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IMPACT OF PHOTOPERIOD ON CIRCADIAN CARBOHYDRATE AND AMYLASE RHYTHMS IN THE DIGESTIVE SYSTEM OF SILK-WORM, *BOMBYX MORI*

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ABSTRACT
The impact of photoperiod on circadian carbohydrate and amylase rhythms was studied in the digestive system of *Bombyx mori* under 12h light -dark cycle (LD), continuous light (LL) and continuous dark (DD) conditions. The peaks in phase response curves were interpreted as carbohydrate (CS) and amylase synthetic (AS) cycles in gut wall and carbohydrate uptake (CU) and amylase release (AR) cycles in gut content while the interval between peaks and troughs as duration of such cycles. The carbohydrate rhythm included 6 CS cycles of 4h duration each under LD, 8 cycles of 3h duration each under LL and 7 cycles of 3.4h duration each under DD in gut wall and 8 CU cycles of 3h duration each under LD and DD and 6 cycles of 4h duration each under LL in gut content. The amylase rhythm maintained 7 AS cycles of 3.4h duration each under LD and DD and 9 cycles of 2.6h each under LL in gut wall and 8 AR cycles of 3h duration each under LD and LL and 6 cycles of 4.0h duration each under DD in gut content. The changes in the duration of these cycles were accompanied by appropriate changes in the free running time of both carbohydrate and amylase rhythms.

INTRODUCTION

The digestive system is the route through which the silkworm, *Bombyx mori* takes-up the mulberry diet for its complete digestion, absorption, assimilation and final conversion to silk. This is made possible by the active secretion of digestive enzymes and several other biochemical constituents into the gut lumen from the gut wall cells (Cermenati *et al*., 2007; Anand *et al*., 2010). The chief source of energy, for silkworm, however, is the carbohydrates present in the mulberry leaf which includes starch, composed of repeated units of D-glucose connected by α-1, 4 glycosidic linkages that are cleaved during digestion by specific enzymes known as α-amylases (Jenner, 1982; Strobl *et al*., 1998). In silkworm, the amylases are synthesized and secreted by the glandular epithelial cells of the gut wall at regular intervals (Baker, 1983; Terra and Ferreira, 2005). As *B. mori* feeds on the starch-rich mulberry leaves, its survival largely depends on the effectiveness of α-amylase it produces (Titarenko and Chrispeels, 2000) much as it does in other animals. For effective digestion, the production and secretion of α-amylase should be synchronized with dietary uptake of carbohydrates in a time dependent circadian fashion. Despite the availability of wealth of literature on its physical properties and chemical activity of α-amylase (Fisher and Stein, 1960; Podoler and Applebaum, 1971; Kanekatsu, 1972; Baker, 1983 and Abraham *et al*., 1992), no effort has so far been made to examine its rhythmic nature in the gut lumen and gut cells. The well established circadian clock mechanism (Naidoo *et al*., 1999; Tawata and Ichikawa, 2001; Giebultowicz, 2001; Froy *et al*., 2003; Sharma, 2003; Sehadova *et al.,* 2004) has not been correlated with biochemical manifestations except for some preliminary reports on circadian protein and amino acid rhythms in *B. mori* (Sailaja and Sivaprasad, 2010, a, b., Sailaja and Sivaprasad, 2011; Sailaja *et al.*, 2011; Sivaprasad and Sailaja, 2011). The present study aims at analyzing the rhythmic changes in the levels of total carbohydrates and α-amylase activity, under the premise that theirs rhythmic nature follows a light-dependent circadian cyclic path which is fine tuned by the photoperiod.

MATERIALS AND METHODS

The pure Mysore x CSR_2 hybrid variety of the Silkworm *Bombyx mori,* reared under standard environmental conditions of 28ºC, 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 12h light and 12h dark conditions. After third moult, the larvae were divided into three batches and reared separately under three different photoperiodic conditions viz., 12h light and 12h dark cycle (LD), continuous light (LL) and continuous dark (DD), but fed uniformly five times a day as usual. Circadian rhythmicity in the levels total carbohydrates and α-amylase activity of the silkworm gut was analyzed for a 25h period, spanning in between day 5 and day 6 of fifth instar development. The gut wall tissue was isolated every hour by mid dorsal dissection of silkworm larvae in ice cold *B. mori*

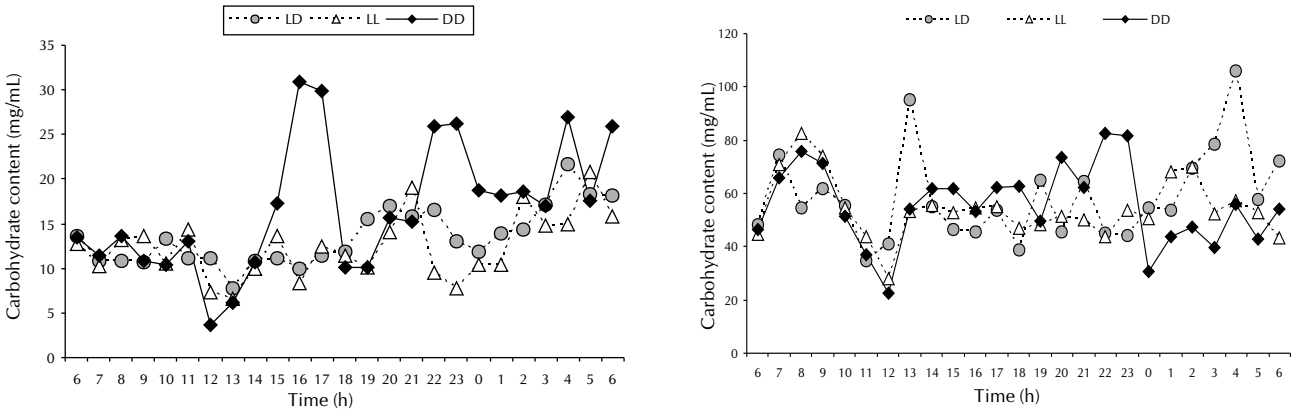

Figure 1: Phase response curves (PRCs) of the 24h circadian carbohydrate rhythms (from 6AM on day 5 to 6AM on day 6) in the gut wall (A) and gut content (B) of fifth instar larva of *Bombyx mori,* under12h light: 12hr dark cycle (LD), continuous light (LL) and continuous dark (DD) conditions. (p <0.001)

