Biolife 1(2):-17-29, 2013
AN INTERNATIONAL QUARTERLY JOURNAL OF BIOLOGY & LIFE SCIENCES
ISSN (online): 2320-4257
www.biolifejournals.com
BIOLIFE ORIGINAL ARTICLE

Impact of Photoperiod on the Circadian Cellulose and Cellulase Rhythms in the Digestive System of Silkworm, *Bombyx Mori*

Bhuvaneswari E, Madhavi R, Thulasi N And Sivaprasad S*

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

ABSTRACT

Circadian cellulose and cellulase rhythms were studied in the digestive system of *Bombyx mori* under 12 hr light-dark cycle (LD), continuous light (LL) and continuous dark (DD) conditions. Since, the symbiotic microbes are associated with cellulose digestion in the silkworm; both the rhythms are presumed to have microbial origin. The rhythms were analyzed in terms of phase response curves and interpreted as cellulose synthetic cycles (CS cycles) and cellulase enzyme synthetic cycles (CES cycles) in gut wall and cellulose uptake cycles (CU cycles) and cellulase enzyme release cycles (CER cycles) in gut lumen. The cellulose rhythm in the gut wall maintained 7 CS cycles each with a duration of 3.4 hr and the 24 hr free running time remained unchanged under altered photoperiodic conditions. In gut lumen, the rhythm showed 7 CU cycles, each with a duration of 3.4 hr under LD and 6 CU cycles of 4.0 hr each under LL and DD, with the result, the 24 hr free running period of the rhythm was clock shifted to 28 hr both under LL and DD. The cellulase rhythm in the gut wall maintained 7 CES cycles with a duration of 3.4 hr each under LD and DD and 9 cycles each with a duration of 2.7 hr under LL. The 24 hr free running time of the cellulase rhythm of LD and DD, was thus advanced to 19 hr under LL. The cellulase in gut lumen maintained 6 CER cycles with a duration of 4.0 hr each, under LD, LL and DD and hence the 24 hr free running time of the rhythm was not affected. Further analysis of data showed that cellulose synthesis was stimulated by light cues, while its uptake from gut lumen through enhanced microbial cellulase activity was favoured by both diet and light cues.

Key words : *Bombyx mori*, Circadian cellulase rhythm, Circadian cellulose rhythm, Digestive system, Photoperiod.

INTRODUCTION

Cellulose is the primary structural component of plant cells and accounts for half of the carbon in the biosphere (Voet *et al*., 1998). It is a linear polymer of D-glucose residues linked by ß (1-4) glycosidic linkages (Anand *et al*., 2010; Pauchet *et al*., 2010). The mulberry leaf, the staple food of *B.mori*, consists of about 121 g/ $kg^{-1}$of cellulose and 107 g/ kg^{-1}hemi cellulose and thus represents the chief dietary source of carbohydrates for the silkworm (Kandylis *et al*., 2009; Anand *et al*., 2010). In the digestive system of silkworm, the cellulose is digested to glucose by a series of enzymes, known as cellulases, which break the ß (1-4) linkages and liberate individual glucose units (Voet *et al*., 1998). Since, the cellulase synthesizing genes are absent in the silkworm, the cellulose digestive functions are taken over by the symbiotic gut bacteria, of the genus *Proteus*, *Klebsiella*, *Citrobacter* and other cellulolytic bacteria. The microbial community synthesizes three types of cellulases, namely, C_1- cellulases (Cellobiohydrolases), C_x- cellulases (Endoglucanases) and cellobioses (ß-

glucosidases) in an ambient alkaline medium in the silkworm gut (Martin, 1983; Gringorten *et al*., 1993; Genta *et al*., 2003; Anand *et al*., 2010; Shi *et al*., 2011and Nagaraju Mareedu and Sunitha Devi Gudamani).

Like any other biochemical constituent of the digestive system, the cellulose and cellulase levels are subjected to modulation by circadian clocks. Despite the availability of a wealth of literature on its physical and chemical properties of these two biochemical constituents, and the clock gene expression patterns (Froy *et al*., 2003; Sharma, 2003; Satyanarayana *et al*., 2004; Sehadova *et al*., 2004; Iwai *et al*., 2006), no efforts have since been made to examine their rhythmic nature in the digestive system of *B.mori*. Nevertheless, our previous investigations largely focused on this potential area of research and made significant contributions in elucidating the clock-shifting influence of light on biochemical rhythms in *B.mori* (Sailaja and Sivaprasad, 2010 a, 2010 b; Sailaja and Sivaprasad, 2011; Sailaja *et al*., 2011; Sivaprasad and Sailaja, 2011; Bhuvaneswari and Sivaprasad, 2012 a; Bhuvaneswari and Sivaprasad, 2012 b; Bhuvaneswari and Sivaprasad, 2013).

