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Photoperiod-induced clock-shifting in the circadian protein and amino acid rhythms in the larval fat body of silkworm, *Bombyx mori*

B. Sailaja and S. Sivaprasad*

Department of Zoology, Smt. N.P.S. Government College for Women, Chittoor-517 002 (A.P), INDIA
*Corresponding author. E-mail: sivaprasadzoology@yahoo.co.in

Abstract: The photoperiod-induced clock-shifting in the free running time of the circadian protein and amino acid rhythms was studied in the larval fat body of *Bombyx mori*. The analysis of peaks and troughs of phase response curves of the rhythm revealed that the fourth and fifth instar larvae grown under normal 12 h light and 12 h dark cycle (LD) showed 7 protein synthetic cycles, while those reared under continuous light (LL) recorded 9.5 cycles in fourth instar and 8 in fifth instar. Under continuous dark (DD), the protein rhythm maintained 8 cycles in fourth instar and 7.5 cycles in fifth instar. Clearly, both LL and DD conditions advance the 24-h free running time of the protein rhythm by durations ranging from 1.6 to 6.5 h. Comparative analysis of protein and amino acid rhythms shows that the photoperiod modulates the free running time of the former by altering the rate of amino acid mobilization.

Keywords: *Bombyx mori*, Circadian amino acid rhythm, Circadian protein rhythm, Fat body, Photoperiod

INTRODUCTION

Like the mammalian liver, insect fat body is a dynamic metabolic tissue that performs multiple physiological functions in energy and intermediary metabolisms (Scott *et al.*, 2004; Arrese and Soulages, 2010). Its constituent cells; trophocytes, urocytes and mycetocytes play unique roles in metabolism (Pajio, 2010). While the trophocytes synthesize and store the biochemical constituents of carbohydrate, protein and lipid metabolisms, the mycetocytes facilitate vitamin B synthesis and symbiotic cellulose digestion and urocytes help in storage-excretion of uric acid (David and Ananthakrishnan, 2006). The proteomic analysis of the fat body in *Bombyx mori* revealed the presence of 722 proteins involved in larval growth, development and metamorphosis that are synthesized and released at regular intervals (Hou *et al.,* 2007). Moreover, the insect fat body is known to contain endogenous pacemakers that modulate locomotory and feeding behaviours (Kanyan Xu *et al.,* 2008) probably by causing rhythmic changes in its biochemical profiles including proteins. Evidently, the fat body protein levels are subjected to rhythmic changes under the influence of light-sensitive endogenous circadian clock mechanism.

The economically important insect *Bombyx mori* has been viewed as a powerful model for the study of circadian rhythms next only to *Drosophila*. Most of the circadian studies focused on the identification, isolation and cloning of silk genes (Ishikawa and Suzuki, 1985; Kimura *et al.*, 1985; Obara and Suzuki, 1988; Michaille *et al.*, 1989; Durand *et al.*, 1992; Fukuta, *et al.*, 1993; Gizelak, 1995), determination of their expression patterns and identification of their products (Hall, 2003; Sharma, 2003; Iwai *et al.*, 2006) and the identification of tissue-specific endogenous pace makers or circadian clocks (Sehadova *et al.*, 2004; Reppert, 2006; Dolezel *et al.*, 2008). Obviously, these studies attempted to elucidate the molecular and genetic mechanisms without taking into cognizance its impact on the tissue-specific biochemical profiles. In order to bridge this gap, we made an attempt to analyze circadian changes in the fat body protein and amino acid profiles in the silkworm larvae during the fourth and fifth instars under the influence of altered photoperiodic conditions with a view to ascertain instar-specific and photoperiodic-specific clock-shifting in the free running time of the circadian protein rhythm.

MATERIALS AND METHODS

The present study was carried out on the Pure Mysore x CSR_2 hybrid variety of the silkworm *Bombyx mori,* reared under standard environmental conditions of 28^0 C, 85 % RH, as per Krishnaswamy (1986). After hatching from the eggs, the larvae were reared on M_5 variety of mulberry laves five times a day, at 6AM, 10 AM, 2 PM, 6PM and 10 PM under 12 h light and 12 h dark cycle. After the third moult, the larvae were divided into three batches, reared and fed separately under three different photoperiodic conditions, viz., 12 h light and 12 h dark cycle (LD), continuous light (LL) and continuous dark (DD) throughout the development of the fourth and fifth larval instars.

The fat body tissue was isolated every hour by dissecting the silkworm larvae in ice-cold Silkworm Ringer (Yamaoka *et al*., 1971).The circadian changes in the levels of total and soluble proteins was analyzed in 1% tissue homogenates in distilled water by the method of Lowry *et al.* (1951) on hourly basis from 8 A. M onwards for a period of 25 h that spans in between 3rd and 4th day of fourth instar and 5th and 6th day of fifth instar. The rhythmic changes in the levels of structural proteins were obtained by subtracting the levels of soluble proteins from total proteins. The circadian changes in the levels of amino acids were assayed on bi-hourly basis by the method of Moore and Stein (1954) as described by Colowick and Kaplan (1957) in 10% homogenates of the tissue in 10% TCA, during the same period.

RESULTS

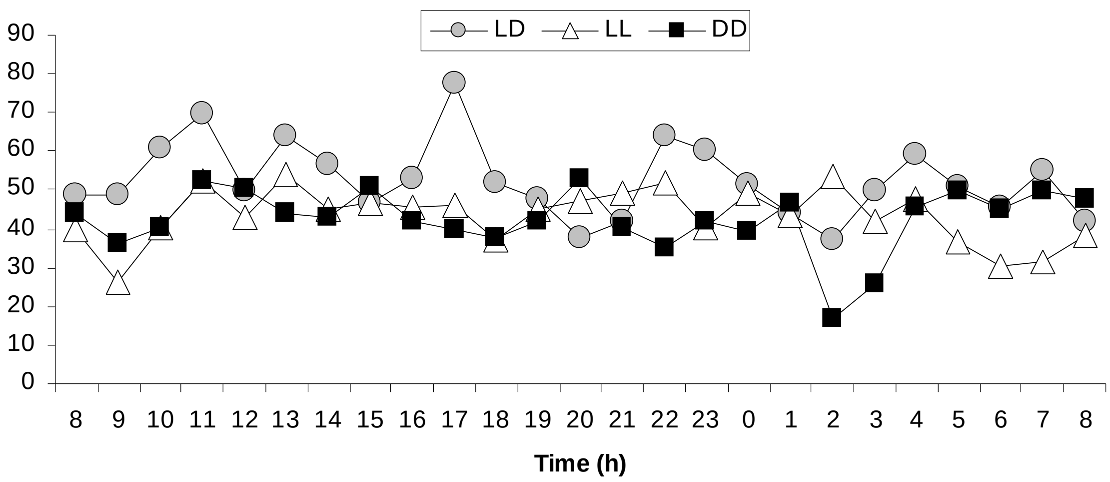
The 24-h changes in the protein and amino acid rhythms is designated the *free running period* or *tau* and shown in the form of phase response curves with its characteristic peaks (elevated points) and troughs (low points) in figures 1 to 6. The circadian data was analyzed in terms of intervals between peaks and troughs and presented in tables 1 to 5. The protein and amino acid levels were expressed in mg / g wet weight of tissue.

