of fifth instar larva of *Bombyx mori*, under12 h light: 12 h dark cycle (LD), continuous light (LL) and continuous dark (DD) conditions. (p values: < 0.001)

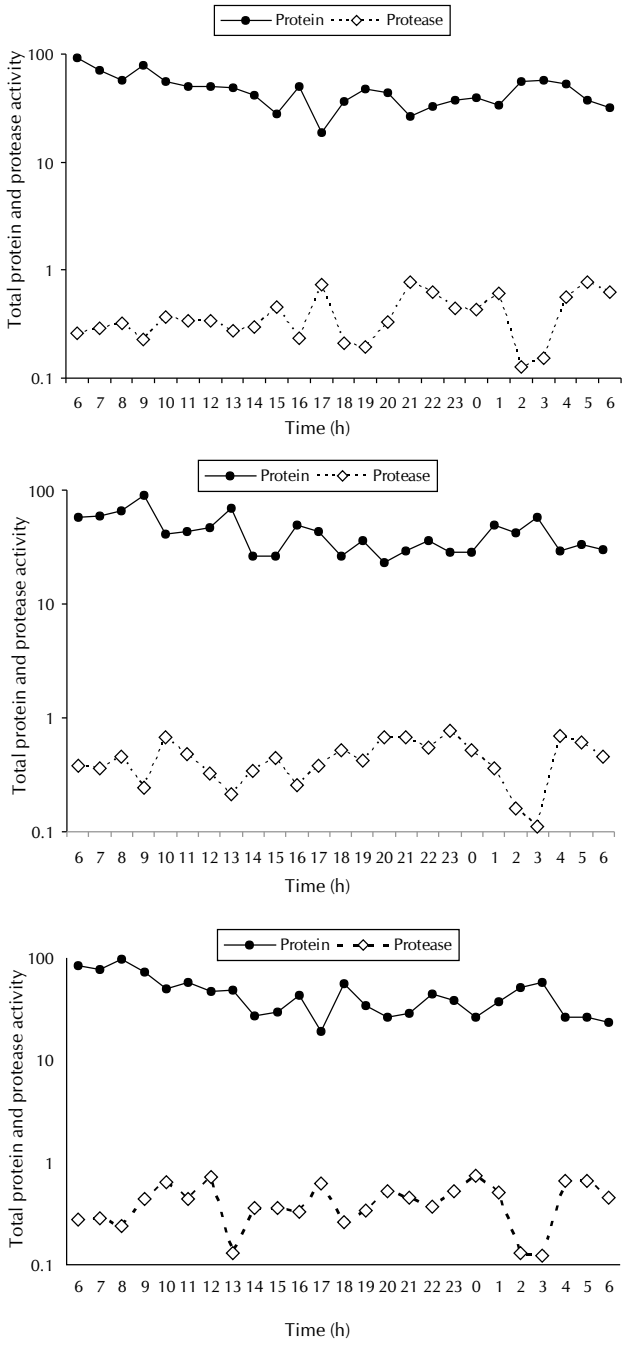

Figure 3: Circadian changes in protein profiles and protease activity in the gut wall of the fifth instar larva of *Bombyx mori*, under (A) 12 h light: 12 h dark cycle (LD), (B) continuous light and (C) continuous dark (DD) conditions. The values expressed in mg per gm-wet weight of tissue, represent 24 h (6 AM on day-5 to 8A.M on day 6) free running time of the circadian rhythm. (p values: < 0.001)