MATERIALS AND METHODS

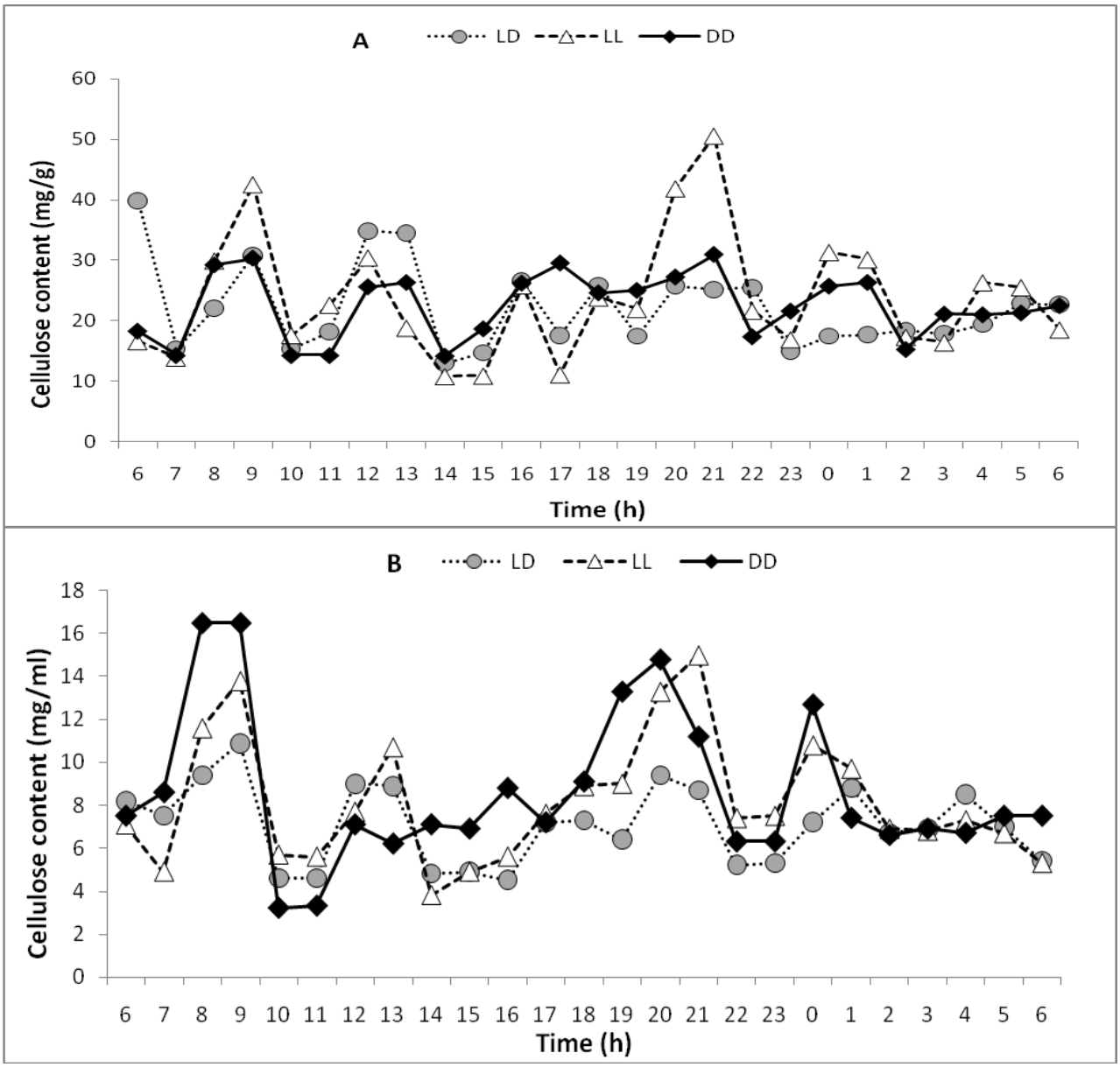
The Pure Mysore x CSR_2 hybrid variety of the Silkworm *Bombyx mori,* reared under standard environmental conditions of 28°C, 85% relative humidity (Krishnaswami, 1986), was used as the test species in the present study. After hatching, the worms were feed with M_5 variety of mulberry leaves, five times a day at 6 AM, 10 AM, 2 PM, 6 PM and 10 PM, under normal 12 hr light and 12 hr dark conditions. After third moult, the larvae were divided into three batches and reared separately under three different photoperiodic conditions viz., 12 hr light and 12 hr 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 cellulose and cellulase activity in the digestive system of silkworm was analyzed for a period of 25 hr spanning in between day 5 and day 6 of the 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 25 hr). At the same time the digestive juice was extracted from the gut through a hypodermic syringe by inserting it into its lumen. The digestive fluid was collected in a test tube and kept under ice-cold conditions till the mulberry leaf pieces were settled at the bottom and later, the supernatant was decanted and used for the biochemical assay. Hour- to- hour changes in cellulose levels of the gut wall and gut content were estimated by the method of Updegroff (1969) in 5% homogenate of the gut wall tissue and 1:9 diluted gut digestive juice in acetic nitric reagent.

The cellulose levels computed using standard glucose, were expressed as mg glucose/g wet weight of tissue or 1 ml of digestive juice. Likewise, hour to hour changes in the cellulase activity was estimated by the method of Miller (1959) in 5% homogenate of the gut wall and 1: 9 diluted digestive juice in 0.05M phosphate buffer. The enzyme activity was computed using glucose standard and expressed in µ moles of glucose/ mg cellulose/ hour. The whole experiment lasted for two consecutive days encompassing 12:12 hr 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 as experimental samples.

RESULTS

The circadian cellulose and cellulase rhythms of the gut wall and gut content under three photoperiodic conditions LD, LL and DD were projected in phase response curves (PRCs) which were then analyzed in terms the number of peaks (elevated points) and troughs (low



Note: The values expressed in mg glucose /g wet weight of tissue, represent the 24 hr (from 6 AM on day 5 to 6 AM on day 6) free running time of the circadian rhythm (P values: <0.001).

Fig. 1A & B: **Phase response curves (PRCs) of the 24 hr circadian cellulose rhythms in the gut wall (A) and gut content (B) of fifth instar larva of *Bombyx mori*, under12 hr light: 12 hr dark cycle (LD), continuous light (LL) and continuous dark (DD) conditions.**

points) and intervals between them as shown in figures 1 to 4.

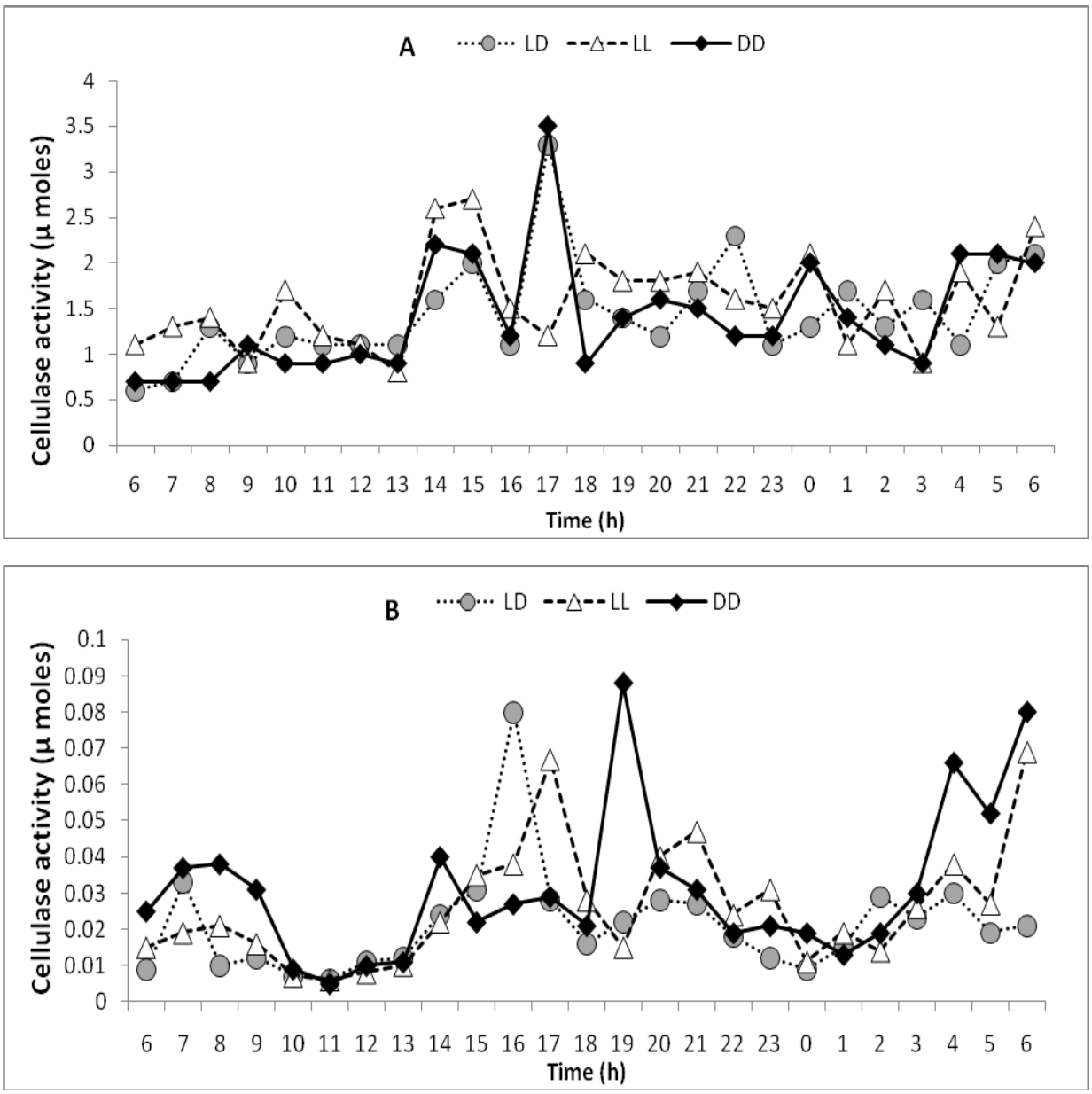
Circadian cellulose rhythms

Gut wall:

In the gut wall, the cellulose levels showed 7 peaks and 6 troughs under LD during the 24 hr free running period of the rhythm (Fig. 1A). The peaks occurred at 09 hr (~31 mg), 12-13 hr (~35 mg), 16 hr (~27 mg), 18 hr (~26 mg), 20-22 hr (~26 mg) and next day at 05-06 hr (~23 mg). The troughs were recorded at 07 hr (~15 mg), 10 hr (~15 mg), 14 hr (~13 mg), 17 hr (~18 mg), and 19 hr (~ 18 mg) and at 23 hr (15 mg). Under LL, the cellulose rhythm showed 7 peaks and 7 troughs during the 24 hr free running period. The

peaks appeared at 06 hr (~17 mg), 09 hr (~43 mg), 12 hr (~30 mg), 16 hr (~26 mg), 21 hr (~51 mg), 00 hr (~31 mg) and next day at 04 hr (~26 mg), the troughs were recorded at 07 hr (~14 mg), 10 hr (~18 mg), 14-15 hr (~11 mg), 17 hr (~11 mg), 23 hr (~17 mg) and next day at 02-03 hr (~17 mg) and 06 hr (~19 mg).

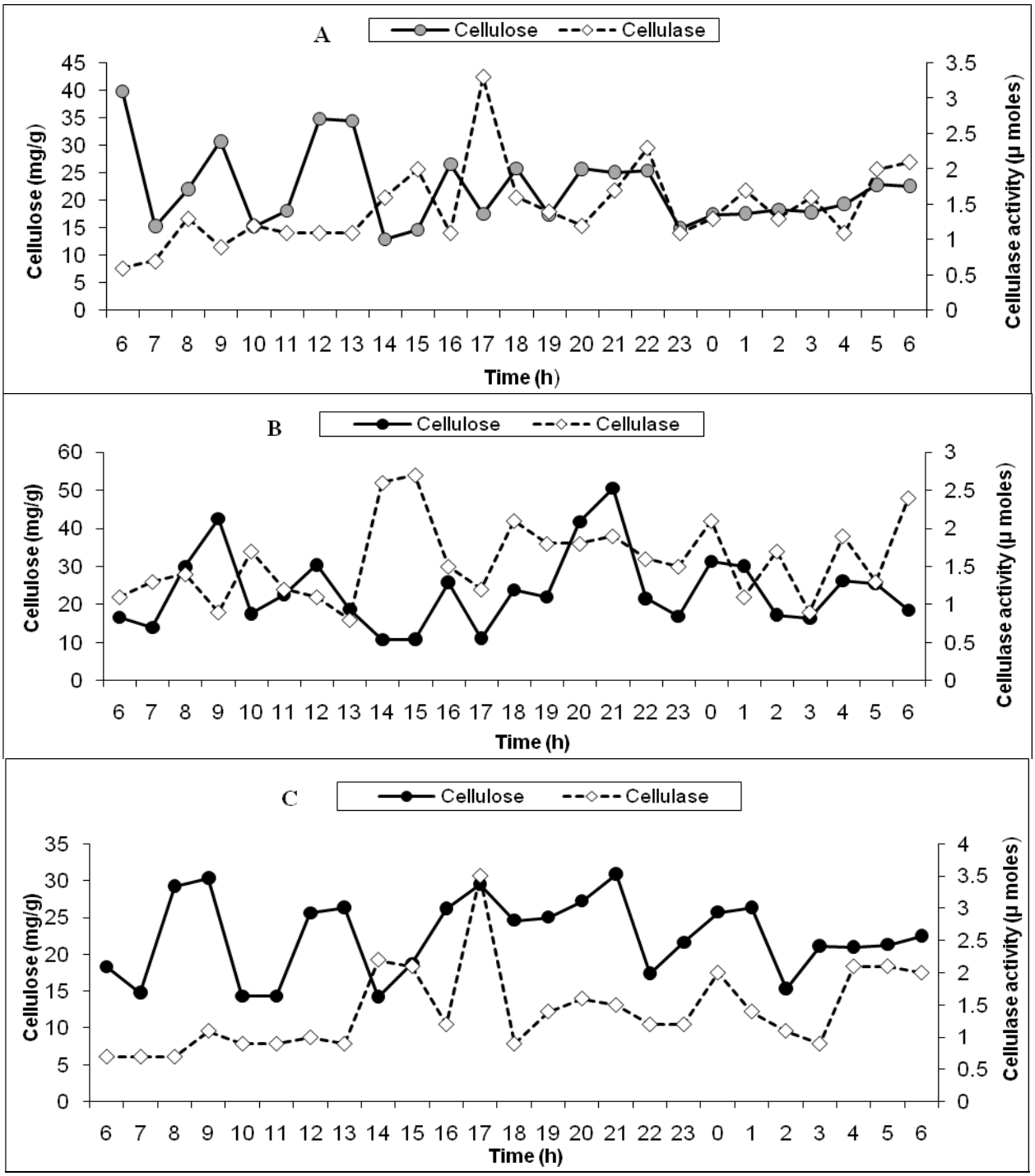
Under DD, the cellulose rhythm showed 7 peaks and 6 troughs. While, the peaks appeared at 06 hr (~18 mg), 09 hr (~30 mg), 13 hr (~26 mg), 17 hr (~30 mg), 21 hr (~31 mg) and next day at 01 hr (~26 mg) and 06 hr (~23mg), troughs occurred at 07 hr (~15 mg), 10-11 hr (~14 mg), 14 hr (~14 mg), 18-19 hr (~25 mg), 22 hr (~17



