



Impact of photoperiod on circadian sucrose and sucrose rhythms in the digestive system of silkworm, *Bombyx mori*.

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Received: April 29, 2013; Revised received: May 27, 2013; Accepted: June 10, 2013

Abstract: The impact of photoperiod on circadian sucrose and sucrose rhythms were analyzed in the digestive system of *Bombyx mori* under 12 hr light-dark cycle (LD), continuous light (LL) and continuous dark (DD). The rhythmic changes were interpreted as synthetic cycles in gut wall and release or uptake cycles in gut lumen. The gut wall comprised 6 sucrose synthetic cycles (SS cycles) under LD, LL and 5 under DD. The 24 hr rhythm of LD and LL was clock shifted to 28.8 hr under DD. In gut content, the sucrose rhythm showed 7 sucrose uptake cycles (SU cycles) under LD, 6 under LL and 5 under DD and the 24 hr rhythm of LD was clock shifted to 28.0 hr under LL and 34 hr under DD. In the gut wall sucrose rhythm maintained 7 SES cycles under LD and DD and 9 cycles under LL and its 24-hr rhythm is advanced to 18.2 hr. In the gut lumen 5 SER cycles under LD, 8 under LL and 6 under DD and its rhythm is advanced to 15 hr under LL and 20 hr under DD. Further analysis of data showed that LD favoured both synthesis and uptake of sucrose while LL, favoured the sucrose synthesis and its release.

Keywords: *Bombyx mori*, Circadian sucrose rhythm, Circadian sucrose rhythm, Gut, Photoperiod.

INTRODUCTION

Sucrose is a non-reducing disaccharide and the product of photosynthesis in all plants (Sumida and Ueda, 2007). It is the principal dietary sugar of the silkworm, *Bombyx mori*, as the mulberry leaf, the sole food of this insect, contains significant amount of sucrose; about 72 mg/ g dry weight (Kuribayashi *et al.*, 1990). The fresh mulberry leaves are the rich source of sucrose and its strength stimulates feeding in the silkworm larvae compared to other dietary carbohydrates such as pectin, xylan and starch (Sumida and Ueda, 2007). The dietary sucrose represents the chief energy source for metabolism in insects and its excess concentration contributes to the accumulation of glycogen and trehalose their tissues by inactivating glycogen phosphorylase (Thompson and Redak, 2000; Thompson *et al.*, 2001; Thompson *et al.*, 2002; Sumida and Ueda, 2007). Further, it functions as a cryoprotectant and membrane stabilizer and regulates the water flux across the gut wall (Thompson, 2003).

Sucrase is a carbohydrase that hydrolyses sucrose to glucose and fructose and it is widely distributed in the different regions of the digestive system (Kanekatsu *et al.*, 1992). In *Bombyx mori*, it occurs in three distinct forms with different molecular weights and pH ranges. Sucrase-I (MW: 31000; pH 6.5) is produced by the columnar midgut cells and has a large affinity to sucrose while, sucrase- II (MW: 66000; pH 6.5) and sucrase- III (MW: 96000; pH 6.6), are produced by the goblet cells, predominantly in the posterior part of the midgut.

Together with maltase, lactase and glucoamylase, sucrase- II forms a complex and hydrolyses different sugars (Kanekatsu *et al.*, 1992). Though, sucrases have been extensively studied with reference to their properties, characterization and developmental changes their rhythmic nature in the gut has not been established (Sumida *et al.*, 1990; Kanekatsu *et al.*, 1992; Sumida *et al.*, 1994). Further, the availability of large volume of data on circadian clock mechanism, circadian genes and their products in *Drosophila* and *Bombyx mori* (Shimizu *et al.*, 2001; Williams and Sehgal., 2001; Hall, 2003; Sharma, 2003; Satyanarayana *et al.*, 2004; Iwai *et al.*, 2006; Peschel *et al.*, 2009), suggest that many physiological and biochemical processes in insects are under the control of light-sensitive endogenous circadian clocks, that are fine tuned to work in a time schedule of 24 hr (Fonagy, 2009; Hirayama and Sessone- Corsi, 2009). More importantly, the recent reports on circadian physiology of *B. mori*, emanated from our laboratory (Sailaja and Sivaprasad, 2010 a, 2010 b; Sailaja and Sivaprasad, 2011; Sailaja *et al.*, 2011; Sivaprasad and Sailaja, 2011; Bhuvanewari and Sivaprasad, 2012a,b) provides impetus for similar investigations. The present study aims at analyzing circadian changes in the profiles of sucrose and sucrase in the digestive system of *B. mori* under altered photoperiodic conditions.

MATERIALS AND METHODS

The Pure Mysore x CSR₂ 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 fed 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 sucrose and sucrase activity of the silkworm gut was analyzed for a 25 hr period, spanning in between day 5 and day 6 of fifth instar larval development. The gut wall tissue was isolated every hour, by mid dorsal dissection of silkworm larvae in ice cold B. *mori* 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 juice so collected, was kept in a test tube under ice cold conditions till the mulberry leaf pieces were settled at the bottom and later, the supernatant was decanted and used for the assay. Hour- to- hour changes in the levels of sucrose were estimated by the method of Plumer (1978) in 5% homogenate of the gut wall tissue and 1:19 diluted gut content (digestive fluid) in distilled water, using Anthrone reagent. The sucrose levels, computed by 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 sucrase activity was estimated by the method of Ishaaya Swirski (1970) in 5% homogenate of the gut wall and 1: 9 diluted digestive juice in 0.05M acetate buffer using DNS (Dinitro-salicylic acid) reagent. The enzyme activity was computed using glucose as standard and expressed in μ moles of glucose/ mg sucrose/ 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 sucrose and sucrase rhythms of the gut wall and gut content under three photoperiodic conditions LD, LL and DD were projected in phase response curves (PRCs) and presented in figs.1- 4. The PRCs were analyzed in terms the number of peaks (elevated points) and troughs (low points) and intervals between peaks and troughs and the relevant details are shown in Tables 1- 6.

