Original Research Paper

Volume -10 | Issue - 4 | April - 2020 | PRINT ISSN No. 2249 - 555X | DOI : 10.36106/ijar

Physiology

IMPACT OF HONEY-ENRICHED MULBERRY DIET ON THE DIGESTIVE METABOLISM OF THE SILKWORM, *BOMBYX MORI*

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ABSTRACT The impact of honey-enriched mulberry diet on digestive metabolism of *Bombyx mori* has been studied during fifth instar larval development. The biochemical data on digestive substrates (proteins, carbohydrates, trehalose and cellulose) and digestive enzymes (protease, α-amylase, trehalase and cellulase), have been analyzed in terms of developmental changes vis-à-vis the impact of hone-enriched mulberry diet on such changes. The growth trends in the midgut compartments of gut wall and gut content indicate that all digestive substrates tend to accumulate in the gut wall cells, while the activity levels of corresponding digestive enzymes tend to decline throughout larval development. The positive growth trends in digestive substrates and negative growth trends in enzyme activities indicate that the silkworm digestive metabolism follows Hutchinson's investment principle and accordingly accumulates matter in gut wall as energy reserves for future use. The honey-enriched mulberry diet showed positive impact on matter accumulation in gut wall and digestibility of gut enzymes.

KEYWORDS : *Bombyx mori,* Digestive metabolism, Honey, Midgut.

INTRODUCTION

Bombyx mori uses the dietary mulberry leaf as the source of raw material for silk production. The digestive system is the route through which the dietary inputs are processed and channelized for silk production in the silk gland. In essence, the digestive system supplies finer raw materials and energy reserves required for the silk synthesis. This it does so, by actively synthesizing and secreting digestive enzymes, enhancing their digestibility in the gut lumen and by facilitating the absorption of the digested food materials into the gut wall and their final transportation to silk gland for silk production (Takano and Akai, 1978). Thus, in ***B. mori*** the silk productivity largely depends on the efficacy of the digestive metabolism.

The insect gut acts as a transient region for dietary proteins, carbohydrates, lipids and other biochemical constituents, whose concentration varies as a function of synthesis and secretion of digestive juices and the relative ability of the gut to uptake such digested materials. The gut wall and gut lumen act as two functional compartments for effectively discharging the functions of digestion and absorption. In this endeavour, the glandular epithelium of gut wall synthesizes and releases digestive enzymes such as proteases, amylases and lipases into the gut lumen at regular intervals, while the latter, with its vast majority of ingested food materials like proteins, lipids, carbohydrates, cellulose, pectin, vitamins, minerals and ions represents the major site of digestion (Terra and Ferreira, 2005). Obviously, the mulberry diet of silkworm is subjected to chemical treatment in its midgut, before it is processed for silk production in the silk gland (Cermenati *et al.*, 2007). Obviously, the enhanced rate of digestive and oxidizing enzymes in silkworm larval midgut helps in the utilization of more food materials and efficient consumption of digested food material, ultimately leading to superior economic traits of sericulture.

In view of the importance of digestive metabolism in the larval growth, metamorphosis and silk production, the silkworm digestive physiology has emerged as an important area of research in sericulture (eg. Sarangi and Anitha, 2007; Manjula *et al*., 2010; Buvaneswari and Sivaprasad, 2013; Bhuvaneswari *et al*., 2013). Further, the role of honey in enhancing the digestive function has been studied with reference to silkworm growth, metabolism and silk production (Thulasi and Sivaprasad, 2015; Madhavi *et al*., 2018). However, no effort has since been made to analyze the impact of honey on silkworm digestive metabolism with reference to the rules governing the growth and body mass accumulation. Present investigation was taken up against this backdrop.

MATERIAL AND METHODS

Experimental design: The Pure Mysore x CSR_2 hybrid variety of the silkworm ***Bombyx mori,*** reared under standard environmental conditions of 28°C and relative humidity of 85%, was used as the test species. 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. The third instar larvae were divided into two zero dose control (ZDC) and honey-fed experimental (HFE) batches, each comprising 100 worms and the 2 PM diet in respect of HFE batch was replaced by the 2% honey-enriched mulberry leaf as per Madhavi *et al*., (2018).

Assay of biochemical constituents: Biochemical assays on proteins, carbohydrates, trehalose and cellulose and their respective enzymes were carried out on gut wall and gut content, simultaneously in the larvae of both ZDC and HFE batches. The gut wall tissue was isolated by mid-dorsal dissection the silkworm larvae in ice cold Silkworm Ringer while, the gut content was extracted from the gut through a hypodermic syringe by inserting it into its lumen. The total proteins were estimated by the method of Lowry *et al.,* (1951) in 1% homogenate of gut wall in distilled water and 1 ml of gut content diluted with 9 ml of distilled water and the values were expressed as mg /g wet weight of the tissue or 1 ml of gut content. The Protease activity was estimated by the method of Davis and Smith (1955) in 5% homogenate of gut wall in ice cold distilled water and 1:19 diluted gut content in distilled water and the enzyme activity was expressed in µ moles of tyrosine / mg protein /h. The levels of total carbohydrates were estimated by the method of Carroll *et al.,* (1956) in 5% homogenate of gut wall in 10% trichloroacetic acid (TCA) and 1:19 diluted gut content in 10% TCA and the values were expressed as mg / g wet weight of tissue or 1 ml of gut content. The α-amylase activity was estimated by the method of Bernfeld (1955) in 2% homogenate of gut wall in 0.05M acetate buffer and in 1:9 diluted gut content in acetate buffer and the values were expressed in µ moles of maltose/mg carbohydrates/h. Trehalose levels were estimated by the method of Roe (1955) in 2% homogenate of gut wall in ice-cold distilled water and 1:19 diluted gut content in ice-cold distilled water and values were expressed as mg glucose / g wet weight of tissue or 1 ml of gut content. The trehalase activity was estimated by the method of Dahlman (1971) in 5% homogenate of gut wall in 0.05M phosphate buffer and 1:9 diluted gut content in phosphate buffer and the values were expressed in µ moles of glucose/ mg trehalose / h. Cellulose levels were estimated by the method of Updegroff (1969) in 5% homogenate of gut wall in an acetic nitric reagent and 1:9 diluted gut content in acetic nitric reagent and the values were expressed as mg glucose/ g wet weight of tissue or 1 ml of gut content. The cellulase activity was estimated by the method of Miller (1959) in 5% homogenate of gut wall in 0.05M phosphate buffer and 1:9 diluted gut content in phosphate buffer and the values were expressed in µ moles of glucose / mg cellulose / h.