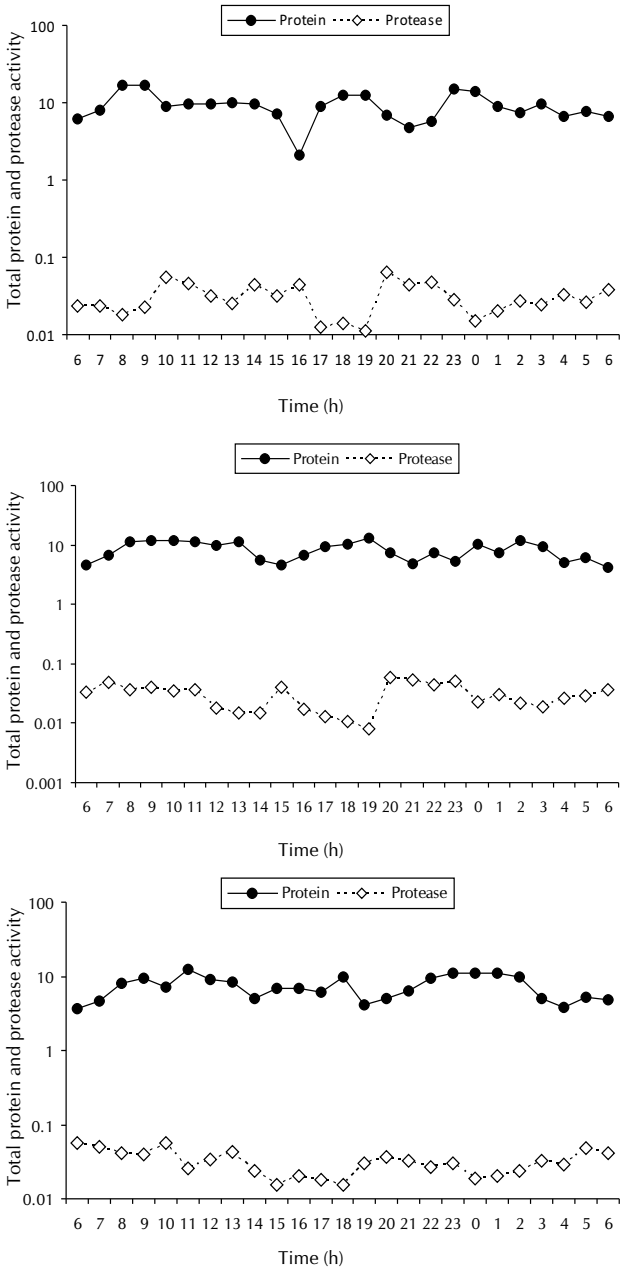

Figure 4: Circadian changes in protein profiles and Protease Activity in the Gut content of the fifth instar larva of *Bombyx mori*, under (A) 12h light: 12h dark cycle (LD), (B) continuous light and (C) continuous dark (DD) conditions. The values expressed in mg per ml of tissue, represent 24h (6 AM on day-5 to 8A.M on day 6) free running time of the circadian rhythm. (p values: < 0.001)

Circadian Protein Rhythms

Gut wall

Under LD the total protein rhythm of the gut wall showed 6 peaks and 6 troughs during the 24h free running period of the rhythm (Fig.1.A). The first peak occurred early at 06 hr with a total protein value ~91 mg/g wet wt. of tissue, subsequent peaks occurred at 09h (~ 79 mg), 16h (~ 51 mg) 19h (~ 48 mg), 00h (~ 40 mg) and next day again at 03h (~ 58 mg). Troughs occurred at 08 hr (~ 58 mg), 15h (~28 mg), 17h (19 mg) 21h (~27 mg) and next day at 01h (~34 mg) and again at 06h (~32 mg). Under LL, the total protein rhythm showed 7 peaks and 7 troughs during the 24h free running period. Peaks appeared at 09h (~91 mg), 13h (~ 69 mg), 16h (~49 mg), 19h (~35 mg), 22h (~37 mg) and next day at 01h (~49 mg) and again at 03h (~58 mg). Troughs occurred at

Table 1: Interval between peaks (Table 1 A)and troughs (Table 1B) of protein levels in the gut wall of the fifth instar larva of *Bombyx mori* during the free running period of the rhythm under 12 h light/ dark cycle (LD), continuous light (LL) and continuous dark (DD) conditions

Photo period	No of peaks	Interval between peaks in h 1-2	2-3	3-4	4-5	5-6	6-7	Mean interval in h
LD	6	3	7	3	5	3	-	3.5
LL	7	4	3	3	3	3	2	2.6
DD	7	2	3	5	2	4	5	3.0

Photo period	No of troughs	Interval between troughs in h 1-2	2-3	3-4	4-5	5-6	6-7	Mean interval in h
LD	6	7	2	4	4	5	-	3.7
LL	7	4	5	3	2	4	6	3.4
DD	7	3	4	3	4	3	5	3.1

Table 3: Interval between peaks (Table 3 A) and troughs (Table 3 B) of protein levels in the gut content of the fifth instar larva of *Bombyx mori* during the free running period of the rhythm under 12 h light/ dark cycle (LD), continuous light (LL) and continuous dark (DD) conditions

