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Research Article

Impact of honey-enriched mulberry diet on the energy metabolism of the silkworm, *Bombyx mori*

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Abstract
The present study was taken-up with a view to clearly define the role of oxidative phosphorylation vis-a-vis transdeamination in *Bombyx mori* metamorphosis, under the influence of honey-enriched mulberry diet. Therefore, the study examined the accumulation and utilization patterns of carbohydrate (glycogen, trehalose, glucose) and non-carbohydrate energy reserves (proteins, amino acids) in its fat body during larval, pupal and adult stages. In accordance with Hutchinson's investment principle, the energy reserves invested during larval stage are partly used in pupal stage and those invested during larval and pupal stages are used in adult stage. Their utilization patterns are correlated with the activity levels of succinate (SDH) and glutamate (GDH) dehydrogenases and aspartate (AAT) and alanine (ALAT) aminotransferases and changes thereof were interpreted in terms of glycolytic oxidative phosphorylation and non-glycolytic transdeamination. The trends in mass incorporation rates vis-à-vis enzyme activities indicated that the metabolism-related energy needs of all metamorphic events are majorly met through a gluconeogenetic mechanism called transdeamination, while the behavioural-related energy demands of larval and pupal stages are fulfilled through glycolytic-based oxidative phosphorylation. The activity trends further indicated that AAT plays major role in meeting the energy needs of larva and pupa, while GDH predominantly meets the energy requirements of reproduction in adults. The honey-enriched mulberry diet showed stage-specific and pathway-specific impacts on energy metabolism. It positively reinforced the energy metabolism in larval stage, but showed no significant effect in pupal and adult stages. Similarly, it showed more promising effect on glycolytic-oxidative phosphorylation and null or neutral effect on transdeamination.

Keywords: Aminotransferases, *Bombyx mori*, Dehydrogenases, Energy metabolism, Energy reserves

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INTRODUCTION

The silkworm metamorphosis is an energy intensive process. It is closely associated with energy metabolism and brings about energy-dependent metamorphic changes in its morphology, behaviour, anatomy, histology, embryology, physiology and biochemistry (Merkey *et al.,* 2001; Hemalatha *et al*.,2014).Obviously, during insect metamorphosis, the larval and pupal tissues are reorganized and restructured into adult organs through a variety of energy intensive processes such as histolysis, histogenesis, differentiation and morphogenesis (Siva Prasad, 2015).During actively feeding larval stages, insects acquire the basic raw materials in the form of storage proteins, carbohydrates (glycogen and trehalose), fats and amino acids and deposits them in body tissues in accordance with the Hutchinson's investment principle, that serve as future energy reserves for metamorphic activities such as the morphogenesis, ecdysis, formation of haemocytes and chitin, silk protein synthesis, vitellogenesis, metabolism, lipid transport and sexual maturation (Hutchinson *et al*., 1999; Mirth and Riddiford, 2007). In this context, the fat body acts as the physiological energy reservoir of metamorphosis and multi-functional organ of metabolism that mobilizes energy reserves by integrating signals from neighbouring tissues (Cheng *et al*., 2006). Accordingly, the silkworm acquires the energy mass from the mulberry diet during larval stage and invests them in its fat body cells for meeting the future energy demands of growth, metabolism, silk production and repro-

duction (Tibbets and Matinez del Rio, 2007). The investment of energy matter varies as a function of nutritional status of the mulberry leaf. With a dual objective of enhancing dietary mass investments in body tissues and to improve the sericultural productivity, several sericulturists fed the silkworm with exogenous nutrient-enriched mulberry leaf and obtained positive results (eg. Ramakrishna and Baskar, 2009; Kumar and Balasubramanium, 2013, Thulasi and Sivaprasad, 2013, 2014). One of the most promising exogenous nutrients emerged from such studies is the honey, the multi-factorial nutrient-cum-medicine (Thulasi and Sivaprasad, 2015; Madhavi *et al*., 2018, 2020).