Statistical analysis: The experimental data were statistically analyzed by mean, standard deviation (SD), percent change and test of

significance using M.S. Excel platform and online software packages (www, Graph pad. com / quick calcs / index cfm / and www.percent change com / index php). With a view to assess day-to-day changes and to arrive at meaningful conclusions, the digestive parameters were analyzed in terms of an innovative growth parameter called Compound Periodical Growth Rate (CPGR) as given by Sivaprasad (2012).

RESULTS

The growth trends in the levels of different biochemical constituents of digestive are analyzed in terms of overall growth rates (OGRs) and compound periodical growth rates (CPGRs) and presented in tables 1 to 4 and figures 1 and 2.

Total proteins and protease activity

The levels of total proteins and protease activity showed contradictory growth trends in both the compartments of the midgut. While proteins levels recorded elevatory growth trends, the enzyme activity showed clear declining trends throughout the fifth instar.

Gut wall: In the gut wall tissue, the levels of total proteins increased from 13.98 mg/g to 70.31 mg/g, representing a CPGR of 30.89% in the ZDC batch and from 10.88 mg/g to 61.82 mg/g, with a higher CPGR of 33.58% in the HFE batch during the 7-day period of fifth instar. Interestingly, the rate of increase in protein levels in the first half was more pronounced than that in the second half of fifth instar. In the first half, the protein levels registered an OGR of about 310% (CPGR: 60.03%) in the ZDC batch and a higher OGR of about 348% (CPGR: 68.84%) in the HFE batch. In the second half, their levels grew by about 23% (CPGR: 7.06%) in the ZDC batch and by about 27% (CPGR: 8.26%) in the HFE batch (Table 1). In contrast, the protease activity in gut wall cells declined from 0.72 µm/mg proteins/h to 0.26 µm /mg proteins /h, representing a CPGR of -10.91% in ZDC batch and from 0.77 µm/ mg proteins/ h to 0.29 µm/ mg proteins/ h, representing a CPGR of -15.52% in the HFE batch. The rate of decline in enzyme activity was more conspicuous in the second half compared to that in the first half of fifth instar. In the first half, the protease activity registered a decline of about 22% (CPGR: 8.04%) in the ZDC batch and of about 21% (CPGR: 7.47%) in the HFE batch. But in the second half, the enzyme activity witnessed a higher drop of about 54% (CPGR: 22.57%) in the ZDC batch and about 52% (CPGR: 21. 95%) in the HFE batch (Table 1).

Table 1: Impact of honey-enriched mulberry diet on total proteins and protease activity in the midgut of *Bombyx mori* during fifth instar development

Stage	Statistical tool	Total Proteins (mg/g or mg/ml)				Protease Activity (µm/mg protein/h)			
		ZDC Batch		HFE Batch		ZDC Batch		HFE Batch	
		Gut wall	Gut content	Gut wall	Gut content	Gut wall	Gut content	Gut wall	Gut content
1	2	3	4	5	6	7	8	9	10
Day 1 (Early)	Mean S.D (±)	13.98 0.525	1.04 0.100	10.88 0.89	0.81 0.073	0.72 0.051	0.28 0.009	0.77 0.017	0.29 0.008
Day 4 (Mid)	Mean OGR (%) S.D (±) CPGR (%)	57.29 309.7 1.29* 60.03	8.45 712.5 0.073* 101.04	48.73 347.8 1.45* 68.84	7.95 881.4 0.15* 114.11	0.56 -22.22 0.022** -8.04	0.05 -81.27 0.005* -43.69	0.61 -20.78 0.008* -7.47	0.06 -76.09 0.006* -40.86
Day 7 (Late)	Mean OGR (%) S.D (±) CPGR (%)	70.31 22.73 1.11* 7.06	10.25 21.31 0.089* 6.65	61.82 26.86 1.22* 8.26	9.91 24.65 0.17* 7.62	0.26 -53.57 0.013* -22.57	0.02 -62.26 0.0* -26.32	0.29 -52.45 0.013* -21.95	0.03 -50.91 0.005* -20.63
Overall CPGR %		30.89	46.42	33.58	51.80	-10.91	-35.59	-15.52	-28.79

** Statistically significant (P value <0.001); ** statistically not significant.*

Protein values represent the mean ± standard deviation (SD) of four separate observations. For each observation tissue from 10 to 15 larvae was pooled. The percent change (PC) for each period was calculated taking its previous value as the control, while CPGR was separately calculated for two successive periods in fifth instar (from early to mid and from mid to late) and for whole instar (from day 1 to day 7).

Gut content: In the lumen compartment of midgut, the levels of total proteins increased from 1.04 mg/g to 10.25 mg/g, representing a CPGR of 46.42% in the ZDC batch and from 0.81 mg/g to 9.91 mg/g, with a higher CPGR of 51.80% in the HFE batch during the 7-day period of fifth instar. Like that of the gut wall, the rate of increase in protein levels of gut content in the first half was more significant, compared to that in the second half of the fifth instar. In the first half, its protein levels registered an OGR of about 713% (CPGR: 101.04%) in the ZDC batch and a higher OGR of about 881% (CPGR: 114.81%) in the HFE batch. In the second half, their levels grew by about 21% (CPGR: 6.65%) in the ZDC batch and by about 25% (CPGR: 7.62%) in the HFE batch (Table 1). During fifth instar growth, the protease activity in gut content dropped from 0.28 µm/mg proteins/h to 0.02 µm/mg proteins/h, representing a CPGR of -35.59% in ZDC batch and from 0.29 µm/mg proteins/h to 0.0.03 µm/mg proteins/h, representing a CPGR of -28.79% in the HFE batch. The declining rate was more pronounced in the first half compared to that in the second half of instar. In the first half, the protease activity registered a decline of about 81% (CPGR: 43.69%) in the ZDC batch and of about 76% (CPGR: 40.86%) in the HFE batch. In the second half, the enzyme activity dropped by about 62% (CPGR: 26.32%) in the ZDC batch and by about 51% (CPGR: 28.79%) in the HFE batch (Table 1).

Total carbohydrates and α-amylase activity

The levels of total carbohydrates and α-amylase activity showed opposing growth trends in gut wall and gut lumen in both ZDC and HFE batches.

