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

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Abstract: Circadian trehalose and trehalase rhythms were studied 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 cycles in gut lumen. The trehalose rhythm of gut wall comprised 8 trehalose synthetic cycles (TS cycles) under LD and LL and 7 under DD. The 24 hr trehalose rhythm of LD and LL was clock shifted to 27.2 hr under DD. The trehalose rhythm included 4 TR cycles under LD, 5 under LL and DD in the gut lumen and the 24 hr rhythm of LD was clock shifted to 19.2 hr under LL and DD. In the gut wall trehalase rhythm maintained 8 trehalase enzyme synthetic cycles (TES cycles) under LD, 10 LL and 7 under DD and the 24 hr rhythm of LD was clock shifted to 19.2 hr under LL and 27.2 hr under DD. In the gut lumen it included 4 TER cycles under LD and DD, 5 under LL and its 24-hr rhythm was advanced to 19.2 hr. Further analysis of data showed that LD favours trehalose synthesis, while LL and DD favour trehalase synthesis.

Keywords: *Bombyx mori*, Circadian trehalose rhythm, Circadian trehalase rhythm, Digestive system, Photoperiod

INTRODUCTION

Trehalose is the principal insect sugar that accounts for 23% of the total carbohydrate content of the body. It is a disaccharide comprising two glucose units joined by glycosidic linkages and is synthesized in the presence of trehalose phosphate synthase (Thompson, 2003). Principally, trehalose is synthesized and stored in the fat body and to a lesser extent in the gut wall cells and circulates in the haemolymph (Kanamori, 2010). Trehalose has been implicated in a multitude of functions in the insect body. It serves as energy source for metabolism, stabilizes protein and cell membrane structures against ill effects of high temperature and oxygen radicals as a cryoprotectant and regulates feeding mechanism as a signaling molecule (Nath, 2000; Banaroudji *et al*., 2001; Goto *et al*., 2001; Elbein *et al*., 2003; Thompson, 2003; Khani *et al*., 2007; Bolat, 2008; Han *et al*., 2008; Tang *et al*., 2008; Behroozi, 2010).

Trehalase is a metabolically active enzyme that hydrolyses trehalose to glucose and it is widely distributed in the tissues of *Bombyx mori* and other insects (Saito, 1960; Egorova and Khomidov, 1991). The activity levels of this enzyme facilitate homeostatic control of haemolymph trehalose levels in the silkworm (Yanagawa, 1979). Trehalase occurs in two inter convertible forms; a membrane bound trehalase-I (M.W: 73 KDa, pH 6.5) and a freely soluble, trehalase-II (M.W: 140 KDa, pH :5.5) in the digestive system of insects and the later has a predominant role in metamorphosis (Sumida and Yamashita, 1977; Huang *et al*., 2006). These two trehalases have been extensively studied with reference to their purification, properties, metabolism, cellular localization and homeostatic regulation (Saito, 1960; Nijhout, 1994; Terra and Ferreira, 2005; Becker *et al*., 1996; Oda *et al*., 2000; Thompson, 2003). In the silkworm tissues, the levels of trehalose and trehalase vary as function of the ongoing homeostatic mechanisms under the influence of hyper and hypo trehalosemic factors (Sutherland, 1972; Orchard *et al*., 1993; Roeder, 1999; Iwami, 2000; Leevers, 2001; Rulifson *et al*., 2002). It is likely that their levels could be altered in a circadian fashion under the control of peripheral circadian clocks. The available data on circadian clock mechanism in *Drosophila* and *B. mori* suggests that the physiological and biochemical processes are modulated by endogenous circadian oscillators under the influence of light and dark signals (Naidoo *et al*., 1999; Giebultowicz, 2001; Shimizu *et al*., 2001; Froy *et al*., 2003; Hall, 2003; Sharma, 2003; Sehadova *et al*., 2004; Iwai *et al*., 2006; Peschel *et al*., 2009). This has been demonstrated in our previous reports with reference to some biochemical constituents (Sailaja and Sivaprasad, 2010, a, b., Sailaja and Sivaprasad, 2011; Sailaja *et al*., 2011; Sivaprasad and Sailaja, 2011; Bhuvaneswari and Sivaprasad, 2012). However, no effort has since been made to study the impact of photoperiod on the circadian trehalose and trehalase rhythms in the digestive system of *B. mori*. The present study concentrates on this aspect.

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MATERIALS AND METHODS

The Pure Mysore x CSR_2 hybrid variety of the Silkworm *B. mori*, reared under standard environmental conditions of 28° C, 85% relative humidity (Krishnaswami, 1986), was taken as the test species for the present study. After hatching, the worms were feed with M_5 variety of mulberry leaves, five times 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 trehalose and trehalase activity in the two compartments of digestive system (gut wall and gut lumen) of silkworm was analyzed for a period of 25 hr spanning in between day 5 and day 6 of fifth instar development. The gut wall tissue was isolated every hour by mid-dorsally dissecting the silkworm larvae in ice cold silkworm Ringer (Yamaoka *et al*., 1971) starting from 6 AM on day 5 through 6 AM on day 6 (*i.e.* for 25 hr). At the same time the gut content was extracted through a hypodermic syringe by inserting it into the lumen of the gut. The gut content, 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 biochemical assay.

Hour- to- hour changes in trehalose of the gut wall and gut content were estimated by the method of Roe (1955) in 2% homogenate of the gut wall tissue and 1:19 diluted gut content in ice-cold distilled water, using Anthrone reagent. The trehalose levels computed by using standard glucose were expressed as mg glucose/g wet weight of tissue or 1 ml of gut content. Likewise, hour to hour changes in the trehalase activity was estimated by the method of Dahlman (1971) in 5% homogenate of the gut wall and 1: 9 diluted digestive juice in ice cold phosphate buffer using DNS (Dinitro-salisylic acid) reagent. The enzyme activity was computed using glucose as standard and expressed in µ moles of glucose/ mg trehalose/ 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 were treated as the experimental samples.