Fourth instar larval rhythms

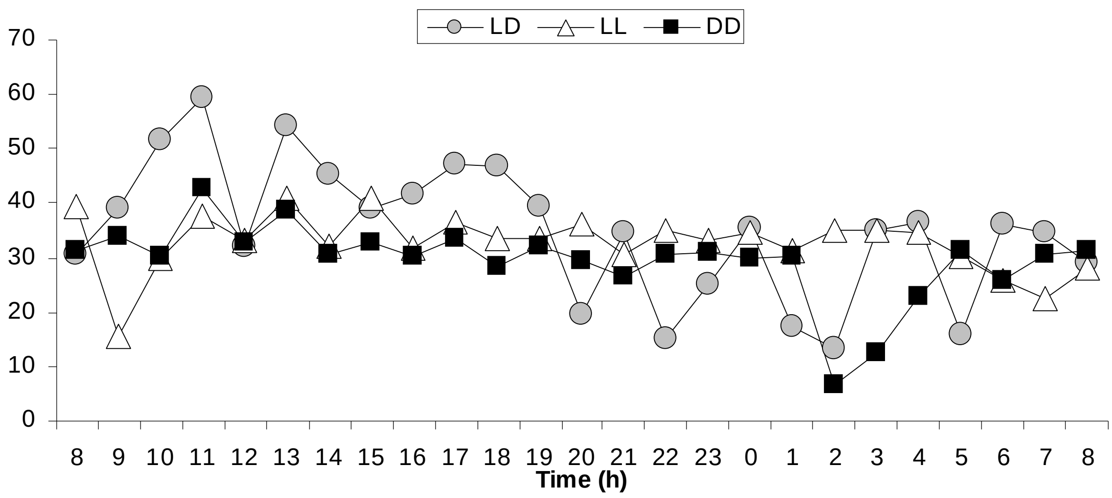
Total proteins: Under 12:12 light: dark cycle (LD), the free running time of the total protein rhythm of the fat body showed 6 peaks and 6 troughs (Fig. 1A; Tables 1 and 2). The peaks occurred at 11 h (~70 mg / g wet wt. of tissue), 13 h (~64 mg) 17 h (~76 mg), 22 h (64 mg) and next day at 04 h (~59 mg) and 07 h (~55 mg). Similarly, troughs appeared at 08 h (~49 mg), 12 h (~50 mg), 15 h (~46 mg), 20 h (~38 mg) and next day at 02 h (~37 mg) and 06 h (~45 mg). Under continuous light (LL), 10 peaks and 9 troughs were recorded in the *tau* of the total protein rhythm. Peaks in the protein levels were recorded at 8 h (~40 mg), 11h (~52 mg), 13 h (~54 mg), 15 h (~47 mg), 17 h (~46 mg), 22 h (52 mg), 00 h (~49 mg) and next day at 02 h (~54 mg), 04 h (~48 mg) and 08 h (~38 mg). Similarly, under LL, troughs were recorded at 09 h (~26 mg), 12 h (43 mg), 14 h (~45 mg), 16 h (~ 45 mg), 18 h (~ 37 mg), 23 h (~40 mg) and next day at 01h (~44 mg), 03 h (~42 mg) and 06 h (~30 mg). Under continuous dark (DD) the total protein content of the fat body recorded 8 peaks and 8 troughs during the 24 h free running period of the rhythm. Peaks occurred at 08 h (~44 mg), 11 h (~52 mg), 15 h (~ 51 mg), 20 h (~53 mg), 23 h (~42 mg) and next day at 01 h (~47 mg), 05 h (~50 mg) and 07 h (~50 mg). Likewise, under DD, troughs appeared at 09 h (~36 mg), 14 h (~43 mg), 16 h (42 mg), 18 h (~38 mg), 22 h (~35 mg) and next day at 02 h (~ 17 mg), 06 h (~45 mg) and 08 h (~48 mg). Though, the individual intervals between any two peaks or troughs ranged from 2 to 6 h, the mean interval between any two peaks or troughs stood at 3.5 h under LD, ~2.4 h under LL and 2.9 under DD (Fig. 1 A; Tables 1 and 2).

Soluble proteins: Under LD, the 24-h *tau* of the soluble protein rhythm showed 7 peaks and 8 troughs (Fig. 1B; Tables 1 and 2). Peaks appeared at 11 h (~59 mg), 3 h (~54 mg), 18 h (~47 mg), 21 h (~35 mg), 00 h (~35 mg) and next day at 04 h (~37 mg) and 06 h (~36 mg). Troughs occurred at 08 h (31 mg), 12 h (~32 mg), 15 h (~39 mg), 20 h (~ 20 mg), 22 h (~15 mg) and next day at 02 h (~13 mg), 05 h (~16 mg) and 08 h (~29 mg). Under LL, the 24-h free running time of the soluble protein rhythm recorded 10 peaks and 9 troughs. Peaks in soluble protein levels occurred at 08 h (~ 39 mg), 11 h (~38 mg), 13 h (~41 mg), 15 h (~ 41 mg), 17 h (~37 mg), 20 h (~36 mg), 22 h (35 mg), 00 h (~35 mg) and next day at 02-03-04 h (~35 mg each) and 08 h (~28 mg). Similarly, troughs in their levels appeared at 09 h (~15 mg), 12 h (~ 33 mg), 14 h (~32 mg), 16 h (~32 mg), 18-19 h (~ 34 mg each), 21h (~30 mg), 23 h (~33 mg) and next day at 01h (~ 31 mg) and at 07 h (~22 mg). Under DD, the 24-h free running time of the soluble protein rhythm recorded 9 peaks and 9 troughs. Peaks appeared at 09 h (~34 mg), 11 h (~ 43 mg), 13 h (~ 39 mg), 15 h (~33 mg), 17 h (~ 33 mg), 19 h (~32 mg), 22-23 h (~31 mg each) and next day at 05 h (~31 mg) and 08 h (~31 mg). Similarly, troughs occurred at 08 h (~32 mg), 10h (~ 30 mg), 12 h (~33 mg), 14 h (~30 mg), 16 h (~30 mg), 18 h (~ 28 mg), 21 h (~ 26 mg) and next day at 02 h (~7 mg) and 06 h (~26 mg). While the individual intervals between any two peaks or any two troughs ranged from 2 to 6 h, their combined mean interval stood at ~2.9 h under LD, ~2.3 h under LL and ~2.6 under DD (Fig. 1B; Tables 1 and 2).

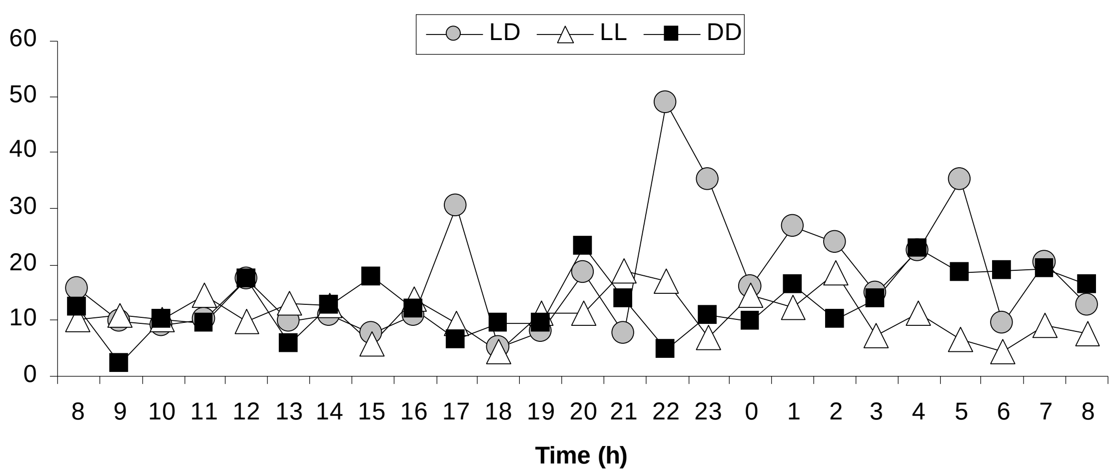
Structural proteins: Under LD, the 24-h free running time of the structural protein rhythm showed 8 peaks and 8 troughs (Fig. 1C; Tables 1 and 2). Peaks appeared at 08 h (~15 mg), 12 h (~17 mg), 17 h (~30 mg), 20 h (~18 mg), 22 h (~49 mg) and next day at 01h (~27 mg) 05 h (~35 mg) and 07 h (~20 mg). Similarly, troughs were observed at 09-10 h (~9 mg each), 15 h (~7.5 mg), 18 h (~ 5.2 mg), 21 h (~7 mg), 00 h (~16 mg) and next day at 03 h (~15 mg), 06 h (~9 mg) and 08 h (~13 mg). Under LL, the structural protein rhythm recorded 9 peaks and 9 troughs. Peaks occurred at 09 h (~11 mg), 11 h (~ 15 mg), 13 h (~ 13 mg), 16 h (~ 14 mg), 21 h (~19 mg), 00 h (~14 mg) and next day again at 02 h (~19 mg), 04 h (~11 mg) and 07 h (~9 mg). At the same time troughs were recorded at 08 h (~10 mg), 10 h (~10 mg), 12 h (~ 9.8 mg), 15 h (~ 6 mg), 18 h (~4 mg), 23 h (~7 mg) and again next day at 01 h (~12 mg), 03 h (~7 mg) and 06 h (~4 mg). Under DD, the structural protein rhythm recorded 8 peaks and 8 troughs during the 24-h free running time. Peaks appeared at 08 h (~12 mg), 12 h (~17 mg), 15 h (~18 mg), 20 h (~23 mg), 23 h (11 mg) and next day again at 01h (~16 mg), 04 h (~23 mg) and 07 h (~19 mg). Similarly, troughs occurred at 09 h (~2 mg), 13 h (~6 mg), 17 h (~6.5 mg), 22 h (4.6 mg), 00 h (~10 mg) and