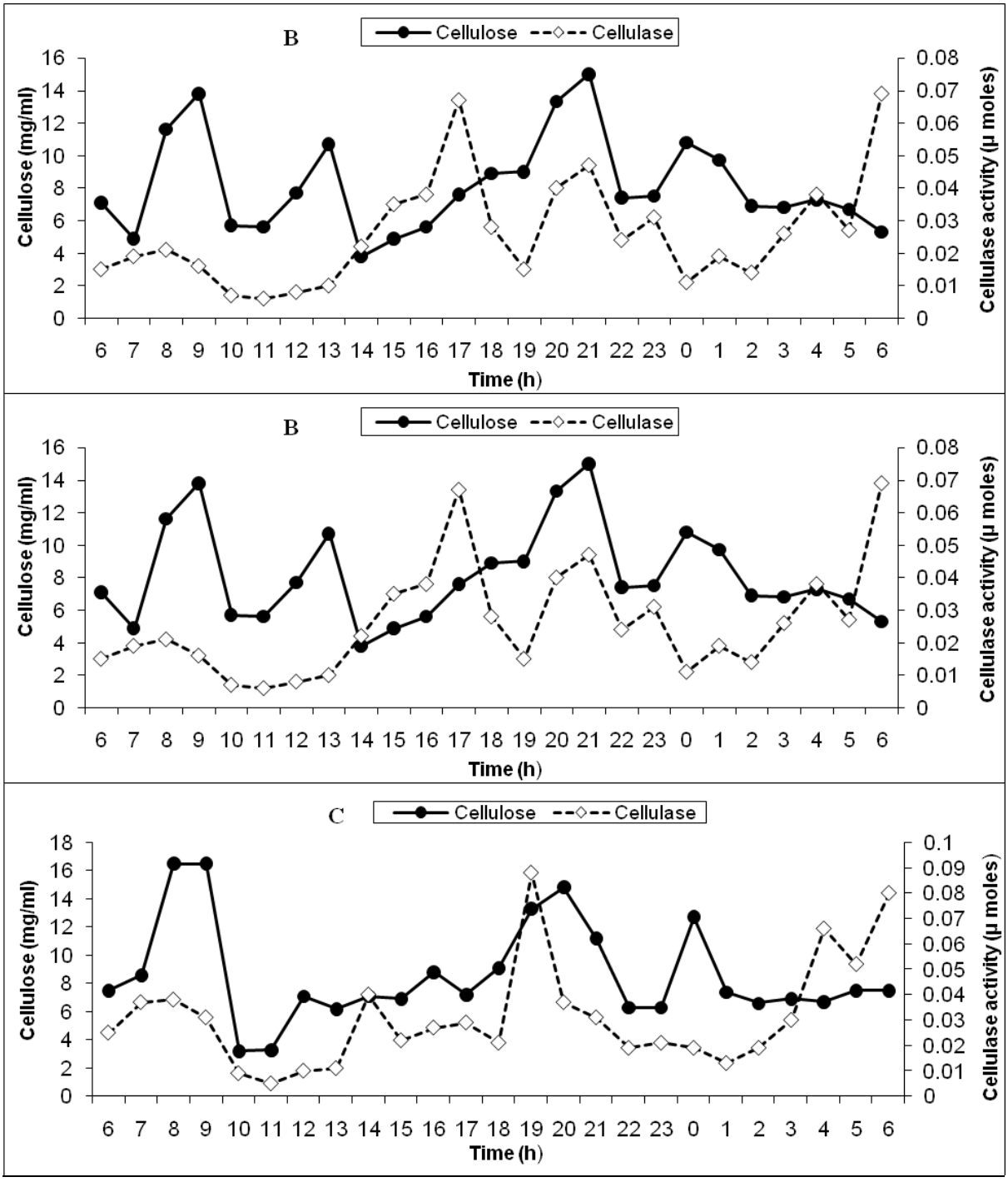
Note: The values expressed in µ moles of glucose /mg cellulose/ hr), represent the 24 hr (from 6 AM on day 5 to 6 AM on day 6) free running time of the circadian rhythm (P values: <0.001).

Fig. 2A & B: Phase response curves (PRCs) of the 24 hr circadian cellulase rhythms in the gut wall (A) and gut content (B) of fifth instar larva of *Bombyx mori*, under12 hr light: 12 hr dark cycle (LD), continuous light (LL) and continuous dark (DD) conditions.

mg) and next day at 02 hr (~15 mg). The mean intervals between peaks and troughs varied considerably. It was about 2.9 hr under LD, 3.1 hr under LL and 3.4 hr under DD between two peaks and that between troughs was 2.7 hr under LD, 3.0 hr under LL and 2.8 hr under DD. In order to minimize the gap between two peaks and troughs, the time intervals were computed in terms of combined mean intervals of both peaks and troughs, and the same was about 2.8 hr under LD and 3.1 hr both under LL and DD.



Note: The values expressed in mg glucose per gm wet weight of tissue in case of cellulose and µ moles of glucose formed/ mg cellulose/ hr in case of cellulase, represent the 24 hr (6 AM on day-5 to 6 A.M on day-6) free running time of the circadian rhythm. (P values: <0.001).



Note: The values expressed in mg glucose per ml of tissue in case of cellulose and µ moles of glucose formed/ mg cellulose/ hr in case of cellulase, represent the 24 hr (6 AM on day-5 to 6 A.M on day-6) free running time of the circadian rhythm. (P values: <0.001)

Fig. 4-A, B & C: Circadian changes in cellulose profiles and cellulase activity in the gut content of the fifth instar larva of *Bombyx mori*, under (A) 12 hr light: 12 hr dark cycle (LD), (B) continuous light (LL) and (C) continuous dark (DD) conditions.

Gut content:

Under LD, the cellulose levels of the gut content showed 7 peaks and 7 troughs during the 24 hr free running period of the rhythm (Fig. 1B). The first peak appeared at 06 hr with a cellulose value of ~8 mg/ml of digestive juice. Subsequent peaks occurred at 09 hr (~11 mg), 12-13 hr (~9 mg), 17-18 hr (~7 mg), 20 hr (~9 mg), and next day at 01 hr (~9 mg) and 04 hr (~9 mg). Likewise, troughs were recorded at 07 hr (~8 mg), 10-11 hr (~5 mg), 16 hr (~5 mg), 19 hr (~6 mg), 22-23 hr (~5mg), and next day at 02-03 hr (~7 mg) and 06 hr (~5 mg). Under LL, the cellulose rhythm showed 6 peaks and 6 troughs. The peaks appeared at 06 hr (~7 mg), 09 hr (~14 mg), 13 hr (~11 mg), 21 hr (15 mg), 00 hr (~11 mg) and next day at and 04 hr (~7 mg) and the troughs appeared at 07 hr (~5 mg), 10-11 hr (~6 mg), 14 hr (~4 mg), 22-23 hr (~8 mg), and next day at 02-03 hr (~7 mg) and 06 hr (~5 mg). Under DD, the cellulose rhythm showed 6 peaks and 6 troughs. The peaks appeared at 08-09 hr (~17 mg), 12 hr (~7 mg), 16 hr (~9 mg), 20 hr (~15 mg), 00 hr (~13 mg) and next day at 05-06 hr (~8 mg), troughs occurred at 06 hr (~8 mg), 10-11 hr (~3 mg), 13 hr (~6 mg), 17 hr (~7 mg), 22-23 hr (~6 mg) and next day at 04 hr (~7 mg). The interval between peaks was about 2.9 hr under LD, 3.7 hr under LL and 3.3 hr under DD and that between troughs was about 2.9 hr under LD, and 3.3 hr under both LL and DD. The computed combined mean interval of both peaks and troughs was about 2.9 hr under LD, 3.5 hr under LL and 3.3 hr under DD (Fig 2A & B).