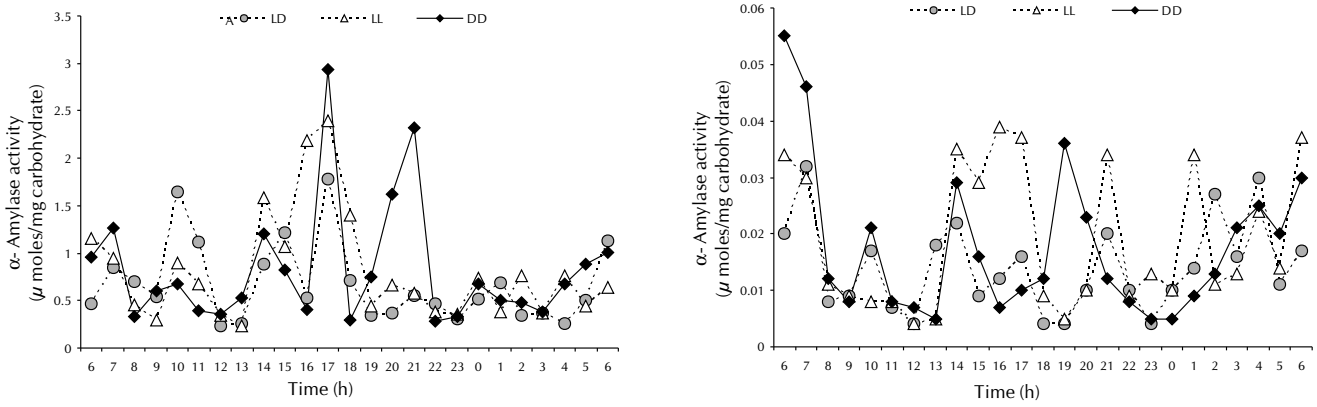

Figure 2: Phase response curves (PRCs) of the 24h circadian *á*-amylase rhythms (from 6AM on day 5 to 6AM on day 6) in the gut wall (A) and gut content (B) of fifth instar larva of *Bombyx mori*, under12h light: 12h dark cycle (LD), continuous light (LL) and continuous dark (DD) conditions. (p <0.001)

Ringer (Yamaoka *et al*., 1971) starting from 6 AM on day 5 through 6 AM on day 6 (*i.e.* for 25h). 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- h changes in the levels of the total carbohydrates were estimated by the method of Caroll *et al.* (1956) in 5% homogenate of the gut wall tissue and 1:19 diluted gut content (digestive juice) in 10% TCA, using Anthrone reagent. The carbohydrate levels computed by using standard glucose were expressed as mg glucose/g wet weight of tissue or 1mL of digestive juice. Likewise, h to hour changes in the α-amylase activity was estimated by the method of Bernfeld (1955) in 2% homogenate of the gut wall and 1: 9 diluted digestive juice in 0.05M acetate buffer using DNS (Dinitro-salisylic acid) reagent. The enzyme activity was computed using glucose as standard and expressed in μ moles of maltose/mg carbohydrates/h. 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 carbohydrate and α-amylase 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 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 carbohydrate rhythms

Gut wall

Under LD, the total carbohydrate rhythm of the gut wall showed 6 peaks and 6 troughs during the 24h free running period of the rhythm (Fig.1A). The first peak occurred early at 06 h with a total carbohydrate value ~ 14 mg/g wet wt. of tissue, while the subsequent peaks occurred at 10h (~ 13 mg), 15h (~ 11 mg), 20h (~ 17 mg), 22h (~ 17mg) and next day again at 04h (~ 22mg). Troughs occurred at 07-09h (~ 11mg), 13h (~ 8mg), 16h (~ 10mg), 21h (~16mg), 00h (~12mg) and next day at 05-06h (~18mg). Under LL, the total carbohydrate