Gut wall: In the gut wall tissue, the levels of total proteins increased from 11.09 g/mg to 15.56 mg/g, representing a meager CPGR of 5.65% in the ZDC batch and from 13.73 mg/g to 19.49 mg/g, with a relatively higher CPGR of 6.01% in the HFE batch. Interestingly, the increasing trends in carbohydrate levels followed a slow phase in the first half and a faster phase in the second half of the instar. In the first phase, their levels registered an OGR of about 14% (CPGR: 4.36%) in the ZDC batch and a lower OGR of about 6% (CPGR: 1.88%) in the HFE batch. In the second half, their levels grew at a rate of about 22% (CPGR: 6.95%) in the ZDC batch and by about 34% (CPGR: 10. 31%) in the HFE batch (Table2).

Table 2: Impact of honey-enriched mulberry diet on total carbohydrates and α-amylase activity in the midgut of *Bombyx mori* during fifth instar development

Stage	Statistical tool	Total carbohydrates (mg glucose /g or ml)				α-amylase Activity *(µ moles of maltose /mg carbohydrate / hr)*			
		ZDC Batch		HFE Batch		ZDC Batch		HFE Batch	
		Gut wall	Gut content	Gut wall	Gut content	Gut wall	Gut content	Gut wall	Gut content
1	2	3	4	5	6	7	8	9	10
Day 1 (Early)	Mean S.D (±)	11.19 0.21	117.80 1.15	13.73 0.39	165.90 1.47	2.11 0.18	0.09 0.007	2.78 0.15	0.09 0.006

Day 4 (Mid)	Mean OGR (%) S.D (±) CPGR (%)	12.72 13.67 0.36* 4.36	137.20 16.47 1.11* 5.21	14.52 5.75 0.38* 1.88	185.7 11.90 4.39* 3.83	0.63 -70.14 0.039* -33.16	0.02 -77.78 0.0005* 39.43	1.04 -62.77 0.042* -27.95	0.02 -77.78 0.0006* 39.43
Day 7 (Late)	Mean OGR (%) S.D (±) CPGR (%)	15.56 22.33 0.32* 6.95	45.55 -66.95 1.73* -30.76	19.49 34.23 0.38* 10.31	56.00 -69.84 1.57* -32.94	0.12 -80.95 0.029* -42.46	0.02 0.0 0.0005* 0.0	0.60 -42.03 0.009* -16.75	0.02 0.0 0.0005* 0.0
Overall CPGR %		5.65	-14.65	6.01	-16.56	-37.99	-22.17	-22.55	-22.17

** Statistically significant (P value <0.001); ** statistically not significant.*

Carbohydrate values represent the mean ± standard deviation (SD) of four separate observations. For each observation tissue from 10 to 15 larvae was pooled. The percent change (PC) for each period was calculated taking its previous value as the control, while CPGR was separately calculated for two successive periods in fifth instar (from early to mid and from mid to late) and for whole instar (from day 1 to day 7).

In contrast, the α-amylase activity in gut wall declined significantly from 2.11 µm/mg carbohydrates/h to 0.12 µm/mg carbohydrates/h, representing a CPGR of -37.99% in ZDC batch and from 2.78 µm to 0.60 µm, representing a CPGR of -22.55% in the HFE batch. The rate of decline in enzyme activity was maximal in the second half compared to that in the first half of instar. In the first half, the α-amylase activity registered a decline of about 70% (CPGR: 33.16%) in the ZDC batch and about 63% (CPGR: 27.95%) in the HFE batch. In the second half, it registered a higher drop of about 81% (CPGR: 42.46%) in the ZDC batch and a lower drop of about 42% (CPGR: 16.75%) in the HFE batch (Table 2).

Gut content: The carbohydrate levels decreased from 117.80 g/mg to 45.55 mg/g, representing a CPGR of -14.65% in the ZDC batch and from 165.90 mg/g to 56.0 mg/g, with a more negative CPGR of -16.56% in the HFE batch during fifth instar. Surprisingly in a significant move, their levels registered elevatory trends in the first half and declining trends in the second half of the instar. Clearly, in the first half, their levels registered an OGR of about 16% (CPGR: 5.21%) in the ZDC batch and a lower OGR of about 12% (CPGR: 3.83%) in the HFE batch. However, in the second half, their levels registered a drop of about 67% (CPGR: 30.76%) in the ZDC batch and by about 70% (CPGR: 32.94%) in the HFE batch (Table 2). The α-amylase activity in gut content dropped from 0.09 µm/mg carbohydrates/h to 0.02 µm, representing a CPGR of -22.17% both in the ZDC and HFE batches. The rate of decline in its activity was more rapid in the first half and almost zero in the second half. In the first rapid phase, the α-amylase activity registered a decline of about 78% (CPGR: 39.43%) in both ZDC and HFE batches. However, its activity becomes zero during the second half of the instar (Table 2).

Trehalose and trehalase activity

Trehalose levels increased continuously during fifth instar, but the activity levels of its hydrolyzing enzyme, trehalase declined sharply by the instar-end.

Gut wall:

In the gut wall cells, the accumulation of trehalose levels increased from 22.10 g/mg to 79.10 mg/g, representing a daily increasing rate (CPGR) of 23.68% in the ZDC batch and from 28.10 mg/g to 86.50 mg/g, representing a lower CPGR of 20.61% in the HFE batch. Interestingly, the growth in the levels of trehalose was more pronounced in the first half compared to that in the second half of fifth instar. Clearly, in the first phase trehalose levels registered an OGR of about 145% (CPGR: 34.86%) in the ZDC batch and a lower OGR of about 116% (CPGR: 29.34%) in the HFE batch. In the second half, its levels grew at a rate of about 46% (CPGR: 13.43%) in the ZDC batch and at about 42% (CPGR: 12.47%) in the HFE batch (Table 3).