Circadian cellulase rhythms

Gut wall:

Under LD, the cellulase activity showed 7 peaks and 7 troughs in the gut wall during the 24 hr free running period of the rhythm (Fig. 2A). Peaks appeared at 08 hr (1.3 µ moles), 15 hr (2.0 µ moles), 17 hr (3.3 µ moles), 22 hr (2.3 µ moles) and next day at 01 hr (1.7 µ moles), 03 hr (1.6 µ moles) and 06 hr (2.1 µ moles), troughs appeared at 06 hr (0.6 µ moles), 09 hr (0.9 µ moles), 16 hr (1.1 µ moles), 20 hr (1.2 µ moles), 23 hr (1.05µ moles) and next day at 02 hr (1.3 µ moles) and 04 hr (1.1µ moles). Under LL, the rhythm showed 9 peaks and 9 troughs during the 24 hr free running time. The first peak appeared at 08 hr (1.4 µ moles), 10 hr (1.7 µ moles), 15 hr (2.7 µ moles), 18 hr (2.1 µ moles), 21 hr (1.9 µ moles), 00 hr (2.8 µ moles) and next day at 02 hr (1.7 µ moles), 04 hr (1.9 µ moles) and 06 hr (2.4 µ moles).Troughs occurred at 06 hr (1.1 µ mole), 09 hr (0.9 µ moles), 13 hr (0.8 µ moles), 17 hr (1.2 µ moles), 19-20 hr (1.8 µ moles), 23 hr (1.5 µ moles) and next day at 01 hr (1.1 µ moles), 03 hr (0.9 µ moles) and 05 hr (1.3 µ moles). Under DD, the cellulase activity rhythm showed 7 peaks and 7 troughs during the 24 hr free running period. The peaks appeared at 09 hr (1.1 µ moles), 12 hr (1.0 µ moles), 14 hr (2.2 µ moles), 17 hr (3.5 µ moles), 20 hr (1.6 µ moles), 00 hr (2.0 µ moles) and next day at 04-05 hr (2.1 µ moles), while the troughs occured at 06-08 hr (0.7 µ moles), 10-11 hr (0.9 µ moles), 13 hr (0.9 µ moles), 16 hr (1.1 µ moles), 18 hr (0.9 µ moles), 22-23 hr (1.2 µ moles) and next day at 03 hr (0.9 µ moles). The interval between peaks was about 3.1 hr under LD, 2.4 hr under LL and 2.7 hr under DD and that between troughs was about 3.1 hr under LD and 2.4 hr under both LL and DD. The combined mean interval of peaks and troughs was about 3.1 hr under LD, 2.4 hr under LL and 2.6 hr under DD (Fig 3A, 3B.

Gut content:

Under LD, the cellulase activity showed 6 peaks and 6 troughs in the gut content during the 24 hr free running period of the rhythm (Fig. 2B). Peaks appeared at 07 hr (0.033 µ moles), 16 hr (0.080 µ moles), 20 hr (0.028 µ moles) and next day at 02 hr (0.029 µ moles), 04 hr (0.030 µ moles) and 06 hr (0.021 µ moles) and troughs occured at 06 hr (0.009 µ moles), 11 hr (0.006 µ moles), 18 hr (0.016 µ moles), 00 hr (0.009 µ moles) and next day at 03 hr (0.023 µ moles) and 05 hr (0.019 µ moles). Under LL, the rhythm showed 6 peaks and 6 troughs in the gut content during the 24 hr free running period. The peaks appeared at 08 hr (0.021 µ moles), 17 hr (0.067 µ moles), 21 hr (0.047µ moles), 23 hr (0.031 µ moles) and next day at 04 hr (0.038 µ moles) and at 06 hr (0.069 µ moles) and troughs occurred at 06 hr (0.015 µ moles), 11 hr (0.006 µ moles), 19 hr (0.015 µ moles), 22 hr (0.024 µ moles), 00 hr (0.011 µ moles) and next day at 05 hr (0.027 µ moles). Under DD, the cellulase activity rhythm

showed 6 peaks and 6 troughs in the gut content during the 24 hr free running period of the rhythm. The peaks appeared at 08 hr (0.038µ moles), 14 hr (0.040 µ moles), 17 hr (0.029µ moles), 19 hr (0.088 µ moles) and next day at 04 hr (0.066 µ moles) and 06 hr (0.080µ moles), the troughs occured at 06 hr (0.025µ moles), 11 hr (0.005 µ moles), 15 hr (0.022 µ moles), 18 hr (0.021µ moles) and next day at 01 hr (0.013 µ moles) and 05 hr (0.052 µ moles). The interval between peaks was about 3.8 hr under LD, 3.7 hr, both under LL and DD and that between troughs was about 3.7 hr under LD, 3.8 hr both under LL and DD. The combined mean interval between peaks and troughs was about 3.8 hr under all the three photoperiodic conditions (Fig; 4A, 4B and 4C).

DISCUSSION

In the silkworm, the digestive system acts as an organ of synthesis, storage, secretion and absorption for a variety of biochemical constituents and digestive products. The carbohydrate- rich mulberry leaf, on which it feeds, is the major source of cellulose in its gut (Ross *et al*., 1991; Aruga, 1994). The dietary cellulose is digested by the cellulases derived from the symbiotic microbial fauna that thrives and functions effectively in an alkaline medium of digestive fluid in the gut lumen (Gringorten *et al*., 1993; Pytelkova *et al*., 2009). Nevertheless, it has been suggested that concentration of cellulose and the digestive action of cellulase are modulated in the two compartments of the digestive system (gut wall and digestive fluid), synergetically both by the host organism and the symbiotic microbes (Hongoh, 2008; Liu *et al*., 2008). Thus, the digestive system of *B.mori* acts as a dual functional system, at least for the synthesis and hydrolysis of the cellulose, in which both these functions are shared by the two stakeholders, namely the silkworm and microbes. The profiles of cellulose and cellulase vary in a cyclic fashion under the control of light sensitive circadian clocks as demonstrated in *B.mori* and other insects (Falcon, 1999; Kostal and Shimada, 2001; Saunders, 2002; Syrova *et al*., 2003; Sailaja and Sivaprasad, 2010 a, 2010 b; Sailaja and Sivaprasad, 2011; Sailaja *et al*., 2011; Sivaprasad and Sailaja, 2011; Bhuvaneswari and Sivaprasad, 2012 a; Bhuvaneswari and Sivaprasad, 2012 b; Bhuvaneswari and Sivaprasad, 2013). The circadian data on cellulose and cellulase activity in the digestive system of *B.mori* are depicted in the form of phase response curves (PRCs), and the peaks and troughs thereof are systematically analyzed (Fig. 1 to 4 and Tables 1 to 6). The number of peaks in cellulose rhythms was interpreted in terms of the number of cellulose synthetic cycles (CS cycles) in the gut wall and number of cellulose uptake cycles (CU cycles) in the gut content. Similarly, the number of peaks in cellulase activity was interpreted in terms of cellulase enzyme synthetic cycles (CES cycles) in the gut wall and cellulase enzyme release cycles (CER cycles) in the gut content. In both the compartment of digestive system (gut wall and gut lumen), the height of peaks was interpreted in terms of intensity of synthetic/ release cycles and the mean peak value (MPV) in terms of the average levels of cellulose or cellulase activity maintained during the 24 hr free running time of the rhythm. At the same time, the combined mean interval between peaks and troughs was considered as the time taken for completion of each synthetic/ release/ uptake cycles of cellulose and cellulase (Tables 5 and 6).