(A). Fat body total protein rhythm in IV instar (mg/g).



(B). Fat body soluble protein rhythm in IV instar (mg/g)

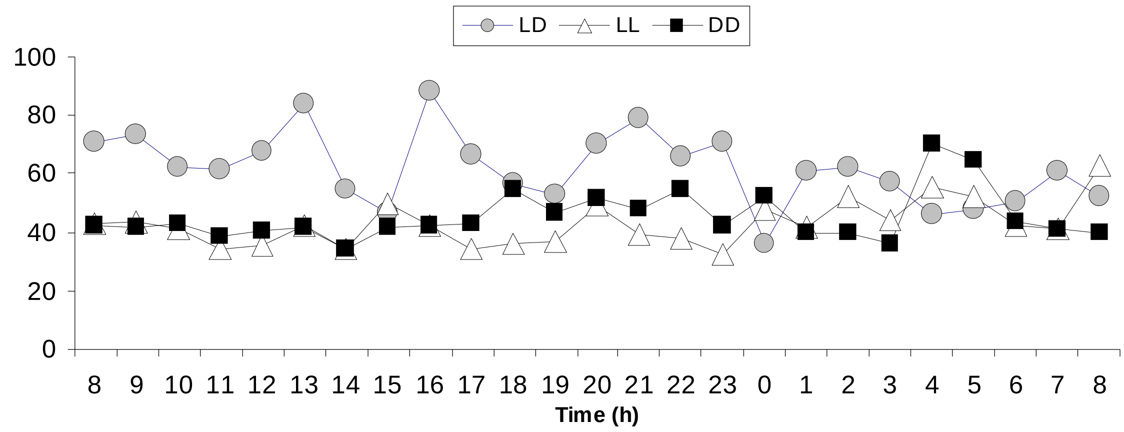


(C) Fat body structural protein rhythm in IV instar (mg/g)

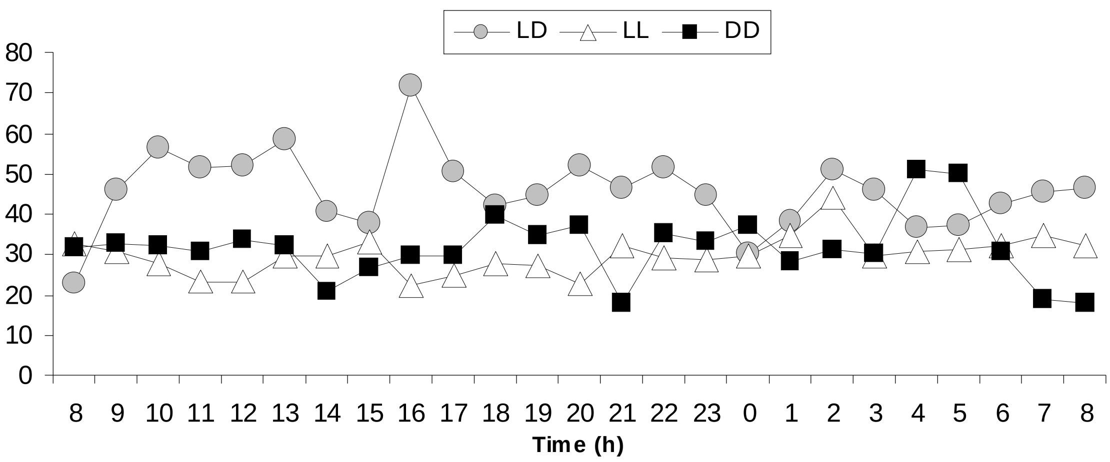
Fig. 1. *Phase response curves (PRCs) of the 24-h circadian protein rhythms (from 8AM on day 3 to 8 AM on day 4) in the fat body of the fourth instar larva of Bombyx mori, under 12h light: 12h dark cycle (LD); continuous light (LL) and continuous dark (DD).A. Total proteins; B. soluble proteins and C. structural proteins. (P values: <0.001).*

next day at 02 h (10 mg), 05 h (~18 mg) and 08 h (~16 mg). The individual intervals between any two peaks or troughs ranged from 2 to 6 h, their mean interval was ~2.9 h under LD, ~2.6 h under LL and 2.9 h under DD (Fig. 1C; Tables 1 and 2).

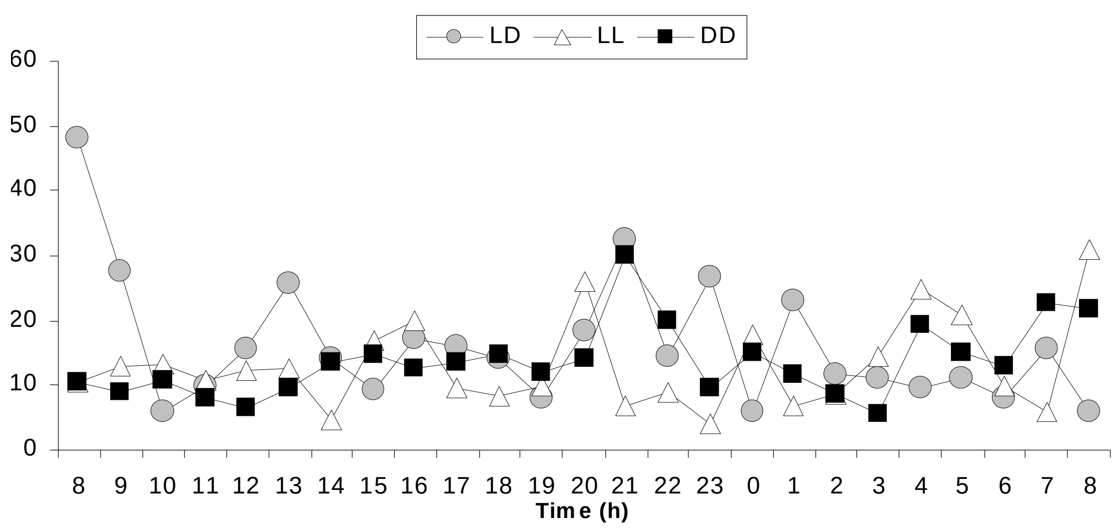
Free amino acids: In the fourth instar larva, the free amino acid rhythm of the fat body maintained relatively higher levels under LL and DD conditions compared to LD (Fig.