Table 3: Impact of honey-enriched mulberry diet on gut trehalose and trehalase activity in the midgut of *Bombyx mori* during fifth instar development

Stage	Statistical tool	Trehalose (mg glucose /g or ml)				Trehalase Activity *(µ moles of glucose /mg trehalose/ h)*			
		ZDC Batch		HFE Batch		ZDC Batch		HFE Batch	
		Gut wall	Gut content	Gut wall	Gut content	Gut wall	Gut content	Gut wall	Gut content
1	2	3	4	5	6	7	8	9	10
Day 1 (Early)	Mean S.D (±)	22.10 ±0.49	26.80 ±0.32	28.10 ±0.59	33.60 ±0.47	2.78 0.10	0.17 ±0.01	2.47 ±0.10	0.14 ±0.006
Day 4 (Mid)	Mean OGR (%) S.D (±) CPGR (%)	54.20 145.24 ±0.44* 34.86	40.60 51.49 ±0.36* 14.85	60.8 116.37 ±0.75* 29.34	45.70 36.01 ±0.67* 10.80	1.10 -60.43 ±0.03* -26.59	0.02 -88.23 ±0.0005* -51.00	1.09 -55.47 ±0.20* -23.87	0.02 -85.71 ±0.0005* -47.72
Day 7 (Late)	Mean OGR (%) S.D (±) CPGR (%)	79.10 45.94 ±0.39* 13.43	56.10 38.18 ±0.28* 11.38	86.50 42.27 ±0.46* 12.47	62.30 36.32 ±0.47* 10.88	0.62 -43.64 ±0.017* -17.4	0.01 -50.00 ±0.0005* -20.63	0.69 -36.70 ±0.013* -14.14	0.01 -50.00 ±0.0008* -20.63
Overall CPGR %		23.68	13.10	20.61	10.84	-22.13	-37.64	-19.15	-35.59

** Statistically significant (P value <0.001); ** statistically not significant.*

Trehalose values represent the mean ± standard deviation (SD) of four separate observations. For each observation tissue from 10 to 15 larvae was pooled. The percent change (PC) for each period was calculated taking its previous value as the control, while CPGR was separately calculated for two successive periods in fifth instar (from early to mid and from mid to late) and for whole instar (from day 1 to day 7).

In contrast, the trehalase activity declined significantly throughout the fifth instar in the gut wall, from an initial activity level of 2.78 µm/mg glucose/h to 0.62 µm, representing a CPGR of -22.13% in ZDC batch and from 2.47 µm to 0.69 µm, representing a CPGR of -19.15% in the HFE batch. The rate of decline in enzyme activity was more pronounced in the first half compared to that in the second half of instar. Significantly, in the first half, the trehalase activity registered a decline of about 60% (CPGR: -26.59%) in the ZDC batch and about 55% (CPGR: 23.87%) in the HFE batch. Subsequently in the second half, its activity dropped by about 44% (CPGR: -17.4%) in the ZDC batch and by about 37% (CPGR: -14.14%) in the HFE batch (Table 3).

Gut content: In gut content, the rate of ingestion of dietary trehalose becomes slower than that in the gut wall. During fifth instar, its levels increased from 26.80 mg/g to 56.10 mg/g, representing a CPGR of 13.10% in the ZDC batch and from 33.60 mg/g to 62.30 mg/g, with a higher CPGR of 10.84% in the HFE batch. The elevation in its levels during the first half was more pronounced than that in the second half. Clearly, during the first 4 days, their levels increased by about 51% (CPGR: 14.85%) in the ZDC batch and by 36% (CPGR: 10.80%) in the HFE batch. In the second half, its levels grew by about 38% (CPGR: 11.38%) in the ZDC batch and by about 36% (CPGR: 10.88%) in the HFE batch (Table3). The trehalase activity levels dropped all through the fifth instar from a value of 0.17 µm/mg glucose/h to 0.01 µm, representing a CPGR of -37.64% in the ZDC batch and 0.14 µm to 0.01 µm, with a CPGR of -5.59 in the HFE batch. The rate of decline in its activity was more rapid in the first half and slower in the second half. In the first rapid phase, the trehalase activity registered a decline of about 89% (CPGR: -51.0%) in the ZDC batch and about 86% (CPGR:-47.72%) in the HFE batch. In the second slower phase, its activity

declined by about 50% (CPGR: -20.63%) both in the ZDC and HFE batches (Table 3).

Cellulose and cellulase activity

The levels of the plant sugar, cellulose increased during the fifth instar, but the activity levels of its hydrolyzing enzyme, cellulase declined sharply by the instar-end.

Gut wall:

In the gut wall the of cellulose levels increased from 9.91 mg/g to 17.4 mg/g, at a daily growth rate (CPGR) of 11.20% in the ZDC batch and from 12.05 mg/g to 22.03 mg/g, representing a lower CPGR of 11.0% in the HFE batch during fifth instar. Surprisingly, its levels showed positive growth during the first half, but slumped significantly during the second half of instar. Clearly, in the first phase, its levels rose from 9.91 mg/g to 27.55 mg/g and registered an OGR of about 178% (CPGR: 40.61%) in the ZDC. Similarly, its levels increased from 12.05 mg/g to 30.15 mg/g and registered a lower OGR of about 150% (CPGR: 33.76%) in the HFE batch. In the second half, its levels showed a downward trend and registered negative growth rates, both in ZDC (OGR:-37%; CPGR: -14.20%) and HFE (OGR:-27%; CPGR: -9.93.0%) batches (Table 4).

Table 4: Impact of honey-enriched mulberry diet on gut cellulose and cellulase activity in the midgut of *Bombyx mori* during fifth instar development

Stage	Statistical tool	Cellulose (mg glucose /g or ml)				Cellulase Activity *(μ moles of glucose /mg cellulose/ h)*			
		ZDC Batch		HFE Batch		ZDC Batch		HFE Batch	
		Gut wall	Gut content	Gut wall	Gut content	Gut wall	Gut content	Gut wall	Gut content
1	2	3	4	5	6	7	8	9	10
Day 1 (Early)	Mean S.D (±)	9.91 0.34	2.29 0.099	12.05 0.38	2.57 0.13	1.25 0.13	0.04 0.006	1.19 0.078	0.05 0.006
Day 4 (Mid)	Mean OGR (%) S.D (±) CPGR (%)	27.55 178.0 0.19* 40.61	9.50 314.8 0.12* 60.68	30.15 150.2 0.31* 35.76	10.25 298.8 0.13* 58.59	1.05 -16.0 0.018* -5.65	0.03 25.0 0.0* -9.14	1.04 -12.61 0.017* -4.39	0.04 -11.11 0.0** -7.17
Day 7 (Late)	Mean OGR (%) S.D (±) CPGR (%)	17.4 -36.84 0.36* -14.20	3.46 -63.58 0.14* -28.59	22.03 -26.93 0.33* 9.93	4.59 -55.22 0.15* -23.49	0.51 -51.24 0.025* -21.39	0.01 -66.66 0.005* -30.66	0.59 -43.27 0.017* -17.22	0.03 -40.00 0.01* -9.14
Overall CPGR %		11.2	7.12	11.0	10.2	-13.7	-20.63	-11.04	-8.16

** Statistically significant (P value <0.001); ** statistically not significant.*

Cellulose values represent the mean ± standard deviation (SD) of four separate observations. For each observation tissue from 10 to 15 larvae was pooled. The percent change (PC) for each period was calculated taking its previous value as the control, while CPGR was separately calculated for two successive periods in fifth instar (from early to mid and from mid to late) and for whole instar (from day 1 to day 7).