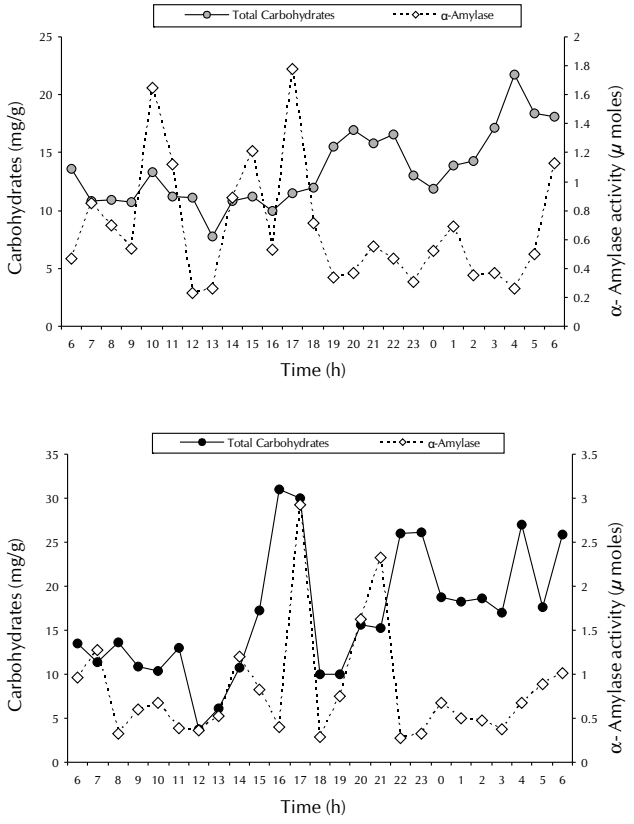

Figure 3: Circadian changes in total carbohydrate profiles and *á*-amylase activity in the gut wall of the fifth instar larva of *Bombyx mori*, under (A) 12h light: 12h dark cycle (LD), (B) continuous light and (C) continuous dark (DD) conditions. The values expressed in mg per gm wet weight of tissue, represent the24h (6 AM on day-5 to 6 A.M on day 6) free running time of the circadian rhythm. ($p < 0.001$)

rhythm showed 8 peaks and 8 troughs during the 24h free running period. While the peaks occurred at 06h (~ 13 mg), 09h (~14mg), 11 hr (~14 mg), 15h (~14 mg), 17h (~12 mg), 21h (~19 mg) and next day at 02h (~18 mg) and 05h (~ 21 mg), the troughs occurred at 07h (~10 mg), 10h (~11 mg), 13h (~7 mg), 16h (~8 mg), 19h (~10mg), 23h (~8 mg) and next day at 03-04h (~15 mg) and again one at 06h (~ 6mg). Under DD, the total carbohydrate rhythm showed 7 peaks and 6 troughs during the 24h free running period. Peaks appeared at 06h (~14 mg), 08h (~14 mg), 11h (13mg), 16h (~31 mg), at 22-23h (~26 mg) and next day at 04h (27mg) and again one at 06h (~26mg), troughs occurred at 07h (~11mg), 10h (~10 mg), 12h (~4 mg), 18-19h (~ 10mg) and next day at 03h (~17mg) and again at 05 h (~18mg). At the same, the intervals between peaks and troughs varied from one photoperiodic condition to the other. The interval between peaks was about 3.7h under LD, 2.9h under LL and 3.4h under DD and that between troughs was 3.5h under LD, 2.9 h under LL and 3.7h under DD. The combined mean interval of peaks and troughs was roughly about 3.6h under both LD and DD and 2.9h under LL (Tables 1A, 1B and 5).

Gut Content

Under LD the total carbohydrate rhythm of the gut content showed 8 peaks and 8 troughs during the 24h free running period of the rhythm (Fig. 1B). The peaks appeared at 07h (~ 74 mg), 09 h (~ 62mg), 13h (~95mg), 17h (~54mg), 19h (~65mg), 21 (~ 65mg) and next day again at 04h (~ 106mg) and 06h (~ 72mg), while the troughs were recorded at 06 hr (~ 48mg), 08 hr (~ 55mg), 11h (~ 35mg) 15-16h (~ 46mg), 18h (~39mg), 20 hr (~ 46mg), 23h (~ 44mg) and next day at 05h (~ 58mg). Under LL, the total carbohydrate rhythm showed 6 peaks and 7 troughs during the 24h free running period. The peaks occured at 08h (~ 83mg), 14h (~ 55mg), 20h (~ 52mg), 23h (~ 54mg) and next day at 02h (~ 70mg) and 04h (~ 57mg). Troughs appeared at 06h (~ 45mg), 12h (~ 28mg), 18h (~ 47mg), 22h (~ 44mg), 00h (~ 51mg) and next day at 03h (~ 52mg) and 06h (~ 44mg). Under DD, the total carbohydrate rhythm showed 8 peaks and 8 troughs during the 24h free running time. Peaks appeared at 08 h (~ 76mg), 14-15h (~ 62mg), 17-18h (~ 63mg), 20h (~ 73mg), 22h (~ 83mg) and next day at 02h (~ 47mg), 04h (~ 56mg) and 06h (~ 54mg). Troughs appeared at 06h (~ 47mg), 12h (~ 22mg), 16h (~ 53mg), 19h (~ 50mg), 21h (~ 62mg), 00h (~ 31mg) and next day at 03h (~ 40mg) and 05h (~ 43mg). The interval between peaks was about 2.9h under LD, 3.3h under LL and 2.8h under DD and that between troughs was about 3.0h under LD, 3.4h under LL and 2.9h under DD. The combined mean interval of peaks and troughs was about 2.9h under both LD and DD and 3.4h under LL (Tables 2A, 2B and 5).

Circadian amylase rhythms

Gut wall

Under LD the rhythm of α-amylase activity showed 7 peaks and 7 troughs in the gut wall during the 24h free running period of the rhythm (Fig. 2A). Peaks appeared at 07h (0.85μ moles) 10h (1.65μ moles), 15h (1.21μ moles), 17 hr (1.78μ moles), 21h (0.55μ moles) and next day at 01h (0.69 μ moles) and 06h (1.13 μ moles) and troughs appeared at 06h (0.47 μ moles), 09h (0.54 μ moles), 12h (0.23 μ moles), 16h (0.53 μ moles), 19h (0.34μ moles), 23h (0.31μ moles) and next day at 04h (0.26 μ moles). Under LL the rhythm showed 9 peaks and 8 troughs during the 24h free running time. The peaks appeared at 06h (1.15μ moles), 10h (0.90 μ moles), 14h (1.58 μ moles), 17h (2.40 μ moles), 20h (0.66 μ moles), 00h (0.74 μ moles) and next day at 02h (0.76μ moles), 04h (0.76μ moles) and 06h (0.68μ moles), while the troughs appeared at 09h (0.29μ mole), 13h (0.23μ moles), 15h (1.07μ moles), 19h