RESULTS

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

Circadian trehalose rhythm

Gut wall: Under LD, the trehalose rhythm of the gut wall showed 8 peaks and 8 troughs during the 24 hr free running period of the rhythm (Fig.1A). The first peak occurred at 07 hr with trehalose value of 109 mg/g wet wt. of tissue. Subsequent peaks occurred at 09 hr (~95 mg), 12-13hr (~88 mg), 15 hr (~66 mg), 19 hr (~75 mg) and next day at 01 hr (~122 mg), 03 hr (~88 mg) and 06 hr (~56 mg). Troughs occurred at 06 hr (~67 mg), 08 hr (~53 mg), 10 hr (~45 mg), 14 hr (~34 mg), 17 hr (~32 mg), 20 hr (~35 mg) and next day at 02 hr (~73 mg) and 04-05 hr (~36 mg). Under LL, the trehalose rhythm showed 8 peaks and 7 troughs during the 24 hr free running period. Peaks appeared at 07 hr (~66 mg), 10 hr (~75 mg), 12 hr (~130 mg), 15 hr (~56 mg), 18 hr (~56 mg), 21 hr (~78 mg) and next day at 02 hr (~76 mg) and 06 hr (~76 mg). Troughs occurred at 08 hr (~27 mg), 11 hr (~44 mg), 14 hr (~44 mg), 16 hr (~28 mg), 19 hr (~42 mg), 00 hr (~58 mg) and next day at 04 hr (~47 mg). Under DD, the trehalose rhythm showed 7 peaks and 7 troughs during the 24 hr free running period. The Peaks appeared at 07 hr (~104 mg), 11 hr (~68 mg), 16 hr (~58 mg),19 hr (~85 mg), 22 hr (~65 mg) and next day at 01 hr (~88 mg), 05 hr (~65 mg), the troughs occurred at 06 hr (~57 mg), 08 hr (~24 mg), 15 hr (~37 mg), 17 hr (~38 mg), 20 hr (~37 mg), 00 hr (~56 mg) and next day at 03-04 hr (~58 mg).The interval between peaks was 2.8 hr under LD, 2.9 hr under LL and 3.1 hr under DD and that between troughs was 2.6 hr under LD, 2.7 hr both under LL and DD. The combined mean interval between peaks and troughs was roughly about 2.7 hr under both LD, 2.8 hr under LL and 2.9 hr under DD (Table 1A, B and Table 5).

Gut content: Under LD the trehalose rhythm of the gut content showed 4 peaks and 5 troughs during the 24 hr free running period of the rhythm (Fig.1B). The peaks occurred at 09 hr (~38 mg), 14 hr (~38 mg), 19 hr (~38mg) and 22 hr (~48 mg), and the troughs were recorded at 06 hr (~9 mg), 12 hr (~17 mg), 18hr (~22 mg) 20 hr (~31 mg) and next day at 04 hr (~15 mg). Under LL, the trehalose rhythm showed 5 peaks and 6 troughs. The peaks occured at 08-10 hr (~27 mg), 16 hr (~38 mg), 20 hr (~39 mg), 22 hr (~44 mg) and next day at 01 hr (~26 mg). The troughs appeared at 06 hr (~11 mg), 11-12 hr (~19 mg), 18 hr (~ 27 mg), 21 hr (~ 34 mg), 00 hr (~ 23 mg) and next day at 05 hr (~ 13 mg). Under DD, the trehalose rhythm showed 5 peaks and 6 troughs during the 24 hr free running period of the rhythm. The peaks occured at 07-08 hr (~ 27 mg), 10 hr (~ 26 mg), 15 hr (~ 34 mg), 17 hr (~ 39 mg) and at 00 hr (~ 53 mg), the troughs occured at 06 hr (~ 14 mg), 09 hr (~ 23 mg), 12 hr (~ 17 mg), 16 hr (~ 31 mg), 18 hr (~ 29 mg) and next day at 02 hr (~ 35 mg). The