In contrast, the cellulase activity declined from an activity level of 1.25 μm/mg glucose/h to 0.51 μm, representing a CPGR of -13.70% in ZDC batch and from 1.19 μm to 0.59 μm, representing a CPGR of -11.04% in the HFE batch during fifth instar. The rate of decline in enzyme activity was slow in the first half and rapid in the second half of instar. Significantly, in the first half, its activity registered a decline of about 16% (CPGR: -5.65%) in the ZDC batch and about 13% (CPGR: 4.39%) in the HFE batch. Subsequently in the second half, its activity dropped more rapidly by about 51% (CPGR: -21.39%) in the ZDC batch and by about 43% (CPGR: -17.22%) in the HFE batch (Table 4).

Gut content: The rate of uptake of dietary cellulose into the gut lumen increased from 2.29 mg/g to 3.46 mg/g, representing a CPGR of 7.12% in the ZDC batch and from 2.57 mg/g to 4.59 mg/g, with a higher CPGR of 10.20% in the HFE batch. Actually, its levels increased in the first half, but declined in the second half of instar. During the first four days its levels recorded an impressive OGR of about 315% (CPGR: 60.68%) in ZDC batch and a lower OGR of 299% (CPGR: 58.59%) in the HFE batch. But in the second half, its levels slumped significantly and registered an OGR of about -64%. (CPGR:-28.59%) in the ZDC batch and an OGR of about -55% (CPGR:-23.49%) in the HFE batch (Table 4). The cellulase activity levels dropped from 0.04 μm/mg glucose/h to 0.01 μm, representing a CPGR of -20.63% in the ZDC batch and from 0.05 μm/mg glucose/h to 0.03 μm with a CPGR of -8.16% in the HFE batch. The rate of decline in its activity was slower in the first half and faster in the second half of fifth instar. In the first slow phase, its activity registered a decline of about 25% (CPGR: -9.14.0%) in the ZDC batch and about 11% (CPGR:-7.17%) in the HFE batch. In the second faster phase, its activity declined by about 67% (CPGR: -30.66%) in the ZDC batch and about 40% (CPGR:-9.14%) in the HFE batch (Table 4).

DISCUSSION

The gut wall and gut lumen compartments have clearly defined digestive functions. While the former performs secretary, absorptive and storage functions, the latter performs digestive function. While discharging its function, the gut wall secretes digestive enzymes (proteases, carbohdrases, lipases) and ions (H^+, K^+) (Kalaivani *et al*., 2013), while the gut lumen forms a virtual chemical soup that comprises gut wall secretions and pieces of mulberry diet (Cermenati *et al*., 2007). The present study highlights two important facets of digestive metabolism in ***B. mori***. Firstly, the digestive substrates (eg. total carbohydrates, total proteins, trehalose and cellulose) tend to accumulate in the gut wall and secondly, the activity levels of the corresponding digestive enzymes (eg. α-amylase, protease, trehalse and cellulase) tend to decline both in gut wall and gut lumen. Thus, while the digestive substrates follow consolidation phases, the digestive enzymes show retardation phases during fifth instar. Further, except for total carbohydrates, the consolidation of all other digestive substrates (proteins, trehalose, cellulose), occurs in two phases; an exponential log phase and a slow lag-phase during fifth instar development.

Developmental changes in protein digestion

Protein digestion generates the much needed amino acid pool required for metabolism, metamorphosis, silk production and reproduction in silkworm (Sarangi and Anitha, 2007). The present study highlights opposing growth trends in protein mass accumulation in gut wall vis-à-vis protein digestion in both gut wall and gut lumen.

Gut wall: The glandular epithelium of the gut wall synthesizes and stores over 96 proteins including endogenous digestive enzymes and proteins involved in cell growth, metabolism, immunity, heat shock treatment, muscle contraction, carcinoma control, carotenoid binding and antimicrobial activity (Kajiwara *et al*., 2005). The consolidation of protein pool in this compartment continues throughout the fifth instar at a steady rate of 30.89% per day. Further, our study highlights that protein consolidation occurs in two distinct phases; an early exponential log phase and a late delayed lag phase. In the log phase, that occurs in the first half (from early to mid) of fifth instar, proteins are consolidated exponentially with an OGR of about 310%, at a daily turnover rate of 60.03%. In the ensuing lag phase that occurs in the second half (from mid to late) of the instar, the rate of protein accumulation slows down and proceeds at a low OGR of just 23% in at a lower CPGR of 7.06% (Table 1). The growth trends in the gut wall proteolytic activity are contrary to those of proteins and involve an early delayed lag phase and a late exponential log phase. Though, the day-to-day retardation rates in proteolytic activity are maintained at a CPGR of 10.91% during fifth instar, the drop was significantly low (OGR: -22%; CPGR: -8.04%) in the early lag phase and remarkably higher (OGR: -53%; CPGR: -22.57%) in the late log phase (Table 1). Obviously, the function of protein accumulation takes precedence over

the function of protein digestion in the gut wall.

Gut content: About 77 proteins including dietary proteins and secretary enzymes are directly or indirectly involved in the digestive metabolism of the midgut in silkworm (Hui-Peng Yao *et al*., 2009; Anand *et al*., 2010). Apparently, the protein inputs into the gut content from the gut wall and dietary sources, increased at a day-to-day rate (CPGR) of 46.42%, representing 15.53 additional percentage points over that of those of the gut wall. The protein addition to the gut content also occurred in similar log and lag phases at different rates. Firstly, in the log phase (from day 1 to day 4), the proteins were added at an exponential rate of over 712% (CPGR: 101. 04%), while its rate declined significantly in the subsequent lag phase (from day 4 to day 7) and recorded extremely lower growth rates in terms of both OGR (21%) and CPGR (6.65%) during fifth instar growth (Table 1). This indicates that the silkworm feeds voraciously during the first four days, ingesting more and more mulberry proteins into its midgut, but its feeding rate declines significantly during the latter phases of fifth instar resulting in lower addition of dietary proteins. The digestibility of proteolytic enzymes in the gut lumen seems to decline during fifth instar, as evidenced by continuous retardation in protease activity at a daily declining rate of 35.59% during the period. The drop in its digestibility also involves an early log phase and a late lag phase. In the log phase, the enzyme activity registered a significant down fall with an OGR of about -81% (CPGR:-43.69%) and in the follow-up lag phase, it registered a lower drop rate about 62% (CPGR: 26.32%), representing a fall of 19 percentage points in its digestibility (Table1). It seems probable that the protein accumulation in the gut wall is not contributed much by dietary inputs of fifth instar, but possibly by those from earlier instars, a point that requires further investigation.

