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PHOTOPERIOD INDUCED INSTAR-SPECIFIC CLOCK-SHIFTING IN THE CIRCADIAN PROTEIN AND AMINO ACID RHYTHMS IN THE LARVAL HAEMOLYMPH OF THE SILKWORM, *BOMBYX MORI*

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ABSTRACT
The instar-specific and photoperiod-modulated clock-shifting in the free running time of the circadian protein and amino acid rhythms was studied in the larval haemolymph of *Bombyx mori*. The combined mean in the peaks and troughs in the phase response curves (PRC) of protein rhythms were interpreted in terms of protein releasing cycles (PR cycles) into haemolymph. The larvae grown under normal 12 hr light and 12 hr dark (LD) condition showed seven PR cycles in fourth instar and six in fifth instar, resulting in shifting the 24 hr free running time of the rhythm to 28 hr in fifth instar. Under continuous light (LL) the protein rhythm recorded six cycles in fourth instar and nine in fifth instar, but in continuous dark (DD), it maintained eight cycles in both the instars. In fourth instar, the 24 hr free running time of the rhythm is re-scheduled to operate at 28 hr under LL and 21 hr under DD, while in fifth instar it is set at ~16 hr under LL and 19 hr under DD. 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.

INTRODUCTION

The haemolymph is the chief circulating fluid in the haemocoel of insects. It bathes all tissues and organs in the insect body and transports nutrients, hormones and metabolic wastes (Gilbert and Chino, 1974). It serves as a transient mobile biochemical repository for proteins, amino acids, carbohydrates and lipids (David and Ananthakrishnan, 2006). Of all constituents, the haemolymph proteins assume significance as they represent the products of gene expression. Their levels show generic-specific, tissue-specific and stage-specific variations during insect metamorphosis (Hou *et al.*, 2007, 2010; Bakkappa and Subramanya, 2010). Since 1950s, the haemolymph proteins have been extensively studied with a view to ascertain their role in silkworm development. In a pioneering study, Telfer and Williams (1953) identified female-specific vitellogen proteins in the haemolymph of *Hyalophora cecropia*. In 1980s two more haemolymph storage proteins; SP-1 and SP-2 and 30 K stage-specific larval proteins were identified and their functional roles have been established (Tojo *et al.*, 1980; Fujii *et al.*, 1989). In recent studies, the proteomic technology was applied to silkworm research for analysing tissue-specific protein profiles during metamorphosis (Li *et al.*, 2006; Zhang *et al.*, 2007; Hou *et al.*, 2007, 2010).

The plasma protein profiles depend on the rate of protein synthesis in the tissues bathed by it and the quantum of proteins released into it from time to time (Haunerland, 1996). As such, its volume and composition vary in a stage-dependent and time-dependent manner during larval growth and metamorphosis, apparently caused by the ongoing protein synthetic activity coupled with the operation of an exchange shuttle between the haemolymph and other tissues (Siva Prasad and Sailaja, 2009). Since, it is known that tissue-specific endogenous pacemakers modulate circadian behaviour of insects (Sehadova *et al.*, 2004; Reppert, 2006; Xu *et al.*, 2008), it is presumed that the biochemical constituents of haemolymph in *Bombyx mori* vary as a function of circadian endogenous clock mechanism. The genetic and molecular investigations on the silkworm chronobiology primarily centred round the identification of circadian clock genes, determination of their expression patterns and functional analysis of their products (Ishikawa and Suzuki, 1985; Gizelak, 1995; Sehadova *et al.*, 2004; Iwai *et al.*, 2006). But, no effort has since been made to ascertain the rhythmic changes in biochemical constituents. In our earlier investigations (Sailaja and Sivaprasad, 2010a, 2010b, 2011), we analysed the circadian protein and amino acid rhythms in the silk gland and fat body of *B. mori*, which is a well-studied insect model for chronobiology-based investigations. The present study brings forth similar investigations on the silkworm haemolymph proteins and free amino acids with a view to find instar-specific and photoperiod-specific clock-shifting in their circadian profiles.

MATERIALS AND METHODS

The circadian protein and amino acid rhythms were examined

in the Pure Mysore x CSR_2 hybrid variety of the silkworm *Bombyx mori,* reared under standard environmental conditions of 28ºC, 85 % RH, as per Krishnaswami (1986). After hatching the worms were reared on M_5 variety of mulberry leaves with 5 feeds per day at 6AM, 10 AM, 2 PM, 6 PM and 10 PM under 12 hr light and 12 hr dark cycle. After the third moult, the larvae were divided into three batches and reared and fed separately under three different photoperiodic conditions, viz., 12 hr light and 12 hr dark cycle (LD), continuous light (LL) and continuous dark (DD) during fourth and fifth instars.

The haemolymph was, collected from the silkworms by cutting their telson with fine scissors. The circadian protein rhythm as reflected in the levels of total, soluble and structural proteins, was estimated by the method of Lowry *et al*. (1951), in the 1 mL of haemolymph diluted with 9 ml of distilled water. Hourly changes in protein profiles were analyzed for a period of 25 hr that spans in between 3rd and 4th day of fourth instar and 5th and 6th day of fifth instar, starting from 08 hr on the first day to 08 h on the second day. At the same time 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 1:9 diluted haemolymph in 10% TCA. For analyzing instar-specific changes in the rhythm, the larvae reared under LD condition of fourth instar was treated as control and that of fifth instar as experimental and for analyzing photoperiod-specific changes, the worms reared under LD condition of both fourth and fifth instar were treated as the control and those reared under LL and DD as experimental samples.

RESULTS

The 24 hr circadian rhythmicity in the profiles of haemolymph proteins and amino acids is designated the free running period or tau and shown in the form of phase response curves in Fig. 1 to 5, with characteristic peaks (elevated points) and troughs (low points). The circadian data was analyzed in terms of the number of peaks and troughs and intervals between them are presented in Tables 1 to 5. The protein and amino acid levels were expressed in mg / mL of haemolymph.