Amidst nutritional studies, the energetics of insect growth, mass accumulation and metamorphosis have become hot research topics in entomology (Llandres *et al*., 2015; Maino and Kearney, 2015). It was earlier believed that sugars have greater say in the silkworm energy metabolism because of their high solubility and rapid mobilization (eg.Inagaki and Yamashita, 1986). However, most of the subsequent investigations anticipated the involvement of non-carbohydrate reserves like lipids and amino acids in energy production, especially under demanding situations like the ecdysis, metamorphosis, long-term flight, chill, stress and starvation (Arrese and Soulages, 2010). Importantly, some recent studies highlighted the importance of an alternative energy generation process called transdeamination, which generates glucose from non-carbohydrate sources during pupal-adult metamorphosis in silkworm (Hemalatha *et al*.,2016; Siva Prasad and Bhuvaneswari, 2018). Therefore, analyzing the role of carbohydrate and non-carbohydrate reserves of fat body in relation to the energy metabolism of *Bombyx mori* assumes importance. It raises the following questions; 1) how does the silkworm use the energy derived from the oxidation of glucose via glycolysis and tricarboxylic acid cycle? 2) What is the role of transdeamination in its metamorphosis? 3) How does the fat body regulate these two energy generating pathways and maintains homeostasis?, and 4) How does the honey-enriched mulberry diet influence its energy metabolism? The present study intends to address these issues by analyzing metamorphic changes in the levels of carbohydrate and non-carbohydrate reserves, together with the activity levels of key enzymes of glycolytic oxidative phosphorylation and non-glycolytic transdeamination under the impact of honey-enriched mulberry diet.

MATERIALS AND METHODS

The Pure Mysore x CSR_2 hybrid variety of the silkworm *B. mori,* reared under standard environmental conditions of 28°C and relative humidity of 85% (Krishnaswami, 1986),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 12h light and 12h dark conditions. After the second molt, the third instar larvae were divided into two batches; the zero dose control (ZDC) and honey-fed experimental (HFE), each comprising 100 worms. The ZDC batch was given five feedings per day as usual, while in respect of HFE batch, the 2.00 P.M diet was replaced by 2% honey-enriched mulberry leaf, but normal feeding pattern continued at other timings. Before feeding, the honey-enriched mulberry leaf was prepared by soaking it in the honey solution at a minimum effective concentration of 2% in distilled water as determined in the present study (Madhavi *et al*., 2018). The soaked mulberry leaf was dried under cool weather conditions and fed to the larvae of HFE batch. The impact of honey-fortified mulberry leaf on energy metabolism has been studied in the fat body tissues of both the batches simultaneously. The fat body tissue was isolated by mid-dorsal dissection of *B. mori* in ice cold Silkworm Ringer (Yamaoka *et al*., 1971). Biochemical assays were carried out on the fat body samples obtained from the thoracic and abdominal segments of fifth instar larval, pupal and adult stages. Glucose levels were estimated by the method of Mendal *et al* (1954) in 5% fat body homogenate in methanol and the values expressed as mg/g wet weight of tissue. Trehalose content was estimated by the method of Roe (1955) in 1% homogenate of the fat body, prepared in ice cold distilled water and the values were expressed as mg/g wet weight of tissue. The glycogen content was estimated by the method of Carroll *et al.,* (1956) in 1% fat body homogenate in 5% trichloroacetic acid and the values were expressed in mg/gm wet weight of tissue. The total protein levels were estimated by the method of Lowry *et al*., (1951) in 1% fat body homogenate in distilled water and the values were expressed as mg/g wet weight of fat body tissue. Free amino acids were estimated by the method of Moore and Stein (1954) in 5% fat body homogenate in 10% trichloroacetic acid and the values were expressed in mg/ gm wet weight of fat body tissue. The activities of aspartate aminotransferase (AAT) and alanine amino transferase (ALAT) were estimated by the method of Reitman and Frankel (1957) in 5% fat body homogenate in distilled water and the enzyme activity was expressed as µ moles of pyruvate formed/ mg protein/h. The succinate dehydrogenase (SDH) activity was estimated by the method of Nachalas *et al.,* (1960) in the 5% fat body homogenate in 0.25N sucrose solution and the enzyme activity was expressed in µ moles of formazan formed/mg protein/h. The glutamate dehydrogenase activity was estimated by the method of Lee and Lardy (1965) in 5% fat body homogenate in ice-cold 0.25M su-

crose solution and the enzyme activity was expressed as µ moles of formazan /mg protein/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, all the energy parameters were analyzed in terms of an innovative growth parameter called Compound Periodical Growth Rate (CPGR) as given by Sivaprasad (2012).

RESULTS

Transitional changes in the levels of carbohydrate and non-carbohydrate energy reserves of vis-à-vis the activity levels of key enzymes involved in energy metabolism during larval-pupal-adult metamorphosis in *B. mori*, under conditions of zero dose control (ZDC) and honey-fed experimental (HFE) are presented in tables 1 to 4 and figures1 to 3.