(A). Fat body total protein rhythum in V instar (mg/g)



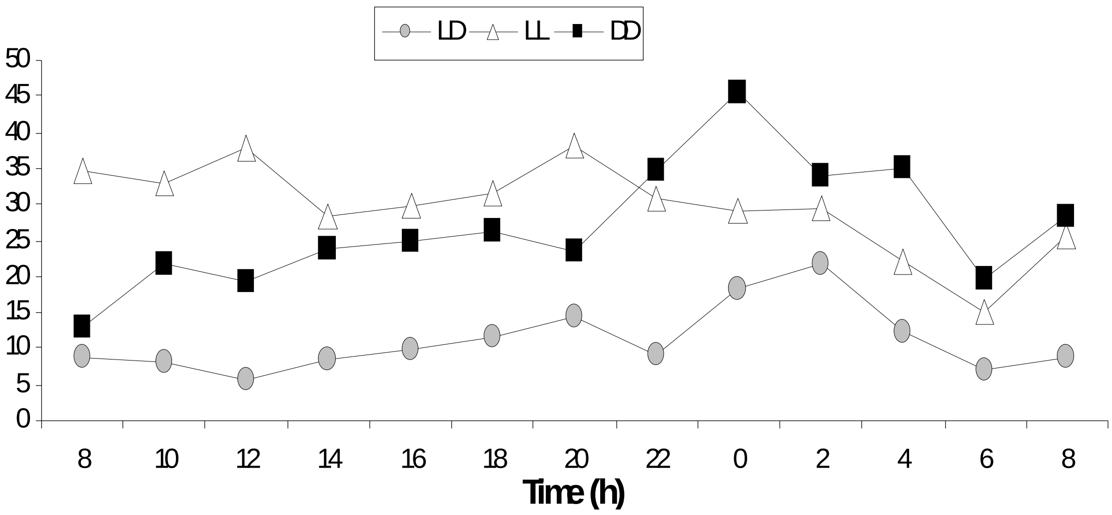
(B). Fat body soluble protein rhythum in V instar (mg/g)



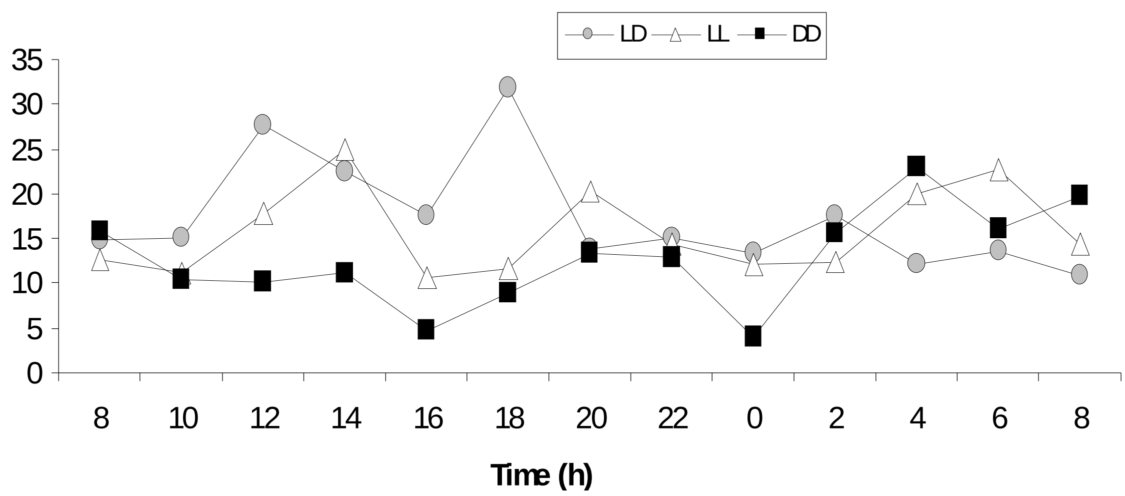
(C). Fat body structural protein rhythum in V instar (mg/g)

Fig. 2. *Phase response curves (PRC) of the 24-h circadian protein rhythm (from 8AM on day 5 to 8 AM on day 6) in the fat body of fifth instar larva of Bombyx mori, under 12h light: 12h dark cycle (LD); continuous light (LL) and continuous dark (DD) conditions . A. Total proteins; B. soluble proteins and C. structural proteins. (P values: <0.001).*

3A). Under LD, their levels ranged from ~ 6 mg to ~22 mg during the free running time, showing peaks at 08 h (8.7 mg), 20 h (~15 mg) and next day at 2 h (~22 mg). Similarly, troughs in their levels were recorded at 12 h (~6 mg), 22 h (~9 mg) on day-1, and again next day at 06 h (~7 mg). Under LL, significantly a higher rhythm (~15 to ~38 mg) is maintained in their levels, with peaks at 12 h (~38 mg) 20 h (~38 mg) and again next day at 8 h (~26 mg). Similarly, troughs in free amino acid levels were observed at 14 h (~28 mg), 00 h (~29 mg) and again, next day at 06 h (~15



(A). Fat body amino acid rhythm- IV instar (mg/g)



(B). Fat body amino acid rhythm- V instar (mg/g)

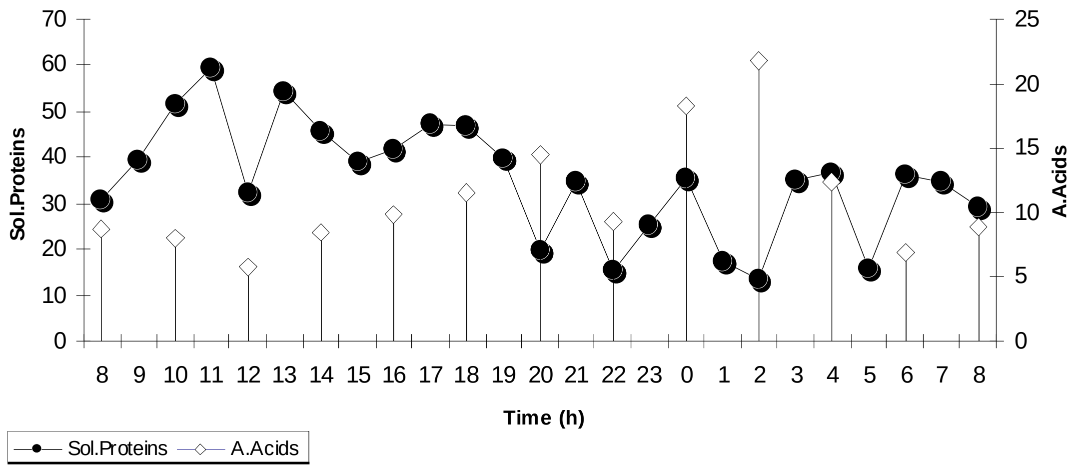
Fig. 3. *Phase response curves (PRC) of the 24-hr circadian amino acid rhythms in the fat body of (A) fourth instar (from 8AM on day-3 to 8 AM on day-4) and (B) fifth instar (from 8AM on day-5 to 8 AM on day-6) larvae of Bombyx mori, under 12h light: 12h dark cycle (LD); continuous light (LL) and continuous dark (DD) conditions (Source: Table 4.13). (P values: < 0.001).*

mg). Under DD, the range in amino acid rhythm varied in between ~13 to 46 mg, with peaks at 10 h (~22 mg), 18 h (~26 mg), 00 h (46 mg) on day-1 and 04 h (~ 35 mg) and 06 h (~28 mg) on day-2. Similarly, troughs in their levels were observed at 08 h (~13 mg), 12 h (~19 mg), 20 h (~24 mg) and again next day at 02 h (~34 mg) and 06 h (~22 mg).