Photo period	No of peaks	Interval between peaks in h 1-2	2-3	3-4	4-5	5-6	6-7	Mean interval in h
LD	6	5	5	4	4	2	-	3.3
LL	7	3	6	3	2	2	3	2.7
DD	5	2	7	7	4	-	-	4.0

Photo period	No of troughs	Interval between troughs in h 1-2	2-3	3-4	4-5	5-6	6-7	Mean interval in h
LD	6	4	6	5	5	2	-	3.7
LL	7	9	6	2	2	3	2	3.4
DD	5	4	4	5	9	-	-	4.4

06h (~ 58 mg), 10h (~41 mg), between14-15h (~27 mg), 18h (~26 mg), 20h (~23 mg), 23-00h (~28 mg) and next day at 04-06h (~33 mg). Under DD, the total protein rhythm showed 7 peaks and 7 troughs. Peaks appeared at 06h (~85 mg), 08h (~97 mg), 11h (~58 mg) 16h (~43 mg) 18h (~56 mg), 22h (~45 mg) and next day at 03h (~57 mg). Troughs appeared at 07h (~76 mg), 10h (~50 mg), 14h (~27 mg), 17h (~19 mg), 20-21h (26-29 mg), 00h (26 mg) and next day at 04-05h (~26 mg).

Gut Content

Under LD the total protein rhythm of the gut content showed 6 peaks and 6 troughs during the 24h free running period of the rhythm (Fig. 2.A). The first peak appeared at 08-09h (~17 mg), 13-14h (~10 mg) 18-19h (~12 mg), 23h (~15 mg) and next day again at 03h (~10 mg) and 05h (~8 mg). Troughs were recorded at 06h (~6 mg), 10h (~ 9 mg), 16h (~2 mg) 21h (~5 mg) and next day at 02h (~7 mg). Under LL, the protein rhythm showed 7 peaks and 7 troughs during the 24h free running period. Peaks appeared at 08-10h (~12 mg), 13h (~ 11 mg), 19h (~13 mg), 22h (~8 mg), 00h (~11 mg) and next day at 02h (~12 mg) and 05h (~6 mg). Troughs appeared at 06h (~5 mg), 15h (~ 5 mg), 21h (~ 5 mg), 23h (5 mg) and next day at 01h (~7 mg), 04h (~5 mg) and 06h (~ 4 mg). Under DD, the total protein rhythm showed 5 peaks and 5 troughs during the 24h free running time. Peaks appeared at 09h (~9 mg), 11h (~12 mg), 18h (~10 mg), 23-01h (~11 mg) and next day at 05h (~5 mg). Troughs appeared

Table 2: Interval between peaks (Table 2A) and troughs (Table B) of protease activity in the gut wall of the fifth instar larva of *Bombyx mori* during the free running period of the rhythm under 12 h light/ dark cycle (LD), continuous light (LL) and continuous dark (DD) conditions

Photo period	No of peaks	Interval between peaks in h 1-2	2-3	3-4	4-5	5-6	6-7	Mean interval in h
LD	6	2	5	2	4	8	-	3.5
LL	7	2	5	3	2	3	5	2.9
DD	6	2	5	3	4	5	-	3.2

Photo period	No of troughs	Interval between Troughs in h 1-2	2-3	3-4	4-5	5-6	6-7	Mean interval in h
LD	6	3	4	3	3	7	-	3.3
LL	7	2	4	3	3	8	3	3.3
DD	6	5	5	4	5	3	-	3.7

Table 4: Interval between peaks (Table 4A) and troughs (Table 4B) of protease activity levels in the gut content of the fifth instar larva of *Bombyx mori* during the free running period of the rhythm under 12 h light/ dark cycle (LD), continuous light (LL) and continuous dark (DD) conditions

Photo period	No of Peaks	Interval between Peaks in h 1-2	2-3	3-4	4-5	5-6	6-7	Mean interval in h
LD	7	4	2	5	6	2	2	3.0
LL	7	2	6	5	3	2	5	3.3
DD	7	4	3	7	3	4	2	3.3

Photo period	No of troughs	Interval between troughs in h 1-2	2-3	3-4	4-5	5-6	6-7	Mean interval in h
LD	7	5	2	4	5	3	2	3.0
LL	7	2	6	5	3	2	3	3.0
DD	7	2	4	7	3	4	2	3.1

at 06h (~4 mg), 10h (~7 mg), 14h (~5 mg), 19h (~4 mg) and the last one at 04h (~4 mg).