Fourth instar larval circadian rhythms

Total proteins

Under 12:12 light / dark cycle (LD) the haemolymph total protein rhythm showed 6 peaks and 6 troughs during the 24 hr free running period of the circadian rhythm or *tau*. Peaks in the levels of total proteins occurred at 8 hr (~13 mg / mL of haemolymph), 12 hr (~10 mg),14 hr (~9 mg), 22 hr (~20 mg) and next day at 02 hr (18 mg) and 6 hr (~11 mg). Troughs in their levels were observed at 11hr (~9 mg),13 hr (~8 mg), 15 hr (~8 mg), 00 hr (~10 mg) and next day at 04-05 hr (~9 mg) and 08 hr (~10 mg). Under continuous light (LL), 7 peaks and 7 troughs were recorded during the free running time of the rhythm. Peaks appeared at 11hr (~4 mg), 14 hr (4.3 mg), 16 hr (~5 mg), 18 hr (~4 mg), 20 hr (~9 mg), 22-23 hr (~9 mg each) and next day at 07 hr (~7 mg). Troughs under LL were recorded at 13 hr (~2 mg), 15 hr (~4 mg), 17 hr (~4 mg), 19 hr (~4 mg), 21 hr (~8.5 mg) and next day at 01hr (5.7 mg) and 08 hr (~5 mg). Under continuous dark (DD) the total protein rhythm recorded 7 peaks and 7 troughs. Peaks occurred at 09 hr (2.7 mg), 14 hr (~3.5 mg), 16 hr (~3.7 mg), 20 hr (~8.2 mg), 23 hr (~10 mg) and next day at 01hr (~8 mg) and 03 hr (13.5 mg). Troughs appeared at 10 hr (2.5 mg), 15 hr (2.8 mg), 17 hr (3 mg), 21-22 hr (~8 mg), 00 hr (4.7 mg) and next day at 02 hr (5.5 mg) and 08 hr (4.4 mg) (Fig. 1A).

Soluble proteins

Under LD, the 24 hr *tau* of the haemolymph soluble protein rhythm showed 8 peaks and 6 troughs. Peaks appeared at 08 hr (~9.7 mg) 11hr (~7.7 mg), 13 hr (~7.1 mg), 17 hr (11 mg), 22 hr (13.5 mg) and next day at 03 hr (~9 mg), 06 hr (~8 mg) and 08 hr (~8 mg). Troughs were recorded at 09 hr (7.8 mg), 12 hr (~7 mg), 19 hr (~10 mg), 23-00-01hr (~ 8 mg each) and again next day at 04-05 hr (~7.8 mg) and 07 hr (~6.6 mg). Under LL, 6 peaks and 6 troughs were recorded during the free running time of the soluble protein rhythm. Peaks occurred at 11 hr (~3 mg), 14 hr (~3 mg), 20 hr (~7 mg), 22 hr (~6.7 mg) and next day at 02 hr (~5.3 mg) and 07 hr (~5.7 mg). Troughs were recorded at 13 hr (~1.5 mg), 17 hr (~2.5 mg), 21 hr (~5.6 mg), 00-01hr (~5.0 mg each) and next day at 03-04 hr (~5 mg) and 08 hr (~3.8 mg). Under DD, the soluble protein rhythm showed 8 peaks and 8 troughs. Peaks appeared at 08 hr (1.6 mg), 12 hr (~2.2 mg), 14 hr (~2.3 mg), 18 hr (~3 mg), 23hr (~6.4 mg) and next day at 01hr (~5 mg), 03 hr (~5.4 mg) and 07 hr (~4.2 mg). Troughs occurred at 09 hr (~1.5 mg), 13 hr (~1.6 mg), 15 hr (~1.6 mg), 19 hr (~2.9 mg), 00 hr (~4.1 mg) and next day at 02 hr (~4.2 mg), 05 hr (3.4 mg) and 08 hr (~3.3 mg) (Fig. 1 B).

Structural proteins

Under LD, the free running time of the haemolymph structural protein rhythm showed 8 peaks and 8 troughs. Peaks appeared at 08-09-10 hr (~3 mg each), 12 hr (~2.6 mg), 14 hr (~2.2 mg), 16 hr (~2.1 mg), 19 hr (~3.5 mg), 22 hr (6.8 mg) and again next day at 02 hr (~ 9.7 mg) and 07 hr (~3.6 mg). Troughs were recorded at 11 hr (~1.3 mg), 13 hr (~ 0.9 mg), 15 hr (~0.6 mg), 17hr (~1.7 mg), 20 hr (~2.7 mg), 00h (~1.8 mg) and next day again at 03 hr (~1.2 mg) and 08 hr (~1.6 mg). Under LL, the *tau* recorded 6 peaks and 6 troughs. Peaks occurred at 09 hr (~1.9 mg), 16 hr (~ 2 mg), 21 hr (~ 2.9 mg), 23 hr (~2.1 mg) and next day again at 04 hr (~0.6 mg) and 07-08 hr (~1.2 mg). Troughs were recorded at 08 hr (~1.7 mg), 11-12-13 hr (~0.9 mg each), 18 hr (~1.5 mg), 22 hr (~2 mg) and next day at 03 hr (~0.6 mg) and 05 hr (0.5 mg). Under DD the rhythm recorded 8 peaks and 8 troughs. Peaks appeared at 09 hr (~1.3 mg), 11 hr (~0.9 mg), 13 hr (~1.6 mg), 16 hr (~1.3 mg), 20 hr (~3 mg), 23 hr (~3.3 mg) and next day again at 01 hr (~2.8 mg) and 03 h (~ 8 mg). Troughs occurred at 08 hr (~1.1 mg), 10 hr (~0.7 mg), 14 hr (~1.2 mg), 17 hr (~ 0.5 mg), 21-22 hr (~2.5 mg each), 00 hr (~0.5 mg) and next day again at 02h (~1.3 mg) and 07-08 hr (~1.1 mg each). During the remaining period of the free running time, the rhythms in the levels of total, soluble and structural proteins maintained minor ups and downs, which are of least significance (Fig. 1C).