Circadian sucrose rhythms

Gut wall: Under LD, the sucrose rhythm of the gut wall showed 6 peaks and 7 troughs during the 24 hr free running period of the rhythm (Fig.1A). The first peak occurred early at 07 hr with a sucrose value ~15 mg/g wet wt. of tissue, while the subsequent peaks occurred at 09 hr (~13 mg), 14 hr (~23 mg), 18 hr (~19 mg), 20-23 hr (~27 mg) and next day again at 02 hr (~24 mg). Troughs occurred at 06 hr (~11 mg), 08 hr (~11 mg), 10 hr (~09 mg), 16 hr (~18 mg), 19 hr (~15 mg), 00 hr (~15 mg) and next day at 04-05 hr (~18 mg). Under LL, the sucrose rhythm showed 6 peaks and 7 troughs during the 24 hr free running period. While the peaks occurred at 07 hr (~19 mg), 09-10 hr (~16 mg), 14 hr (~16 mg), 18 hr (~26 mg), 23 hr (~22 mg) and next day at 02 hr (~18 mg), the troughs occurred at 06 hr (~14 mg), 08 hr (~10 mg), 11-13 hr (~11 mg), 15 hr (~11 mg), 19 hr (~14 mg) and next day at 01 hr (~10 mg) and at 06 hr (~12 mg). Under DD, the rhythm showed 5 peaks and 6 troughs during the 24 hr free running period. While the peaks occurred at 07-11 hr (~14 mg), 15 hr (~24 mg), 21 hr (~34 mg) and next day at 03 hr (~13 mg) and at 05 hr (~18 mg), troughs occurred at 06 hr (~9 mg), 12-13 hr (~10 mg), 17 hr (~17 mg) and next day at 01 hr (~10 mg), 04 hr (~10 mg) and at 06 hr (~15 mg). At the same, the intervals between peaks and troughs varied from one photoperiodic condition to the other. The interval between peaks was about 2.7 hr under LD, 3.0 hr under LL and 3.6 hr under DD and that between troughs was 3.1 hr under LD and LL and 3.8 hr under DD. The combined mean interval of peaks and troughs was roughly about 2.9 hr under LD, 3.1 hr under LL and 3.7 hr under DD (Tables 1A, B and 5).

Gut Content: Under LD the sucrose rhythm of the gut content showed 7 peaks and 7 troughs during the 24 hr free running period of the rhythm (Fig. 1B). The peaks appeared at 07 hr (~37 mg), 10 hr (~25 mg), 13 hr (~45mg), 15 hr (~41 mg), 17 hr (~48 mg), 20 hr (~88 mg) and next day at 02 hr (~55 mg), while the troughs were recorded at 06 hr (~13 mg), 08 hr (~13 mg), 12 hr (~17 mg) 14 hr (26 mg), 16 hr (~37mg), 19 hr (~37 mg) and next day at 03 hr (~33 mg). Under LL, the sucrose rhythm showed 6 peaks and 6 troughs during the 24 hr free running period. The peaks occurred at 07 hr (~39 mg), 10 hr (26 mg), 13 hr (~48 mg), 21 hr (~65 mg), 23 hr (~46 mg) and next day at 02 hr (~34 mg), while the troughs appeared at 09 hr (~23 mg), 11 hr (~16 mg), 17 hr (~31 mg), 22 hr (~34 mg) and next day at 01 hr (~20 mg) and 06 hr (~22 mg). Under DD, the sucrose rhythm showed 5 peaks and 6 troughs during the 24 hr free running time. Peaks appeared at 07-08 hr (~33 mg), 10 hr (~26 mg), 13 hr (45 mg), 17 hr (~56 mg) and at 20 hr (~78 mg), and the troughs were recorded at 06 hr (~11 mg), 09 hr (~18 mg), 11 hr (~18 mg), 14 hr (~36 mg), 18 hr (~37 mg) and next day at 03 hr (~21 mg). The interval between peaks was about 2.7 hr under LD, 3.2 hr under