Developmental changes in carbohydrate digestion

The chief source of carbohydrates for silkworm is the mulberry leaf. The carbohydrate digestion generates monosaccharides like glucose that could be used by the silkworm as an immediate source of energy. The productivity of sericulture largely depends on the utilization of carbohydrate reserve food materials through enhanced activity of α-amylase that releases individual glucose molecules from the linear glucose chains (Sashindran Nair *et al*., 2004).

Gut wall: The gut wall cells absorb dietary glucose from the gut lumen and accumulate it in two types of storage sugars called trehalose and glycogen, which meet the energy demands of metamorphosis in silkworm (Thompson, 2003; Shivakumar and Shamitha, 2011). As evidenced from the findings of the present study, carbohydrate reserves accumulate in the gut wall continuously during the entire period of fifth instar at a steady rate (CPGR) of 5.65%. Though, clear log and lag phases are not discernable in their accumulation, the overall growth trends indicate that the rate of their accumulation is slower (OGR: 14%; CPGR:4. 36%) during the first four days and faster (OGR:22%; CPGR:6.95%) during the last four days of fifth instar (Table 5. 2). The rate of their accumulation varies as a function of digestibility of α-amylase, which cleaves the α-1, 4 glycosidic linkages of D-glucose units of starch (Terra and Ferreira, 200). Obviously, the reserve carbohydrates of gut wall are subjected to intracellular digestion by the activity of selective amylases. Nevertheless, the amylase activity of gut wall showed a clear declining trend throughout the fifth instar and registered a daily reduction of 37.99% in its activity. Of course, the retardation of enzyme activity manifested in an early lag phase with reduced rates (OGR: -70%; CPGR:-33.16%) during the first four days and in a log phase with more reduced rates (OGR: -81%; CPGR:-37.09%) during the last four days of fifth instar (Table 2). Clearly, the continuous elevatory trends in carbohydrate levels vis-à-vis the declining growth trends in its α-amylase activity indicate two points. Firstly, the gut wall carbohydrates are not predominantly used by the silkworm as a source of energy for larval growth and development. Second, even if it uses carbohydrates in tracer quantities, the larva explores other alternative energy sources towards the end of fifth instar.

Gut content: The uptake of dietary carbohydrates is directly related to the quantum of mulberry leaf consumed and the rate at which it is consumed (Narayanaswamy and Shankar 2010). As evident from the present study, the dietary uptake of carbohydrates decreased by the instar-end at a daily turnover rate of -14.65% during fifth instar larval growth. Nevertheless, its uptake is positive during the first four days of instar with an overall increase of about 16% at a daily turnover rate of 5.21% per day (Table 2). Surprisingly, the dietary uptake of carbohydrates decreased dramatically (OGR:-67%) towards instar-end at a dropping rate of 30.76% per day, indicating similar declines in the rate of consumption of mulberry leaf during the latter half of fifth instar. For effective digestion, the production and secretion of α-amylase should be synchronized with dietary uptake of carbohydrates. Soon after its release, the amylase becomes active in the alkaline medium of gut content and hydrolyzes the starch present in dietary carbohydrate (Abraham *et al.*, 1992). However, contrary to the accumulatory trends in dietary carbohydrates, the α-amylase activity showed a declining trend (OGR:-78% and COGR:-39.43% during the first four days, but becomes almost null and void (OGR=CPGR=0) towards the end of fifth instar (Table 2). Thus, detectable change in enzyme activity is not clear during the last four days of fifth instar, further substantiates the fact that carbohydrate reserves are not effectively utilized as a source of energy during the latter half of fifth instar.

Developmental changes in trehalose digestion

Trehalose is the principal insect sugar and a disaccharide that accounts for 23% of the total carbohydrates in the body. It is synthesized and stored in the fat body and to a lesser extent in the gut wall cells in the presence of trehalose phosphate synthase (Thompson, 2003). The trehalose is degraded to glucose by an enzyme called trehalase, which occurs in two forms; trehalase-I and trehalase-II and are used both as a means of energy source and for the maintenance of homeostasis in the midgut (Mitsumasu *et al*., 2005).

Gut wall: It is known that most of the mulberry carbohydrates are digested and absorbed as glucose molecules that finally assimilated in the form of storage sugar called trehalose in the gut wall (Shivakumar and Shamitha, 2011). The current study shows that trehalose accumulation continues at a daily increasing rate of 23.68% during fifth instar. Interestingly, this increasing phase (log phase) lasts for first four days of instar and thereafter it follows a declining path (lag phase). In the log phase, its increase represents a 3-fold hike with an impressive OGR of about 145% at a daily increasing rate (CPGR) of 34.86% during fifth instar larval growth. But, in the lag phase its levels recorded a lower overall (46%) and day-wise (13.43%) growth rates (Table 3). In contrast, the activity of trehalase, its hydrolyzing enzyme, declined continuously at a daily rate of 22.13% during fifth instar larval growth, with an early log phase and a late lag phase. In the log phase its activity registered an exponential growth of about 60% at a daily declining rate of 26.59%, but in the lag phase, its activity levels fell moderately by about 44% at a daily declining rate of 17.4% at the instar-end (Table 3). The increasing rates of trehalose accumulation on one hand and the slower enzymatic activity of trehalase on the other indicates that the gut wall trehalose is not immediately made available as a long-term energy source for metamorphosis, but it is used for the sustenance of peristalsis and digestive metabolism during larval growth (Azuma and Yamashita, 1985).