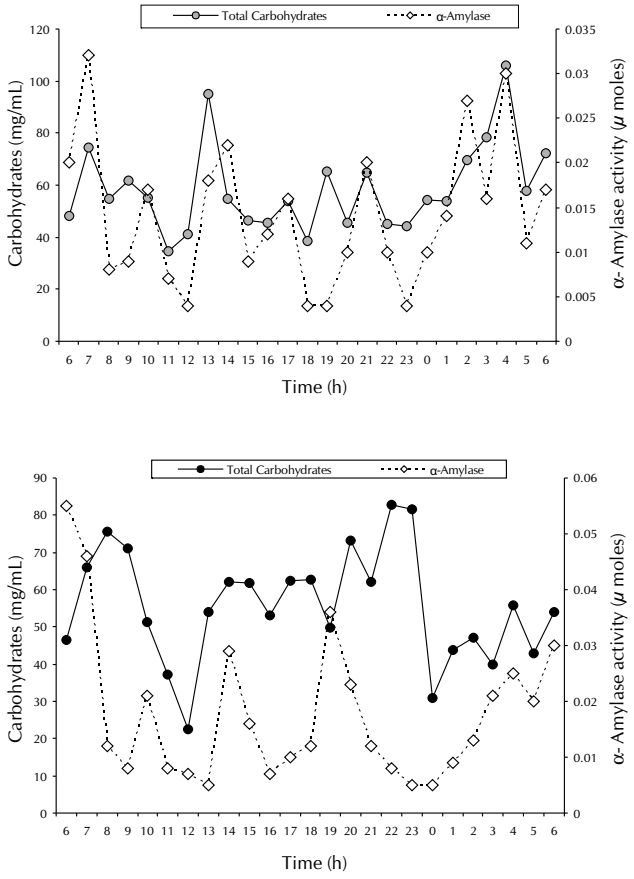

Figure 4: Circadian changes in total carbohydrate profiles and á-amylase activity in the gut content of the fifth instar larva of *Bombyx mori,* under (A) 12h light: 12h dark cycle (LD), (B) continuous light and (C) continuous dark (DD) conditions. The values expressed in mg per ml of tissue, represent the24h (6 AM on day 5 to 6 A.M on day 6) free running time of the circadian rhythm. (p <0.001)

(0.44μ moles), 22-23h (~ 0.38μ moles) and next day at 01h (0.38μ moles), 03h (0.37μ moles) and at 05h (0.44μ moles). Under DD, the α-amylase activity rhythm showed 7 peaks and 7 troughs during the 24h free running period. The peaks appeared at 07h (1.27μ moles), 10h (0.68μ moles), 14h (1.20μ moles), 17h (2.93μ moles), 21h (2.32μ moles), 00h (0.67μ moles) and next day at 06h (1.01μ moles). The troughs appeared at 06h (0.96μ moles), 08h (0.33μ moles), 11-12h (~ 0.36μ moles), 16h (0.40μ moles), 18h (0.29μ moles), 22h (0.28μ moles) and next day at 03h (0.38μ moles). The interval between peaks was about 3.3h under LD, 2.7h under LL and 3.3h under DD and that between troughs was about 3.1h under LD, 2.5h under LL and 3.0h under DD. The combined mean interval of peaks and troughs was about 3.2h under both LD and DD and 2.6h under LL (Tables 3A, 3B and 6).

Gut content

Under LD, the α-amylase activity showed 8 peaks and 8 troughs in the digestive juice (gut content) during the 24 h free running period of the rhythm (Fig.2B). Peaks appeared at 07h (0.032 μ moles) 10h (0.017μ moles), 14h (0.022μ moles), 17h (0.016μ moles), 21h (0.020μ moles) and next day at 02h (0.027μ moles), 04h (0.030μ moles) and 06h (0.017μ moles) and troughs appeared at 06h (0.020μ moles), 08h (0.008μ moles), 12h (0.004μ moles), 15h (0.009μ moles), 18-19h (0.004μ moles), 23h (0.004μ moles) and next day at 03h (0.016μ moles) and 05h (0.011μ moles). Under LL the enzyme activity showed 8 peaks and 7 troughs in the gut content during the 24h free running period of rhythm. Peaks appeared at 06h (0.034μ moles), 14h (0.035μ moles), 16h (0.039μ moles), 21h (0.034μ moles), 23h (0.013μ moles) and next day at 01h (0.034μ moles), 04h (0.024 μ moles) and again at 06h (0.037μ moles) and the troughs occurred at 12h (0.004μ moles), 15h (0.029μ moles), 19h (0.005μ moles), 22h (0.009μ moles), 00h (0.010μ moles) and next day at 02h (0.011μ moles) and again at 05h (0.014μ moles). Under DD the α-amylase activity rhythm showed 6 peaks and 5 troughs in the gut content during the 24h free running period. While peaks appeared at 06h (0.055μ moles), 10h (0.021μ moles), 14h (0.029μ moles),

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

Photo period	No. of peaks	Interval between peaks in h							Mean interval in h
		1-2	2-3	3-4	4-5	5-6	6-7	7-8	
LD	6	4	5	5	2	6	-	-	3.7
LL	8	3	2	4	2	4	5	3	2.9
DD	7	2	3	5	7	5	2	-	3.4

Photo period	No. of troughs	Interval between troughs in h							Mean interval in h
		1-2	2-3	3-4	4-5	5-6	6-7	7-8	
LD	6	4	3	5	3	6	-	-	3.5
LL	8	3	3	3	3	4	5	2	2.9
DD	6	3	2	7	8	2	-	-	3.7

Table 2 (A and B): Interval between peaks (Table 2 A) and troughs (Table 2 B) in the levels of total carbohydrates in the gut content of the fifth instar larva of *Bombyx 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.