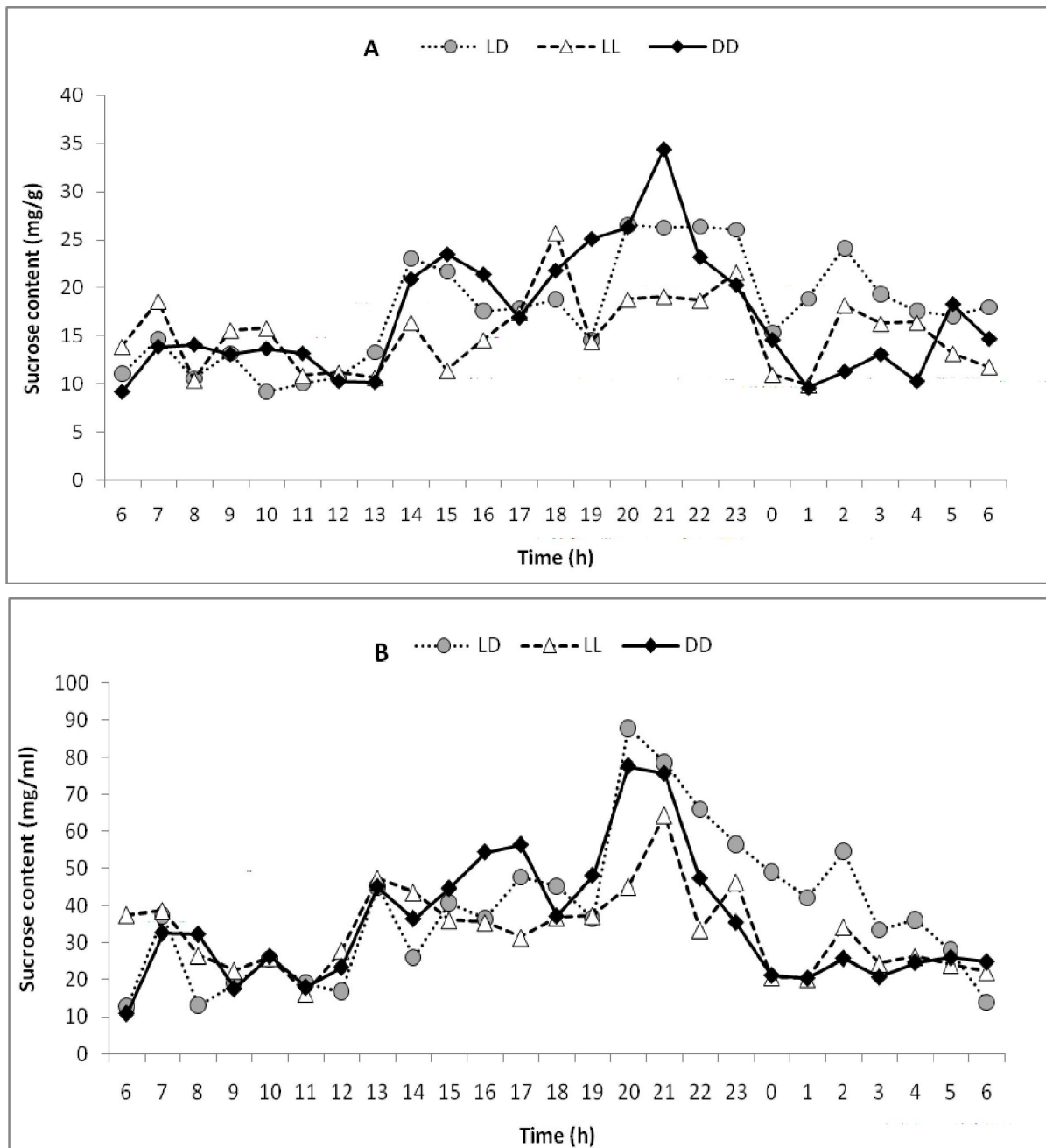


Fig. 1. Phase response curves (PRCs) of the 24 hr circadian sucrose rhythms in the gut wall (A) and gut content (B) of fifth instar larva of *Bombyx mori*, under 12 hr light: 12 hr dark cycle (LD), continuous light (LL) and continuous dark (DD) conditions. The values expressed in mg glucose/g wet weight 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).

LL and 2.4 hr under DD and that between troughs was about 3.0 hr under LD and 3.5 hr under LL and DD. The combined mean interval of peaks and troughs was about 2.9 hr under LD, 3.4 hr under LL and 3.0 hr under DD (Tables 2A, B and 5).

Circadian sucrose rhythms

Gut wall: Under LD the rhythm of sucrose 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 (1.03 μ moles), 15 hr (1.18 μ moles), 17 hr (2.26 μ moles), 21 hr (0.96 μ moles), 00 hr (1.29 μ moles)

and next day at 02 hr (1.08 μ moles) and 06 hr (0.98 μ moles), and troughs appeared at 06 hr (0.82 μ moles), 13 hr (0.37 μ moles), 16 hr (0.81 μ moles), 19 hr (0.71 μ moles), 23 hr (0.49 μ moles) and next day at 01 hr (0.88 μ moles) and 04 hr (0.38 μ moles). Under LL the rhythm showed 9 peaks and 8 troughs during the 24 hr free running time. The peaks appeared at 06 hr (1.06 μ moles), 10 hr (1.03 μ moles), 14 hr (1.96 μ moles), 18 hr (1.05 μ moles), 20 hr (0.95 μ moles), 00 hr (2.54 μ moles) and next day at 02 hr (1.31 μ moles), 04 hr (1.0 μ moles) and 06 hr (0.95 μ moles), while the troughs appeared at 09 hr (0.65 μ mole), 13 hr

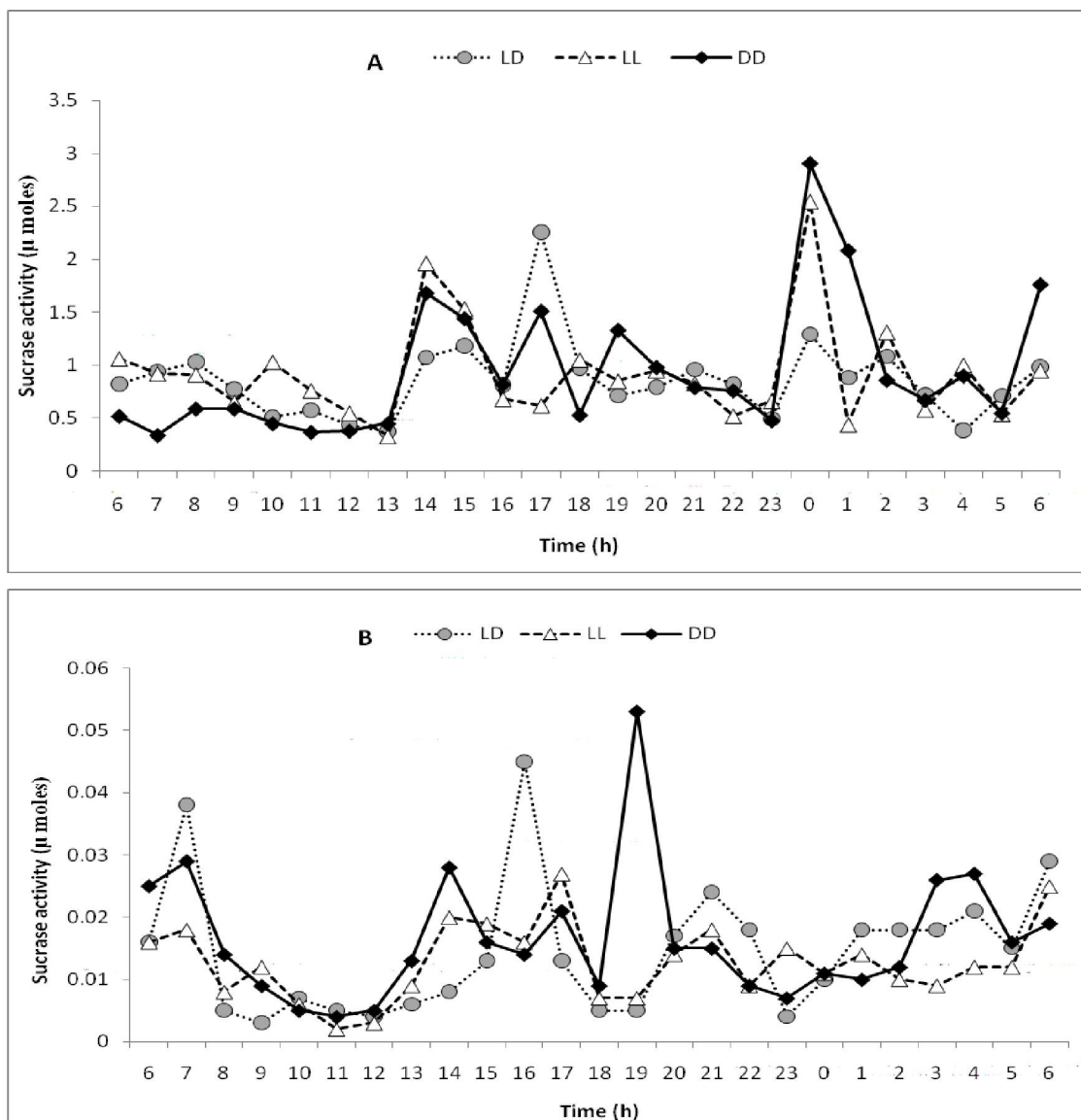


Fig. 2. Phase response curves (PRCs) of the 24 hr circadian sucrose rhythms in the gut wall (A) and gut content (B) of fifth instar larva of *Bombyx mori*, under 12 hr light: 12 hr dark cycle (LD), continuous light (LL) and continuous dark (DD) condition. The values expressed in μ moles of glucose/mg sucrose/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).