Free amino acids

The free amino acid rhythm of the haemolymph maintained relatively higher levels under LL and DD conditions compared to LD (Fig. 3A). Under LD, their levels ranged from ~ 32 to ~146 mg / mL of haemolymph during the free running time,

showing peaks at 10 hr (~65 mg), 16 hr (~146 mg) and next day at 02 hr (~124 mg) and troughs at 08 hr (56 mg), 12 hr (~39 mg), 20 hr (~55 mg) on day-1, and next day again at 08 hr (~32 mg). Under LL, significantly a higher rhythm (~45 to ~153 mg / mL) is maintained during the *tau*, with peaks in FAA levels at 08 hr (~150 mg), 22-02 hr (~153 mg each) and next day again at 06 hr (~135 mg) and troughs at 16 hr (~45 mg), 04 h (~89 mg) and again, next day at 08 hr (~64 mg). Under DD, the amino acid rhythm ranged from ~81 to 154 mg, with peaks at 14-16 hr (~137 mg each) on day-1and next day at 02-04 hr (~154 mg each) and at 08 hr (150 mg) and troughs at 08 hr (~81 mg), 18 hr (~121 mg) on day-1 and again next day at 06 hr (~129 mg).

Fifth instar larval circadian rhythms

Total proteins

Under LD, the total protein rhythm of haemolymph showed 6 peaks and 6 troughs. Peak appeared at 8 hr (15.3 mg / mL), 13 hr (~24 mg), 17 hr (~26 mg), 20 hr (28 mg), 23 hr (~25 mg) and next day again at 06 hr (~18 mg). Troughs occurred at 11 hr (~13 mg), 15 hr (15 mg), 19 hr (~25 mg), 21 hr (~25 mg) and next day at 04 hr (~14 mg) and 08 hr (~15 mg). Under LL, the protein rhythm recorded 10 peaks and 9 troughs**.** Peaks were recorded at 08 hr (15 mg), 10 hr (~14 mg), 12 hr (~19 mg), 15 hr (~19 mg), 18 hr (~19 mg), 22 hr (~18 mg), 00 hr (~18 mg) and next day at 04 hr (~21 mg), 06 hr (~23 mg) and at 08 hr (~19 mg). Troughs were observed at 09 hr (~12 mg), 11 hr (~12 mg), 14 hr (~17 mg), 17 hr (~18 mg), 19 hr (~15 mg), 23 hr (~15 mg) and next day at 01hr (~16 mg), 05 hr (~19 mg) and again at 07 hr (~17 mg). Similarly, the rhythm recorded 8 peaks and 7 troughs under DD. Peaks occurred at 08 hr (~14 mg), 10 hr (~12 mg), 15 hr (~17 mg), 17 hr (~19 mg), 00 hr (~17 mg) and next day 03 hr (~18 mg), 05 hr (~19 mg) and again at 08 hr (~17 mg). Troughs appeared at 09 hr (12 mg), 11 hr (~11 mg), 16 hr (~14 mg), 20 hr (~12 mg), 01hr (~16 mg) and next day at 04 hr (~17 mg) and again at 06 hr (~17 mg) (Fig. 2A).

Soluble proteins

The haemolymph soluble protein rhythm showed 6 peaks and 6 troughs under 12 LD. Peaks appeared at 08 hr (~11 mg), 13 hr (~21 mg), 17 hr (16 mg), 20 hr (~16 mg), 23 hr (~17 mg) and next day at 07 hr (~15 mg), while 6 troughs occurred at 09 hr (~11 mg), 16 hr (~12 mg), 18 hr (15 mg), 21 hr (~15 mg) and next day at 05 hr (~11 mg) and 08 hr (~11 mg). Under LL, the soluble protein rhythm showed 9 peaks and 9 troughs. While the peaks appeared at 08 hr (~11 mg), 10 hr (~11 mg), 12 hr (~17 mg), 15 hr (17 mg), 18 hr (~16 mg), 20 hr (~14 mg), 00 hr (~14 mg) and next day at 03 hr (~15 mg) and 06 hr (~18 mg), the troughs were recorded at 09 hr (~10 mg), 11 hr (~8 mg), 14 hr (~14 mg), 16 hr (~11 mg), 19 hr (~13 mg), 23 hr (~13 mg) and next day again at 01 hr (~12 mg), 04 hr (14 mg) and at 07-08 hr (~12 mg each). Under DD, the rhythm projected 8 peaks and 7 troughs. Peak occurred at 08 hr (~12 mg),10 hr (~9 mg), 14 hr (13 mg), 17 hr (~16 mg), 19 hr (~15 mg), 22 hr (~14 mg) and next day at 01 hr (~16 mg) and 07 hr (~13 mg). At the same time troughs appeared at 09 hr (~9 mg), 11 hr (~9 mg), 16 hr (~12 mg), 20 hr (~9 mg), 23 hr (~11 mg) and next day at 06 hr (~12 mg) and 08 hr (~11 mg). Though, the individual intervals between any two peaks or troughs ranged from 2 to 6 hr, the computed mean value stood at ~3 hr under LD, 2.5 hr under LL and ~2 hr under DD (Fig. 2B).

Structural proteins

Under LD, the phase response curve of structural protein rhythm showed 6 peaks and 5 troughs. Peaks appeared at 08 hr (~4 mg), 14 hr (~7 mg), 17 hr (~10 mg), 20 hr (~12 mg) and next day at 06 hr (~6 mg) and 08 hr (4 mg). Troughs were observed at 11 hr (~1 mg), 15 hr (~3 mg), 19 hr (9.6 mg) and next day at 03 hr (~2 mg) and 07 hr (~1 mg). Under LL, 9 peaks and 8 troughs were observed in the structural protein rhythm. Peaks appeared at 08 hr (~3.6 mg), 11 hr (~4.2 mg), 14 hr (~2.9 mg), 16 hr (~7.4 mg), 22 hr (~3.6 mg) and next day at 01hr (~4 mg), 04 hr (~7 mg),06 hr (~5.7 mg) and at 08 hr (~6.5 mg). As regards troughs, they appeared at 09 hr (~2.3 mg), 12 hr (~2.6 mg), 15 hr (~2.5 mg), 20 hr (~2 mg), 23 hr (~1.8 mg) and next day again at 02 hr (~1.8 mg), 05 hr (~5.2 mg) and at 07 hr (~5.5 mg). Under continuous dark (DD) the structural protein rhythm displayed 8 peaks and 8 troughs. Peaks appeared at 09 hr (~3.4 mg), 12 hr (~3.2 mg), 15 hr (~3.7 mg), 18 hr (3.4 mg), 20 hr (~3.3 mg), 23 hr (~5 mg) and next day 05 hr (6.6 mg) and 08 hr (6.6 mg), while the troughs occurred at 08 hr (2.2 mg), 11 hr (~2.2 mg), 13 hr (~1.6 mg), 16 hr (~1.9 mg), 19 hr (~ 2.7 mg), 21-22 hr (~2 mg each) and again next day at 01-02 hr (~2.3 mg each) and at 07 hr (~4 mg) (2 C). Though, the individual intervals between any two peaks or troughs ranged from 2 to 6 hr, the mean interval between any two peaks or troughs stood at 2.8 hr under LD, 2.6 hr under LL and ~3 hr under DD (Fig. 2C).