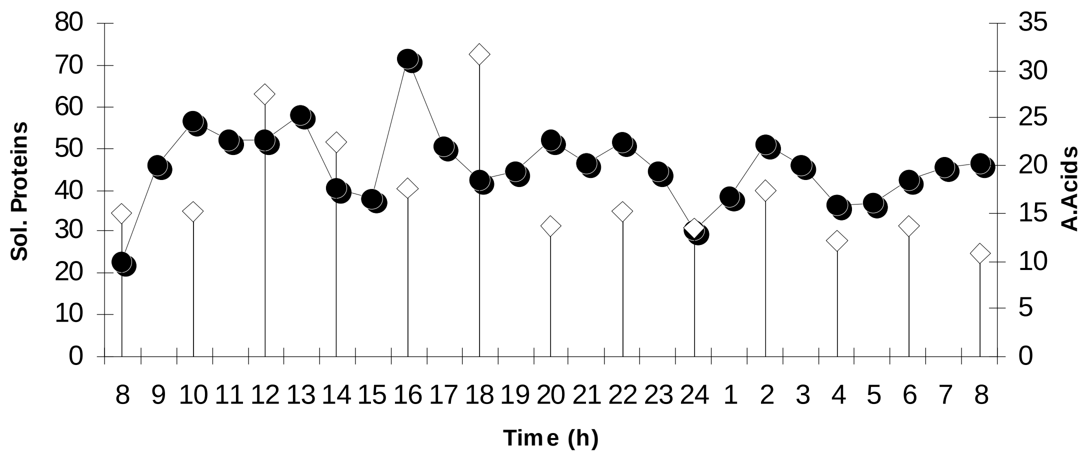
Fifth instar larval rhythms

Total proteins: Under 12:12 light / dark cycle (LD), the total protein rhythm of the fat body showed 7 peaks and 7 troughs (Fig. 2 A; Tables 3 and 4). Peaks appeared at 09 h (73 mg / g wet wt. of tissue) 13 h (~84 mg), 16 h (~88 mg), 21h (~79 mg), 23 h (~71 mg) and next day at 02 h (~62 mg) and 07 h (~61 mg). Similarly, troughs occurred at 10 h (~62 mg), 15 h (~47 mg), 19 h (~53 mg), 22 h (66 mg), 00 h (36 mg) and next day at 04 h (~46 mg) and 8 h (~52 mg). Under LL, the total protein rhythm showed 8 peaks and 7 troughs. Peaks were recorded at 09 h (~44 mg), 13 h (42 mg), 15 h (~50 mg), 20 h (~49 mg), 00 h (~48 mg) and next day at 02 h (~52 mg), 04 h (~55 mg) and 08 h (63 mg). Troughs occurred at 11h (34 mg), 14 h (~ 34 mg), 17 h (~34 mg), 23 h (~32 mg) and next day at 01 h (~41 mg), 03 h (~44 mg) and 06-07 h (~41 mg each). Similarly, the phase response curve of the total protein rhythm recorded 7 peaks and 7 troughs under DD. Peaks occurred at 10 h (~43 mg), 13 h (~42 mg), 18 h (~ 54 mg), 20 h (~51 mg), 22 h (~55 mg), 00 h (~52 mg) and next day again at 04 h (~70 mg). Similarly, troughs appeared at 11 h (~39 mg), 14 h (~34 mg), 19 h (~47 mg), 21 h (~48 mg), 23 h (~43 mg) and next day at 03 h (~36 mg) and 08 h (~40 mg). Though, the individual intervals between any two peaks or troughs ranged from 2 to 6 h, the mean interval between any two peaks or troughs stood at 3.1 h under LD, ~2.8 h under both LL and DD (Fig. 2 A; Tables 3 and 4).

Soluble proteins: The free running time of the 24-h soluble protein rhythm showed 7 peaks and 7 troughs under LD (Fig. 2 B; Tables 3 and 4). The peaks appeared at 10 h (~56 mg), 13 h (~58 mg), 16 h (~72 mg), 20 h (~52



(A). Fat body soluble proteins v/s amino acids (mg/g) in IV instar under LD.

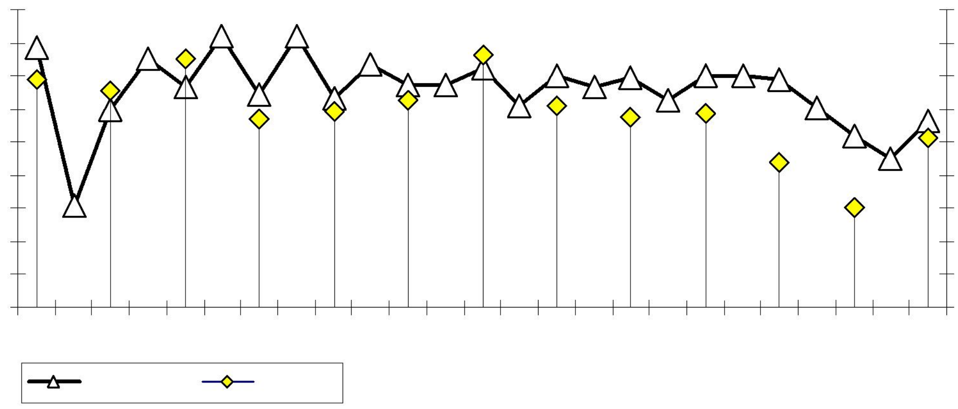


(B). Fat body soluble proteins v/s amino acids (mg/g) in V instar under LD.

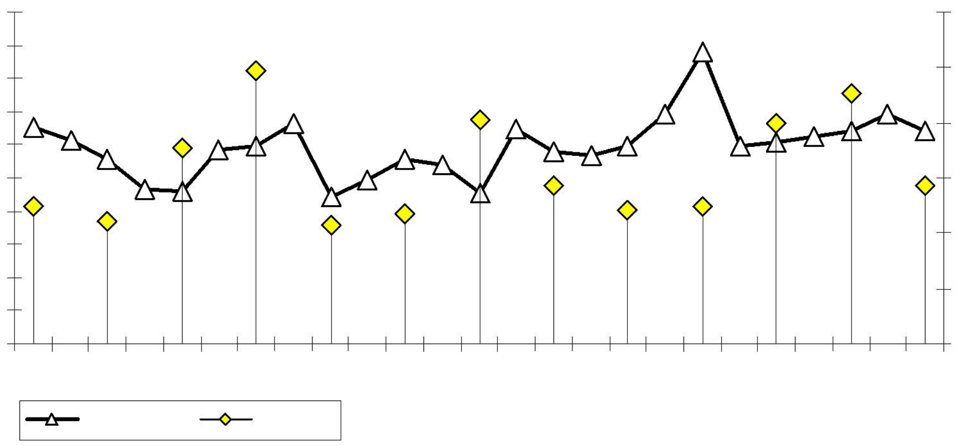
Fig.4. *Circadian changes in profiles of soluble proteins and free amino acids in the fat body of Bombyx mori during fourth (A) and fifth (B) instar larval stages, under 12 h light: 12hr dark cycle (LD). The values expressed in mg per gm-wet weight of tissue, represent 24-h free running time of respective circadian rhythms.(P values: <0.001).*

mg), 22 h (~52 mg) and next day at 02 h (~51 mg) and 08 h (~46 mg). The troughs occurred at 08 h (~23 mg), 11-12 h (~52 mg each), 15 h (~38 mg), 18 h (~42 mg), 21 h (~46 mg), 00 h (~ 30 mg) and next day at 04 h (~36 mg). Under LL, the soluble protein rhythm showed 7 peaks and 7 troughs**.** The peaks appeared at 08 h (~33 mg), 13 h (~29 mg), 15 h (~33 mg), 18 h (~28 mg), 21 h (~32 mg), 02 h (~44 mg) and next day at 02 h (~44 mg) and 07 h (~35 mg). The troughs appeared at 11-12 h (~23 mg), 14 h (~30 mg), 16 h (~22 mg), 20 h (~23 mg), 23 h (~28 mg), and next day at 03 h (~30 mg) and 08 h (~32 mg). Under DD, the phase response curve of the soluble protein rhythm showed 7 peaks and 7 troughs. The peaks occurred at 10 h (32 mg), 12 h (~34 mg), 18 h (~40 mg), 20 h (~37 mg), 22 h (~35 mg), 00 h (~37 mg) and next day at 04 h (~51 mg). The troughs appeared at 11 h (~31 mg), 14 h (~21 mg), 19 h (~35 mg), 21 h (~18 mg), 23 h (~33 mg) and next day at 01-02-03 h (~30 mg each) and 07-08 h (~18 mg each). Though, the individual intervals between any two peaks or troughs ranged from 2 to 6 h, the mean interval between any two peaks or troughs stood at 2.9 h under LD, 3.1 h under LL and ~2.6 h under DD (Fig. 2 B; Tables 3 and 4).