(0.33 μ moles), 16-17 hr (~ 0.68 μ moles), 19 hr (0.85 μ moles), 22 hr (0.52 μ moles), and next day at 01 hr (0.44 μ moles), 03 hr (0.58 μ moles) and at 05 hr (0.54 μ moles). Under DD, the rhythm showed 7 peaks and 6 troughs during the 24 hr free running period. The peaks appeared at 06 hr (0.52 μ moles), 08-09 hr (0.59 μ moles), 14 hr (1.68 μ moles), 17 hr (1.51 μ moles), 19 hr (1.33 μ moles), 00 hr (2.90 μ moles) and next day at 06 hr (1.76 μ moles), the troughs appeared at 07 hr (0.34 μ moles), 11 hr (0.37 μ moles), 16 hr (0.82 μ moles), 18 hr (0.53 μ moles), 23 hr (0.48 μ moles) and next day at 05 hr (0.55 μ moles). The interval between peaks was about 3.1 hr under LD, 2.7 hr under LL and 3.3 hr under DD and that between troughs was about 3.1 hr under LD, 2.4 hr under LL and 3.7 hr under DD. The combined mean interval of peaks and troughs was about 3.1 hr under LD, 2.6 hr under LL and

3.5 hr under DD (Tables 3A, B and 6).

Gut content: Under LD, the sucrose activity showed 5 peaks and 5 troughs in the gut content during the 24 hr free running period of the rhythm (Fig. 2B). Peaks appeared at 07 hr (0.038 μ moles), 16 hr (0.045 μ moles), 21 hr (0.024 μ moles) and next day at 04 hr (0.021 μ moles) and 06 hr (0.029 μ moles) and troughs appeared at 06 hr (0.016 μ moles), 09 hr (0.003 μ moles), 18-19 hr (0.005 μ moles), 23 hr (0.004 μ moles) and next day at 05 hr (0.015 μ moles). Under LL the sucrose activity showed 8 peaks and 8 troughs in the digestive juice during the 24 hr free running period of rhythm. Peaks appeared at 07 hr (0.018 μ moles), 09 hr (0.012 μ moles), 14 hr (0.020 μ moles), 17 hr (0.027 μ moles), 21 hr (0.018 μ moles), 23 hr (0.015 μ moles), and next day at 01 hr (0.014 μ moles) and 06 hr (0.025 μ moles) and the troughs occurred at 06 hr (0.016 μ moles), 08 hr

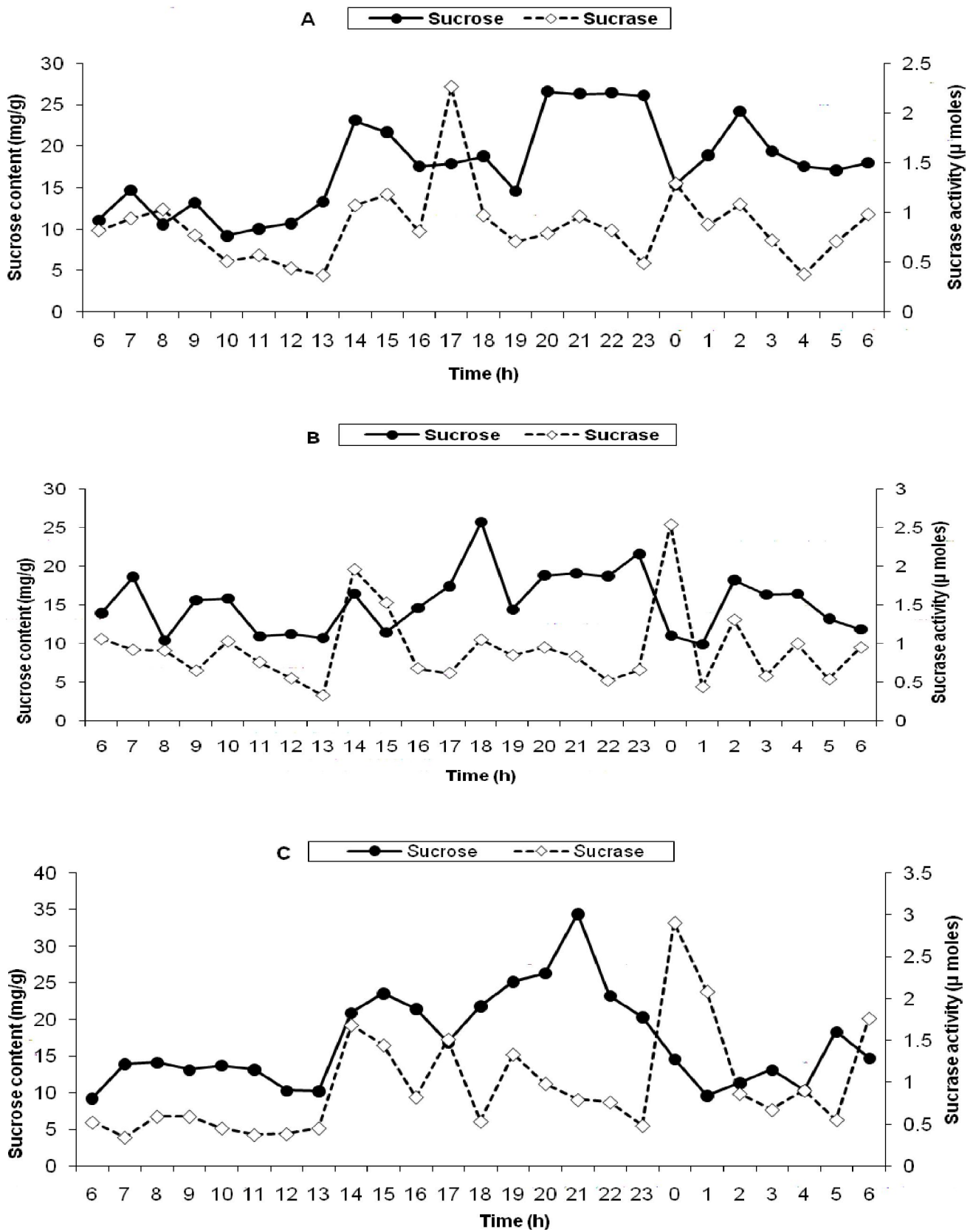


Fig. 3. Circadian changes in sucrose profiles and sucrase activity in the gut wall of the fifth instar larva of *Bombyx mori*, under (A) 12 hr light: 12 hr dark cycle (LD), (B) continuous light (LL) and (C) continuous dark (DD) conditions. The values expressed in mg glucose per gm wet weight of tissue in case of sucrose and μ moles of glucose formed/ mg sucrose/ hr in case of sucrase, 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).