Carbohydrate energy reserves

Glucose: This monosaccharide is the immediate source of energy. Its levels in the silkworm fat body showed elevatory growth trends during larval and pupal stages and declining trends during larval-pupal and pupal-adult transitional periods (Table 1). During the fifth instar larval growth, its levels increased from 3.67 mg/g wet weight of tissue to 6.43 mg/g in ZDC and to 9.53 mg/g in HFE, representing an overall growth rate (OGR) of 75% in the former and 160% in the latter. In terms of compound periodical growth rates, the elevation accounts for 9.80% in ZDC and 17.24% in HFE. Upon entry into the pupal stage, the glucose levels were dropped by about 13% in the former and about 31% in the latter. However subsequently, during the 9-day period of pupal life, its levels increased from 5.56 mg/g to 9.12 mg/g in ZDC and from 6.54 mg/g to 9.95 mg/g in HFE. Thus, it registered an OGR of about 64% (CPGR: 6.38%) in the former and 52% (CPGR: 5.39%) in the latter. But, upon transformation of pupa to adult, the glucose levels slumped significantly and registered negative CPGRs in both the sexes. Thus, the silkworm recorded a higher drop of 52.3% in males and a lower drop of 35.7% in females. However, in HFE, its levels dropped by 47% in males and about 33.7% in females (Cols. 3, 4; Table 1).

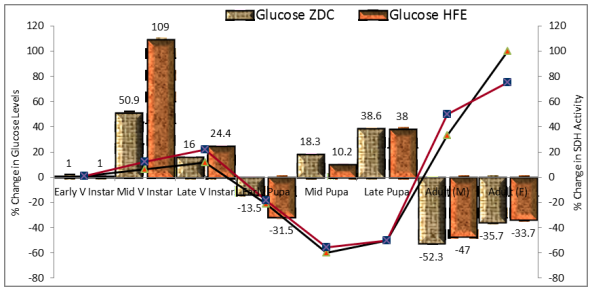

Fig.1. *Percent growth in the levels of glucose and succinate dehydrogenase activity in the fat body of B. mori, under ZDC (zero dose control) and HFE (honey -fed experimental) conditions during larva-pupal-adult metamorphosis (Source: Tables 1and4)*

Glycogen: The dietary glucose is converted to glycogen and stored in the fat body. The glycogen levels displayed steadily increasing growth trends in fifth instar and staggered growth trends in pupal and adult stages. During the 7-day period of fifth instar, glycogen levels increased from 46.7 mg/g wet weight of tissue to 176.3 mg/g in ZDC and to 211.9 mg/g in HFE, representing an overall growth rate (OGR) of 277% in the former and 354% in the latter. In terms of compound periodical growth rates, the elevation accounts for 24.78% in ZDC and 28.68% in HFE. Upon entry into the pupal stage, its levels were dropped by about 40% in the former and by 44% in the latter. But in mid pupal stage its levels dramatically increased by about 104% in ZDC and 86% in HFE. But, thereafter, its levels registered a downward trend continuously throughout the late pupal stage and during pupal-adult transition. At the end of pupal stage the glycogen levels slumped uniformly by about 64% in both control and experimental batches. Nevertheless, during the entire pupal period, it witnessed negative CPGRs both in ZDC (-4.07%) and HFE (-4.87%). The downfall in their levels continued with much more clarity during pupal-adult transformation, where in its levels were dropped uniformly by about 68% in males of both control and experimental batches, but with regard to females, its levels fell by about 51% in the former and 46% in the latter (Table 1).

Trehalose: Trehalose is another stored form of energy reserve in the fat body. Compared to glucose and glycogen, silkworm maintains higher levels of trehalose deposits in its fat body. Its growth trends were similar to those of glucose and glyco-

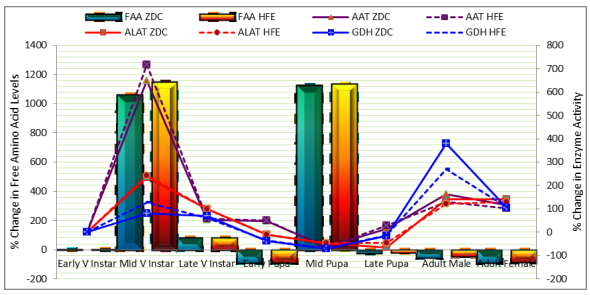

Fig.2. *Percent growth in free amino acids (FAA) and activity levels of aspartate (AAT) and alanine (ALAT) aminotransferases and glutamate dehydrogenase in the fat body of B. mori, under ZDC (zero dose control) and HFE (honey-fed experimental) conditions during larva-pupal-adult metamorphosis (Source: Tables 2,3 and 4).*

Table 1. Impact of honey-enriched mulberry diet on carbohydrate energy reserves of fat body in *B. mori* during larval-pupal-adult metamorphosis.