Structural proteins: Under LD, the phase response curve of structural protein rhythm showed 7 peaks and 7 troughs (Fig. 2 C; Tables 3 and 4). The peaks appeared at 8 h (~48 mg), 13 h (~26 mg), 16 h (~ 17 mg), 21 h (~32 mg), 23 h (~27 mg) and next day at 01h (~23 mg) and 07 h (~16 mg). With regard to troughs, they occurred at 10 h (~6 mg), 15 h (~9 mg), 19 h (~8 mg), 22 h (~15 mg), 00 h (~6 mg) and next day at 04 h (10 mg) and 08 h (~6 mg). Under LL, 8 peaks and 8 troughs were observed in the structural protein rhythm. Peaks appeared at 10 h (~13 mg), 13 h (~13 mg), 16 h (~20 mg), 20 h (~26 mg), 22 h (~9 mg), 00 h (~18 mg) and next day at 04 h (~25 mg) and 08 h (~31 mg). As regards troughs, they appeared at 08 h (~10 mg), 11 h (~11 mg), 14 h (~5 mg), 18 h (~8 mg), 21 h (~7 mg), 23 h (~4 mg) and next day at 01 h (~7 mg) and 07 h (~6 mg). Under DD, the structural protein rhythm displayed 7 peaks and 7 troughs. As regards peaks, they appeared at 10 h (~11 mg), 15 h (~15 mg), 18 h (~15 mg), 20 h (~14 mg), 00 h (15 mg) and next day at 04 h (~19 mg) and 07 h (~23 mg). The troughs occurred at 09 h (9 mg), 12 h (~7 mg), 16 h (~13 mg), 19 h (~12 mg), 23 h (~10 mg) and next day at 03 h (~6 mg) and 06 h (~13 mg). Though, the individual intervals



(A). Fat body soluble proteins v/s amino acids (mg/g) in IV instar under LL.



(B). Fat body soluble proteins v/s amino acids (mg/g) in V instar under LL.

Fig. 5. *Circadian changes in profiles of soluble proteins and free amino acids in the fat body of Bombyx mori during fourth (A) and fifth (B) instar larval stages, under continuous light (LL). The values expressed in mg per gm-wet weight of tissue, represent 24-h free running time of respective circadian rhythms. (P values: <0.001).*

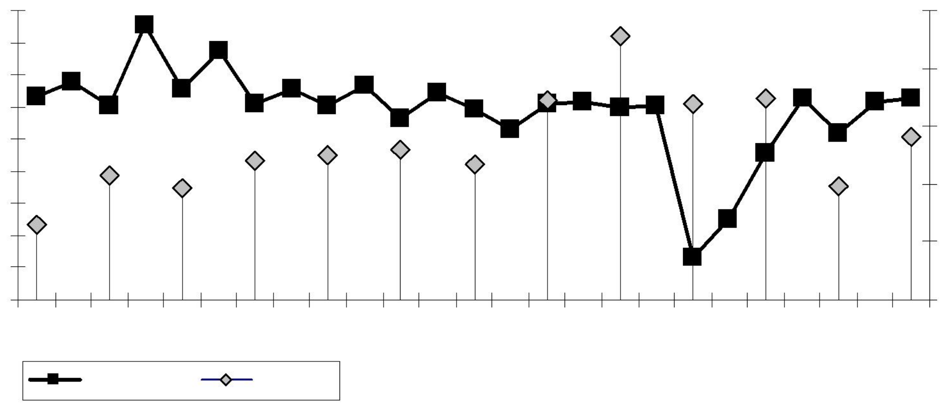
between any two peaks or troughs ranged from 2 to 6 h, the mean interval between any two peaks or troughs stood at 3.2 h under LD, ~2.9 h under LL and ~3h under DD (Fig. 2 C; Tables 3 and 4).

Free amino acids: The fifth instar circadian amino acid rhythm differs slightly from that of the fourth instar (Fig. 3 B), in which higher levels of free amino acids (~11 mg to ~32 mg) were recorded under LD compared to LL (~11 mg to 25 mg) and DD (~4 mg 23 mg). Under LD, peaks in free amino acid levels were observed at 12 h (~28 mg), 18 h (~32 mg), 22 h (~15 mg), 02 h (~17 mg) and again next day at 06 h (~14 mg) and troughs were observed at 08-10 h (~15 mg each) 16 h (~18 mg), 20 h (~14 mg), 00 h (13 mg), and next day at 04 h (~12 mg) and 06 h (11 mg). Under LL, free amino acid levels recorded peaks at 14 h (~25 mg), 20 h (~20 mg) and again next day at 06 h (23 mg) and troughs at 08 h (~13 mg), 16 h (~11 mg) and 00 h (~12 mg) and next day at 08 h (14 mg). Similarly, under DD, peaks in their levels were observed at 08 h (~16 mg), 14 h (~11 mg) and 20-22 h (~13 mg each) and again next day at 04 h (~23 mg) and 06 h (~20 mg) while troughs were observed at 10 h (~10 mg) 16 h (~5 mg), 00 h (~4 mg) and next day at 06 h (~16 mg).

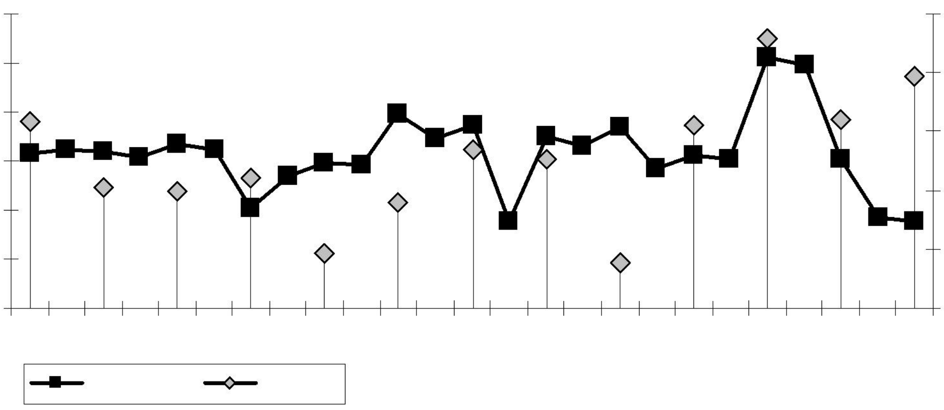
In both fourth and fifth instars, the free running period of the protein and free amino acid rhythms maintained constant fluctuations that are not statistically significant to be counted as peaks and troughs under all the three photoperiodic conditions; LD, LL and DD (Figs. 1, 2 and 3).

DISCUSSION

The silkworm fat body being a major site of metabolism, synthesizes and stores over 177 proteins that includes nine glycolysis related proteins, several metabolically related proteins like diacylglycerol binding protein, triacylglycerol lipase, putative hydrolases, cytoskeleton proteins, defence proteins, heat shock proteins, 30 K proteins and actin (Hou *et al.*, 2007). The current study demonstrates that the fat body proteins in *B. mori* show time-bound circadian changes that are probably triggered by tissue-specific endogenous pace makers, much like that in other insects (Shimizu *et al.*, 2001; Sehadova *et al.*, 2004; Iwai *et al.*, 2006; Kyung *et al.*, 2006). Though, the rhythm of particular protein is not examined in the present study, it might relate to a multitude of proteins that are synthesized from time to time during larval development.