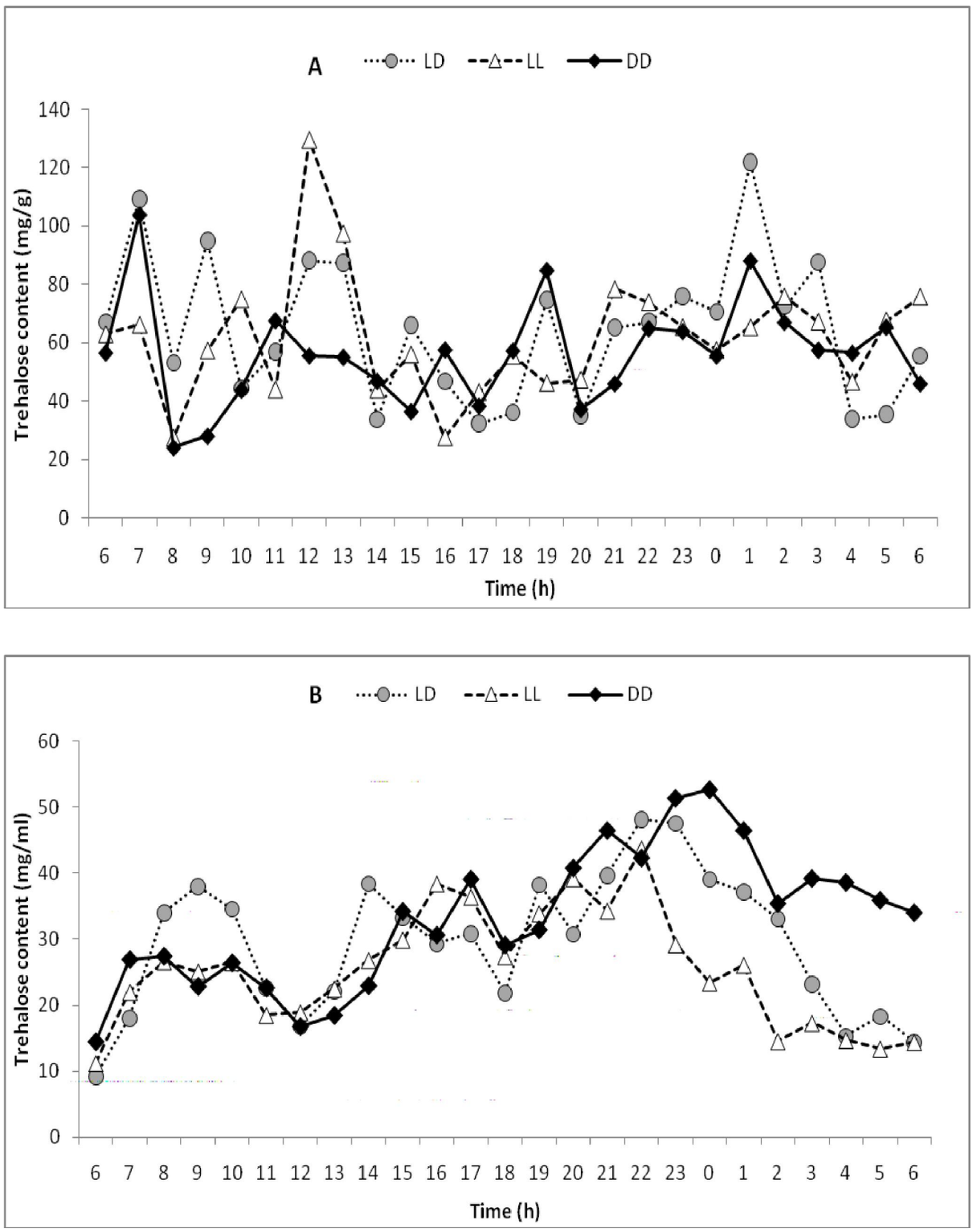

Fig. 1. *Phase response curves (PRCs) of the 24hr circadian trehalose rhythms in the gut wall (A) and gut content (B) of fifth instar larva of Bombyx mori, under12hr light: 12hr dark cycle (LD), continuous light (LL) and continuous dark (DD) conditions. The values expressed in mg glucose /g wet weight of tissue, represent the 24 hr (from 6AM on day 5 to 6AM on day 6) free running time of the circadian rhythm (P values: <0.001).*

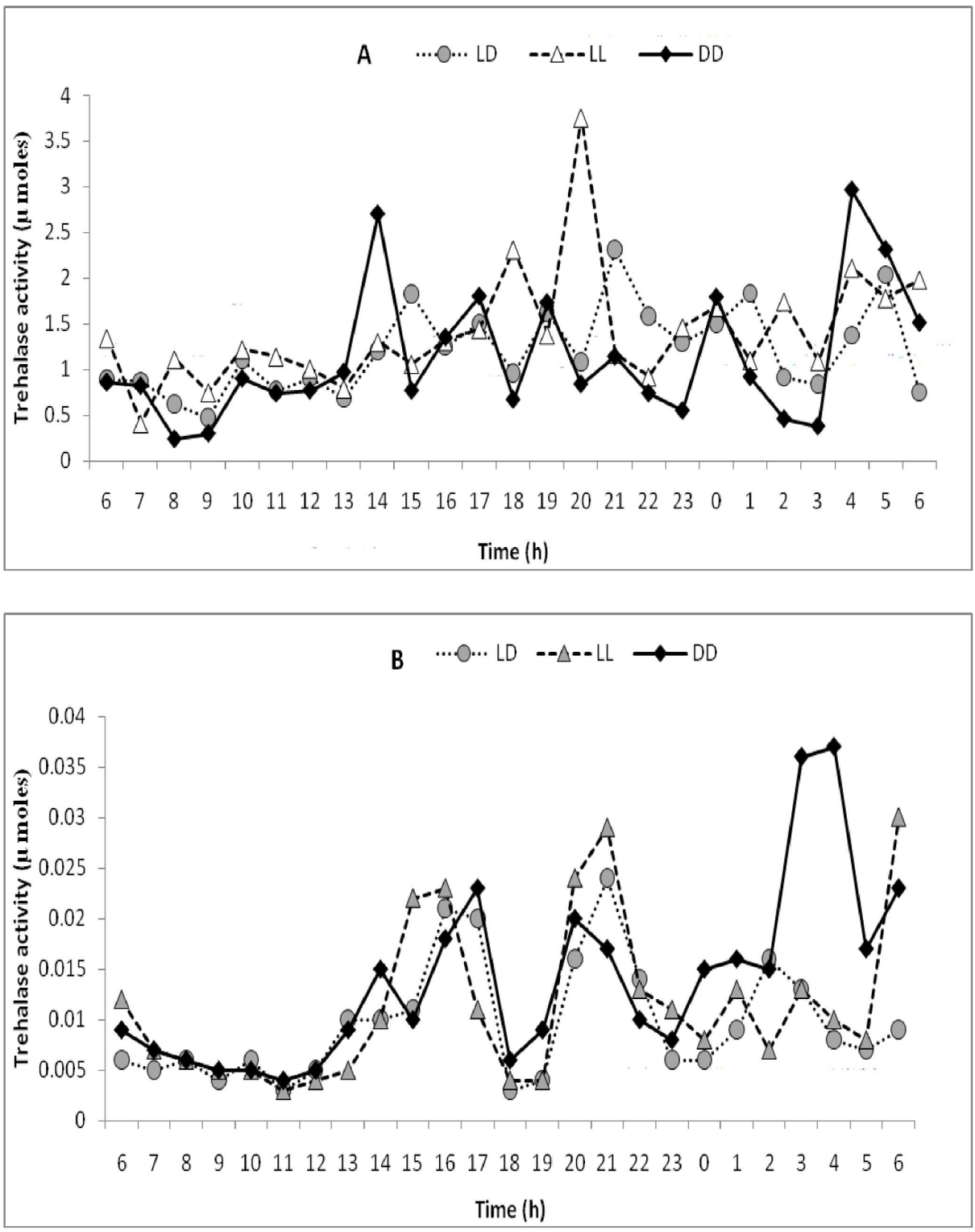

Fig. 2. *Phase response curves (PRCs) of the 24hr circadian trehalase rhythms in the gut wall (A) and gut content (B) of fifth instar larva of Bombyx mori, under12hr light: 12hr dark cycle (LD), continuous light (LL) and continuous dark (DD) condition. The values expressed in µ moles of glucose /mg trehalose/ hr), represent the 24 hr (from 6AM on day 5 to 6AM on day 6) free running time of the circadian rhythm (P values: <0.001).*