Free amino acids

The circadian amino acid rhythm during fifth instar is opposite to that of the fourth instar (Fig. 3B) as higher levels of free amino acids (~98 mg to ~155 mg) were recorded under LD compared to LL (~64 mg to 129 mg) and DD (~50 mg 154 mg). Under LD, peaks in FAA levels were observed at 10-14 hr (~110-114 mg), 22-02 hr (~150-155 mg) and again next day at 06 hr (~154 mg), while troughs were observed at 08 hr (~98 mg), at 16 hr (~107 mg) and next day at 04 hr (~117 mg) 08 hr (~108 mg). Under LL, peaks in FAA levels occurred at 10 hr (~79 mg), 14 hr (~90 mg), 20 hr (119 mg) and again next day at 04-06 hr (~128 mg each) and troughs appeared at 08 hr (~65 mg), 12 hr (~64 mg) 16 hr (~77 mg), 22-02 hr (~105 each) and next day at 08 hr (101 mg). Similarly, under DD, peaks in FAA levels occurred at 08 hr (~137 mg), 12 hr (~135 mg) 22 hr (~124 mg) and again next day at 04-08 hr (~151-154 mg), while troughs were observed at 10 hr (~95 mg) 16 hr (~57 mg) and again at 00 hr (~50 mg).

During the remaining period of the free running time, the total, soluble and structural protein rhythms vis-vis that of free amino acids maintained minor ups and downs, which are of least significance to be counted as peaks and troughs.

DISCUSSION

The haemolymph of *B. mori* acts as a flowing reservoir for ~298 proteins that includes juvenile hormone binding proteins, RNA binding proteins, paralytic peptide-binding protein, gesolin (actin-binding protein), aldose reductase, low-molecular weight lipoproteins, carboxylesterases, zinc-finger

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

Protein type	Photo-period	No. of peaks	Interval between peaks in hour 1-2	2-3	3-4	4-5	5-6	6-7	7-8	8-9	9-10	Mean interval in hour
Total proteins	LD	6	4	2	8	4	4	-	-	-	-	3.7
	LL	7	3	2	2	2	2	8	-	-	-	2.7
	DD	7	5	2	4	3	2	2	-	-	-	2.6
Soluble proteins	LD	8	3	2	4	5	5	3	2	-	-	3
	LL	6	3	6	2	4	5	-	-	-	-	3.3
	DD	8	4	2	4	5	2	2	4	-	-	2.9
Structural proteins	LD	8	2	2	2	3	3	4	5	-	-	2.6
	LL	6	7	5	2	5	3	-	-	-	-	3.7
	DD	8	2	2	3	4	3	2	2	-	-	2.3

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

Protein type	Photo-period	No. of troughs	Interval between troughs in hour 1-2	2-3	3-4	4-5	5-6	6-7	7-8	8-9	9-10	Mean interval in hour
Total proteins	LD	6	2	2	9	4	3	-	-	-	-	3.3
	LL	7	2	2	2	2	4	7	-	-	-	2.7
	DD	7	5	2	3	2	2	6	-	-	-	2.9
Soluble proteins	LD	6	3	7	4	3	2	-	-	-	-	3.2
	LL	6	4	4	3	2	4	-	-	-	-	2.8
	DD	8	4	2	4	5	2	3	3	-	-	2.9
Structural proteins	LD	8	2	2	2	3	4	3	5	-	-	2.3
	LL	6	3	5	4	5	2	-	-	-	-	3.2
	DD	8	2	4	3	4	2	2	5	-	-	2.8

Table3: Interval between peaks (elevated points) of protein levels in the haemolymph of the fifth instar larva of *Bombyx mori* during the free running period of the rhythm under 12 hr light/ dark cycle (LD), continuous light (LL) and continuous dark (DD) conditions

Protein type	Photo-period	No. of peaks	Interval between peaks in hour 1-2	2-3	3-4	4-5	5-6	6-7	7-8	8-9	9-10	Mean interval in hour
Total proteins	LD	6	5	4	3	3	7	-	-	-	-	3.7
	LL	10	2	2	3	3	4	2	4	2	2	2.4
	DD	8	2	5	2	7	3	2	3	-	-	3.0
Soluble proteins	LD	6	6	5	5	3	3	8	-	-	-	4.0
	LL	9	2	2	3	3	2	4	3	3	-	2.4
	DD	8	2	4	3	2	3	3	6	-	-	2.9
Structural proteins	LD	6	6	3	3	10	2	-	-	-	-	4.0
	LL	9	3	3	2	6	3	3	2	2	-	2.7
	DD	8	3	3	3	2	3	6	3	-	-	2.9

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

Protein type	Photo-period	No. of troughs	Interval between troughs in hour 1-2	2-3	3-4	4-5	5-6	6-7	7-8	8-9	9-10	Mean interval in hour
Total proteins	LD	6	4	4	2	7	4	-	-	-	-	3.5
	LL	9	2	3	3	2	4	2	4	2	-	2.4
	DD	7	2	5	4	5	3	2	-	-	-	3.0
Soluble proteins	LD	6	7	2	3	8	3	-	-	-	-	3.8
	LL	9	2	3	2	3	4	2	3	3	-	2.4
	DD	7	2	5	4	3	7	2	-	-	-	3.3
Structural proteins	LD	5	4	4	8	4	-	-	-	-	-	4.0
	LL	8	3	3	5	3	3	3	2	-	-	2.8
	DD	8	3	2	3	3	2	3	5	-	-	2.6

proteins, imaginal disc growth factor, gesolin, glyoxylase reductase, hydroxypyruvate isomerase, aminoacylase, trypsin inhibitor, transferrin protein, serine proteases, chymotrypsin inhibitor, hemolin, prophenoloxidase, 30 K proteins, stage-specific proteins (early, mid and late larval and pupal), βN-acetylglucosaminidase and a multitude of other unidentified proteins (Hou *et al*., 2010). Their functions range from silk formation to haemocyte production, ecdysis to eclosion, metabolism to metamorphosis, injury to immunity, locomotion to cocoon-spinning, digestion to respiration, tissue degeneration to organ growth, and from heat shock control to gene expression (Li *et al.,* 2006; Kiuchi *et al*., 2008; Choi *et al*., 2008; Nakahara *et al.,* 2009). Most of these proteins are produced in tissues bathed by the haemolymph and