Stage	Statistical Tool	Glucose (mg /g wet wt.)		Glycogen (mg /g wet wt.)		Trehalose (mg /g wet wt.)	
		ZDC	HFE	ZDC	HFE	ZDC	HFE
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
Early V Instar (Day 1)	Mean	3.67	3.67	46.7	46.7	114.50	114.5
	S.D (±)	0.075	0.075	0.93	0.93	2.86	2.86
Mid V Instar (Day 4)	Mean	5.54	7.66	82.7	98.2	183.50	204.8
	P.C (%)	50.9	108.7	77.0	110.3	60.26	78.9
	S.D (±)	0.124*	0.094*	0.70*	1.67*	1.95*	3.30*
Late V Instar (Day 7: Late)	Mean	6.43	9.53	176.3	211.9	200.10	290.3
	P.C (%)	16.0	24.4	113.1	115.7	9.05	41.7
	S.D (±)	0.042*	0.080*	1.11*	1.59*	3.47*	3.75*
OGR- Larval (%)		75.20	159.67	277.57	353.72	74.75	153.53
CPGR-Larval (%)		9.80	17.24	24.78	28.68	9.75	16.77
Early Pupa (Day 1)	Mean	5.56	6.54	105.8	117.8	121.9	152.3
	P.C (%)	-13.53	-31.37	-39.98	-44.40	-39.08	-32.69
	S.D (±)	0.15	0.15	1.14	1.14	1.55	1.55
Mid Pupa (Day 5)	Mean	6.58	7.21	216.0	219.1	174.3	202.1
	P.C (%)	18.3	10.24	104.1	85.99	42.9	65.8
	S.D (±)	0.05*	0.1*	0.71*	0.57*	2.84*	3.35*
Late Pupa (Day 9)	Mean	9.12	9.95	75.9	79.0	190.5	222.0
	P.C (%)	38.6	38.0	-64.8	-63.9	9.29	9.84
	S.D (±)	0.08*	0.03*	0.81*	0.59*	2.65*	1.49**
OGR- Pupal (%)		64.02	52.14	-28.26	-32.93	56.27	45.76
CPGR-Pupal (%)		6.38	5.39	-4.07	-4.87	5.74	4.82
Adult-Male	Mean	4.35	5.27	24.2	25.4	114.8	138.4
	CPGR (%)	-52.3	-47.0	-68.1	-67.8	-39.7	-30.1
	S.D (±)	0.091*	0.117*	1.058*	0.588*	2.84*	2.02*
Adult-Female	Mean	5.86	6.60	37.0	42.5	147.3	175.3
	CPGR (%)	-35.7	-33.7	-51.3	-46.2	-22.7	-11.4
	S.D (±)	0.145*	0.126*	0.50*	0.66*	1.71*	2.84

**Statistically significant (P<0.001), ** Statistically not significant.*
Values, expressed as mg / g wet weight of fat body tissue, 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 CPGRs were separately calculated for the larval, pupal and adult stages, based on the total duration of each stage (7 days for larva, 9 days for pupa and 2 days for adult).

gen, projecting increasing trends in fifth instar and pupal stages and falling trends at larval-pupal and pupal-adult transitions. From early to late fifth instar, its levels increased from about 114 mg/g wet weight of tissue to 200 mg/g in ZDC and to 290 mg/g in HFE, representing an overall growth rate (OGR) of 75% in the former and 153% in the latter. In terms of compound periodical growth rates, the elevation accounts for 9.75% in ZDC and 16.77% in HFE. Upon entry into the pupal stage, its levels were dropped by about 39% in the former and by 33% in the latter. But in mid pupal stage its levels dramatically increased by about 43% in ZDC and by 66% in HFE. The upward trend extended to late pupal stage, whereby, its levels recorded a growth rate of just over 9% in both control and experimental batches. Thus, during pupal life, the trehalose levels grew at an OGR of about 56% (CPGR: 5.74%) in ZDC and 46% (CPGR: 4.82%) in the HFE. However, its levels recorded a downfall of about 40% in males and 23% in females of ZDC and about 30% in males and 11% in females of HFE (Table 1).

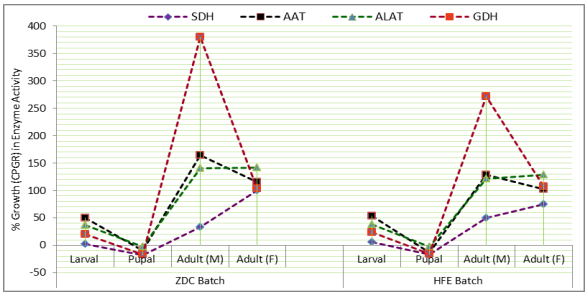

Fig.3. *Compound periodical growth rates in the activity levels of aspartate (AAT) and alanine (ALAT) aminotransferases and glutamate (GDH) and succinate (SDH) dehydrogenases in the fat body of B. mori, under ZDC (zero dose control) and HFE (honey-fed experimental) conditions during larva-pupal-adult metamorphosis (Source: Tables 3 and4)*