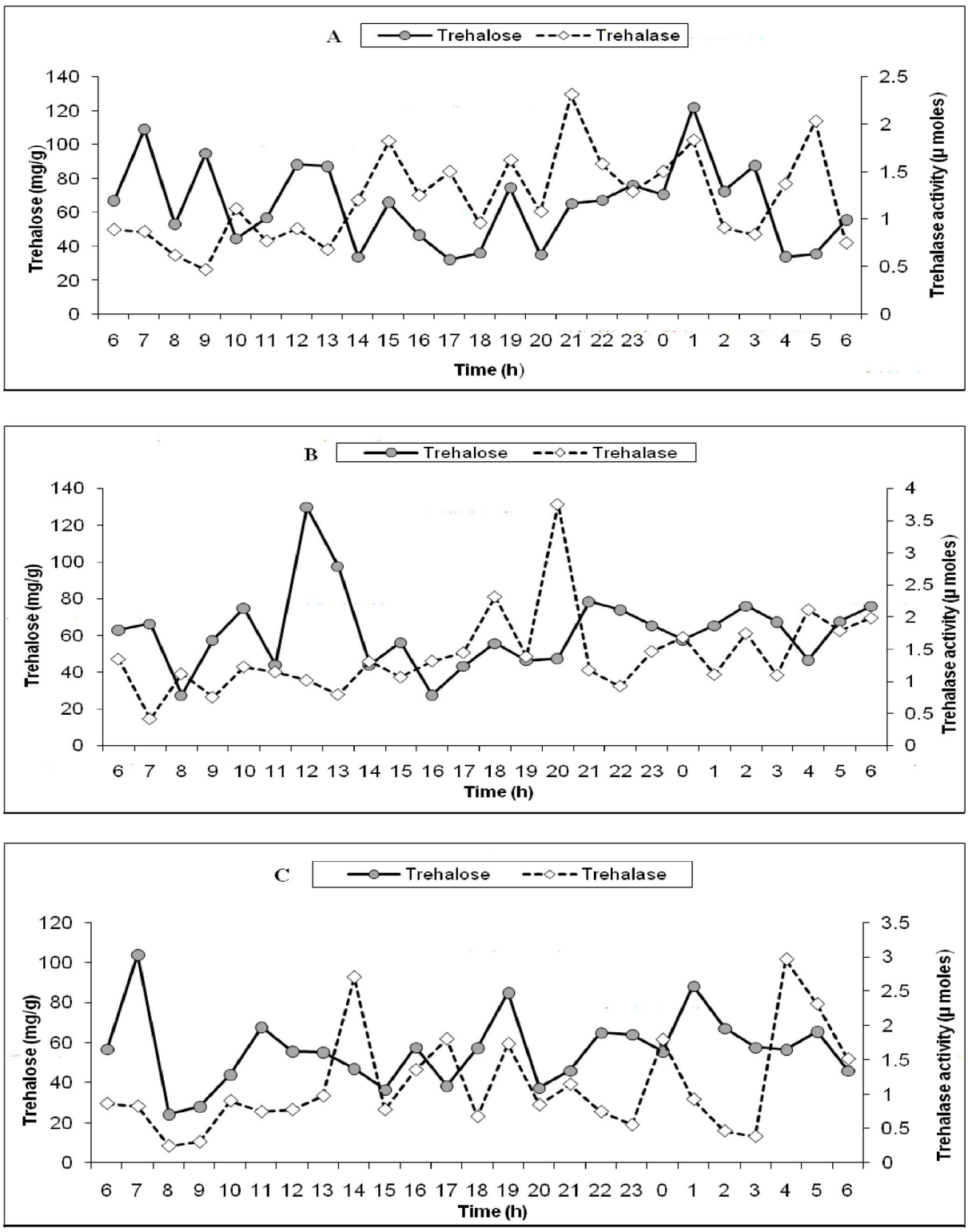

Fig. 3. *Circadian changes in trehalose profiles and trehalase activity in the gut wall of the fifth instar larva of Bombyx mori, under (A) 12hr light: 12hr dark cycle (LD), (B) continuous light (LL) and (C) continuous dark (DD) conditions. The values expressed in mg glucose per gm wet weight of tissue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