Photo period	No. of peaks	Interval between troughs in h							Mean interval in h
		1-2	2-3	3-4	4-5	5-6	6-7	7-8	
LD	8	2	4	4	2	2	7	2	2.9
LL	6	6	6	3	3	2	-	-	3.3
DD	8	6	4	2	2	4	2	-	2.8

Photo period	No. of troughs	Interval between troughs in h							Mean interval in h
		1-2	2-3	3-4	4-5	5-6	6-7	7-8	
LD	8	2	3	5	2	2	3	6	3.0
LL	7	6	6	4	2	3	3	-	3.4
DD	8	6	4	3	2	3	3	2	2.9

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

Photo period	No. of peaks	Interval between peaks in h								Mean interval in h
		1-2	2-3	3-4	4-5	5-6	6-7	7-8	8-9	
LD	7	3	5	2	4	4	5	-	-	3.3
LL	9	4	4	3	3	4	2	2	2	2.7
DD	7	3	4	3	4	3	6	-	-	3.3

Photo period	No. of troughs	Interval between troughs in h							Mean interval in h
		1-2	2-3	3-4	4-5	5-6	6-7	7-8	
LD	7	3	3	4	3	4	5	-	3.1
LL	8	4	2	4	4	2	2	2	2.5
DD	7	2	4	4	2	4	5	-	3.0

Table 4 (A and B): Interval between peaks (Table 3 A) and troughs (Table 3 B) in the activity levels of *á*-Amylase in the gut content of the fifth instar larva of Bombyx mori during the free running time of the circadian rhythm under 12h light/ dark cycle (LD), continuous light (LL) and continuous dark (DD) conditions

Photo period	No. of Peaks	Interval between Peaks in h							Mean interval in h
		1-2	2-3	3-4	4-5	5-6	6-7	7-8	
LD	8	3	4	3	4	5	2	2	2.9
LL	8	8	2	5	2	2	3	2	3.0
DD	6	4	4	5	9	2	-	-	4.0

Photo period	No. of troughs	Interval between troughs in h							Mean interval in h
		1-2	2-3	3-4	4-5	5-6	6-7	7-8	
LD	8	2	4	3	4	4	4	2	2.9
LL	7	3	4	3	2	2	3	-	2.4
DD	5	4	3	8	5	-	-	-	4.0

Table 5: Comparative analysis of the phase response curves of the carbohydrate rhythm in the gut wall and gut content of the fifth instar larva of *Bombyx mori*, in terms of mean number of peaks and troughs and the mean interval between them, under 12h light / dark cycle (LD), continuous light (LL) and continuous dark (DD) conditions.

Parameter	Gut wall			Gut content		
	LD	LL	DD	LD	LL	DD
No. of peaks	6	8	7	8	6	8
No. of troughs	6	8	6	8	7	8
Mean interval b/w peaks (h)	3.7	2.9	3.4	2.9	3.3	2.8
Mean interval b/w troughs (h)	3.5	2.9	3.7	3.0	3.4	2.9
Combined mean interval b/w peaks and troughs (h)	3.6	2.9	3.6	2.9	3.4	2.9
Probable no. of CS/CU cycles	6	8	7	8	6	8
Approximate time taken for each CS/CU cycles (h)	4.0(24/6 = 4)	3.0(24/8 = 3.0)	3.4(24/7 = 3.4)	3.0(24/8 = 3.0)	4.0(24/6 = 4)	3.0(24/8 = 3.0)
Free running time of rhythm (h)	24(4x6 = 24)	18.0(3x6 = 18)	20.4(3.4x6 = 20.4)	24(3x8 = 24)	32(4x8 = 32)	24(3x8 = 24)
Mean peak value	15.6	15.6	21.4	74.2	61.7	64.2

CS Cycles: Carbohydrate synthetic cycles; CU cycles: Carbohydrate uptake cycles; **Note**: The combined mean intervals between peaks and troughs are roughly equal to the calculated time taken for completion of each CS / CU cycles

Table 6: Comparative analysis of the phase response curves of the *á*-amylase rhythm in the gut wall and gut content of the fifth instar larva of *Bombyx mori*, in terms of mean number of peaks and troughs and the mean interval between them, under 12h light / dark cycle (LD), continuous light (LL) and continuous dark (DD) conditions

Parameter	Gut wall			Gut content		
	LD	LL	DD	LD	LL	DD
No. of Peaks	7	9	7	8	8	6
No .of Troughs	7	8	7	8	7	5
Mean interval b/w Peaks (h)	3.3	2.7	3.3	2.9	3.0	4.0
Mean interval b/w Troughs (h)	3.1	2.5	3.0	2.9	2.4	4.0
Combined mean interval b/w Peaks and Troughs (h)	3.2	2.6	3.2	2.9	2.7	4.0
Probable no. of AS/AR cycles	7	9	7	8	8	6
Approximate time taken for each AS/AR cycles (h)	3.4(24/7 = 3.4)	2.6(24/9 = 2.6)	3.4(24/7 = 3.4)	3.0(24/8 = 3)	3.0(24/8 = 3)	4.0(24/6 = 4)
Free running time of Rhythm (h)	24(3.4x7 = 24)	18.2(2.6x7 = 18.2)	24(3.4x7 = 24)	24(3x8 = 24)	24(3x8 = 24)	32(4x8 = 32)
Mean Peak value	1.12	1.07	1.44	0.023	0.031	0.033

AS cycles: Amylase synthetic cycles; AR cycles: Amylase release cycles; **Note**: The combined mean intervals between peaks and troughs are roughly equal to the calculated time taken for completion of each AS / AR cycles

19h (0.036μ moles) and next day at 04h (0.025μ moles) and 06h (0.030μ moles), the troughs appeared at 09h (0.008μ moles), 13h (0.005μ moles), 16h (0.007μ moles), 23-00h (0.005μ moles) and next day at 05h (0.020μ moles). The interval between peaks was about 2.9h under LD, 3.0h under LL and 4.0h under DD and that between troughs was about 2.9h under LD, 2.4h under LL and 4.0h under DD. The combined mean interval between peaks and troughs was about 2.9h under LD, 2.7h under LL and 4.0h under DD (Tables 4A, 4B and 6).