Circadian protease rhythms

Gut wall

Under LD the protease activity rhythm showed 6 peaks and 6 troughs in the gut wall during the 24h free running period of the rhythm (Fig.1.B). Peaks appeared at 08h (0.33 μ moles / mg protein) 10 hr (0.37 μ moles), 15h (0.45 μ moles), 17h (0.73 μ moles), 21h (0.77 μ moles) and next day at 05h (0.77 μ moles). Troughs appeared at 06 hr (0.26 μ mole) 09h (0.23 μ moles), 13h (0.27 μ moles), 16h (0.23 μ moles), 18-19h (~0.21 μ moles) and the sixth one at next day at 02h (0.13 μ moles). Under LL the protease activity rhythm showed 7 peaks and 7 troughs in the gut wall during the 24h free running period. Peaks appeared at 08h (0.46 μ moles) 10h (0.68 μ moles), 15h (0.45 μ moles), 18h (0.52 μ moles), 20h (0.68 μ moles), 23h (0.76 μ moles) and next day at 04h (0.69 μ moles). Troughs appeared at 07h (0.27 μ mole) 09h (0.24 μ moles), 13h (0.21 μ moles), 16h (0.26 μ moles),19h (0.42 μ moles), and next day at 03 h (0.11 μ moles) and 06h (0.46 μ moles). Under DD, the protease activity rhythm showed 6 peaks and 6 troughs in the gut wall. Peaks appeared at 10h (0.64 μ moles) 12h (0.72 μ moles), 17h (0.62 μ moles), 20h (0.52 μ moles), 00h (0.74 μ moles) and the last one next day at 04-05h (~0.67 μ moles). Troughs appeared at 08 hr (0.24 μ moles), 13h (0.13 μ moles), 18h (0.26 μ moles), 22h (0.37 μ moles), and

Table 5: Comparative analysis of the phase response curves of the protein and protease rhythms in the gut wall of the fifth instar larvae of *Bombyx mori*, in terms of mean number of peaks and troughs and the mean interval between them, under 12 h light / dark cycle (LD), continuous light (LL) and continuous *dark (DD) conditions*

Parameter	Total Proteins			Protease Activity		
	LD	LL	DD	LD	LL	DD
No. of peaks	6	7	7	6	7	6
No .of troughs	6	7	7	6	7	6
Mean interval b/w peaks	3.5	2.6	3.0	3.5	~3.0	~3.0
Mean interval b/w troughs	~4.0	~3	~3.0	~3.0	~3.0	~4.0
Combined mean interval of peaks and troughs	3.5	~3.0	3.0	~3.0	3.0	3.5
Probable no. of PS/ES cycles	6	7	7	6	7	6
Time taken for each PS/ES cycles	4.0(24/6=4)	3.4(24/7=3.4)	3.4(24/7=3.4)	4.0(24/6=4)	3.4(24/7=3.4)	4.0(24/6=4)
Free running time of rhythm	24(4x6=24)	20.4(3.4x6=20.4)	20.4(3.4x6=20.4)	24(4x6=24)	20.4(3.4x6=20.4)	24(4x6=24)
Mean peak value of Proteins (mg/g)	60.9	55.5	62.8	0.57	0.61	0.65

Table 6: Comparative analysis of the phase response curves of the protein and protease rhythms in the gut content of the fifth instar larvae of *Bombyx mori*, in terms of mean number of peaks and troughs and the mean interval between them, under 12hr light / dark cycle (LD), continuous light (LL) and continuous dark (DD) conditions

Parameter	Total Proteins			Protease Activity		
	LD	LL	DD	LD	LL	DD
No. of Peaks	6	7	5	7	7	7
No .of Troughs	6	7	5	7	7	7
Mean interval b/w Peaks	~3.0	~3.0	4.0	3.0	~3.0	~3.0
Mean interval b/w Troughs	~4.0	~3.0	~4.0	3.0	3.0	~3.0
Combined mean interval of Peaks and Troughs	~3.0	3.0	4.0	3.0	3.0	3.0
Probable no. of PR/ER cycles	6	7	5	7	7	7
Time taken for each PR/ER cycles	4.0 (24/6=4)	3.4 (24/7=3.4)	4.8 (24/5=4.8)	3.4 (24/7=3.4)	3.4 (24/7=3.4)	3.4 (24/7=3.4)
Free running time of Rhythm	24 (4x6=24)	20.4 (3.4x6=20.4)	28.8 (4.8x6=28.8)	~24 (3.4x7=23.8)	~24 (3.4x7=23.8)	~24 (3.4x7=23.8)
Mean Peak value of Proteins (mg/g)	11.95	13.1	9.61	0.044	0.040	0.040

Source: Fig. 2 A &B

next day at 02- 03h (~0.13μ moles) and 06h (0.46 μ moles).