Table 5: Comparative analysis of the phase response curves of the protein rhythm in the haemolymph 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 LD	LL	DD	Fifth Instar LD	LL	DD
Average number of peaks	~7	~6	~8	6	~9	8
Average number of troughs	~7	~6	~8	~6	~9	~7
Mean interval between peaks	~3 hr	3.2 hr	2.6 hr	~4 hr	2.5 hr	~3 hr
Mean interval between troughs	~3 hr	~3 hr	~3 hr	3.8 hr	2.5 hr	~3 hr
Combined mean interval of peaks and troughs	3 hr	3.1 hr	2.8 hr	~4 hr	2.5 hr	3 hr
Probable number of protein releasing cycles	7		6	8	6	9 7.5
Time taken for each releasing cycle	3.4 hr (24/7 = 3.4)	4 hr (24/6 = 4)	3 hr (24/8 = 3)	4 hr (24/6 = 4)	2.7 hr (24/9 = 2.7)	3.2 hr (24/7.5 = 3.2)
Free running time of the rhythm	~24 hr (3.4x7 = 23.8)	28 hr (4x7 = 28)	21 hr (3x7 = 21)	28 hr (4x7 = 28)	~19 hr (2.7x7 = 18.9)	22.4 hr (3.2x7 = 22.4)

Please note that instar-specific changes in the rhythm were computed taking the larvae reared under LD condition of fourth instar as control and that of fifth instar as experimental and for analyzing photoperiod-specific changes, the worms reared under LD condition of both fourth and fifth instar were treated as the controls and those reared under LL and DD as experimental samples

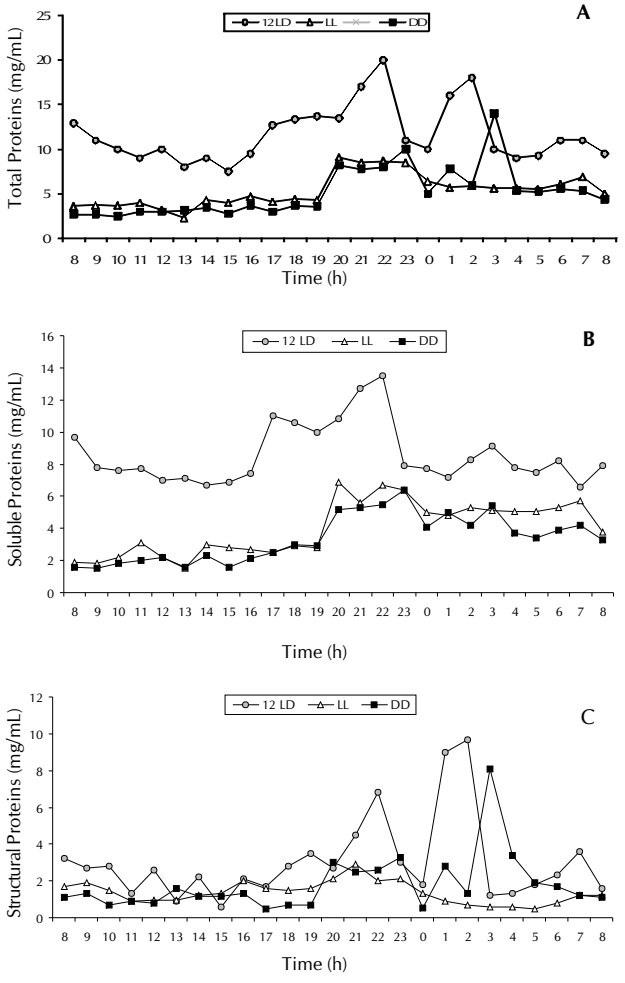

Figure 1: Phase response curves (PRCs) of the 24 hr circadian protein rhythms (from 8AM on day 3 to 8 AM on day 4) in the haemolymph of fourth instar larva of *Bombyx mori*, under12h light: 12 hr dark cycle (LD), continuous light (LL) and continuous dark (DD) conditions. A. Total proteins; B. soluble proteins and C. structural proteins (p < 0.001)

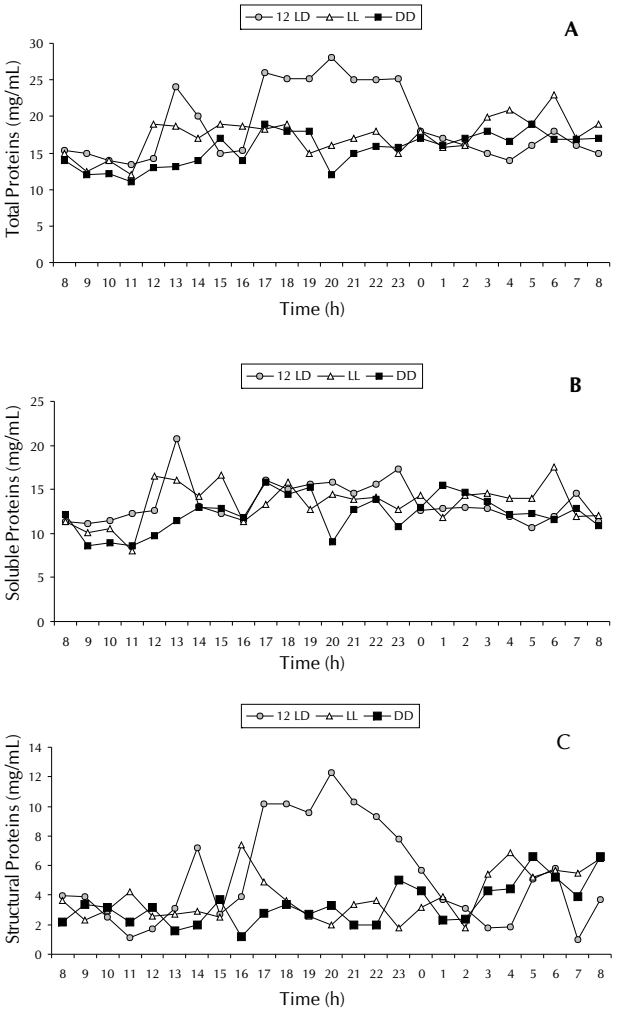

Figure 2: Phase response curves (PRCs) of the 24hr circadian structural protein rhythms (from 8AM on day 5 to 8 AM on day 6) in the haemolymph of fifth instar larva of *Bombyx mori,* under 12h light: 12h dark cycle (LD); continuous light (LL) and continuous dark (DD) conditions. A. Total proteins; B. soluble proteins and C. structural proteins (p < 0.001)