Non-carbohydrate energy reserves

Total Proteins: The total protein profiles of the fat body represent the proteins synthesized and stored by it during metamorphosis. The rate of protein accumulation is similar to that of glycogen. Obviously, it shows increasing trends in fifth instar and mid pupal stages and falling trends at early pupal, late pupal and adult stages. From early to late fifth instar, its levels increased from 32.4 mg/g

Table 2. Impact of honey-enriched mulberry diet on non-carbohydrate energy reserves in fat body of *B. mori* during larval-pupal-adult metamorphosis.

Stage	Statistical Tool	Total proteins (mg / gm wet wt.)		Total free amino acids *(mg/g wet wt).*	
		ZDC	HFE	ZDC	HFE
(1)	(2)	(3)	(4)	(5)	(6)
Early V Instar (Day 1)	Mean	32.4	32.4	1.48	1.48
	S.D (±)	0.99	0.99	0.42	0.42
Mid V Instar (Day 4)	Mean	48.2	54.2	17.2	18.5
	P.C (%)	48.7	67.2	1062	1150
	S.D (±)	1.08*	1.55*	0.61*	0.56*
Late V Instar (Day 7: Late)	Mean	55.8	59.8	31.6	33.5
	P.C (%)	15.7	10.3	83.7	81.1
	S.D (±)	0.95*	0.86*	0.52*	0.99*
OGR- Larval (%)		72.22	84.56	2035.30	2163.50
CPGR-Larval (%)		9.48	10.75	66.56	68.19
Early Pupa (Day 1)	Mean	41.6	43.56	3.11	3.21
	PC (%)	-25.44	-27.15	-90.15	-90.41
	S.D (±)	±0.94	±0.94	±0.46	±0.46
Mid Pupa (Day 5)	Mean	100.4	104.1	38.1	39.7
	P.C (%)	141.3	138.98	1125.0	1136.70
	S.D (±)	1.10*	0.98*	0.40*	0.41*
Late Pupa (Day 9)	Mean	68.1	71.4	29.7	31.4
	P.C (%)	-32.2	-31.41	-22.0	-20.90
	S.D (±)	1.55*	1.16*	0.29*	0.42*
OGR- Pupal (%)		63.70	63.91	854.98	878.19
CPGR-Pupal (%)		6.35	6.37	32.59	32.99
Adult-Male	Mean	38.6	43.0	14.0	16.6
	CPGR (%)	-43.3	-39.8	-52.8	-47.1
	S.D (±)	1.52*	0.75*	0.47*	0.33*
Adult-Female	Mean	42.3	45.5	3.37	4.80
	CPGR (%)	-37.8	-36.3	-88.6	-84.7
	S.D (±)	0.77*	0.55*	0.39*	0.57*

**Statistically significant (P<0.001), ** Statistically not significant.*
Values, expressed as mg / g wet weight of fat body tissue, 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 CPGRs were separately calculated for the larval, pupal and adult stages, based on the total duration of each stage (7 days for larva, 9 days for pupa and 2 days for adult).

wet weight of tissue to 55.8 mg/g in the ZDC and to 59.8 mg/g in the HFE, representing an overall growth rate (OGR) of 72% in the former and 85% in the latter. In terms of compound periodical growth rates, the elevation accounts for 9.48% in ZDC and 10.75% in HFE. Upon entry into the pupal stage, its levels were dropped by about 25% in the former and 27% in the latter. But in mid pupal stage its levels dramatically increased by about 141% in ZDC and 139% in HFE. But, subsequently in the late pupal stage its levels dropped significantly by about 32% in ZDC and 31% in the HFE. Nonetheless, its levels registered an OGR of about 64%, and recorded almost similar CPGRs (6.35%) in both the control and experimental batches during pupal growth. However, its levels recorded a downfall of about 43% in males and 42% in females of ZDC and about 40% in males and 36% in females of HFE (Cols. 3, 4; Table2).