DISCUSSION

In the silkworm and other insects, the gut acts as an organ of synthesis, storage, secretion and absorption for a multitude of biochemical constituents including the products of digestion (Nagata and Yashitake, 1989; Narayanaswamy and Shankar, 2010). The present study on the circadian profiles of total carbohydrates and α-amylase activity confirms the existence 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) and their rhythmic changes are modulated by light-sensitive circadian clocks (Edmunds, 1998; Dunlap, 1999; Sehadova *et al.*, 2004; Iwai *et al.*, 2006; Weng *et al.*, 2009). The analysis of phase response curves in terms of number of peaks, interval between peaks and troughs, height of peaks and mean peak value provide meaningful insights into the photoperiod modulated rhythmic changes in carbohydrate and amylase profiles (Figs. 1 and 2 and Tables 1 to 6). Keeping in view the recent reports emanated from our laboratory (Sailaja and Sivaprasad, 2010, a, b., Sailaja and Sivaprasad, 2011; Sailaja *et al.*, 2011; Sivaprasad and Sailaja, 2011; Bhuvaneswari and Sivaprasad, 2012), the number of peaks in carbohydrate rhythm were analyzed in term of the number of carbohydrate synthetic cycles (CS cycles) in the gut wall and number of carbohydrate uptake cycles (CU cycles) in the gut content. Similarly, the number of peaks in amylase activity was interpreted in terms of amylase synthetic cycles (AS cycles) in the gut wall and amylase release cycles (AR cycles) in the gut content. In both the compartments (gut wall and gut lumen) of digestive system, the height of peaks were interpreted in terms of intensity of synthetic/release/uptake cycles and the mean peak value in terms of the average levels of carbohydrates or amylase activity maintained during the 24h free running time of the biochemical rhythm. Likewise, the combined mean intervals between peaks and troughs were considered as the time taken for completion of synthetic/ uptake/ release cycles, the durations of which are almost similar to those obtained by dividing the 24h free running time of the rhythm with the number of such cycles in a day (Tables 5 and 6).

Circadian carbohydrate rhythm

Gut wall

Mulberry leaf, the sole food of *B. mori* comprises pectin, xylan, sucrose, cellulose and starch and it undergoes chemical treatment within the digestive system and get converted to simple monosaccharides like glucose which are later transported to the gut wall and stored as in the form of storage sugars called trehalose and glycogen (Yamashita and Hasegawa, 1974; Thompson, 2003; Anand *et al.*, 2010). Obviously, the carbohydrate turnover in the gut wall cells involves the synthesis of these two storage carbohydrates (trehalose and glycogen) in a cyclic fashion, that is represented as carbohydrate synthetic cycles (CS cycles) in the current report (Fig. 1A). The carbohydrate rhythm maintains 6 CS cycles under LD with 4h duration each, 8 cycles under LL with duration of 3h each and 7 cycles under DD with duration of 3.4h each. Thus, both LL and DD conditions modulate carbohydrate synthesis by reducing duration of CS cycles; the LL does so by 60 min (from 4h to 3h) and DD by 36 min (from 4h to 3.4h). Due to shortening of the duration of all CS cycles in a day, the normal 24h free running time of carbohydrate rhythm under LD is clock-shifted to 18 hr under LL and 20.4 hr under DD (Table 5). Clearly, within free running time of the rhythm, the gut wall cells are able to accomplish two additional rounds (CS cycles) under LL and one under DD.

Further analysis of peaks in terms of their height reveals that the intensity of carbohydrate synthesis increases, more particularly during the scotopic (dark) period of the day and such intense phases are associated with the accumulation of storage carbohydrates in the gut wall cells. For instance, the carbohydrate rhythm under LD includes two active synthetic phases, one at19- 22h (15.5 to16.9mg) and other at 03-06h

(17.1to21.7mg). Similarly two active synthetic cycles appear under LL condition at 20-21h (14.1 to 19.1mg) and 02-06h (14.7 to 20.8mg). But in DD condition three such active phases appear 15-17h (17.2 to 30.9mg), 22-23hr (25.9 to 26.2mg) and 04-06h (17.6 to 27.0mg). The photoperiodic conditions not only altered the number of peaks (Iwai *et al*., 2006) but also the mean peak value (MPV) of the metabolite during the operation of its rhythm. For instance, the MPV of carbohydrates is significantly high under DD (21.4mg) moderate under LD and LL (15.6mg), a fact that indicates that dark period is essential for the synthesis of storage carbohydrates much like those of proteins as suggested in our recent report (Bhuvaneswari and Sivaprasad, 2012). The light condition seems to have disturbing effect on the synthetic activity, through it causes more number of CS cycles that are less intense than those in darkness.