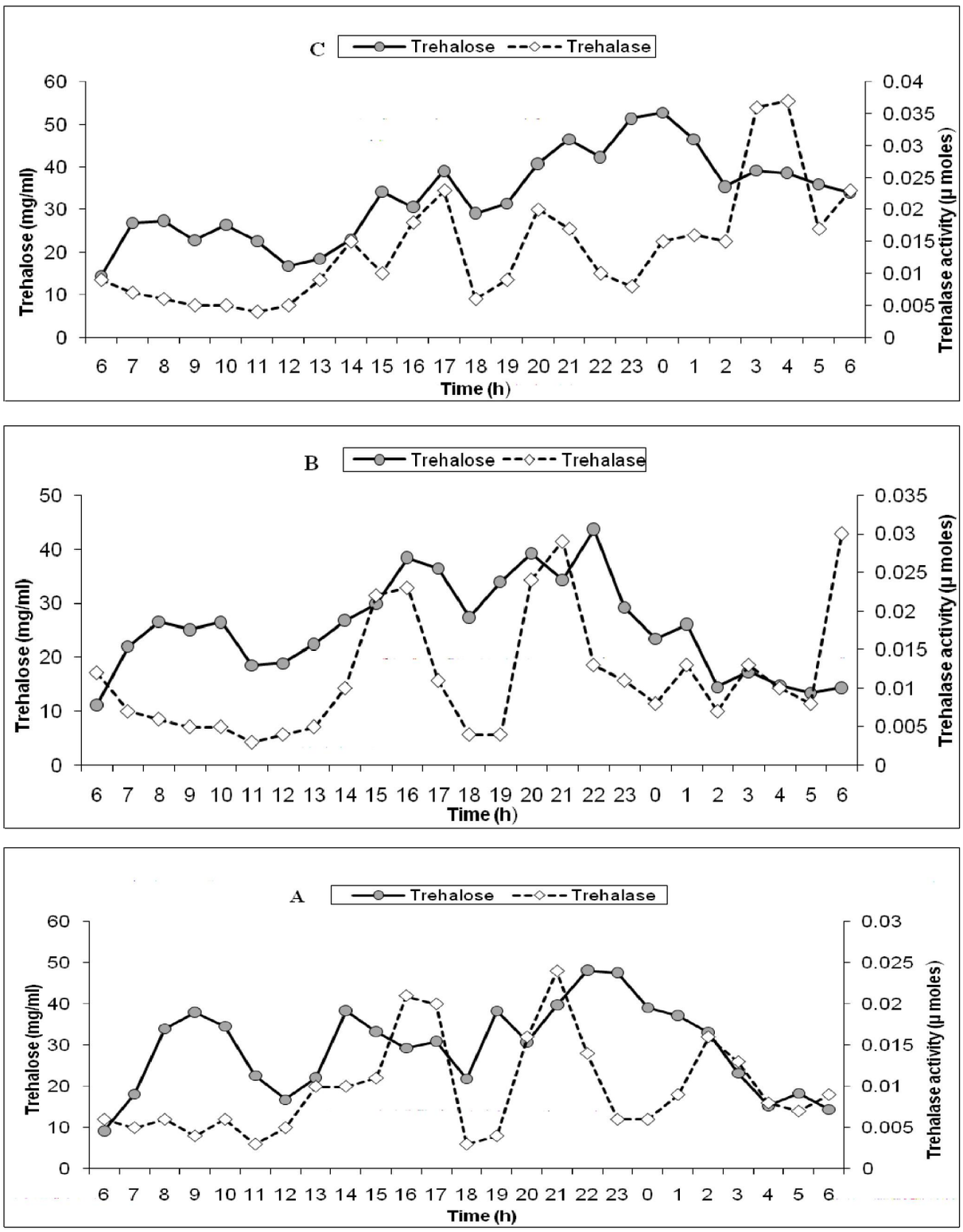

Fig. 4. *Circadian changes in trehalose profiles and trehalase activity in the gut content of the fifth instar larva of Bombyx mori, under (A) 12hr light: 12hr dark cycle (LD), (B) continuous light (LL) and (C) continuous dark (DD) conditions. The values expressed in mg glucose per ml of tissue in case of trehalose and ì moles of glucose formed/ mg trehalose/ hr in case of trehalase, 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 trehalose in the gut wall of the fifth instar larva of *B.mori* during the free running time of the circadian rhythm under 12 hrs light/ dark cycle (LD), continuous light (LL) and continuous dark (DD) conditions**.**

A.

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	
LD	8	2	3	2	4	6	2	3	2.8
LL	8	3	2	3	3	3	5	4	2.9
DD	7	4	5	3	3	3	4	-	3.1

B.

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	
LD	8	2	2	4	3	2	6	2	2.6
LL	7	3	3	2	3	4	4	-	2.7
DD	7	2	7	2	3	4	3	-	2.7

Source: Fig. 1A.

interval between peaks was about 3.3 hr under LD, 3.0 hr under LL and 3.2 hr under DD and that between troughs was about 4.4 hr under LD, 3.7 hr under LL and 3.3 hr under DD. The combined mean interval between peaks and troughs was about 3.9 hr under LD, 3.4 hr under both LL and DD (Table 2A, B and Table 5).

Circadian trehalase rhythm

Gut wall: Under LD the rhythm of trehalase activity showed 8 peaks and 8 troughs in the gut wall during the 24 hr free running period of the rhythm (Fig.2A). Peaks appeared at 06-07 hr (~ 0.89 µ moles), 10 hr (1.11 µ moles), 15 hr (1.82 µ moles), 17 hr (1.5 µ moles), 19 hr (1.62 µ moles), 21 hr (2.31 µ moles) and next day at 01 hr (1.83 µ moles) and 05 hr (2.03 µ moles) and troughs appeared at 09 hr (0.47 µ moles), 13 hr (0.68 µ moles), 16 hr (1.25 µ moles), 18 hr (0.96 µ moles), 20 hr (1.08 µ moles), 23 hr (1.29 µ moles) and next day at 03 hr (0.84 µ moles) and 06 hr (0.75 µ moles). Under LL the rhythm showed 10 peaks and 9 troughs during the 24 hr free running time. The first peak appeared at 06 hr (1.34 µ moles), 08 hr (1.11 µ moles), 10 hr (1.22 µ moles), 14 hr (1.30 µ moles), 18 hr (2.31 µ moles), 20 hr (3.75 µ moles), 00 hr (1.68 µ moles) and next day at 02 hr (1.74 µ moles), 04 hr (2.11 µ moles) and 06 hr (1.98 µ moles) and the troughs occured at 07 hr (0.41 µ mole), 09 hr (0.75 µ moles), 13 hr (0.79 µ moles), 15 hr (1.06 µ moles), 19 hr (1.38 µ moles), 22 hr (0.92 µ moles) and next day at 01 hr (1.10 µ moles), 03 hr (1.09 µ moles) and at 05 hr (1.78 µ moles). Under DD, the trehalase activity rhythm showed 7 peaks and 7 troughs during the 24 hr free running period (Fig.2A). The peaks appeared at 06 hr (0.86 µ moles) 14 hr (2.70 µ moles), 17 hr (1.80 µ moles), 19 hr (1.73 µ moles), 21 hr (1.14 µ moles), 00 hr (1.79 µ moles) and next day at 04 hr (2.96 µ moles) and the troughs occured at 08 hr (0.24 µ moles), 15 hr (0.77 µ moles), 18 hr (0.67 µ moles), 20 hr (0.84 µ moles), 23 hr (0.55 µ moles) and next day at 03 hr (0.38 µ moles) and 06 hr (1.51 µ moles). The interval between peaks was about 2.8 hr under LD, 2.4 hr under LL and 3.1 hr under DD and that between troughs was about 2.5 hr under LD, 2.4 hr under LL and 3.0 hr under DD. The combined mean

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

A.

Photo period	No. of peaks	Interval between peaks in hours				Mean interval in hours
		1-2	2-3	3-4	4-5	
LD	4	5	5	3	-	3.3
LL	5	6	4	2	3	3.0
DD	5	2	5	2	7	3.2

B.