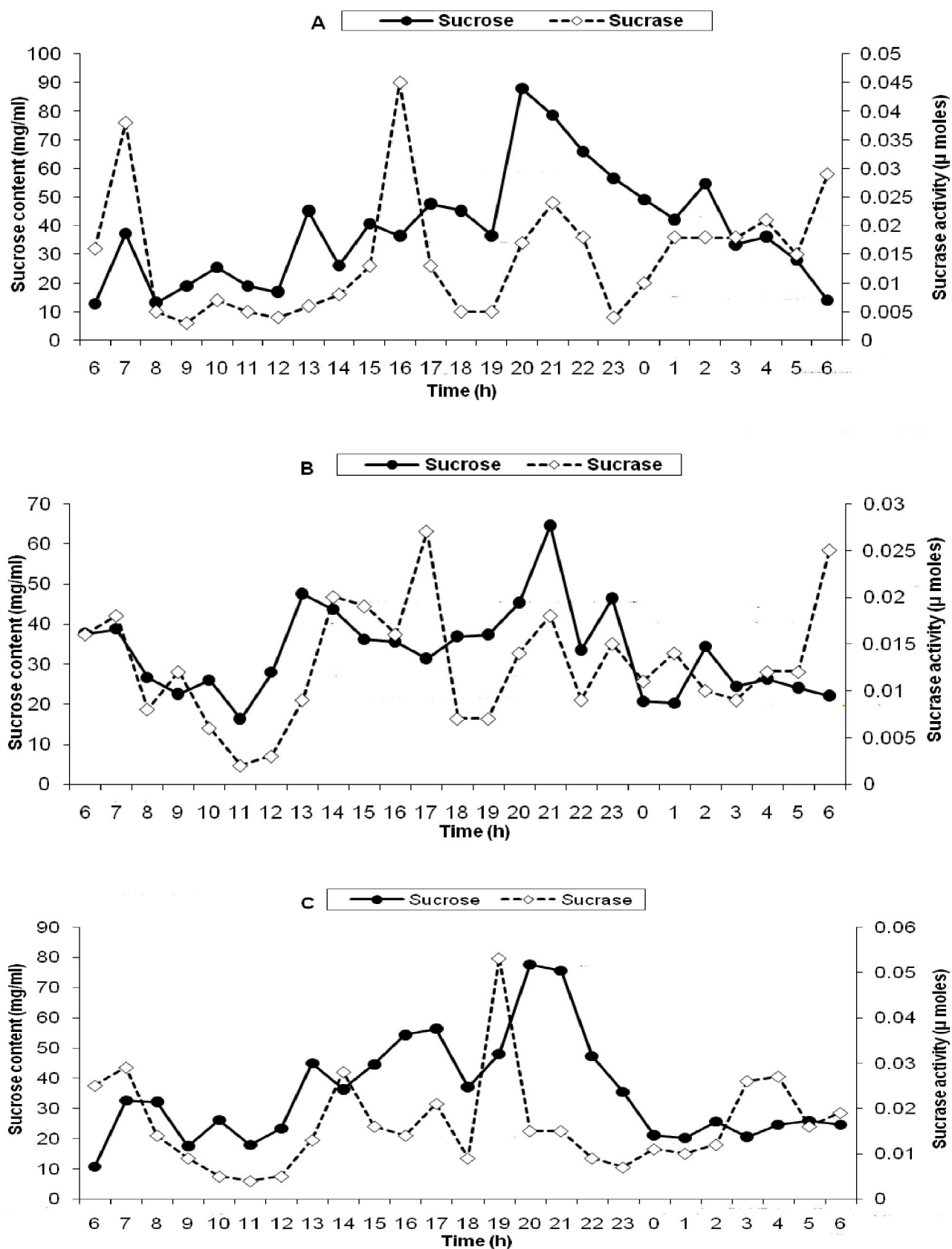


Fig. 4. Circadian changes in sucrose profiles and sucrase activity in the gut content of the fifth instar larva of *Bombyx mori*, under (A) 12 hr light: 12 hr dark cycle (LD), (B) continuous light (LL) and (C) continuous dark (DD) conditions. The values expressed in mg glucose per ml of tissue in case of sucrose and μ moles of glucose formed/mg sucrose/hr in case of sucrase, 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).

Table 1 (A and B). Interval between peaks (A) and troughs (B) in the levels of sucrose in the gut wall of the fifth instar larva of *B. mori* during the free running time of the circadian rhythm under 12 hr light/ dark cycle (LD), continuous light (LL) and continuous dark (DD) conditions.

Photo period	No. of peaks	Interval between peaks in hours					Mean interval in hours
		1-2	2-3	3-4	4-5	5-6	
LD	6	2	5	4	2	3	2.7
LL	6	2	4	4	5	3	3.0
DD	5	4	6	6	2	-	3.6

(A)

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	
LD	7	2	2	6	3	5	4	3.1
LL	7	2	3	2	4	6	5	3.1
DD	6	6	4	8	3	2	-	3.8

(B)

Source: Fig. 1 A.

Table 2 (A and B). Interval between peaks (A) and troughs (B) in the levels of sucrose in the gut content of the fifth instar larva of *B. mori* during the free running time of the circadian rhythm under 12 hr light/ dark cycle (LD), continuous light (LL) and continuous dark (DD) conditions.

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	
LD	7	3	3	2	2	3	6	2.7
LL	6	3	3	8	2	3	-	3.2
DD	5	2	3	4	3	-	-	2.4

(A)

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	
LD	7	2	4	2	2	3	8	3.0
LL	6	2	6	5	3	5	-	3.5
DD	6	3	2	3	4	9	-	3.5

(B)

Source: Fig. 1 B.