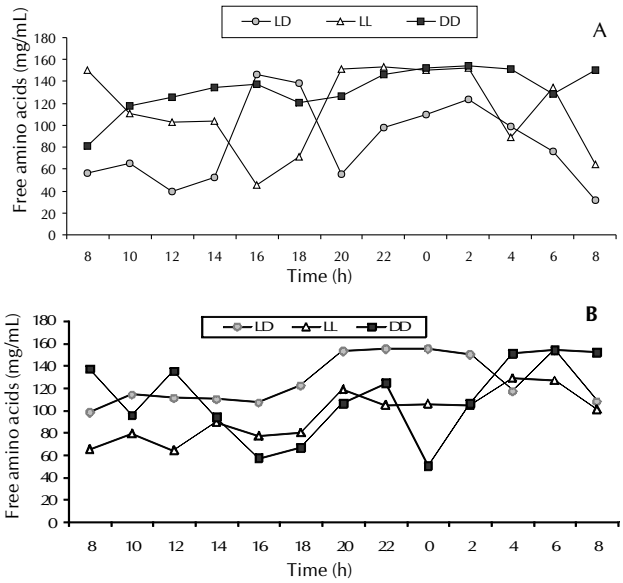

Figure 3: Phase response curves (PRC) of the 24-hr circadian amino acid rhythms in the haemolymph of (A) fourth instar (from 8AM on day-3 to 8 AM on day-4) and (B) fifth instar (from 8AM on day-5 to 8 AM on day-6) larvae of *Bombyx mori*, under 12h light: 12h dark cycle (LD); continuous light (LL) and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

sequestered in the haemolymph at regular intervals during the day and known to display stage-specific (larval, pupal and adult) variation in the silkworm (Kawaguchi *et al*., 1993). The haemolymph proteins represent whole or a part of 93 silk gland proteins, 177 fat body proteins, and 278 skeletal muscle proteins (Takasu *et al.,* 2005; Kyung *et al.,* 2006; Zhang *et al*., 2007). Their levels rise and fall in a rhythmic fashion during the 24 hr free running time and appear in the form of peaks and troughs in the phase response curves (Figs.1 and 2).

In our previous reports on silkworm silk gland and fat body tissues (Sailaja and Sivaprasad, 2010a, 2010b, 2011), we interpreted the peaks and troughs of protein rhythms in terms of protein synthetic cycles (PS cycles) with alternating phases of transcription and translation. In a similar fashion, it is presumed that the peaks and troughs in the haemolymph protein rhythms indicate the operation of a dynamic exchange mechanism in which proteins shuttle in between tissues and haemolymph at frequent intervals during metamorphosis. This exchange shuttle probably reflects the prevalence of a series of protein releasing cycles (PR cycles), each corresponds to the combined mean interval of one peak and one trough that are synchronised with the tissue-specific protein synthetic cycles. It is likely that the peaks reflect the timing of protein release into the haemolymph and the troughs, the timing of protein uptake from it. Thus, similar to transcription and translation phases of protein synthetic cycles in tissues, each PR cycle in haemolymph includes the events of protein release and uptake that occur on a continuous basis throughout the metamorphosis. In the releasing phase, proteins synthesized in the silkgland, muscle ,fat body and other tissues are released into the haemolymph, which remain there for a specific period of time and in the uptake phase they are absorbed by the needy tissues. The preset study demonstrates that the number

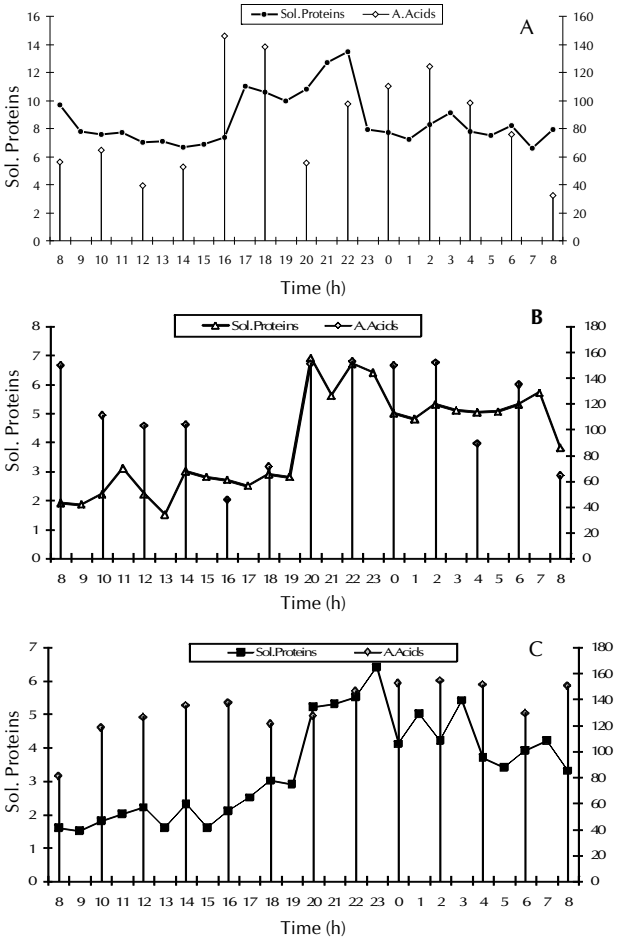

Figure 4: Circadian changes in profiles of soluble proteins and free amino acids in the haemolymph of the fourth instar larva of *Bombyx mori*, under (A)12hr light: 12hr dark cycle (LD), (B): continuous light and (C) continuous dark (DD) conditions. The values expressed in mg per g-wet weight of tissue, represent 24 hr (8 AM on day-3 to 8AM on day 4) free running time of the circadian rhythm

of PR cycles varies in stage-specific and photoperiod-specific fashions in *B. mori* with alternating releasing and uptake phases.