(A). Fat body soluble proteins v/s amino acids (mg/g) in IV instar under.



(B). Fat body soluble proteins v/s amino acids (mg/g) in V instar under DD.

Fig. 6. *Circadian changes in profiles of soluble proteins and free amino acids in the fat body of Bombyx mori during fourth (A) and fifth (B) instar larval stages, under continuous dark (DD). The values expressed in mg per gm-wet weight of tissue, represent 24-h free running time of respective circadian rhythms. (P values: <0.001).*

As reported in our previous reports for the silk gland (Sailaja and Sivaprasad, 2010 a, b), the peaks and troughs of the phase response curves (PRCs) of the fat body protein rhythm probably indicate two vital stages of gene expression; translation (protein synthesis) and transcription, and the combined mean interval between a peak and trough reflects the duration of each protein synthetic cycle. Though, the ups and downs in protein profiles were earlier linked with mechanisms such as the shuttling of clock related proteins between nucleoplasm and cytoplasm, intracellular proteolysis (Chen and You, 2004; Ciechanover, 2005) and protein consolidation processes such as gelation, adhesion and crystallization (Inoue *et al.*, 2000; Takasu *et al.*, 2005). Though photoperiod-specific clock-shifting is reflected in the protein rhythm, it is not so with reference to larval stage, as both the fourth and fifth instar larval stages depicted 7 PS cycles under LD condition. Nevertheless, the longer duration of fifth instar (7 or 8 days) ensures the synthesis of additional proteins in about 49 or 56 PS cycles (7 x 7 = 49 or 7 x 8 = 56) compared to short duration of the fourth instar (4 days) that shows only 28 PS cycles (7 x 4 = 28). Thus, large number of PS cycles during the fifth instar development contributes to heavy toll of proteins (Figs. 1 and 2) that are associated with metabolic events of larval-pupal transformation. This obviously is sustained by the recruitment of more and more genes in the fat body similar to that in the silk gland (Sailaja and Sivaprasad, 2010 a, b). It is not known as to which genes are expressed and which proteins are synthesized during the protein rhythm. More studies on silkworm proteomics and genomics are needed to elucidate this fact.

Photoperiod-specific clock-shifting: Our findings suggest that the photoperiod has clock-shifting effect on the free running time of the fat body protein rhythm that significantly alters the number of PS cycles either by extending or by retarding the duration of each cycle in both the instars examined (Table 5). In the fourth instar, the number of PS cycles, which was 7 under LD, increased to 9.5 under LL and 8 under DD. At the same time the duration of each cycle, which was about 3.4 h under LD, is reduced to 2.5 h under LL (a reduction of 0.9 h or 54 m) and to 3.0 h under DD (a reduction of 0.4 h or 24 m). The shift in the protein rhythm during fifth instar is more or less similar to that of fourth instar. The number of PS cycles, which was 7 under LD, increased to 7.5 under LL,

Table 1. Interval between peaks (elevated points) of protein levels in the fatbody 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									Mean interval in hours
			1-2	2-3	3-4	4-5	5-6	6-7	7-8	8-9	9-10	
Total Proteins	LD	6	2	4	5	6	3	-	-	-	-	3.3
	LL	10	3	2	2	2	5	2	2	2	4	2.4
	DD	8	3	4	5	3	2	4	2	-	-	2.9
Soluble proteins	LD	7	2	5	3	3	4	2	-	-	-	2.7
	LL	10	3	2	2	2	3	2	2	2	4	2.2
	DD	9	4	2	2	2	2	3	6	3	-	2.7
Structural Proteins	LD	8	4	5	3	2	3	4	2	-	-	2.9
	LL	9	4	2	3	5	3	2	2	3	-	2.7
	DD	8	4	3	5	3	2	3	3	-	-	2.9

Source: Figs. 1, 2,3

but remained unchanged under DD. The duration of each cycle is maintained at 3.4 h under both LD and DD, but declined by 0.2 h or 12 m (from 3.4 h to 3.2 h) under LL (Table 5). Accordingly, when the growing silkworm larvae are exposed to different photoperiodic cues, the circadian protein rhythm shifts from the standard 24 hr-pattern. In the fourth instar larvae, the shift results in decreasing the free running time from normal 24 h to 17.5 h under LL and 21 h under DD, thus advancing the rhythm by 6 h and 30 m (24-17.5 = 6.5 hr) under LL and by 3 h (24 – 21 = 3 h) under DD. But in the fifth instar larva, the 24 h-free running time is clock-shifted to 22. 4 h (22 h, 24 m) under LL, but remained the same under DD (Table 5). The increase in the number of PS cycles under LL and DD conditions observed in B. mori is in close agreement with the earlier observations in crickets and other insects that more proteins are synthesized under altered conditions of photoperiod (Kenny and Saunders, 1991; Koga *et al.*, 2005; Peschel *et al.*, 2009).

As projected for the silk gland (Sailaja and Sivaprasad, 2010 a, b), the photoperiod-induced changes in the fat body protein rhythm reflect corresponding changes in the timing of gene expression. Obviously, the genes in the fat body cells express 7 times under LD, 9.5 times under LL and 8 times under DD at intervals of 3.4 h, 2.5 h and 3 h respectively under the three photoperiodic conditions. Similarly, in the fifth instar, they express 7 times under LD and DD (at 3. 4 h intervals) and 8 times under LL (at 3 h intervals). Thus, the fat body maintains a constant or altered rhythm even in darkness (DD), probably by taking cues from the diet that acts as an alternative *zeitgeber* (time giver) to light and quickly resets the clock in peripheral organs (Damiola *et al.*, 2000; Stokkan *et al.*, 2001; Kita *et al.*, 2002) such as the fat body. Since, the silkworms, reared under DD were also fed 5 times a day, they carried through the rhythm much

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

Protein type	Photo-period	No. of troughs	Interval between troughs in hours									Mean interval in hours
			1-2	2-3	3-4	4-5	5-6	6-7	7-8	8-9	9-10	
Total Proteins	LD	6	4	3	5	6	4	-	-	-	-	3.7
	LL	9	3	2	2	2	5	2	2	3	-	2.3
	DD	8	5	2	2	4	4	4	2	-	-	2.9
Soluble proteins	LD	8	4	3	5	2	4	3	3	-	-	3.0
	LL	9	3	2	2	2	2	2	2	6	-	2.3
	DD	9	2	2	2	2	2	3	5	4	-	2.4
Structural Proteins	LD	8	5	3	3	3	3	3	2	-	-	2.8
	LL	9	2	2	3	3	5	2	2	3	-	2.4
	DD	8	4	4	5	2	2	3	3	-	-	2.9

Source: Figs. 1, 2,3

Table 3. Interval between peaks (elevated points) of protein levels in the fat body of the fifth 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									Mean interval in hours
			1-2	2-3	3-4	4-5	5-6	6-7	7-8	8-9	9-10	
	LD	7	4	3	5	2	3	5	-	-	-	3.1
Total	LL	8	4	2	5	4	2	2	4	-	-	2.9
Proteins	DD	7	3	5	2	2	2	4	-	-	-	2.6
	LD	7	3	3	4	2	4	6	-	-	-	3.1
Soluble	LL	7	5	2	3	3	5	5	-	-	-	3.3
proteins	DD	7	2	6	2	2	2	4	-	-	-	2.6
Structural	LD	7	5	3	5	2	2	6	-	-	-	3.3
Proteins	LL	8	3	3	4	2	2	4	4	-	-	2.8
	DD	7	5	3	3	3	4	3	-	-	-	3.0

Source: Figs. 4, 5, 6

like those reared under LD and LL, a fact that indicates the existence of at least two-oscillators in the fat body of *B. mori*, that respond separately for light and dark cues as observed in *Drosophila* (Forster, 2000). The studies on insect clock genes and their expression timings (Syrova *et al*., 2003; Grima *et al*., 2004;Hardin, 2004; Shafer *et al*., 2004; Stoleru *et al*., 2004) and on some physiological parameters (Koga *et al*., 2005; Kanyan Xu, 2008) lend credibility to the existence of peripheral oscillators in *B. mori*. Hopefully, our findings (Sailaja and Sivaprasad, 2010 a, b) on the silk gland protein rhythms could provide further conclusive proof for the clock-shifting nature of the light in stage-specific and tissue-specific manners in the fat body of silkworm. While, the scoto (dark) phase causes elevation in peak heights, the photo (light) phase increases the number of peaks during the free running time of the protein rhythm (Fig. 2 A, B, C). Evidently, the intensity (height of peaks) and frequency (number of peaks) of silk gene expression in *B. mori* are independently modulated by the dark and light cues respectively, for which conclusive proof is not available. Nevertheless, the photoperiod undoubtedly causes behavioural adjustments by resetting the timing of the expression of the circadian clock genes in the fat body.