Photo period	No. of troughs	Interval between troughs in hours					Mean interval in hours
		1-2	2-3	3-4	4-5	5-6	
LD	5	6	6	2	8	-	4.4
LL	6	5	6	3	3	5	3.7
DD	6	3	3	4	2	8	3.3

Source: Fig. 1B.

Table 3 (A and B). Interval between peaks (A) and troughs (B) in the activity levels of trehalase 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.

A.

Photo period	No. of peaks	Interval between peaks in hours									Mean interval in hours
		1-2	2-3	3-4	4-5	5-6	6-7	7-8	8-9	9-10	
LD	8	3	5	2	2	2	4	4	-	-	2.8
LL	10	2	2	4	4	2	4	2	2	2	2.4
DD	7	8	3	2	2	3	4	-	-	-	3.1

B.

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	8	4	3	2	2	3	3	3	-	2.5
LL	9	2	4	2	4	3	3	2	2	2.4
DD	7	6	3	2	3	4	3	-	-	3.0

Source: Fig. 2A

interval between peaks and troughs was about 2.7 hr under LD, 2.4 hr under LL and 3.1 hr under DD (Table 3A, B and Table 6).

Gut content: Under LD, the trehalase activity showed 4 peaks and 4 troughs in the gut content during the 24 hr free running period of the rhythm (Fig.2B). Peaks appeared at 16 hr (0.021 µ moles), 21 hr (0.024 µ moles) and next day at 02 hr (0.016 µ moles), and 06 hr (0.009 µ moles), and troughs appeared at 06 hr (0.006 µ moles), 18-19 hr (~0.004µ moles), 23-00 hr (0.006 µ moles) and next day 05 hr (0.007 µ moles).Under LL the rhythm showed 5 peaks and 5 troughs, the peaks appeared at 15-16 hr (~ 0.023 µ moles), 21 hr (0.029 µ moles) and next day at 01 hr (0.013 µ moles), 03 hr (0.013 µ moles) and 06 hr (0.030 µ moles) and the troughs at 06 hr (0.012 µ moles), 18-19 hr (0.004 µ moles), 00 hr (0.008 µ moles) and next day at 02 hr (0.007 µ moles) and 05 hr (0.008 µ moles). Under DD the trehalase activity rhythm showed 4 peaks and 4 troughs during the 24 hr free running period. The peaks appeared at 17 hr (0.023µ moles), 20 hr (0.020 µ moles) and next day at 04 hr (0.037 µ moles) and 06 hr (0.023µ moles) and the troughs appeared at 06 hr (0.009µ moles), 18 hr (0.006 µ moles), 23 hr (0.008 µ moles) and next day at 05 hr (0.017 µ moles). The interval between peaks was about 3.5 hr under LD and 3.0 hr under both LL and DD and that between troughs was about 5.3 hr under LD, 4.6 hr under LL and 5.8 hr under DD. The combined mean interval between peaks and troughs was about 4.4 hr under both LD and DD and 3.8 hr under LL (Table 4A, B and Table 6).

DISCUSSION

Trehalose is the most common blood sugar in insects. It is synthesized from the glucose derived from the dietary carbohydrates such as the pectin, xylan, cellulose, sucrose and starch present in the mulberry leaf and later transported to the gut wall, where it is stored either in the same form or in the form of glycogen (Yamashita, 1965; Yamashita and Hasegawa, 1974; Anand *et al*., 2010).

Table 4 (A and B). Interval between peaks (A) and troughs (B) in the activity levels of trehalase 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.

A.

Photo period	No. of Peaks	Interval between peaks in hours				Mean interval in hours
		1-2	2-3	3-4	4-5	
LD	4	5	5	4	-	3.5
LL	5	5	4	2	3	3.0
DD	4	3	7	2	-	3.0

B.

Photo period	No. of troughs	Interval between troughs in hours					Mean interval in hours
		1-2	2-3	3-4	4-5	5-6	
LD	4	12	4	5	-	-	5.3
LL	5	13	5	2	3	-	4.6
DD	4	12	5	6	-	-	5.8

Source: Fig. 2B.

Table 5. Comparative analysis of the phase response curves of the trehalose rhythm in the gut wall and gut content of the fifth instar larva of *B.mori*, under 12 hr 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	8	8	7	4	5	5
No. of troughs	8	7	7	5	6	6
Mean interval b/w peaks (hr)	2.8	2.9	3.1	3.3	3.0	3.2
Mean interval b/w troughs (hr)	2.6	2.7	2.7	4.4	3.7	3.3
Combined mean interval b/w peaks and troughs (hr)	2.7	2.8	2.9	3.9	3.4	3.4
Probable no. of TS/TR cycles	8	8	7	4	5	5
Approximate time taken for each TS/TR cycles (hr)	3.0 (24/8=3.0)	3.0 (24/8=3.0)	3.4 (24/7=3.4)	6.0 (24/4=6.0)	4.8 (24/5=4.8)	4.8 (24/5=4.8)
Free running time of rhythm (hr)	24 (3x8=24)	24 (3x8=24)	27.2 (3.4x8=27.2)	24 (6x4=24)	19.2 (4.8x4=19.2)	19.2 (4.8x4=19.2)
Mean peak value	87.2	76.6	76.0	40.7	34.7	35.9

Source: Fig. 1A and B; TS Cycles: Trehalose synthetic cycles; TR cycles: Trehalose release cycles