Instar-specific clock shifting

The instar-specific clock shift in the protein rhythm occurs when the fourth instar larva develops into fifth instar larva under normal LD conditions. The number of PR cycles decreased from ~7 to ~6 when the larva moved from fourth to fifth instar. At the same time, the duration of each cycle is enhanced from 3.4 to 4 hr, indicating high rate of protein synthesis and protein release during fourth instar compared to fifth instar (Table 5). This difference occurs due to the delay in the operation of each PR cycle in fifth instar by 0.6 hr (or 36 m) and total rhythm by 4.2 hr (or 4 hr 12 m) with consequent extension of the free running time of the 24 hr – fourth instar rhythm to 28 hr in the fifth instar (when fourth instar LD rhythm is treated as control). Though, the possible reason for this shift is not in sight, presumably it varies as a function of instar duration. Clearly, the fourth instar with shorter duration (4 days) of larval life has a higher rhythmic rate, both in terms of synthesis and release of proteins, while the fifth instar with

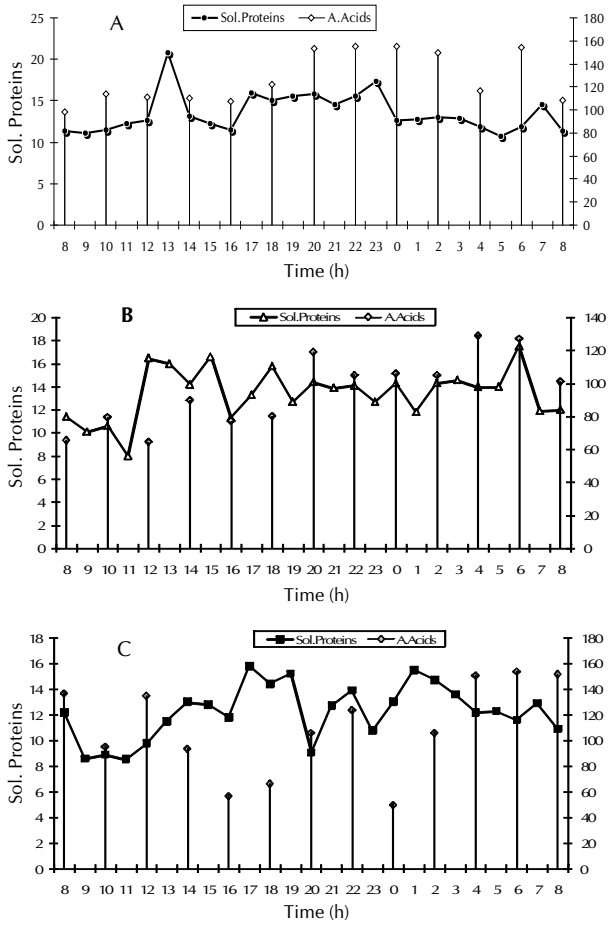

Figure 5: Circadian changes in profiles of soluble proteins and free amino acids in the haemolymph of the fifth instar larva of *Bombyx mori*, under (A)12 hr light: 12 hr dark cycle (LD), (B): continuous light and (C) continuous dark (DD) conditions. The values expressed in mg per g-wet weight of tissue, represent 24 hr (8 AM on day 5 to 8AM on day 6) free running time of the circadian rhythm

longer duration (7 to 8 days) of larval life maintains a slow rhythmic rate. Obviously, the shorter the instar duration the greater will be the rate of protein synthesis and its release into the circulating fluid. However, the decline in the rate is compensated by operation of more number of PS and PR cycles during fifth instar development. Thus, during the free running time of the protein rhythm, the fourth instar larva accomplishes 7 x 4 = 28 cycles, where as the fifth instar larva completes either 42 (6x7) or 56 (6x8) cycles, resulting in the operation of 14 additional cycles, but each one is delayed by 36 m. This delay in PS and PR cycles during fifth instar reflects two points. Synthesis of additional proteins that commensurate with the fifth instar larval growth and development as evidenced by the prevalence of higher peaks in the protein profiles of fifth instar larva, compared to those of fourth instar (Fig. 2A, B, C). These could include several fat body proteins such as aldose reductase (that convert glucose to sorbital in carbohydrate metabolism), glyoxylate reductase (that catalyses glycolate to produce glyoxylate), hydroxyl pyruvate isomerase (that intercoverts aldoses to ketoses of glyoxylate and dicarboxylate metabolism) and amino acylase (that catalyses the conversion of N-acyl-L-amino acids to carboxylate and L-amino acids) which appear in fifth instar and accumulate through pupal stage and help in energy metabolism during pupal-adult metamorphosis (Hou *et al*., 2010). Though, these proteins are not individually identified, their higher levels, nevertheless support the view that additional proteins are added to the haemolymph in a stage-dependent fashion during metamorphosis. Recruitment of more and more genes and their simultaneous expressions that may require additional time during fifth instar development. It is not known as to which genes are expressed and which proteins are synthesized during fourth and fifth instars. But, the indications are that the two silk genes; sericin-2 and fibroin, express actively during fourth and fifth instars respectively (Ishikawa and Suzuki, 1985; Michaille *et al*., 1989). In a similar fashion, ten fat body genes express in a stage-specific way to produce a genetically variable group of lipoproteins that play vital role in energy metabolism during embryonic development in silkworm (Hou *et al*., 2010). Thus, the instar-specific clock shifting of circadian protein rhythm in the haemolymph probably involves changes in the rate and the timing of tissue-specific gene expression or both. More extensive studies are needed to substantiate this assumption.

Photoperiod-specific clock shifting

Light is the principal zeitgeber (time giver) of circadian rhythms. Its impact on the circadian clock mechanism in *Drosophila* has been elucidated (Peschel *et al*., 2009). The present study on haemolymph protein profiles substantiates our earlier findings (Sailaja and Sivaprasad, 2010 a, 2010b, 2011) that the circadian protein rhythms of *B. mori* are subjected to clock-shifting or entrainment by the light cues under altered photoperiodic conditions of LD, LL and DD and this results in changing the number and duration of protein synthetic (PS) and protein release (PR) cycles in the silkworm larvae. Accordingly, when the growing silkworm larvae are deprived of natural light cues (12 hr light and 12 hr dark condition) the free running time of the circadian protein rhythm shifts from the standard 24 hr-pattern in a stage-specific fashion.