Gut content

Under LD the protease activity showed 7 peaks and 7 troughs in the digestive juice during the 24h free running period of the rhythm (Fig. 2.B). Peaks appeared at 10h (0.056 μ moles) 14h (0.045 μ moles), 16h (0.045 μ moles), 20 h (0.065 μ moles) and next day at 02h (0.028 μ moles), 04h (0.033 μ moles) and again at 06h (0.038 μ moles). Troughs occurred at 08h (0.018 μ moles), 13h (0.026 μ moles), 15h (0.032 μ moles), 17-19h (0.014 μ moles), 00h (0.015 μ moles) and next day at 03h (0.024 μ moles) and 05h (0.026 μ moles). Under LL the protease activity showed 7 peaks and 7 troughs in the digestive juice during the 24h free running period of rhythm. Peaks appeared at 07h (0.048 μ moles), 09h (0.041 μ moles), 15h (0.041μ moles), 20h (0.057 μ moles), 23h (0.052 μ moles) and next day at 01h (0.03 μ moles) and 06h (0.037 μ moles). Troughs occurred at 06h (0.034 μ moles), 08h (0.037 μ moles), 12-14h (~0.018μ moles each), 19h (0.008 μ moles), 22h (0.045 μ moles), 00h (0.023μ moles) and next day at 03h (0.019 μ moles). Under DD the protease activity rhythm showed 7 peaks and 7 troughs. Peaks appeared at 06h (0.056 μ moles), 10h (0.056 μ moles), 13h (0.044μ moles), 20h (0.037 μ moles), 23h (0.030 μ moles) and next day at 03h (0.032 μ moles) and 05h (0.049μ moles). Troughs appeared at 09h (0.039μ moles), 11h (0.026 μ moles), 15h (0.016 μ moles), 22h (0.027μ moles), 00h (0.019μ moles) and next day at 04h (0.029 μ moles) and 06h (0.042 μ moles).

DISCUSSION

Insect gut acts as a transient repository for proteins, carbohydrates, lipids and other biochemical constituents and their levels vary as a function of synthesis, secretion and dietary uptake (Nagata and Yashitake, 1989). These three events occur in a circadian fashion under the influence of light and their rhythmicity is probably triggered by tissue specific endogenous pace makers (Shimizu *et al*., 2001; Sehadova *et al*., 2004; Iwai *et al*., 2006; Kyung *et al*., 2006).

Gut wall protein and protease rhythms

The glandular epithelium of the gut wall is the rich source of over 96 proteins that are involved in cell growth, metabolism, immunity, heat shock treatment, muscle contraction, protein degradation, carcinoma control, carotenoid binding and antimicrobial activity (Tabunoki *et al*., 2002; Weng *et al*., 2003; Kajiwara *et al*., 2005; Xia *et al*., 2007; Pandian *et al*., 2008; and Saizhang *et al*., 2011). Prominently, the gut proteins include endogenous digestive enzymes such as amylase, cellulase, hemicellulase, cellobiase, urease, invertase, trehalase, lipase, sucrase, xylanase, pectinase and proteases like trypsin and dipeptidase (Yamashita and Hasegawa, 1974;