Free amino acids: The levels of free amino acids in the fat body have shown exponential growth rates during fifth instar, wherein their levels increased from 1.48 mg/g to 31.6 mg/g in ZDC and to 33.5 mg/g in HFE, representing a massive OGR of about 2035% in ZDC and 2163% in HFE. The analysis of free amino acid data in terms of compound periodical growth rates (CPGRs) has shown that their levels grew by 66.56% per day in the control batch and 68.19% in the experimental batch, indicating their availability for transdeamination throughout fifth instar. However, in early pupal stage, their levels declined by about 90% in both ZDC and HFE. Surprisingly, once again their levels peaked to new heights during mid pupal stage and attained a growth rate of 1125% in ZDC and 1136% in HFE in just five days, but again followed a downward path during late pupal stage, wherein its levels fell by about 22% in the former and 21% in the latter. Further, the declining trends in FAA levels continued through pupal-adult transition in a sex specific fashion. In ZDC, their levels declined by about 53% in males and 89% in females and in HFE it did so by about 47% in males and 85% in females, reflecting the corresponding proportions of their utilization in both the sexes (Cols.5, 6: Table 2)

Key enzymes of energy metabolism

Aspartate aminotransferase (AAT): In general,

Table 3. Impact of honey-enriched mulberry diet on the activity of aspartate aminotransferase (AAT) and alanine aminotransferase (ALAT) in the fat body of *B. mori* during larval-pupal-adult metamorphosis

Stage	Statistical Tool	AAT activity (µ m of pyruvate /mg protein/h)		ALAT activity (µ m of pyruvate /mg protein/h)	
		ZDC	HFE	ZDC	HFE
(1)	(2)	(3)	(4)	(5)	(6)
Early V Instar (Day 1)	Mean	0.06	0.06	0.12	0.12
	S.D (±)	±0.020	±0.020	±0.026	±0.026
Mid V Instar (Day 4)	Mean	0.45	0.49	0.40	0.41
	P.C (%)	650.0	716.6	233.3	241.6
	S.D (±)	±0.031*	±0.022*	±0.017*	±0.022*
Late V Instar (Day 7: Late)	Mean	0.69	0.78	0.80	0.82
	P.C (%)	53.3	59.2	100.0	100.0
	S.D (±)	±0.024*	±0.017*	±0.019*	±0.016*
OGR- Larval (%)		1049.90	1200.00	566.66	583.33
CPGR-Larval (%)		50.24	53.34	37.19	37.75
Early Pupa (Day 1)	Mean	1.01	1.17	0.71	0.73
	PC(%)	46.37	49.99	-11.25	-10.97
	S.D (±)	±0.012	±0.012	±0.027	±0.027
Mid Pupa (Day 5)	Mean	0.38	0.40	0.36	0.39
	P.C (%)	-62.3	-65.81	-49.2	-45.0
	S.D (±)	±0.008*	±0.013*	±0.009*	±0.019*
Late Pupa (Day 9)	Mean	0.44	0.51	0.59	0.63
	P.C (%)	15.78	27.49	-63.8	-46.57
	S.D (±)	±0.019*	±0.012*	±0.015*	±0.012*
OGR- Pupal (%)		-56.43	56.41	-16.90	-13.69
CPGR-Pupal (%)		-9.87	-12.56	-2.29	-1.82
Adult-Male	Mean	1.16	1.17	1.42	1.40
	CPGR (%)	163.6	129.4	140.7	122.2
	S.D (±)	±0.041*	±0.026*	±0.025*	±0.025*
Adult-Female	Mean	0.95	1.03	1.43	1.44
	CPGR (%)	115.9	101.9	142.4	128.6
	S.D (±)	±0.017*	±0.038*	±0.022*	±0.020*

**Statistically significant (P<0.001), ** Statistically not significant.*
Values, expressed as µ moles of pyruvate formed/mg protein/h *of fat body tissue, 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 CPGRs were separately calculated for the larval, pupal and adult stages, based on the total duration of each stage (7 days for larva, 9 days for pupa and 2 days for adult).*

the activity levels of AAT in the fat body projected increasing trends during the entire larval life and early pupal life, declining trends during pupal life and secondary elevatory trends during pupal-adult transition (Cols. 3, 4; Table 3). During the 7-day fifth instar regime, this aminase activity increased from 0.06 µm of pyruvate/mg protein/h to 0.69 µm in ZDC and to 0.78 µm in HFE, representing an OGR of 1050% in the former and 1200% in the latter. In terms of day-wise rates (CPGRs), the elevation in its activity level was 50.24% in ZDC and 53.34% in HFE. During larval-pupal transformation, its activity levels peaked suddenly and in the early pupa it recorded an elevation of about 46% in the former and 50% in the latter. Thereafter, from early to mid pupal stage, its activity dropped significantly both in ZDC (-62%) and HFE (-66%). Surprisingly the downward trend in AAT activity got reversed during the second half of the pupal stage and suddenly peaked to new heights during pupal-adult transformation in a sex-specific fashion. Initially, in the late pupa, its activity witnessed a marginal increase of about 16% in ZDC and 27% in HFE. Upon its entry into the adult stage, the AAT activity in males and females increased respectively by about 164% and 116% in the ZDC and by about 129% and 102% in the HFE (Cols.3,4;Table3).