(0.008 μ moles), 11 hr (0.002 μ moles), 16 hr (0.016 μ moles), 18-19 hr (0.007 μ moles), 22 hr (0.009 μ moles), 00 hr (0.011 μ moles), and next day at 03 hr (0.009 μ moles). Under DD the enzyme activity rhythm showed 6 peaks and 6 troughs in the gut content during the 24 hr free running period. The peaks appeared at 07 hr (0.029 μ moles), 14 hr (0.028 μ moles), 17 hr (0.021 μ moles), 19 hr (0.053 μ moles) and next day at 04 hr (0.027 μ moles) and 06 hr (0.019 μ moles), while the troughs occurred at 06 hr (0.025 μ moles), 11 hr (0.004 μ moles), 16 hr (0.014 μ moles), 18 hr (0.009 μ moles), 23 hr (0.007 μ moles), and next day at 05 hr (0.016 μ moles). The interval between peaks was about 4.6 hr under LD, 2.9 hr under LL and 3.8 hr under DD and that between troughs was about 4.2 hr under LD, 2.5 hr under LL and 3.8 hr under DD. The combined mean interval between peaks and troughs was about 4.4 hr under LD, 2.7 hr under LL and 3.8 hr under DD (Tables 4A, B and 6).

DISCUSSION

In silkworm, the gut acts as an organ of synthesis, storage, secretion and absorption of dietary nutritive materials

including sucrose. This disaccharide is digested by the group of sucrases produced in the glandular epithelium of the midgut and released into the gut lumen and the end products of digestion (glucose and fructose) are absorbed back into the midgut cells. The glucose moieties so absorbed, are converted to storage carbohydrates, namely, glycogen and trehalose (Thompson and Redak, 2000; Thompson *et al.*, 2001; Thompson *et al.*, 2002). The present study on the circadian profiles of sucrose and sucrase activity confirms that their levels are modulated in a circadian pattern under the impact of light signals, similar to those in other biochemical constituents like proteins, free amino acids, carbohydrates, amylase, trehalase, trehalase and protease in different tissues of *B. mori* (Sailaja and Sivaprasad, 2010 a,b; Sailaja and Sivaprasad, 2011; Sailaja *et al.*, 2011; Sivaprasad and Sailaja, 2011; Bhuvaneshwari and Sivaprasad, 2012a,b). The circadian sucrose and sucrase data, presented as peaks and troughs in PRCs (Figs.1- 4 and Tables 1-6) were analyzed in terms of the number of sucrose synthesis cycles (SS cycles) in the gut wall and number

Table 3 (A and B). Interval between peaks (A) and troughs (B) in the activity levels of sucrose in the gut wall of the fifth instar larva of *B. mori* during the free running time of the circadian rhythm under 12 hr light/ dark cycle (LD), continuous light (LL) and continuous dark (DD) conditions.

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	
LD	7	7	2	4	3	2	4	-	-	3.1
LL	9	4	4	4	2	4	2	2	2	2.7
DD	7	2	5	3	2	5	6	-	-	3.3

(A)

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	
LD	7	7	3	3	4	2	3	-	-	3.1
LL	8	4	3	2	3	3	2	2	2	2.4
DD	6	4	5	2	5	6	-	-	-	3.7

(B)

Source: Fig. 2 A.

Table 4 (A and B). Interval between peaks (A) and troughs (B) in the activity levels of sucrose in the gut content of the fifth instar larva of *B. mori* during the free running time of the circadian rhythm under 12 hr light/ dark cycle (LD), continuous light (LL) and continuous dark (DD) conditions.

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	
LD	5	9	5	7	2	-	-	-	-	4.6
LL	8	2	5	3	4	2	2	5	5	2.9
DD	6	7	3	2	9	2	-	-	-	3.8

(A)

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	
LD	5	3	9	4	6	-	-	-	-	4.2
LL	8	2	3	5	2	3	2	3	3	2.5
DD	6	5	5	2	5	6	-	-	-	3.8

(B)

Source: Fig. 2 B.

of sucrose uptake cycles (SU cycles) in the gut content. Similarly, the number of peaks in sucrose activity was interpreted as sucrose enzyme synthetic cycles (SEScycles) in the gut wall and sucrose release cycles (SER cycles) in the gut content. In both the compartments of the 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 (average of all peaks) in terms of the average levels of sucrose or sucrose activity maintained during the 24 hr free running time of the rhythm.

Circadian sucrose rhythm

Gut wall: The sucrose maintains a glucose gradient between the two compartments (gut wall and gut lumen) of digestive system by appropriately altering its synthesis and release (Thompson, 2003). Accordingly, the sucrose rhythm maintains 6 SS cycles under LD and LL with duration of 4.0 hr each and 5 cycles under DD each with duration of 4.8 hr. Thus, DD condition modulates sucrose synthesis by extending the duration of each cycle by 48 min (from 4.0 hr to 4.8 hr). Due to

extension of the duration of all SS cycles in a day, the normal 24 hr free running time of sucrose rhythm under LD and LL is clock-shifted to 28.8 hr under DD (Table 5). Further analysis of the intensity of peaks; in terms of the height indicates that sucrose synthesis is high during scotopic phase compared to photic phase of the day. Evidently, the active synthetic phases occurred thrice (at 14-15 hr, 20-23 hr and 02 hr) under LD, twice each under LL (at 18 hr and 23 hr) and DD (at 14-16 hr and 21 hr). The photoperiod not only altered the number of peaks (Iwai *et al.*, 2006) but also the mean peak value (MPV) of the sucrose during the free running time of the rhythm. Significantly, MPVs maintained higher levels under both LD and DD (~20.6 mg) and moderate levels under LL (19.4 mg). Our findings confirm that dark cues modulate the circadian rhythms by appropriately altering the levels of different biochemical constituents in *B. mori* and other insects (Fonagy, 2009; Weng *et al.*, 2009; Bhuvaneshwari and Sivaprasad, 2012a,b).

Gut content: The mulberry leaf is the rich source of sucrose for the silkworm, *B. mori*, in its gut. It senses the

Table 5. Comparative analysis of the phase response curves of the sucrose rhythm in the gut wall and gut content of the fifth instar larva of *Bombyx mori*, under 12hr light / dark cycle (LD), continuous light (LL) and continuous dark (DD) conditions.