Terra and Ferreria, 1994; Sarangi and Anitha, 2007; Mohamad Sadique *et al*., 2008; Anand *et al*., 2010; Manjula *et al*., 2010). The peaks and troughs observed in the phase response curves of the protein and protease rhythms (Fig. 1A and 1B) of the gut wall are probably indicative of two vital stages of gene expression as reported earlier in the silk gland, fat body and segmental muscle tissues of *Bombyx mori* (Sailaja and Sivaprasad, 2010a, b; Sailaja *et al*., 2011; Sivaprasad and Sailaja, 2011). Accordingly, the peaks represent the translation phases, the troughs depict transcription phases and the mean intervals between peaks and troughs indicate the duration of protein synthetic cycles that are subjected to modulation by the light cues. Since, the light is the principal *zeitgeber* (time giver) of the circadian rhythm (Peschel *et al*., 2009), it either advances or delays its free running time by significantly altering the duration of each PS cycle in a tissue-specific manner. Clearly, the gut wall protein rhythm maintained 6PS cycles under LD, each with 4h duration and 7 cycles under LL and DD, each with a reduced duration of 3.4h. Thus, both LL and DD conditions reduced the duration of each PS cycle by 36 min (from 4h to 3.4h). Consequently, the 24h free running time of circadian protein rhythm under LD is clock shifted to 20.4h under LL and DD conditions in the gut wall (Table 5). With the result, the gut wall cells are able to accomplish one additional round of protein synthesis during the given period of the rhythm.

Within the profile of total proteins of cells in the gut wall, the protease activity maintained similar trends in its rhythm. It included 6 enzyme synthetic cycles (ES cycles) under LD and DD and 7 under LL condition. As in the case of protein rhythm, the durations of each ES cycle lasted for about 04h under LD and DD and for 3.4h under LL condition. Thus, the duration of each cycle of protease activity which was about 24h under LD and DD, gets reduced by ~36 min (from 4 to 3.4h) under LL condition during the free running time of the protease rhythm (Table 5).

Gut content protein and protease rhythms

The gut content represents digestive juice secreted by the epithelial cells of the gut wall and the mulberry leaf juice obtained from the diet. It's biochemical profile include a variety of proteins, lipids and carbohydrates such as the pectin, xylan, cellulose, starch and a multitude of digestive enzymes such as proteases, carbohydrases and lipases emanating both from the gut wall cells and from the endosymbiotic microbes (Hayashiya *et al*., 1976; Kaufman and Klug, 1991; Shinbo *et al*., 1996; Santo Domingo *et al*., 1998; Anand *et al*., 2010). In the gut content (Fig. 2A and 2B) the rhythm maintains 6 protein releasing cycles (PR cycles) under LD, 7 under LL and 5 under DD. Consequently, each cycle runs for about 4.0h under LD, 3.4h under LL and 4.8h under DD. Thus, the duration of each cycle of the protein rhythm is reduced by 36 min (from 4 to 3.4h) under LL, but delayed by 48 min (from 4 to 4.8h) under DD. This change shifts the clock and resets the free running time at 20.4h under LL and 28.8h under DD instead of normal 24h under LD. The clock- shifting so caused results in the accomplishment of one additional PR cycle during the free running time of the rhythm (7 cycles instead of 6) under LL and reduction in their number (from 6 to 5 cycles) under DD. The impact of light on the number and duration of protease releasing cycles, however, remains the same under LD, LL and DD conditions, wherein, the rhythm maintained 7 cycles, each with duration of ~3.4h. Consequently the 24h-free running time of the protease rhythm remained unaffected by changes in the duration of photoperiod (Table 6). As projected for the silk gland, fat body, segmental muscle and haemolymph of silkworm (Sailaja and Sivaprasad, 2010a, 2010b, Sailaja *et al*., 2011; Sivaprasad and Sailaja, 2011), the photoperiod-induced changes in the gut protein rhythm reflect corresponding changes in the timing of gene expression. It is known that the silkworm gut wall is the major site of gene expression in which about 216 genes encode different enzymes, proteins, transferases (Xia *et al*., 2007). Their expression pattern seems to be modulated by the photoperiod-dependent peripheral clock genes such as *Bmper* and *Bmtim* (Soren *et al*., 2001; Iwai *et al*., 2006; Jin *et al*., 2004). If, the photoperiod-induced changes in protein profiles and protease activity levels are an indication of the corresponding gene expression pattern, it is presumed that the gut wall genes express 6 times under LD at 4h intervals and 7 times under LL and DD at 3.4h intervals each. Thus, the gut wall genes maintains a constant rhythmic expression both under light (LL) and dark (DD) conditions, probably by taking cues either from the light and/or from the diet, both of which are identified as alternative *zeitgebers* (time givers) in the peripheral organs of insects (Damiola *et al*., 2000; Stokkan *et al*., 2001; Kita *et al*., 2002). Since, the silkworms reared under DD were also fed 5 times a day, they carried through the rhythm much like those reared under LL, the fact that confirms the existence of at least two-oscillators in the gut wall of *B. mori*, that respond separately for light and dark cues as observed in *Drosophila* (Forster, 2000). Hopefully, our findings (Sailaja and Sivaprasad, 2010 a, b., Sailaja *et al*., 2011; Sivaprasad and Sailaja, 2011) on silkworm protein rhythms provide further conclusive proof for the existence of peripheral clocks that entrain the protein rhythm in a tissue-specific manner in this lepidopteran insect. Evidently, the photoperiod-modulated gut rhythm causes adjustments in feeding times by resetting the timing of the expression of the circadian clock genes in the gut wall. This is substantiated by the fact that the feeding time and production of digestive enzymes are synchronized in the silkworm (Jiang *et al*., 2000; Ueno *et al*., 2006). Exposure of silkworm larvae to different durations of light-dark phases resets the circadian clocks not only by changing the frequency (*i.e.* number) of PS and PR cycles (as noted earlier), but also by altering their intensity (*i.e.* rate) during the free running time of the rhythm. Under each photoperiodic condition the rate of protein synthesis and release may be classified as active or slow. The protein/protease levels higher than those of the mean peak value (MPV = average protein value of peaks) might represent the timings of active protein synthesis and release, while those bellow it, the timing of slow protein synthesis and release. Though these two phases vary significantly under three photoperiodic conditions, those representing higher MPVs are note worthy to be considered as active synthetic phases. For instance, the intensity of the protein synthesis in the gut wall, expressed in terms of mean peak value is high (62.8 mg) under DD, moderate (60.9 mg) under LD and low (55.5 mg) under LL. Similarly, the intensity of protease activity rhythm is higher (0.65 μ moles) under DD, moderate (0.61 μ moles)