Alanine aminotransferase (ALAT): By and large, the activity levels of ALAT projected growth trends similar to those of AAT all through the silkworm metamorphosis, expect for that of pupal life. In general, the activity levels of ALAT in the fat body projected increasing trends during larval and adult lives, but declining trends during pupal life (Cols. 5, 6; Table 3). During the 7-day fifth instar regime, this aminase activity increased from 0.12µm of pyruvate/mg protein/h to 0.80µm in ZDC and to 0.82µm in HFE, representing an OGR of 567% in the former and 583% in the latter. In terms of day-wise rates (CPGRs), the elevation in its activity level was 37.19% in ZDC and 37.75% in HFE.

Table 4. Impact of honey-enriched mulberry diet on the activity of succinate dehydrogenase (SDH) and glutamate dehydrogenase (GDH) in the fat body of*B. mori* during larval-pupal-adult metamorphosis

Stage	Statistical Tool	SDH activity (µ m of formazan /mg protein/h)		GDH activity (µ m of formazan /mg protein/h)	
		ZDC	HFE	ZDC	HFE
(1)	(2)	(3)	(4)	(5)	(6)
Early V Instar (Day 1)	Mean	0.16	0.16	0.11	0.11
	S.D (±)	0.009	0.009	0.017	0.017
Mid V Instar (Day 4)	Mean	0.17	0.18	0.20	0.25
	P.C (%)	6.25	12.5	81.8	127.3
	S.D (±)	0.005*	0.008*	0.009*	0.005*
Late V Instar (Day 7: Late)	Mean	0.19	0.22	0.34	0.40
	P.C (%)	11.8	22.2	70.0	60.0
	S.D (±)	0.005*	0.009*	0.008*	0.005*
OGR- Larval (%)		18.75	37.50	209.9	263.63
CPGR-Larval (%)		2.91	5.45	20.69	24.01
Early Pupa (Day 1)	Mean	0.15	0.18	0.21	0.26
	PC (%)	-21.05	-18.18	-38.23	-35.00
	S.D (±)	0.012	0.012	0.005	0.005
Mid Pupa (Day 5)	Mean	0.06	0.08	0.06	0.08
	P.C (%)	-60.0	-55.55	-71.4	-69.23
	S.D (±)	0.002*	0.002*	0.005*	0.005*
Late Pupa (Day 9)	Mean	0.03	0.04	0.05	0.07
	P.C (%)	-50	-50.0	-16.6	-12.5
	S.D (±)	0.002*	0.002*	0.005*	0.005*
OGR- Pupal (%)		-80.00	-77.77	-76.19	-73.07
CPGR-Pupal (%)		-18.22	-17.14	-16.42	-15.13
Adult-Male	Mean	0.04	0.06	0.24	0.26
	CPGR (%)	33.33	50.0	380.0	271.1
	S.D (±)	0.005*	0.001*	0.017*	0.005*
Adult-Female	Mean	0.06	0.07	0.102	0.145
	CPGR (%)	100.0	75.0	104.0	107.1
	S.D (±)	0.005*	0.003*	0.012*	0.012*

**Statistically significant (P<0.001), ** Statistically not significant*
Values, expressed as µ moles of formazan/mg protein/h *of fat body tissue, 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 CPGRs were separately calculated for the larval, pupal and adult stages, based on the total duration of each stage (7 days for larva, 9 days for pupa and 2 days for adult).*

During larval-pupal transformation, its activity levels dropped suddenly and in the early pupa it recorded a decrease of about 11% each in both the batches. Thereafter, its activity registered significant drops during pupal stage, finally ending with a CPGR of -2.29% in ZDC and 1.82% in HFE. Nevertheless, its activity phenomenally increased again during the period of pupal-adult transformation in a sex-specific fashion. Upon its entry into the adult stage, the AAT activity in males and females increased respectively by about 141% and 142% in ZDC and by about 122% and 123% in HFE (Cols.5,6;Table 3).

Succinate dehydrogenase (SDH): In general, the activity levels of SDH in the fat body projected increasing trends during larval and adult lives, but declining trends during pupal life (Table 4). During the 7-day fifth instar regime, this dehydrogenase activity increased from 0.16µm of formazan/mg protein/h to 0.19 µm in ZDC and to 0.22µm in HFE, representing an OGR of 19% in the former and 37.5% in the latter. In terms of day-wise growth rates (CPGRs), the elevation in its activity during larval life was 2.91% in ZDC and 5.45% in HFE. During larval-pupal transformation, its activity levels dropped suddenly and in the early pupa it recorded a decrease of about 21% in ZDC and 18% in HFE. Thereafter, its activity registered significant drops from early to mid and from mid to late pupal stage, finally ending with a CPGR of -18.22% in ZDC and -17.14% in HFE. Nevertheless, its activity increased significantly again during the period of pupal-adult transformation in a sex-specific fashion. Upon its entry into the adult stage, the SDH activity in males and females increased respectively by about 33% and 100% in ZDC and by about 50% and 75% in HFE (Cols.3,4;Table4).