Parameter	Gut wall			Gut content		
	LD	LL	DD	LD	LL	DD
No. of peaks	6	6	5	7	6	5
No. of troughs	7	7	6	7	6	6
Mean interval b/w peaks (hr)	2.7	3.0	3.6	2.7	3.2	2.4
Mean interval b/w troughs (hr)	3.1	3.1	3.8	3.0	3.5	3.5
Combined mean interval b/w peaks and troughs (hr)	2.9	3.1	3.7	2.9	3.4	3.0
Probable no. of SS/SU cycles	6	6	5	7	6	5
Approximate time taken for each SS/SU cycles (hr)	4.0 (24/6=4)	4.0 (24/6=4)	4.8 (24/5=4.8)	3.4 (24/7=3.4)	4.0 (24/6=4)	4.8 (24/5=4.8)
Free running time of rhythm (hr)	24 (4x6=24)	24 (4x6=24)	28.8 (4.8x6=28.8)	24 (3.4x7=24)	28 (4x7=28)	34 (4.8x7=34)
Mean peak value	20.1	19.4	20.6	48.3	42.9	47.5

Source: Fig. 1A and B; SS cycles: Sucrose synthetic cycles; SU cycles: Sucrose uptake cycles

Table 6. Comparative analysis of the phase response curves of the sucrose rhythm in the gut wall and gut content of the fifth instar larva of *Bombyx mori*, under 12hr light / dark cycle (LD), continuous light (LL) and continuous dark (DD) conditions.

Parameter	Gut wall			Gut content		
	LD	LL	DD	LD	LL	DD
No. of Peaks	7	9	7	5	8	6
No. of Troughs	7	8	6	5	8	6
Mean interval b/w Peaks (hr)	3.1	2.7	3.3	4.6	2.9	3.8
Mean interval b/w Troughs (hr)	3.1	2.4	3.7	4.2	2.5	3.8
Combined mean interval b/w Peaks and troughs (hr)	3.1	2.6	3.5	4.4	2.7	3.8
Probable no. of SES/SER cycles	7	9	7	5	8	6
Approximate time taken for each SES/SER cycles (hr)	3.4 (24/7=3.4)	2.6 (24/9=2.6)	3.4 (24/7=3.4)	4.8 (24/5=4.8)	3.0 (24/8=3)	4.0 (24/6=4)
Free running time of Rhythm (hr)	24 (3.4x7=24)	18.2 (2.6x7=18.2)	24 (3.4x7=24)	24 (4.8x5=24)	15 (3x5=15)	20 (4x5=20)
Mean Peak value	1.25	1.32	1.47	0.030	0.019	0.030

Source: Fig. 2A and B; SES cycles: Sucrase enzyme synthetic cycles; SER cycles: Sucrase enzyme release cycles

environmental cues and stimulates the release of sucraes from the gut wall. The sucraes so released digest the sucrose and contributes to the formation of trehalose, the insect blood sugar (Sumida and Ueda, 2007). The uptake of sucrose, in this fashion, occurs at regular

intervals, referred to as sucrose uptake cycles (SU cycles) in the present report. In the gut content sucrose rhythm maintained 7 SU cycles with a duration of 3.4 hr each under LD, 6 SU cycles under LL with a duration of 4.0 hr each and 5 SU cycles under DD with a duration of 4.8 hr

each. Thus, the 24 hr normal rhythm of LD condition was significantly delayed both under LL and DD conditions. The former extended the duration of each cycle by 36 min (from 3.4 to 4.0 hr) and the latter by 1.4 hr (from 3.4 to 4.8 hr). Consequently, the 24 hr sucrose rhythm under LD, was rescheduled to 28 hr under LL and 34 hr under DD (Table 5). The analysis of the data in terms of the height of peaks indicates that maximum accumulation of sucrose in the gut content is linked to the availability of dark cues. Probably, because of this reason the higher MPVs were recorded under both LD and DD (~ 48 mg) conditions compared to those under LL (42.9 mg). Similar to that of the gut wall cells the gut content also requires the dark cues to uptake sucrose from the gut lumen. This is substantiated by greater accumulation of sucrose in the gut content at 20-21 hr that corresponds to dark hours under the photoperiodic conditions examined (Fig. 1B).

Circadian sucrose rhythm

Gut wall: The glandular epithelium of the midgut wall is the major source of sucrase which digests the dietary sucrose and this process is accompanied by the transfer of monosaccharide moieties from sucrose through a transglucosidation process necessary for the growth and development of silkworm larvae and this enzyme is synthesized and released in response to the stimulant action exerted by the concentration of sucrose in the gut lumen (Kanekatsu *et al.*, 1993; Sumida and Ueda, 2007; Barman and Rajan, 2010). In *B. mori*, the larval gut wall maintained 7 SES cycles under both under LD and DD conditions with a duration of 3.4 hr each and 9 cycles under LL with a duration of 2.6 hr each (Fig. 2A). Thus, LL condition modulates the enzyme synthetic rhythm by reducing the time required for each synthetic cycle by 48 min (from 3.4 to 2.6 hr). Due to shortening of the duration of SES cycles the 24 hr free running time of the rhythm under LD is clock shifted to 18.2 hr under LL but maintained at the same level of 24 hr under DD. The photoperiod not only altered the number of SES cycles, but also the intensity of the enzyme synthesis and its activity levels throughout the free running time of the rhythm. For instance, the active timing of sucrase synthesis as reflected in the height of the peaks, occurred thrice under DD (14-19 hr, 00-01 hr and 06 hr), twice under LL (at 14-15 hr and 00 hr) and once under LD (at 17 hr). Likewise, higher enzyme activity, as reflected in its MPVs, was sustained under DD (1.47 μ moles), while moderate activity was maintained under LL (1.32 μ moles) and low activity under LD (1.25 μ moles) (Table 6). Evidently, the dark condition stimulates the sucrase synthesis in the gut wall cells much like that of amylase synthesis in the digestive system of silkworm (Bhuvaneshwari and Sivaprasad, 2012 b).

Gut content: Sucrase activity remains higher in the gut lumen due to the stimulation and activation brought

about by the concentration of sucrose derived from the ingested food (Sumida *et al.*, 1990). The deficiency of this enzyme in gut content disturbs the sugar metabolism leading to slow growth rate, small body size, under developed silk gland and small cocoon size in *B. mori* (Sashindran Nair *et al.*, 2004; Sumida and Ueda, 2007; Narayanaswamy and Shankar, 2010). The sucrase rhythm in the gut content, represented as sucrase enzyme release cycles (SER cycles) showed 5 cycles under LD, with a duration of 4.8 hr each, 8 cycles under LL with a duration of 3.0 hr each and 6 cycles under DD with a duration of 4.0 hr each. Significantly, the duration of each SER cycle was reduced by 1.8 hr (from 4.8 to 3.0 hr) under LL and by 48 min (from 4.8 to 4.0 hr) under DD. Consequently, the 24 hr sucrase rhythm under LD, has been rescheduled to operate at 15 hr under LL and 20 hr under DD (Table 6). Within the free running time of the rhythm, sucrase recorded active levels in 4 phases under all the three photoperiodic conditions, most of them coinciding with photopic phase of the day. Under LD, SER cycles occurred at 07 hr, 16 hr, 21 hr and 06 hr, while they occurred at 06-07 hr, 14-17 hr, 21-01 hr and 06 hr under LL and at 06-07 hr, 14 hr, 19 hr and 03-04 hr under DD. Surprisingly, the prevalence of higher MPVs under LD and DD (0.030 μ moles) and moderate MPVs under LL (0.019 μ moles), further substantiated the fact that dark cues ensure sustained sucrase activity, throughout the free running time of the rhythm despite the fact that light cues boost it up at regular intervals.