Gut content:

Though trehalose is not a regular constituent of 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 midgut (Anand *et al*., 2010). Though, the addition of trehalose to the gut content showed a day-to-day increasing rate of 13.10% from day 1 to day 7 of fifth instar, its increase has an early rapid log phase and a late slow lag phase. In log phase, its levels are added more rapidly (OGR; 51%) at a daily rate of 14.85%. In the late lag phase the levels of this disaccharide grew moderately about 38% with a daily turnover rate of 11.38% during fifth instar growth (Table 3). The trehalose, so added to the gut content is hydrolyzed by trehalase, which is produced in gut wall and released into the gut lumen along with the other digestive enzymes in tracer amounts. The current study indicates that this enzyme shows reduced activity, at a daily declining rate of 37.64% although the fifth instar larval development, with an early log phase and a late lag phase (Table 3). In the log phase (day 1 to day 4), its activity dropped by 89% at a daily declining rate of 51.0% and in the lag phase (day 4 to day 7), its activity slumped by about 50% at a daily dropping rate of 20.63% during fifth instar development (Table 3). The trehalose digestion in midgut has three important implications, First, the remnants of trehalase activity found in the gut lumen plays an active role extra-cellular digestion of trehalose and the resultant glucose diffuses into the haemolymph and supplies energy for silkworm during starvation and non-feeding times Second, its digestion prevents the wastage of trehalose through egestion in the digestive system and third, the trehalose levels in gut and haemolymph

ensures the regulation of homeostatic mechanism among different tissues and organs during metamorphosis (Azuma and yamashita, 1985; Iwami, 2000).

Developmental changes in cellulose digestion

Cellulose is the chief constituent of mulberry leaf and major dietary source of carbohydrates for the silkworm, but it is not an integral part of silkworm's structure and function (Kandylis *et al*., 2009). It has been substantively proved that the digestive system of silkworm evolved microbial-assisted cellulose hydrolyzing mechanism in the gut lumen and cellulose synthesizing mechanism in the gut wall. The cellulose hydrolyzing mechanism of gut lumen involves digestion of dietary cellulose by a series of enzymes called cellulases that are derived from the symbiotic microbial fauna (eg. *Enterobacteriaceae; Protius vulgaris, Klebsiella pneumonia, Citrobacter freundii*) that thrives and functions effectively in an alkaline medium of digestive fluids (Anand *et al*., 2010). Though, the detailed mechanism of cellulose digestion is not clear, it is believed that the cellulases break the ß (1-4) linkages and liberate individual glucose units (Watanabe and Tokuda, 2010). The cellulose synthesizing mechanism of gut wall involves the maintenance of a community of cellulose synthesizing microbes such as *Escherichia coli* and *Pseudomonas* in its cells, much like that of cellulose- hydrolyzing bacteria in the gut lumen (Romling, 2002). These two bacterial strains do not utilize the dietary cellulose, but synthesizes their own within the gut wall (Anand *et al*., 2010). In fact, the cellulose synthesizing machinery has been evolved as a protective mechanism against the probable cellular damage to the gut wall that might occur due to osmotic imbalances caused by the continuous presence of large quantities of undigested dietary cellulose in the gut lumen. Obviously, the accumulation of cellulose in gut wall helps in establishing a cellulose gradient in midgut that helps in withstanding osmotic pressure differences between the extra cellular (gut lumen) and intracellular (gut cells) spaces apart from sustaining the load bearing function of gut. Thus, the digestive system of ***B. mori*** acts as a dual functional system, at least for the synthesis and hydrolysis of the cellulose in which both these functions are modulated synergistically by the host organism and the symbiotic microbes (Hongoh *et al*., 2008).

Gut wall: If, the intracellular cellulose is the synthetic product of the gut wall microbial fauna (Anand *et al*., 2010), the rate of its de novo synthesis in its cells seems to increase from beginning to the ending of fifth instar at a daily rate of 11.2%. Further, its rate of synthesis follows an early exponential log phase in the first half and a late delayed lag phase in its second half of fifth instar. The synthetic machinery seems to operate effectively in the log phase during which the cellulose levels registered a phenomenal growth of about 178% in gut wall at a day-wise growth rate of 40.61% from early to mid fifth instar. But surprisingly, in the lag phase, its levels fell sharply by about 37% and registered a slower day-wise growth rate of 14.2% (Table 4). The intracellular cellulose, so generated is hydrolyzed by microbial cellulases that percolate into the gut wall from the gut lumen. There seems to be no correlation between the rate of accumulation of substrate (cellulose) and activity of the enzyme (cellulase) in the gut wall of silkworm. As evidenced from the present study, the activity of cellulase declined continuously from early to mid (OGR:-16%; CPGR:-5.65%) and from mid to late (OGR:-51%; CPGR:-21.39%) fifth instar (Table 4).

2. Gut content: The mulberry-derived cellulose of the gut content is hydrolyzed by microbial-derived cellulases in the gut lumen (Kandylis *et al*., 2009). Since, the silkworm is a voracious feeder; the dietary cellulose gets added to the gut content continuously at a low CPGR of 7.12% during the 7-day period of fifth instar and that involves an early exponential log phase and a late delayed.lag phase. In the log phase, its levels increased remarkably by about 315% at a daily growth rate of 60.68% but in the lag phase, its addition to the gut content registered a drop of about 64%, with a negative CPGR of -28.59% during the last four days of fifth instar (Table 4). Probably, such fluctuations in cellulose concentrations between the external (gut lumen) and internal (gut wall) environments seems to have evolved as a complementary protective mechanism that protects the integrity of the gut wall by maintaining the much needed osmotic gradient in the midgut. As far as the hydrolysis of cellulose is concerned, there exists no correlation between the levels of cellulose and cellulase activity in the gut content. Despite a dramatic increase in cellulose levels in the mid instar, the activity levels of cellulase declined at a daily rate of 20.63% all through the fifth instar. Its fall occurred more slowly during the first four days (OGR:25%;CPGR:9.14%) and more rapidly during the last four days (OGR:67%;CPGR:30.66) of the instar (Table 4). This indicates that cellulose and its derivatives are not used as source of energy, at least when the larva prepares for its transformation to pupa.