Circadian cellulose rhythm

***Gut wall*:**

General assumption is that cellulose is not an integral part of animal structure and function. Even in ruminants, insects and certain other invertebrates whose staple food largely comprises cellulose, its digestion is facilitated by symbiotic microbes in different segments of the gut lumen (Nakashima *et al*., 2002; Tokuda *et al*., 2005; Anand *et al*., 2010; Belda *et al*., 2011). The dietary cellulose remains in the gut lumen for a considerable period of time before it is digested and absorbed. Since, the silkworm is a voracious feeder of mulberry leaf, it maintains large quantities of cellulose in its gut almost throughout the day. The continuous presence in its gut of cellulose, obviously, causes osmotic problems between the external (gut lumen) and internal (gut wall) environments, that might

cause damage to the thin gut wall. Probably, such problems are solved by establishing a cellulose gradient in the cellular environment, given the fact that the latter not only helps in withstanding osmotic pressure differences between the extra cellular and intracellular spaces, but also facilitates load bearing function (Voet *et al*., 1998). In silkworms, this is achieved, probably by maintaining, a community of cellulose synthesizing microbes in the gut wall cells, much like that of cellulose-hydrolyzing bacteria in the gut lumen.

Two such potential candidates; *Escherichia coli* and *Pseudomonas* were identified in *B.mori*, based on the comparative genomic studies between cellulose synthesizing and cellulose digesting bacterial groups (Amikam and Benziman, 1989; Romling, 2002). Surprisingly, these two bacterial strains do not utilize the cellulose derived from the dietary source but synthesizes their own within the gut wall cells (Anand *et al*., 2010). The bacterial cellulose has features like high crystallinity, tensile strength and water absorption capacity, fine fiber network, biocompatibility, resistance to degradation and low solubility as compared to plant cellulose (EI- Saied *et al*., 2008). Largely, the gut wall cellulose represents the cellulose synthesized by bacteria, at least in the silkworm. Its rhythm maintains 7 synthetic cycles (CS cycles) in all the three photoperiodic conditions under LD, LL and DD with duration of 3.4 hr each in the gut wall. Thus the 24 hr free running rhythm was not altered by the photoperiod (Table 5). Further analysis of peaks in terms of their height reveals that the intensity of bacterial cellulose synthesis increased during photic phase (light) of the day compared to scotopic (dark) phase. Evidently, intense cellulose synthesis occurred four times (at 08-09 hr, 12-13 hr, 16-17 hr and 20-21 hr) under DD, thrice (at 06 hr, 09 hr and 12-13 hr) under LD and twice (08-09 hr and 20-21 hr) under LL (Fig. 1A). The photoperiod not only altered the intensity but also affected the mean peak values (MPVs) of the cellulose. The MPV of the cellulose was higher under LL (~32 mg), moderate under LD (29.5 mg) and lower under DD (26.3 mg). Obviously, light stimulates cellulose synthesis in the gut wall cells, while the dark condition slowed down the rate of its synthesis in *B, mori* as pre-supposed (Iwai *et al*., 2006; Fonagy, 2009; Kostal and Shimada, 2001; Syrova *et al*., 2003)

Gut content:

The cellulose of the gut content or digestive fluid is derived from the mulberry diet and it is hydrolyzed in the gut lumen by microbial-derived cellulase in the gut lumen (Ross *et al*., 1991; Kandylis *et al*., 2009; Anand *et al*., 2010). The release of cellulose from the diet and its utilization by microbes follows a cyclic path, referred to as cellulose uptake cycles (CU cycles) in this report. The gut content maintains 7 such CU cycles with a duration of 3.4 hr each under LD and 6 CU cycles with duration of 4.0 hr each under both LL and DD. Thus LL and DD conditions modulate the CU cycle by extending the duration of each one by 36 min (from 3.4 hr to 4.0 hr). Hence the 24 hr free running time of LD was clock shifted to 28 hr both under LL and DD conditions. Accordingly, the active release of cellulose occurred five times (at 06-09 hr, 12-13 hr, 20-21 hr, 01 hr and 04 hr) under LD, four times (at 08-09 hr, 13 hr, 20-21hr and 00 hr) under LL and thrice (08-09 hr, 19-21 hr and 00hr) under DD. Nevertheless, the optimal cellulose levels, (represented as higher mean peak values) were maintained both under LL and DD (~11 mg) conditions, compared to those of LD (8.9 mg). Obviously, the cellulose uptake in the gut lumen was triggered by light and dark cues.

Circadian cellulase rhythms

Gut wall:

Since, cellulase is not synthesized by the gut wall cells, but by the symbiotic bacteria residing in the gut wall (Watanabe and Tokuda, 2010), it is presumed that the cellulase rhythm represents that of microbes and it runs on a cyclic path too. As observed in the present study, the microbial cellulase rhythm in the gut wall showed 7 cellulase enzyme synthetic cycles (CES cycles), both under LD and DD conditions, each with a duration of 3.4 hr and 9 cycles under LL, each with a duration of 2.7 hr. Due to reduction in the duration of each CES cycle by 4.2 min (from 3.4

hr to 2.7 hr), the 24 hr free running time of cellulase rhythm of LD and DD condition was reduced to 19 hr under LL (Table 5). Within the rhythm, the microbial enzyme showed three active synthetic phases under LD (at 17 hr, 22 hr and 05-06 hr), four each under LL (at 14-15 hr, 18 hr, 00 hr and 06 hr) and DD (14-15 hr, 17 hr, 00 hr and 04-06 hr). Moreover, they consistently maintained the same MPVs (about 2.0 µ moles) under three photoperiodic conditions, which support that both light and dark cues are necessary not only for the synthesis of cellulase but also for enhancing its activity.

Gut content: The symbiotic microbial community thrives on the cellulose present in the gut content. They release cellulases into the gut lumen at regular intervals and hydrolyze the dietary cellulose in an alkaline medium (Martin, 1983; Shi *et al*., 2011; Thompson, 2003). Hence the cellulase rhythm in the gut content was represented as cellulase enzyme release cycles (CER cycles). During the free running time of its rhythm 6 CER cycles occurred uniformly under all the three photoperiodic conditions (LD, LL and DD), each with duration of 4.0 hr. Notably, the 24 hr free running time of cellulase rhythm remained unaffected by changes in the duration of photoperiod. Nevertheless, the active period of enzyme activity differed from one photoperiodic condition to the other. For instance, higher phases enzyme activity occurred once under LD (16 hr), thrice under LL (17 hr, 20-21hr, and 06 hr) and twice under DD (19 hr, 04-06 hr). However, MPVs of enzyme activity were higher under DD (0.052 µ moles), moderate under LL (0.046 µ moles) and lower under LD (0.037 µ moles) conditions (Table 6). The trends in its activity further suggest that, it was optimally modulated by both light and dark signals.