In the fourth instar, the rhythm maintained seven PS and PR cycles under LD, six under LL and eight under DD. Consequently, the duration of each cycle is maintained at different durations; 3.4 hr under LD, 4 hr under LL and 3 hr under DD. Thus, in the fourth instar larvae, the LL condition resets the normal protein rhythm behind the free running time by 4 hr, while the DD condition keeps it 3 hr ahead of the free running time. Consequently, the 24 hr free running time of the rhythm under LD is re-scheduled to operate at 28 hr under LL and 21 hr under DD (Table 5). In the fifth instar, the impact of the light on the protein rhythm is still more pronounced. While six PS and PR cycles were maintained under LD, it increased to nine under LL and eight under DD. At the same time, the duration of each cycle stood at ~4 hr under LD, 2.7 hr under LL and 3.2 hr under DD (Table 5). Thus, the duration of each cycle of protein rhythm is reduced by 1 hr 18 m (from 4 hr to 2.7 hr) under LL and by 48 m (from 4 hr to 3.2 hr) under DD, resulting in clock-shifting of the 24-hr free running time of the rhythm under LD to ~16 hr under LL and 19 hr under DD. With the result, the haemolymph is able to accomplish 3 additional rounds of PR cycles under LL and 1.5 rounds under DD.

As reported in our earlier findings (Sailaja and Sivaprasad, 2010 a, 2010 b, 2011) the photoperiod- induced clock-shift in protein rhythm reflects similar phase-shift in gene expressions in the silkworm larval tissues. Presumably, in the fourth instar, the genes express 7 times under LD (with 3.4 hr intervals), 6 times under LL (with 4 hr intervals) and 8 times under DD (with 3 hr intervals), while in fifth instar they express 6 times under LD (with 4 hr intervals), 9 times under LL (with 2 hr 42m intervals) and 8 times under DD (with 3 hr 12 m intervals). Though, one-to-one relationship between light and gene expression timings is not available, the circadian clock mechanism observed in *Drosophyla* and other insects point to this fact (Syrova *et al*., 2003; Grima *et al*., 2004; Hardin, 2004; Shafer *et al*., 2004; Stoleru *et al*., 2004). Further, the light undoubtedly has a profound effect on protein biosynthesis in the silkworm as evidenced by increase in protein synthetic and releasing cycles under altered photoperiodic conditions probably by stimulating the production of neuro-humoral factors like juvenile hormone that significantly modulates peripheral oscillators in silkworm tissues (Koga *et al*., 2005) triggering haemolymph protein rhythms.

Protein rhythm versus free amino acid levels

Haemolymph acts as a transient mobile repository for amino acids including glutamine, proline, arginine, lysine, aspartic acid, tyrosine, glutamic acid and histidine (David and Ananthakrishnan, 2006). The protein rhythm requires parallel changes in free amino acid (FAA) levels since they represent the basic raw materials of protein synthesis. Preferably, the levels of soluble proteins represent the newly synthesized proteins, while the free amino acids are derived either from the diet or as products of histolysis that characterise the events of insect metamorphosis. A comparative analysis of the PRCs of the soluble protein and free amino acid rhythms shows that the protein releasing cycles (peaks) are preceded by elevations in the levels of free amino acids notwithstanding some minor deviations that are probably caused by sampling errors (Figs. 4 and 5). The trend is evident in the larval haemolymph of both the instars examined under three photoperiodic conditions. In the fourth instar, the events of PS and PR cycles occur in two or three phases depending on the photoperiodic conditions. Under LD, the PRC of soluble protein rhythm broadly shows three phases; a low profile early phase from 08 to 16 hr, a high profile middle phase from 17 to 22 hr and a low profile late phase from 23 to 08 hr (Fig. 4A). The levels of FAA showed similar trends with relatively low levels (~39 to 65 mg / mL) in the low profile early phase, higher levels in the high profile middle phase (~100 to 146 mg / mL) and again low levels (~32 to 98 mg / mL) in the late phase. Thus, the amino acid rhythm carried through the protein rhythm with a view to ensure uninterrupted supply of the raw materials (free amino acids) required for the tissue-specific protein synthesis during the free running time of the rhythm. But under LL and DD conditions the soluble protein rhythm included only two phases, a low profile early phase from 08 to 19 hr and a long stretched high profile late phase, from 20 hr to 07 hr on the next day (Fig. 4B, C). Thus, both photopic (LL) and scotopic phases seem to trigger active mobilisation of proteins from tissues during the latter half of the day that actually corresponds to the scotopic (dark) phase of normal LD condition. In both these conditions (LL and LD), a clear-cut correlation between amino acid and protein rhythms doesn't exist, as the FAA levels are largely influenced by dietary sources and histolytic events of larval growth and metamorphosis (Sivaprasad and Sailaja, 2009). In the fifth instar, however, the soluble protein rhythm maintained a sustained activity throughout the free running time barring a few early hours of the day during all the photoperiodic conditions; LD, LL and DD. The amino acid profiles maintained by and large the same levels indicating their continuous and unabated release into the haemolymph either from the gut or histolytically derived products from other tissues. But under LL and DD conditions it included only two phases, a short-duration low-profile early phase from 08 to 11 hr and a long-stretched high profile late phase (from 12 hr to 06 hr in case of LL and from 12 to 07 hr in case of DD) with concomitant trends in the levels of FAA. Obviously, the amino acid rhythm in fifth instar continues along the protein rhythm as in fourth instar. Clearly, the impact of light is profound on haemolymph protein and amino acid rhythms. If soluble protein levels represent the profiles of recently synthesized proteins, the rate of protein synthesis and their release into the haemolymph is significantly enhanced under the influence of both photopic (light) and scotopic (dark) conditions, probably by actively mobilizing the free amino acids from diet as well as histolytically derived ones. Although, the amino acid profiles vary independent of protein profiles, nevertheless, the photoperiod causes disturbances in their rhythmicity, more particularly under DD condition as evidenced by the present investigation.

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