Gut content

The gut content in the silkworm represents the digestive juice secreted by the gut wall and dietary nutrients and water emanated from the mulberry leaves. It consists of a variety of food molecules like proteins, carbohydrates (pectin, xylan, cellulose, sucrose, glycogen and trehalose), lipids, vitamins, minerals and different digestive enzymes (Ito, 1972; Anand *et al*., 2010). The presence of large number of biochemical constituents make it virtually a biochemical soup, the composition of which varies from time to time based on dietary inputs and secretory products. It is presumed that the carbohydrate uptake from the diet follows a cyclic path and it is influenced by the quantum of mulberry leaf consumed and the rate at which it is consumed (Sashindran Nair *et al*., 2004; Narayanaswamy and Shankar 2010). The photoperiod seems to cause stimulating effect on these two parameters. In the gut content the carbohydrate rhythm maintained 8 CU cycles each under LD and DD and 6 under LL and the time taken for each cycle was about 3.0h under LD and DD and 4.0h under LL. Thus, light affects carbohydrate uptake from the gut content in two ways. Firstly, it reduces the number of uptake cycles from 8 to 6 and secondly it delays the duration of each cycle by 1h. This combined effect results in shifting the free running time of carbohydrate rhythm from normal 24h-pattern to 32h under LL (Table 5). An in-depth analysis of PRC of total carbohydrates in the gut content (Fig. 1B) reveals the presence of two active phases of carbohydrate uptake from the diet during the free running of the rhythm, much like that of the gut wall. The timing of these active phases changes according to the duration of light exposed (Iwasaki and Thomas, 1997). For instance, the silkworm larvae reared under LD, showed active phases at 13h (95.1mg) and 03-04h (78.4 to106.2mg) and those reared under LL at 07-09h (70.9 to 82.5mg) and 01-02h (68.2 to 70.2mg). Similarly in the larvae reared under DD, the active uptake phases occurred at 07-09h (66.0 to 75.7 mg) and 20-23h (62.2 to 82.8mg). Thus, in all photoperiodic conditions, at least one active phase occurred in light condition and one in dark condition. Further the carbohydrate levels also vary as a function of light duration. For instance, the mean peak values (MPVs) were higher under LD (74.2 mg) moderate under DD (64.2 mg) and lower under LL (61.7mg). Presumably, optimal levels of carbohydrates are maintained in the gut content of *B. mori*, both under light and dark conditions as reported in other insects (Fonagy, 2009).

Circadian amylase rhythm

Gut wall

The gut wall is the major site of gene expression for the synthesis and release of most digestive enzymes including α-amylase. The genes of digestive enzymes are known to express at regular intervals and their products are synthesized and released into the gut lumen in a time bound manner (Xia *et al*., 2007). The production of quality silk and improvements in cocoon economic traits, shell weight and shell percentage depends upon the utilization of carbohydrate reserve food materials through enhanced activity of α-amylase that releases individual glucose molecules from the linear glucose chains (Hirata and Yosuo, 1974; Chatterjiee and Datta, 1992; Sashindran Nair *et al*., 2004). Probably, the α-amylase genes in the gut wall express at regular intervals and triggers amylase synthesis in a cyclic fashion during the free running time of the rhythm. Accordingly, α-amylase rhythm maintains 7 AS cycles under both LD and DD conditions and 9 cycles under LL, each cycle lasting for duration of 3.4h under LD and DD and 2.6h under LL (Fig. 2A). Due to reduction in the duration of each AS cycle by 48 min (3.4h to 2.6 h), the 24 h free running time of amylase rhythm of LD and DD conditions is reduced to 18.2h under LL (Table 6). In tune with rescheduling of amylase rhythm, the number and timing of active synthetic phases also altered. The amylase rhythm showed 3 active synthetic phases under LD, at 10-11h (1.12-1.65μ moles) and 05-06 (0.50-1.13μ moles), one under LL at 14-18h (1.07-2.4μ moles) and two such phases under DD at17h (2.93 μ moles) and 20-21h (1.62 to 2.32 μ moles). Likewise, the levels of enzyme activity represented in the form of mean peak values (MPVs) also altered under three photoperiodic conditions. Significantly, higher enzyme activity (1.44μ moles) was recorded under DD, moderate activity (1.12μ moles) under LD and low activity (1.07μ moles) under LL. Evidently, the dark condition stimulates amylase synthesis in the gut wall cells, similar to those of proteins and protease reported in our recent report (Bhuvaneswari and Sivaprasad, 2012).

Gut content

Soon after the synthesis in the gut wall cells amylase is released into the lumen where it becomes active at an alkaline pH of 9.6-9.8 and breakdown the carbohydrates (Abraham *et al*., 1992). Similar to the release of proteins from tissues into the circulating haemolymph of silk worm (Sailaja *et al*., 2011) the α-amylase rhythm in the gut content maintains amylase release cycles (AR cycles), the number and duration of which vary as a function of the photoperiodic condition. The amylase rhythm included 8 AR cycles under both LD and LL each with duration of 3h and 6 cycles under DD each with duration of 4 hr. Significantly, the DD condition extended the duration each AR cycle by 1h (from 3 to 4h) (Table 6). In addition to rescheduling the free running time of rhythm, the light seems to have stimulating effect on enzyme release from the gut wall cells, in a time-dependent manner (Allada and Emery, 2009). The present study indicates that the amylase rhythm maintains four active releasing phases each under LD and DD conditions five phases under LL at different timings. The active releasing phases occur at 06-07h (0.020-0.032μ moles), 13-14h (0.018 to 0.022μ moles), 20-21h (0.010-0.020μ moles) and 02-04h

(0.016 to 0.030μ moles) under LD condition, at 06-07h (0.030-0.034μ moles), 14-17h (0.029 to 0.039μ moles), 21h (0.034μ moles) 01 hr (0.034μ moles) and at 06 hr (0.037μ moles) under LL conditions, at 06-07h (0.046-0.055μ moles), 14-15h (0.016to 0.029μ moles), 19-20h (0.023-0.036μ moles) and at 04-06h (0.020 to 0.030μ moles) under DD conditions. Our study further confirms that the dark condition ensures greater synthesis and release of amylase into the gut lumen, in order to digest the accumulated carbohydrates during night and day times. This is evidenced by the prevalence of higher MPV in amylase activity under DD (0.033μ moles) and LL (0.031μ moles) conditions compared to that of LD (0.023μ moles).