The digestive system of *Bombyx mori* is principally concerned with the digestion and absorption of dietary nutrients present in the mulberry leaf. The principal nutrients of mulberry leaf include carbohydrates which are digested and absorbed as glucose molecules and finally assimilated in the form of storage sugar called trehalose in the fat body and gut wall cells (Thompson, 2003; Narayanaswamy and Shankar, 2010; Shivakumar and Shamitha, 2011; Lokesh *et al*., 2012). The trehalose, so derived is degraded to glucose by an enzyme called trehalase, and used both as a means of energy source and for the maintenance of homeostasis (Azuma and Yamashita, 1985; Su *et al*., 1993; Nath, 2000). More importantly, trehalose levels in the silkworm tissues are homeostatically controlled by the complementary action of two enzymes, viz, trehalose phosphate synthase that synthesizes trehalose from glucose and trehalase that hydrolyses trehalose to glucose (Candy and Kilby, 1961; Thompson, 2003). The dynamic equilibrium between trehalose and glucose appears to be maintained by light sensitive time giver in a circadian fashion much like that of many other biochemical constituents (Kostal and Shimada, 2001; Iwai *et al*., 2006). Our study on the circadian profiles of trehalose and trehalase activity confirms the exsistence of circadian biochemical rhythms in the *B. mori*, even at the level of digestive system, similar to those in other tissues such as the silk gland, fat body, muscle and haemolymph (Sailaja and Sivaprasad, 2010, a, b., Sailaja and Sivaprasad, 2011; Sivaprasad and Sailaja, 2011; Sailaja *et al*., 2011; Bhuvaneswari and Sivaprasad, 2012). The circadian trehalose and trehalase data, presented as peaks and troughs in PRC s (Figs. 1 and 2 and Tables 1 to 6) are analyzed in the following terms. The number of peaks in trehalose rhythm is interpreted in terms of the number of trehalose synthetic cycles (TS cycles) in the gut wall and number of trehalose release cycles (TR cycles) in the gut content. Similarly, the number of peaks in trehalase activity is interpreted in terms of trehalase enzyme synthetic cycles (TES cycles) in the gut wall and trehalase enzyme release cycles (TER cycles) in the gut content. In both the compartments of digestive system (gut wall and gut lumen), the height of peaks are interpreted in terms of intensity of synthetic/ release cycles and the mean peak value in terms of the average levels of trehalose or trehalase activity maintained during the 24 hr free running time of the rhythm. At the same time the combined mean intervals between peaks and troughs are considered as the time taken for completion of synthetic / release cycles of both trehalose and trehalase (Tables 5 and 6).

Circadian trehalose rhythm

Gut wall: Most of the trehalose found in the epithelial cells of the gut wall is apparently synthesized from the dietary glucose (Thompson, 2003). The trehalose rhythm maintains 8 TS cycles under LD and LL with duration of 3.0 hr each and 7 cycles under DD each with a duration of 3.4 hr. Thus, DD condition modulates trehalose synthesis by extending the duration of each cycle by 24 min (from 3.0 hr to 3.4 hr). Because of the prolongation of the duration of all the TS cycles in a day, the 24 hr free

Table 6. Comparative analysis of the phase response curves of the trehalase rhythm in the gut wall and gut content of the fifth instar larva of *B.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	8	10	7	4	5	4
No .of Troughs	8	9	7	4	5	4
Mean interval b/w Peaks (hr)	2.8	2.4	3.1	3.5	3.0	3.0
Mean interval b/w Troughs (hr)	2.5	2.4	3.0	5.3	4.6	5.8
Combined mean interval b/w Peaks and Troughs (hr)	2.7	2.4	3.1	4.4	3.8	4.4
Probable no. of TES/TER cycles	8	10	7	4	5	4
Approximate time taken for each TES/TER cycles (hr)	3.0 (24/8=3)	2.4 (24/10=2.4)	3.4 (24/7=3.4)	6.0 (24/4=6)	4.8 (24/5=4.8)	6.0 (24/4=6)
Free running time of Rhythm (hr)	24 (3x8=24)	19.2 (2.4x8=19.2)	27.2 (3.4x8=27.2)	24 (6x4=24)	19.2 (4.8x4=19.2)	24 (6x4=24)
Mean Peak value	1.64	1.85	1.85	0.033	0.022	0.026

Source: Fig. 2A and B; TES cycles: Trehalase enzyme synthetic cycles; TER cycles: Trehalase enzyme release cycles

running time of trehalose rhythm under LD and LL is clock-shifted to 27.2 hr under DD (Table 5). Further analysis of peaks interms of their height reveals that the intensity of trehalose synthesis is high during scotopic (dark) phase of the day compared to photic (light) phase. Evidently, intense trehalose synthesis occurred thrice (at 07 hr, 19 hr and 01-02 hr), under DD, twice (at 07-13 hr and 01-03 hr) under LD and only one (at12-13hr) under LL (Fig.1A). In addition, the photoperiod also affects the mean peak value (MPVs; i. e ., the average of all peaks) of trehalose. The MPV of the trehalose is significantly higher under LD (87.2 mg) and moderate under LL and DD (~76.0 mg each).Our study indicates that both light and dark cues are necessary for the maintenance of constant levels of trehalose in tissues during the free running time of the rhythm as presumed by Syrova *et al*., 2003; Iwai *et al*., 2006; and Fonagy, 2009. This obviously, is done by stimulating the synthesis of trehalose during the night and its utilization as energy source during the day. However, the exposure of silkworm larvae to prolonged light and dark conditions disturbs the balance between synthesis and utilization of trehalose as an energy source in metabolism.

Gut content: Gut content is the rich source of proteins, carbohydrates, lipids, digestive enzymes and many other biochemical constituents derived from the mulberry leaves and glandular epithelial cells of the gut wall. Though trehalose is not a regular constituent of the gut content, it is known to be leaked into the gut lumen from the epithelial cells of gut wall and helps in the maintenance of osmotic gradient between the two compartments of digestive system (Wyatt, 1967; Ito, 1972; Anand *et al*., 2010). The trehalose release through leakage obviously follows a cyclic path, referred to as trehalose release cycles (TR cycles) in this report. In the gut content trehalose rhythm maintains 4 TR cycles with duration of 6.0 hr each under LD and 5 TR cycles under LL and DD with duration of 4.8 hr each. Thus, LL and DD conditions modulate the TR cycles by reducing the duration of each TR cycle by 1.2 hr (from 6.0 hr to 4.8 hr). Because of this reason, the free running time of trehalose rhythm runs on 19.2 hr cycle instead of normal 24 hours. Within the rescheduled rhythm, the timing of its active leakage is synchronized with the availability of light. Accordingly, the active leakages occured (at 08-10 hr, 14-15 hr and 22-00 under LD, at 08-10hr, 16-17 hr and 20-23 hr under LL, and at 07-10hr, 15-17 hr and 21-01 hr under DD). Nevertheless, relatively optimal levels (Higher mean peak values) of trehalose (~ 41 mg) were consistently maintained win the gut content under LD compared to those of LL and DD (~ 35 mg each), as in gut wall cells (Table 5). This supports our earlier finding that both light and dark cues are necessary for the maintenance of carbohydrate energy reserves in silkworm tissues, in which such reserves are synthesized in dark phases and predominantly utilized in the light phase of the day (Bhuvaneswari and Sivaprasad, 2012).