The study amply demonstrates that the silkworm digestive metabolism follows Hutchinson's investment principle (HIP), which provides an adoptive explanation for mass accumulations in soft larval tissues as an investment of energy resources for the growth of structural components required for post-embryonic development, silk production and metabolic acceleration (Hutchinson *et al*., 1997). Such a growth mechanism also operates in the digestive system of silkworm and results in greater accumulation of digestive metabolites in the gut wall and other tissues. Nonetheless, its larva accumulates most of the body mass during the fifth and final instar, probably in tune with its voracious feeding habit coupled with high power of digestibility (Hou *et al.,* 2010). Accordingly, larger mass increment in the midgut of silkworm during fifth instar larval development is attributed to the scaling of feeding rate coupled with the length of its larval duration. Thus, *B. mori* accumulates energy reserves in a phased manner during larval stage to be used in the ensuing non-feeding pupal and adult stages.

Honey-enriched mulberry diet enhances digestibility and mass accumulation

Honey is the natural sweetener and multi- factorial nutrient produced by the honey bees. Its chemical composition, which varies as a function of its botanical origin, includes a variety of sugars (82%), proteins, enzymes (eg. diastase, invertase, glucose oxidase, catalase), free amino acids, vitamins (riboflavin, niacin, folic acid, pantothenic acid, vitamin B_6 and ascorbic acid) and a large number of trace elements (Cr, Co, Cu, Fe, Mn and Zn) that are essential for growth and metabolism (Ball David, 2007). Its inherent values of nourishing, healing and prophylaxis make it a rich nutrient-cum-medicine (Eileen De Mars, 2003). The comparative analysis of biochemical data of digestion of the ZDC and HFE batches indicates the mixed impact of honey-enriched mulberry diet on the digestive metabolism of ***B. mori,*** with regards to accumulation of matter in gut cells and digestibility of food substances in the gut lumen.

Digestibility: The term digestibility refers to the ability of the digestive enzymes (eg. protease, α-amylase, trehalase and cellulase) to hydrolyze their respective substrates, both in the gut wall (intracellular digestion) and gut lumen (extracellular digestion). Though the activity levels of all digestive enzymes fall considerably during fifth instar, the present study amply demonstrates the persistence of digestive activity in the midgut of silkworm. The impact of honey-fortified mulberry diet on digestibility appears to be positive in the sense that the declining rates in enzyme activities are minimized to the extent possible in respect of all digestive enzymes except for the protease activity of the gut wall. Nevertheless, the reduction in protease activity was minimized by 6.8 percentage points (35.59-28.79) in gut content of honey-fed larvae (Table 5.1). If, reducing the degree of recession in enzyme activity is considered a positive signal, then the activity levels of all digestive enzymes were considered to have been boosted by the honey-enriched diet. Accordingly, the activity of α-amylase vis-à-vis the digestibility of carbohydrates got enhanced by 15.44 percentage points (37.99-22.55) in the gut wall and by zero percentage points (22.17-22.17) in the gut content (Table 5.2). Similarly, the activity levels of trehalase were boosted by 2.98 percentage points (22.13-19.15) in the gut wall and by 2.05 percentage points (37.64-35.59) in the gut content (Cols. 7 to 10; Table 3) and those of cellulase by 2.66 percentage points (13.70-11.04) in the gut wall and by 12.47 percentage points (20.63-8.16) in the gut content (Table 4). By and large, this kind of positive impact manifested differently, both in the early and late phases of fifth instar. While it occurred more vigorously in the early phase with regard to proteases and α-amylases, it did so in late phase with regard to trehalases and cellulases (Fig 1).

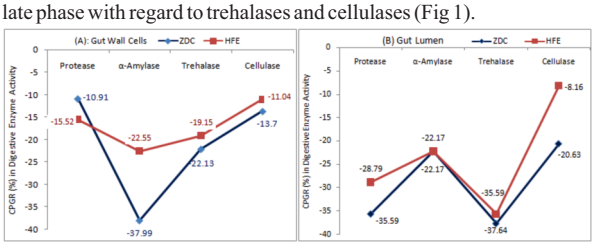

Fig.1: Impact of honey on digestibility of digestive enzymes in the midgut of ***B. mori*** during fifth instar growth (Source: Tables 1 to 4)

Mass accumulation: The gut wall acts as a transitory storage organ for both carbohydrate and non-carbohydrate energy reserves. It absorbs and assimilates the end products of digestion in the form of proteins, and a variety of carbohydrate reserves such as the glycogen, trehalose and cellulose, from which energy is derived and supplied as per emerging metamorphic demands. The accumulation of matter in the silkworm gut wall has been discussed with reference to total proteins, total carbohydrates, trehalose and cellulose (Fig.2). The CPGRs registered under each item clearly illustrate the prevalence of increasing rates of accumulation of these products on day-to-day basis during fifth instar (Tables 1 to 4).

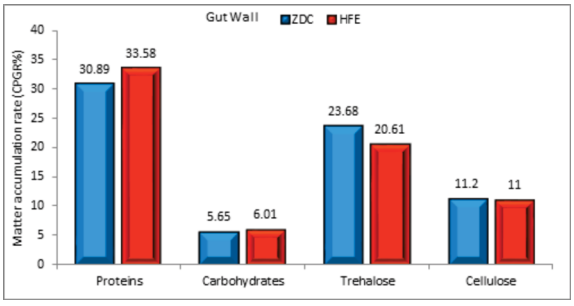

Fig.2: Impact of honey on the rate of accumulation of matter in gut wall cells of ***B. mori*** during fifth instar growth (Source: Tables 1 to 4)

Accordingly, the larvae, which fed on the honey-fortified mulberry leaf (HFE batch) recorded higher accumulation rates of 33.58% in proteins, 6.01% in total carbohydrates, 20.61% in trehalose and 11.0% in cellulose. Compared to the larvae of ZDC batch, this represents a overall increase of 2.9 percentage points (33.58-30.89) in proteins, 0.36 percentage points (6.01-5.65) in carbohydrates and a decrease of 3.07 percentage points (20.61-23.68) in trehalose and 0.2 percentage points (11.0-11.2) in cellulose. Almost, similar growth trends were recorded during the larval development from early to mid and from mid to late of fifth instar stages. Furthermore, our study highlights that the gut wall of silkworm accumulates more of protein and trehalose reserves, compared to those of total carbohydrates and cellulose (Fig.2). It is likely that the silkworm derives its energy mostly from carbohydrate reserves during the active phases of larval development, but stores proteins for future use in pupal and adult stages. Thus, the honey-enriched mulberry diet reinforces not only the accumulation of mass in gut wall cells, ingestion of mulberry leaf in gut lumen but also enhances the digestibility of proteins and carbohydrates in the larval midgut during fifth instar development.

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