Protein rhythm versus free amino acids: Since amino acids are the building blocks of proteins, the protein rhythm rises and falls with the levels of free amino acids (FAA). Since, the soluble proteins represent most of the freshly synthesized proteins; a comparative account of their PRCs vis-a-vis the FAA rhythm help in drawing meaningful conclusions with regard to the instar-specific and photoperiod-specific relationship between these two biochemical constituents. As shown in figures 4, 5 and 6, the protein synthetic phases (peaks) are preceded by elevations in the levels of free amino acids notwithstanding some minor deviations occurred

Table 4. Interval between troughs (low points) of protein levels in the fat body of the fifth instar larva of *Bombyx mori* during the free running period of the rhythm under 12hrs light / dark cycle (LD), continuous light (LL) and continuous dark (DD) conditions.

Protein type	Photo-period	No. of troughs	Interval between troughs in hours									Mean interval in hours
			1-2	2-3	3-4	4-5	5-6	6-7	7-8	8-9	9-10	
Total Proteins	LD	7	5	4	3	2	4	4	-	-	-	3.1
	LL	7	3	3	6	2	2	3	-	-	-	2.7
	DD	7	3	5	2	2	4	5	-	-	-	3.0
Soluble proteins	LD	7	3	3	3	3	3	4	-	-	-	2.7
	LL	7	2	2	4	3	4	5	-	-	-	2.9
	DD	7	3	5	2	2	2	4	-	-	-	2.6
Structural Proteins	LD	7	5	4	3	2	4	4	-	-	-	3.1
	LL	8	3	3	4	3		2	6	-	-	2.9
	DD	7	3	4	3	4	4	3	-	-	-	3.0

Source: Figs.4, 5,6

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

Parameter	Fourth instar			Fifth instar		
	LD	LL	DD	LD	LL	DD
Average number of peaks	7	~10	~8	7	~8	7
Average number of troughs	~7	9	~8	7	~7	7
Mean interval between peaks	~3 h	~2 h	~2.8 h	~3.2 h	3.0 h	~2.7 h
Mean interval between troughs	~3.2 h	~2.3 h	~2.7 h	~3 h	~2.8 h	~2.9 h
Combined mean interval of peaks and troughs	~3 h	~2.2 h	~2.8 h	3.1 h	2.9 h	2.8 h
Probable number of protein synthetic cycles	7	9.5	8	7	7.5	7
Time taken for each protein synthetic cycle	3.4 h (24/7=3.4)	2.5 h (24/9.5=2.5)	3 h (24/8=3)	3.4 h (24/7=3.4)	3.2 h (24/7.5=3.2)	3.4 h (24/7=3.4)
Free running time	24 h (3.4x7=23.8)	17.5 h (2.5x7=17.5)	21 h (3x7=21)	24 h (3.4x7=23.8)	22.4 h (3.2x7=22.4)	24 h (3.4x7=23.8)

Source: Figs. 1, 2, 3 for fourth instar and 5, 6, 7 for fifth instar.

probably due to sampling errors.

Under LD, the FAA pool available at a particular hour, is used up either in the same PS cycle or in the subsequent cycles in the next one or two hours. In the fourth instar, for instance, the rise in the levels of FAA at 8-10 h, 12h, 14-18h, 20 h, 22 h, 02 h and 6-8 h on second day are used-up in the PS cycles occurred at 8-12 h, 13 h, 16-18 h, 21 h, 23-00 h, 3-4 h, and at 6-8 h respectively (Fig. 4 A). Similarly, in the fifth instar, the elevations in the FAA levels at 8 h, 10 h, 12 h, 14-16 h, 18 h, 20-22 h, 00 h, 02 h, and 4-8 h were accompanied by peaks in soluble protein levels at 9 h, 10-11 h, 13 h, 16-17h, 19-20 h, 20-22 h, 1 h, 2-3 h and 4-8 h respectively (Fig. 4 B). Thus, the ongoing PS cycles are sustained by free amino acid inputs from time to time during the free running time of the protein rhythm.

The situation under LL is slightly different. In both the fourth and fifth instars, the FAA levels at a particular hour are proceeded by an increase in the levels of soluble proteins, indicating their utilization within one hour after their availability. As shown in the figure 5 A, the rise in the FAA levels at 8 h, 10 h, 12 h, 14 h, 16 h, 18 h, 20 hrs, 22-00 h, 02-04 h and at 06-08 hr was proceeded by corresponding increase in the levels of proteins at 8 h, 10-11 h, 13 h, 15 h, 17 h, 18-19 h, 20 h, 22-00 h, 02-04 h and next day at 08 h in the fourth instar lava. Similarly, in the fifth instar larval stage, the rise in FAA levels at 08 h, 10-12 h, 16-18 h, 20 h, 22-02h and 04-06 h, were accompanied by elevation in the soluble protein levels at 08 h, 13-15 h, 16-19 h, 21 h, 22-02 h and 04-07 h respectively (Fig. 5 B). The conditions under DD are more or less similar to those of LL (Fig. 6 A, B). While, in fourth instar, the increase in the levels of FAA at 08 h, 10 h, 12 h, 14 h, 16 h, 18 h, 20-22 h, 00 h, 02-04 h and 06-08 h was accompanied by similar upsurge in protein levels at 09 h, 11 h, 13 h, 15 h, 17 h, 19 h, 22-23 h, 01 h, 03-05 h and at 07-08 h respectively. In the fifth instar, the increase in the levels of FAA at 08 h, 10 h, 12 h, 14 h, 16 h, 18 h, 20 h, 22 h, 00 h, 02 h, 04 h and 06-08 h was followed by similar elevations in soluble protein levels at 08-09 h, 09-10 h, 12-13 h, 15 h, 16-17 h, 18 h, 20 h, 22-23 h, 00 h, 02-03 h and 04-05 h respectively. However, the relation between FAA and soluble proteins at 06-08 h is not clear as the protein levels continuously declined despite an increase in the levels of FAA (Fig. 6 B).

Clearly, the photoperiod modulates protein rhythm of the fat body by mobilizing the free amino acids required for protein synthesis from time to time during the free running time of the rhythm. When the silkworms were reared separately, the LL and dark DD conditions advanced the rhythm by favouring quicker utilization of free amino acids in both the instars, but under LD, the light and dark conditions together delayed the operation of protein rhythm by favouring slow utilisation of the amino acid pool. Probably, because of this reason, the number of protein synthetic cycles is increased from 7 to 9.5 under LL and 8 under DD in fourth instar, and to 7.5 under LL in the fifth instar. This results in advancement of the 24-h free running time of the rhythm by 6.5 h (6 hours and 30 minutes) under LL and 3 h under DD in the fourth instar and by 1.6 h (1 hour and 36 minutes) under LL in the fifth instar.

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