Circadian trehalase rhythm

Gut wall: The gut wall is the primary source of synthesis for two forms of trehalase (Tre-1 and Tre-2) that ensures continuous availability of glucose by enzymatic hydrolysis of trehalose and that meets the energy requirements of peristalsis (Azuma and Yamashita, 1985; Su *et al*., 1993; Nath, 2000). As demonstrated in the present study, this enzyme rhythm maintains 8 trehalase enzyme synthetic cycles (TES cycles) under LD with a duration of 3.0 hr each, 10 cycles under LL with a duration of 2.4 hr each and 7 cycles under DD with a duration of 3.4 hr each (Fig. 2A). Thus, LL condition modulates the enzyme rhythm by reducing time required for each synthetic cycle by 36 min (from 3.0 hr to 2.4 hr), while the DD condition does so by extending it by 24 min (from 3.0 hr to 3.4 hr). Due to shortening and extension of the duration of TES cycles the 24 hr free running time of the rhythm is clock shifted to 19.2 hr under LL and 27.2 hour under DD. Clearly within the daily rhythm, the enzyme showed 3 active synthetic phases under LD (at 15 hr, 21-01 hr and 05 hr), one under LL (at 20 hr) and two under DD at (14 hr and 04-05 hr). Irrespective of the timing of its active synthesis the trehalase showed higher activity levels (represented in the form of higher MPVs), both under LL and DD conditions (1.85 µ moles each) compared to that under LD (1.64 µ moles) (Table 6). Evidently, both the light and dark conditions stimulate trehalase synthesis in the gut wall cells, much like that of silk protein synthesis in the silk gland (Sailaja and Sivaprasad, 2010a,b).

Gut content: Trehalase is produced and released into the gut lumen along with the other digestive enzymes in tracer amounts. The remnant of trehalase activity found in the gut content has been implicated in extra-cellular digestion of trehalose that diffuses there from the haemolymph and its recovery back into the system and thus prevents its excretion. It also facilitates the continuous supply of glucose into the haemolymph of non-feeding silkworms (Wyatt, 1967; Gilby *et al*., 1967; Azuma and Yamashita, 1985). Thus trehalose provides energy source during starvation and also facilitates the regulation of homeostatic mechanism among different tissues and organs (Nagasawa *et al*., 1990; Satake *et al*., 1997; Iwami, 2000). The trehalase rhythm in the gut content represented as trehalase enzyme release cycles (TER cycles) showed 4 cycles under both LD and DD each with a duration of 6.0 hr each and 5 cycles under LL each with a duration of 4.8 hr. Notably, the LL condition reduced the duration of each TER cycle by 1.2 hr (from 6.0 hr to 4.8 hr) (Fig. 2B) and thus, supports the view that light stimulates trehalase release (Fonagy, 2009). Due to stimulatory effect of light, the 24 hr trehalase rhythm has been rescheduled to operate on shorter duration of 19.2 hr under LL (Table 6). Within the free running time, irrespective of number of enzyme release cycles, three active release phases (high peaks) occurred at different timings under LD (16-17 hr, 21hr, 02 hr), LL (15-16 hr, 20-21 hr, 06 hr) and DD (17 hr, 20 hr, 03-04 hr). However a higher mean peak value of trehalase activity was observed under LD (0.033 µ moles) followed by a moderate value under DD (0.026 µ moles) and a lower activity under LL (0.022 µ moles). Though the exact mechanism of circadian regulation is not known, it is presumed that the digestive system of *B. mori* maintains an intrinsic time keeping system that comprises light-sensitive peripheral clocks in its gut wall. Probably these clocks modulate circadian digestive rhythm through their ability to detect and respond to the light cues of the environment (Kostal and Shimida, 2001; Saunders, 2002; Syrova *et al*., 2003; Allada and Emery, 2009; Hirayama and Sessone- Corsi, 2009; Iwai *et al*., 2006). This obviously, manifests in the form of circadian changes in the rate of synthesis and release of trehalose and its utilization through trehalase activity in a time dependent manner in *B. mori*.

Trehalose rhythm versus trehalase rhythm: By and large the trehalose and trehalase rhythm showed inverse relationships with each other throughout the free running time in both the compartments of digestive system under three photoperiodic conditions; LD, LL and DD (Figs. 3 and 4). The peaks in one constituent were accompanied by troughs in the other. For example, the peaks in trehalose levels and troughs in trehalase activity in the gut wall at 06-13 hr and 01-06 hr under LD, 06-15 hr and 21-06 hr under LL, 06-13 hr and 18-03 hr under DD and those in the gut content at 06-15 hr, 22-06 hr under LD, 06-02 hr both LL and DD indicate the probable timing of active phases of trehalose synthesis from sucrose - rich mulberry leaves on one hand and its availability through hormone-induced glycogen breakdown in the fat body and haemolymph on the other (Jungries, 1980; Goldsworthy and Gade, 1983; Oda *et al*., 2000; Thompson, 2003). Simultaneously decline in trehalose activity is attributable to the availability of dietary glucose which has an inhibitory effect on the formal (Thompson, 2003). Conversely, troughs in the trehalose levels and peaks in trehalase activity in the gut wall at 14-00 hr under LD, 16-20 hr under LL, 14-17 hr and 04-06 hr under DD (Fig.3A,B,C) and those in the gut content at 16-21 hr under LD, 03-06 hr both under LL and DD (Figs.4A,B,C) reflect the probable role of membrane bound trehalases in maintaining the glucose concentration gradient across the gut by hydrolyzing the trehalose that results from glycogen breakdown under the influence of insulin like peptides (Wyatt, 1967; Nagasawa *et al*., 1990; Satake *et al*., 1997; Satake *et al*., 1999; Iwami, 2000). The circadian changes in the levels of trehalose and trehalase in the silkworm require more correlational studies from physiology and nutrition.

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