Sucrose rhythm versus sucrase rhythm: A comparative analysis of PRCs of circadian sucrose and sucrase rhythms indicate inverse relationship between them during the free running period. This is true in both the compartments of digestive system (gut wall and gut content) under three photoperiodic conditions (LD, LL and DD) examined (Figs. 3 and 4). In this relationship, the peaks (higher levels) in one biochemical parameter were accompanied by troughs (lower levels) in the other. For instance, under LD, the peaks in sucrose levels of gut wall at 07-09 hr, 11-15 hr, 20-02 hr, 02-03 hr and 04-06 hr were coincided with troughs in sucrase activity. At the same time, the peaks in enzyme activity levels at 08 hr, 07 hr and at 00 hr were accompanied by troughs in its substrate levels (Fig. 3A). Similarly, under LL condition, the peaks in sucrase activity at 07 hr, 09 hr, 13 hr, 17-23 hr and 02-05 were by and large coincided with the timing of troughs in sucrose levels. At the same time, the peaks in the enzyme activity at 08 hr, 14-15 hr and 00 hr matched with corresponding troughs in its substrate concentration (Fig. 3B). Under DD, the elevated points in sucrose levels at 07-11 hr, 15-16 hr, 18-23 hr, 03 hr and 05 hr, were coincided with lower points in the sucrase activity (Fig. 3C).

The enzyme (sucrase) and the substrate (sucrose)

relationship continued in more or less similar fashion in the gut lumen, with + or - 1 hr, during the free running time of the respective rhythms. For example, under LD, the peaks in sucrose levels at 07 hr, 10 hr, 13 hr, 17 hr, 20-21 hr and at 02 hr were accompanied by troughs in sucrose activity at 08 hr, 11 hr, 13 hr, 18 hr, 22-23 hr and at 02-03 hr. Conversely, the peaks in enzyme activity at 07 hr, 10 hr, 13 hr, 16 hr, 22 hr, 01-04 hr and 06 hr were accompanied by troughs in its substrate levels at 08 hr, 11-12 hr, 14 hr, 16 hr, 23-01 hr, 03-04 hr and 06 hr respectively. Similarly under LL, the peaks in sucrose levels at 07 hr, 10 hr, 13 hr, 21 hr, 23 hr and 02 hr were accompanied by troughs in enzyme activity at 08 hr, 11 hr, 15-16 hr, 22 hr, 00 hr and 02-03 hr respectively. Under the same light condition, the peaks in sucrose activity at 07 hr, 09 hr, 14 hr, 17 hr, 21 hr, 23 hr, 01 hr, 04 hr and 06 hr were accompanied by troughs in its substrate levels at 08 hr, 09 hr, 15-16 hr, 17 hr, 22 hr, 00-01 hr, 04 hr and 06 hr respectively (Fig. 4B). Likewise, under DD, the peaks in sucrose levels at 07-08 hr, 10 hr, 13 hr, 16-17 hr, 20-21 hr, 02 hr, 04-06 hr were accompanied by troughs in sucrose levels at 08-09 hr, 10-11 hr, 15-16 hr, 18 hr, 22-23 hr and at 05 hr respectively. At the same time, the peaks in sucrose levels at 07 hr, 14 hr, 17 hr, 19 hr, 03-04 hr and 06 hr were accompanied by troughs in its substrate (sucrose) levels at 09 hr, 19 hr, 22-01 hr, 03 hr and 06 hr respectively.

In-depth analysis of free running time of sucrose and sucrose rhythms showed a time dependent and light-sensitive rhythmic changes in their levels, much like carbohydrate and amylase rhythms reported in our previous investigations (Bhuvaneshwari and Sivaprasad, 2012 b). Such changes were more pronounced in the cellular compartment (gut wall) of the gut than in its luminal compartment (gut lumen). Interestingly in the present study, higher sucrose activity levels and lower sucrose levels were observed during the photic phase, while lower sucrose levels and higher sucrose levels were recorded during scotic phase of the day (Fig 3. ABC). For instance, the sucrose rhythm maintained higher MPVs (20.6 mg) throughout its free running time in total darkness and lower MPVs (19.4 mg) under continuous light condition. Further, higher sucrose activity levels and lower sucrose levels were observed at 06-17 hr under LD, 06-15 hr under LL and 01-06 hr under DD. Similarly, lower sucrose activity levels and higher sucrose levels were observed at 19-06 hr under LD, 16-06 hr under LL and 18-22 hr under DD. Obviously, in *B. mori* dark condition stimulates sucrose synthesis and its accumulation in gut wall cells, while the light condition enhances its breakdown and mobilization of its products as observed in some other insects (Kostal and Shimada, 2001; Saunders, 2002; Syrova *et al.*, 2003).

In the gut content, on the other hand, the sucrose and sucrose rhythms are governed by the sucrose content

present in the dietary mulberry leaf, which in turn reinforces its own levels by simulating release of sucrose from the gut wall cells (Kanekatsu *et al.*, 1992; Sumida and Ueda, 2007; Manjula *et al.*, 2010). Thus, the sucrose levels in the gut content represent those derived two sources; mulberry leaf and gut wall. The sucrose activity in the gut lumen is modulated in a light- and dose-dependent fashion like that of amylase activity (Bhuvaneshwari and Sivaprasad, 2012 b). For example, its activity continued throughout the day under LD condition, due to the availability of at least 12 hr light and adequate sucrose from the diet (Fig. 4A). In the presence of continuous light (LL) the sucrose activity was further boosted up, with the result the mean sucrose levels slightly declined during the free running time of its rhythm (Fig. 4B). Contrary to this, the sucrose activity was retarded under continuous dark, resulting in the accumulation of undigested sucrose in the gut lumen, which manifested in the form of higher MPVs in its rhythm (Fig. 4 C). The present study demonstrates that the circadian sucrose and sucrose rhythms of the digestive system in *B. mori* are modulated by internal and external factors; internally by the concentrations of sucrose and sucroses themselves and externally by the photoperiod.

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