Our study further demonstrates that the frequency (number of peaks) and intensity (height of peaks) of circadian carbohydrate and amylase rhythms are also subjected to modulation by the light cues probably through the peripheral circadian clock, located in the gut wall of silkworm much like those of other peripheral clocks (Iwasaki and Thomas, 1997; Peschel *et al*., 2009; Fonagy, 2009). This obviously, manifests in the form of circadian changes in the levels of both the substrate (carbohydrate) and enzyme (α-amylase) activity in different compartments of digestive systems in *B. mori*.

Carbohydrate rhythm versus amylase rhythm

Though it is not possible to establish point-to-point correlation between amylase activity and carbohydrate content, a time-dependent and light-sensitive rhythmic change could be traced in their levels. As stated earlier, the carbohydrate rhythm in the gut wall and gut content connote two things; first the synthesis of storage carbohydrates and their intracellular digestion in the gut wall cells and second, the dietary uptake of carbohydrates and their extracellular digestion in the gut lumen (Horie and Watanabe, 1980; Baker, 1991; Terra *et al*., 1996; Thompson, 2003; Bandani *et al*., 2010).

The impact of light on carbohydrate synthesis and amylase activity in the gut wall is more pronounced than that in the gut content. In the gut wall, the amylase activity and carbohydrate levels are inversely related to each other. Such an inverse relationship also exists in between the photoperiod and the free running time of both carbohydrate and amylase rhythms. Interestingly, higher amylase activity levels and lower carbohydrate level were observed during the photic phase, while lower amylase activity levels and higher carbohydrate levels were recorded during scotic phase of the day (Figs. 3 and 4). Undoubtedly the synthesis and storage of carbohydrates occur during the dark phase and their breakdown during the light phase of the day. Our study confirms the fact that the timing of carbohydrate synthesis and degradation are altered by the duration of the photoperiod (Kostal and Shimida, 2001; Saunders, 2002; Syrova *et al*., 2003). For instance, under LD condition, higher amylase activity levels and lower carbohydrate level were observed during the light phase (09-17h) and lower amylase activity levels and higher carbohydrate levels during the dark phase (19h-06h) of the day. Evidently, more and more reserve carbohydrates are synthesized and stored in the gut wall cells during night time and they are intracellularly digested by amylases, more predominantly during day time (Fig. 3A), though amylase is synthesized in darkness, in response to the accumulated carbohydrate reserves. Under LL condition similar trends in the synthesis and degradation of carbohydrates were observed in which the synthetic (lower amylase activity) phase predominantly seen at 20-05h corresponding to dark hours of the day and their degradative phase (through higher amylase activity) at 14-18h corresponding to light hours of the day (Fig. 3B). The dark conditions completely disturbs the amylase rhythm but sustained the carbohydrate rhythm as evidenced by higher MPV of carbohydrates and lower MPV of amylase activity. Though amylase rhythm is not completely suppressed in darkness, the enzyme activity levels increased between 16h and 21h that overlap both light and dark regimes.

In the gut content the carbohydrate and amylase rhythms are governed by the external supply of carbohydrates through the mulberry diet (Nagaraju and Abraham, 1995; Shivakumar and Shamitha, 2011). Since the silkworm is voracious feeder and it is continuously supplied with mulberry leaves, the carbohydrate rhythm fluctuates with the dietary uptake. The availability of amylase in the gut content depends upon on the quantum of carbohydrates present in the gut lumen. In fact the amylase secretion from the gut wall cells is stimulated by carbohydrate rich diet (Ito, 1972). Because of this reason the carbohydrate levels and enzyme activity levels maintain similar rhythmic trends throughout the day, under all the three photoperiodic conditions (Fig. 4). The analysis of the PRCs of carbohydrate and amylase rhythms exposes one fact, i.e the amylase activity is both light- and diet- dependent. For example, its activity continued throughout the day under LD condition due to the availability of at least 12h. Light and adequate carbohydrate nutrients in the gut (Fig. 4A). In the presence of continuous light (LL) the amylase is further activated, with the result the mean carbohydrate levels slightly declined throughout the free running time of the rhythm (Fig 4B). Contrary to LD and LL in DD condition the amylase activity was kept at lower levels resulting in the accumulation of undigested carbohydrates in the gut content. Because of this reason the carbohydrate rhythm maintained higher MPVs throughout its free running time in total darkness (Fig. 4C).

Thus, our study clearly shows that the carbohydrate rhythm in the silkworm expresses in the form of dark-dependent synthesis and light-dependent degradation of storage carbohydrates. Remarkably, darkness favours the accumulation of carbohydrate reserves in the gut wall cells and in the gut lumen; obviously by stimulating storage synthesis in the former and dietary uptake in the latter. Conversely, the light condition favours the removal of carbohydrate reserves from these two compartments by stimulating the synthesis and release of different carbohydrases. The enhanced amylase activity under light condition bear strong testimony to this and together with other enzymes, it facilitates intracellular degradation of storage carbohydrates (Eg. trehalose and glycogen) in the gut wall cells and extracellular digestion of dietary carbohydrates in the gut lumen.

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