under LL and low (0.57 μ moles) under LD. The rate of protein/ protease release into the gut lumen is likewise affected by light-dark conditions. Higher mean peak value (11.9 mg) was observed under LD condition, moderate (10.3 mg) under LL and low (9.6 mg) under DD, a fact that indicates that both light and dark conditions are necessary for the release of proteins and other digestive enzymes into the gut-lumen soon after their synthesis in the cells of the gut wall. Because of this reason, the release of protease into the gut lumen was not significantly altered by variations in the photoperiod, as the enzyme activity was maintained almost at a constant rate (0.04 μ moles) under the three experimental photoperiodic conditions (LD, LL and DD). The timing of active protein/ protease synthesis varies according to the duration of photoperiod exposed.

Under LD condition active protein synthetic phase were observed at 06h (91.4 mg) and 09h (78.5 mg), while active enzyme (protease) synthetic phases were recorded at 17h (0.73 μ moles), 21h and at 05h (0.77 μ moles each). Similarly active protein release occurred at 08-09h (~17 mg each) and at 23h (15 mg) while higher protease release occurred at 10h (0.056 μ moles) and 20h (0.065 μ moles). Under LL condition active protein synthetic phase occurred at 09h (~91 mg) and 13h (~69 mg), the protease synthetic phases at 10h (0.68 μ moles), 20hr (0.68 μ moles) 23h (0.76 μ moles) and at 04h (0.69 μ moles). Similarly, under this condition active phases of protein release occurred at 08-10h (~12 mg each), 19h (13 mg) and at 02h (12 mg) and those of protease releasing phases at 07h (0.048 μ moles) and 20h (0.057 μ moles) and at 23h (0.052 μ moles). Under DD condition active phases of protein synthesis occurred at 06h (~85 mg) and 08h (~97 mg) and those of protease at 12h (0.72 μ moles) and 00h (0.74 μ moles). Similarly in dark condition, the active phases of protein release occurred at 11h (~12 mg) and 23-01h (~11mg each) and those of protease at 06h, 10h (~0.05 μ moles each) and 05h (~0.05 μ moles). Clearly, the rate of protein synthesis in the gut wall seems to be active during early hours of the photic phase, with 3h interval between two peaks as evidenced by higher peaks at 06 and 09h of the day (Fig. 1A) under normal conditions of LD. Though the same numbers of active peaks were observed under LL and DD conditions, the timing of peaks and the interval between the peaks were slightly altered. While the appearance of peaks is slightly delayed under LL (*i.e.* from 06h to 09h and from 09 to 13h) they occurred in close proximity at 06 and 08h under DD with a reduced interval of 02h between peaks. Thus, a change in duration of photoperiod could significantly alter the rate of protein synthesis not only by shifting the peak hours but also by advancing or delaying the interval between peaks. Similarly, protein releasing phases are active during early (08-09h) and late hours (18-19h) of the photic phase under LD, each lasting for an hour. But in LL condition, a higher protein releasing phase continued for two hours from 08 to 10h in the first phase and for one hour at 19h in the second phase. In darkness (DD) however, the protein release phases shifted to11h and 23-01h, with duration of one hour and two hours respectively. The study indicates that the timing of protease synthesis and release are slightly different from those of total proteins as a whole. While protein synthesis was maximal during early hours of photic phase under LD condition, the protease synthesis seems to be at its peak during the late hours (05h) of the day. Under LL condition however, the active phases not only increased to three but their timing also shifted to different hours (10h, 20h and 23h). A similar trend was recorded under DD with three distinct brisk phases appearing at 12h, 00h and 4-5h of the day. The release of protease from the gut wall cells under LD conditions occurs twice at maximal rate at 10h and 20h one in photopic phase and other in scotopic phase. When the silkworm larvae were reared in LL condition, the number of maximal releasing phases increased by one (from 2 to 3) and occurred at 07, 20 and 23h. In darkness also, the maximal, releasing phases occurred at 06, 10 and 05h. Thus, both LL and DD conditions favour protease release from the gut wall with at least one active enzyme releasing phase occurring during the light regime.