Cellulose rhythm versus cellulase rhythm

A comparative analysis of cellulose and cellulase rhythms showed inverse relationship between the substrate levels and enzyme activity in both the compartments of digestive system under the three photoperiodic conditions examined (Fig. 3 and 4). In such a relationship, the peaks in the cellulose levels were accompanied by troughs in the cellulase activity and vice versa. For instance, in the gut wall, the peaks in the levels of substrate (Cellulose) and troughs in the enzyme (Cellulase) activity occurred almost simultaneously at 06 to 13 hr under LD, 06 to 13 hr and 20-23 hr under LL and 06 to 16 hr and 18 to 06 hr under DD. Conversely, the peaks in cellulase and troughs in cellulose coincided with each other at 14 to 06 hr under LD, 14 to 19 hr and 23-06 hr under LL and 17 hr under DD (Fig. 3A, B, C). Similar inverse relationships were depicted, also in the luminar compartment (gut lumen) of the digestive system. For example, the peaks in cellulose levels and troughs in cellulase activity levels appeared at 06 to 15 hr and 17 hr to 06 hr under LD, 06 to 14 hr and 19 hr to 06 hr under LL and 06 to 18 hr and 20 to 23 hr under DD. At the same time, peaks in enzyme and substrate levels ran counter to each other at 16 hr under LD, 15 to 18 hr under LL and at 19 hr and 04-06 hr under DD (Fig. 4A, B, C).

The study demonstrates that, the maintenance of microbial cellulose and cellulase rhythms are modulated by two different environmental cues. While, the cellulose synthesis is controlled in a light-dependent fashion, the cellulase synthesis and release go in a diet dependent way. As recorded in the present investigation (Fig. 3A), higher levels of cellulose were observed in the gut wall throughout the light regime of the day i.e. from 06 hr to 18 hr under LD. The appearance of higher mean peak value (MPV) of cellulose under LL (~32 mg) and lower MPV under DD (~26 mg) further confirms the role of light in bacterial cellulose synthesis. Surprisingly, as observed in our previous investigations, the reserve carbohydrates are synthesized in *B.mori* under dark regime (Bhuvaneswari and Sivaprasad, 2012 b; Bhuvaneswari and Sivaprasad, 2013), but in contrast to bacterial cellulose synthesis gets a boost under light condition. This difference between animal and bacterial synthesis of cellulose, provides additional proof for the exsistence of microbial cellulose in the gut wall of *B.mori* and its gradient runs counter to the cellulase activity sustained by the same microbial community. The cellulose probably ensures microbial adhesion to gut cells on one hand and protects them from hazardous effects

of ultraviolet radiation (Scott williams and Cannon, 1989). More importantly, as opined by Voet *et al*., (1998), it plays an additional role in the maintenance of osmotic gradient between the intra cellular environment of gut cells and extracellular environment of the gut lumen in the silkworm.

Thus, two cellulose gradients are established in the two compartments of the digestive system in *B.mori* and they modulate the cellulase activity in their respective locations, probably by stimulating its synthesis in gut wall and its release in the gut lumen. The prevalence of higher mean peak values of cellulose- vis-à-vis, cellulase activity during the light regime of the day in the gut wall and diet-dependent variations in the activity levels of cellulase in the gut content provides ample evidence for the existence of such a dynamic and mutual relationship between the circadian cellulose and cellulase rhythms in *B.mori*, that are governed by selective pressures from outside (Romling *et al*., 2000; Zogaj *et al*., 2001; Romling, 2002). In fact, it is not known as to how the circadian clock controls the microbial cellulose and cellulase rhythms within the digestive system of silkworm. Nonetheless, the elucidation of the central mechanism underlying such extraneous rhythms of microbial origin could emerge as a challenging area of research for chronobiologists.

REFERENCES

1. **Amikam, D. and Benziman, M. 1989.** Cyclic diguanylic acid and cellulose synthesis in *Agarobacterium tumefaciens. J. Bacteriol*., 171: 6649-6645.
2. **Anand, A. A. P., Vennison, S. J., Sankar, S. G., Prabhu, D. I. G., Vasan, P. T., Raghuraman,T., Geoffrey, C. J. and Vendan, S. E. 2010.** Isolation and characterization of bacteria from the gut of *Bombyx mori* that degrade cellulose, xylan, pectin and starch and their impact on digestion. *J. Insect. Sci*., 10:107.
   Aruga, H. 1994. Principles of Sericulture. Oxford. pp-97-99.
3. **Belda, E., Pedrola, L., Pereto, J., Martinez-Blanch, F., Montagud, A., Navarro, E., Urchueguia, J., Martinez-Blanch, F., Montagud, A., Navarro, E., Urchueguia, J., Ramon, D., Moya, A. and Porcar, M. 2011.** Microbial diversity in the midguts of field and lab-reared populations of the European corn borer *Ostrinia nubilalis*. *Plos one*. 6: e 21751.
4. **Bhuvaneswari, E. and Sivaprasad, S. 2012 a.** Impact of photoperiod on circadian protein and protease rhythms in the digestive system of silkworm, *Bombyx mori. The Bioscan*. 7: 175-183.
5. **Bhuvaneswari, E. and Sivaprasad, S. 2012 b.** Impact of photoperiod on circadian carbohydrate and amylase rhythms in the digestive system of silkworm, *Bombyx mori. The Bioscan*. 7: 579-588.
6. **Bhuvaneswari, E. and Sivaprasad, S. 2013.** Impact of photoperiod on circadian trehalose and trehalase rhythms in the digestive system of silkworm, *Bombyx mori. J. Appl & Nat. Science*. (In press).
7. **EI- Saied, H., EI- Diwany, A. I., Basta, A. H., Atwa, N. A. and EI- Ghwas, D. E. 2008.** Production and characterization of economical bacterial cellulose. *Bio resources*. 3: 1196-1217.
8. **Falcon, J. 1999.** Cellular circadian clocks in the pineal. *Progress in Neurobiology*. 58: 121-162.
9. **Fonagy, A. 2009.** Insect timing (rhythms) from the point of view of neuroendocrine effector mechanism. *Acta. Phytopathologica et. Entomologica Hungarica.,* 44: 61-73.
10. **Froy, O., Gotter, A. L., Casselman, A. L. and Reppert, S. M. 2003.** Illuminating the circadian clock in monarch butterfly migration. *Science.* 300: 1303-1305.
11. **Genta, F. A., Terra, W. R. and Ferreira, C. 2003.** Action pattern, specificity, lytic activities and physiological role of five digestive beta-glucanases isolated from *Periplaneta americana. Insect Biochem. Mol. Biol.,* 33: 1085-1097.
12. **Gringorten, J. L., Grawford, D. N. and Harvey, W. R. 1993.** High p^H in the ectoperitrophic space of the larval

lepidopteron midgut. *J. Exp. Biol.,* 183: 353-359.

13. **Hongoh, Y., Sharma, V. K., Prakash, T., Noda, S., Toh, H. and Taylor, T. D. 2008.** Genome of an endosymbiont coupling n-2 fixation to cellulolysis within protist cells in termite gut. *Science.* 322: 1108-1109.
14. **Iwai, S., Fukui, Y., Fujiwara,Y. and Takeda, M. 2006.** Structure and expressions of two circadian clock genes, period and timeless in the commercial silkmoth, *Bombyx mori. J. Insect physiol.,* 52: 625-637.
15. **Kandylis, K. Hadjigeorgiou, I. and Harizanis, P. 2009.** The nutritive value of mulberry leaves (*Morus alba*) as a feed supplement for sheep. *The Animal health production.* 41: 17-24.
16. **Kostal, V. and Shimada, K. 2001.** Malfunction of circadian clock in the non-photoperiodic- diapauses mutants of the Drosophilid fly, *Chymomyza costata*. *J. Insect physiol.,* 47: 1269-1274.
17. **Krishnaswami, S. 1986.** New technology of silkworm rearing. Central Sericulture and Training Institute, Mysore, India.
18. **Liu, J. R., Duan, C. H., Zhao, X., Tzen, J. T. C., Cheng, K. J. and Pai, C. K. 2008.** Cloning of a rumen fungal xylanase gene and purification of the recombinant enzyme via artificial oil bodies. *Appl. Microbiol. Biot.,* 79: 225-233.
19. **Martin, M. M. 1983.** Cellulose digestion in insects. *Comp. Biochem. Physiol.,* 75: 313-324.
20. **Miller, G. L., 1959.** Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analytical chemistry.* 31: 426-428.
21. **Nagaraju Mareedu and Sunitha Devi Gudamani. 2012.** Response of skeletal muscle protein and nucleic acid levels to thyroxine injection in fish**.** *Biolife,* 1(1):1-4
22. **Nakashima, K., Watanabe, H., Saitoh, H., Tokuda, G., Azuma, J. I. 2002.** Dual cellulose digesting system of the wood-feeding termite, *Coptotermes formosanus shiraki*. *Insect. Biochem. Mol. Biol.,* 32: 777-784.
23. **Pauchet, Y., Wilkinson, P., Chauhan, R., Ffrench- constant, R. H. 2010.** Diversity of Beetle genes encoding novel plant cell wall degrading enzymes. *Plos one.* 5: e 15635.
24. **Pytelkova, J., Hubert, J., Lepsik, M. Sobotnik, J. and Sendelyca, R. 2009.** Digestive alpha amylases of the flour math, *Ephestia kuehniella*- adaptation to alkaline environment and plant inhibitors. *FEBSJ.,* 276: 3531-3546.
25. **Romling, U., Rohde, A., Olsen, S., Normark, J., Reinkoster, A. 2000.** The checkpoint of multicellular and aggregative behaviour in *Salmonella typhimurium* regulates at least two independent pathways. *Mol. Microbiol.,* 36: 10-23.
26. **Romling, U. 2002.** Molecular biology of cellulose production in bacteria. *Research in microbiology,* 153: 205-212.
27. **Ross. P., Mayer, R. and Benziman, M. 1991.** Cellulose biosynthesis and function in bacteria. *Microbiol. Rev.,* 55: 35-58.
28. **Sailaja, B. and Sivaprasad, S. 2010 a.** Photoperiodic modulation of circadian rhythms in the silk gland protein profiles of *Bombyx mori* and its influence on the silk productivity and quality. *J. Appl & Nat. Science,* 2: 48-56.
29. **Sailaja, B. and Sivaprasad, S. 2010 b.** Photoperiodic modulation of circadian protein rhythms in the silk gland of *Bombyx mori* during fourth instar development. *The Bioscan*. 5: 177-183.
30. **Sailaja, B. and Sivaprasad, S. 2011.** Photoperiod-induced clock shifting in the circadian protein and amino acid rhythms in the larval fat body of silkworm, *Bombyx mori*. *J. Appl & Nat. Science.* 3: 38-50.
31. **Sailaja, B., Bhuvaneswari, E., Kavitha, S. and Sivaprasad, S. 2011.** Photoperiod-induced clock shifting in the circadian protein and amino acid rhythms in the larval haemolymph of the silkworm, *Bombyx mori*. *The Bioscan.* 6: 355-363.
32. **Satyanarayana, S., Zheng, X., Xio, R. and Seghal, A. 2004.** Post translational regulation of *Drosophila* PERIOD protein by protein phosphatase 2A. *Cell*. 116: 603-615.
33. **Saunders, D. S. 2002.** Insect clocks, third edition, Elsevier Science B.V.: Amsterdam. pp-560.

34. **Scott williams, W. and Cannon, R. E. 1989.** Alternative environmental roles for cellulose produced by *Acetobacter xylinum*. *Appl. Environ. Microbiol.,* 55: 2448-2452.
35. **Sehadova, H., Markova, E. P., Sehnal, F. and Takeda, M. 2004**. Distribution of circadian clock related proteins in the cephalic nervous system of the silkworm, *Bombyx mori*. *J. Biol. Rhythms.* 19: 466-482.
36. **Sharma,V. K. 2003.** Adaptive significance of circadian clocks. *Chronobiol. Int.,* 20: 901-919.
37. **Shi, W., Ding, S. Y. and Yuan, J. S. 2011.** Comparison of insect gut cellulase and xylanase activity across different insect species with distinct food sources. *Bioenerg. Res.,* 4: 1-10.
38. **Sivaprasad, S. and Sailaja, B. 2011.** Photoperiod-modulated instar-specific clock-shifting in the circadian protein and amino acid rhythms in the larval segmental muscle of *Bombyx mori*. *J. Appl. & Nat. Science.* 3: 176-188.
39. **Syrova, Z., Dolezel, D., Sauman, I., Hodokova, M. 2003.** Photoperiodic regulation of diapauses in linden bugs: Are period and clock genes are involved? *Cell. Mol. Lif. Sci.,* 60: 2510-2515.
40. **Thompson, S. N. 2003.** Trehalose- the insect blood sugar. *Advances in Insect Physiology*. 31: 206-261.
41. **Tokuda, G., Lo, N. and Watanabe, H. 2005.** Marked variations in patterns of cellulase activity against crystalline- vs carboxy methyl-cellulose in the digestive system of diverse, wood-feeding termites. *Physiological Entomology*. 30: 372-380.
42. **Updegroff, D. M. 1969.** Semimicro determination of cellulose in biological materials. *Anal. Biochem.,* 32: 420-424.
43. **Voet, D., Voet, J. G. and Pratt, C. W. 1988.** Carbohydrates. In Fundamental of Biochemistry: John Wiley and Sons, Linc. pp-204-207.
44. **Watanabe, H. and Tokuda, G. 2010.** Cellulolytic systems in insects. *Ann. Rev. Entamol.,* 55: 609-632.
45. **sYamaoka, K., Hoshino, M. and Hirai, T. 1971.** Role of sensory hairs on the anal papillae in oviposition behaviour of *Bombyx mori*. *J. Insect Physiol.,* 47: 2327-2336.
46. **Zogaj, X., Nimtz, M., Rohde, M., Bokranz, W. and Romling, U. 2001.** The multicellular morphotypes of *Salmonella typhimurium* and *Escherichia coli* produce cellulases as the second component of the extracellular matrix. Mol. *Microbiol.,* 39: 1452-1463.

DOI: https://dx.doi.org/10.5281/zenodo.7185189
Received: 10 April 2013;
Accepted; 27 May 2013;
Available online : 8 June 2013