Protein rhythm versus protease rhythm

A comparative analysis of the PRCs of protein and protease rhythms shows inverse relationship between their levels. The protein levels rise and fall with those of protease activity during the free running time of the rhythm. The higher the peak levels of protein, the lower the protease activity levels and vice versa. By and large the same trend was continued both in the gut wall and gut content under altered photoperiodic conditions of LD, LL and DD (Figs. 3A, B, C and 4A, B, C). In the gut wall for instance, the peaks in protease activity at 08h, 10-12h, 15h, 17h, 21h, 1h and 5-6 h under LD, 10h, 15h, 18h, 20-21h, 23h and 4-5h under LL, 10h, 12h, 17h, 20-21h, 00h and 04-05h under DD are accompanied by troughs in protein values indicating continuous intracellular proteolysis. The proteases are actively synthesized just before feeding time as observed in the present investigation and the synthesis exclusively done by the gut epithelial cells but not by the bacteria residing in the gut wall (Sharma *et al*., 1984). Conversely, the troughs in the levels of protease activity at 06h, 09h, 13-14h, 16h, 18-19h, 23-00h and 02-03h under LD, 06-07h, 09h, 13h, 16h,19h, 22h and 03h under LL and 06-08h, 11h, 13h, 18h, 22h, 02-03h and 06h under DD have resulted in accumulation of more proteins as evidenced by their peaks at respective timings. A notable observation in the trend of protein and protease rhythms is that greater amounts of proteins accumulate during the early hours of the day around 02 to 04h with corresponding decline in protease activity levels (Fig. 3A,B,C) and that these proteins might play crucial role in triggering metamorphic changes in *B. mori*.

In the gut content also, the peaks in the activity levels of enzyme at 10h, 14h, 16h, 20-22h, 02h and 04h, under LD, 11h, 15h, 20-23h, 01h, 04-06h, under LL and 06h, 10h, 13h, 19h, 05h under DD were accompanied by troughs in protein levels indicating corresponding timings of protein digestion in the gut. Conversely, the troughs in the levels of protease activity at 08h, 13h, 15h, 17-19h, 00h, 03h and 05h under LD, 08-09h, 12- 14h, 19h, 00h and 03h under LL and 10h, 13h, 16-18h, 22h and 00h under DD have resulted in accumulation of more proteins as evidenced by their peaks at respective timings. This trend seems to be more pronounced and represented in the form of higher peaks at10-16h, 20-22h and again at 01-06h under LD, at 07-11h, 20-23h and 04-06h under LL at 06-07h, 19-23h and 03-06h under DD (Fig 4A, B, C). Thus, during silkworm larval development the circadian protein and

protease rhythms are modulated by photoperiodic conditions under which the levels of proteins and protease activity fluctuate in an antagonistic and counter balancing manner. Probably, the photoperiod-induced modulation resets the circadian clock in the gut and brings about changes in the free running time of the circadian protein rhythm by effecting corresponding changes in the timings of protein synthesis and digestion.

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