Glutamate dehydrogenase (GDH): By and large, the activity levels of GDH projected growth trends similar to those of SDH, all through the silkworm metamorphosis; with obvious elevatory tends during larval and adult stages, but declining trends during pupal stage (Table 4). During the 7-day fifth

instar regime, the GDH activity increased from 0.11µm of formazan/mg protein/h to 0.34µm in ZDC and to 0.40µm in HFE, representing an OGR of about 201% in the former and 264% in the latter. In terms of day-wise growth rates (CPGRs), the elevations in its activity levelswere20.69% in ZDC and 24.01% in HFE. During larval-pupal transformation, its activity levels dropped suddenly in the early pupa and recorded a decrease of about 38% in ZDC and 35% in HFE. Thereafter, its activity registered significant drops from early to mid and from mid to late pupal stage, finally ending with a CPGR of -16.42% in ZDC and -15.13 % in HFE. Nevertheless, its activity registered manifold increase during the period of pupal -adult transformation in a sex-specific fashion. Upon its entry into the adult stage, the GDH activity in males and females increased respectively by about 380% and 104% in ZDC and by about 271% and 107% in HFE (Cols.5,6;Table4).

DISCUSSION

Insect fat-body is the major centre of energy metabolism in its body. It is widely recognized as an equivalent organ to the mammalian liver and adipose tissue (Arrese and Soulages, 2000). Importantly, it acts as a crucial indicator of fitness by coupling insect metamorphosis with energy metabolism (Aguila *et al.,* 2007). The present study highlights two important facets of energy metabolism in the fat body of *B. mori*. 1) The carbohydrate and non-carbohydrate energy reserves of mulberry diet are invested in the fat body during actively feeding larval stages, in accordance with the Hutchinson's investment principle (Hutchinson *et al*., 1997), 2) The energy reserves, so invested are systematically disinvested and selectively utilized for energy production as per metamorphic demands.

Silkworm energy metabolism reflecting Hutchinson's investment principle: It is customary to analyze mass accumulation in insects in terms of Hutchinson's investment principle (HIP). The underlying concept is that the silkworm metamorphosis is based on the concept of energy reserve and structure and that its larval growth is associated with biomass investment, which becomes the major source of energy for metamorphosis. The HIP has been widely acknowledged. The mechanistic dynamic energy budget (MDEB) model of Maino and Kearney (2015) delineates insect biomass into components of structure (size) and energy reserve (nutritional condition) and predicted that the growth in the former is always accompanied by growth in the latter leading to higher production efficiency in later stages. Llandres *et al*. (2015) confirmatively concluded that the exponential larval growth of lepidopterans is associated with the growth of structural components required for post-embryonic development, silk production and metabolic acceleration.

Investment patterns in energy reserves: The incorporations and depletions patterns of energy reserves into and from the fat body are treated as investments and disinvestments respectively. The dietary glucose is invested in the form of a disaccharide called trehalose and a branched polymer called glycogen and used as a glycolytic fuel to generate energy for metabolism and metamorphosis (Van der Horst, 2003; Yamada *et al*., 2018).The amount of glycogen in the fat body is controlled by glycogenesis and glycogenolysis and the timings of its investments and disinvestments denote timings of glycogenesis and glycogenolysis respectively (Anand and Lorenz, 2008).The non-carbohydrate energy reserves of fat body includes 177 proteins involved in glycolysis, metabolism, cytoskeleton formation, immunity, heat shock mechanism, silk production and muscle contraction and a rich pool of free amino acids (Hou *et al*., 2007; Kasmaei and Mahesha, 2012).

It is known that the carbohydrate and non-carbohydrate energy reserves are incorporated into the fat body predominantly during the final and fifth instar in tune with its exponential body growth coupled with voracious feeding habit and high power of digestibility [Hou *et al.,* 2010; Venugopal Reddy *et al*., 2015). The findings of our study indicate that such investments do occur in pupal stage and are positively reinforced by the honey-enriched mulberry diet. Initially during fifth instar, large quantities of glycogen (277%), trehalose(75%) and glucose (75%)were incorporated into the fat body and their investment rates were further boosted by 77 (354-277%), 78 (153-75%) and 85 (160-75)percentage points respectively under the impact of honey-enriched mulberry diet. Similarly, their daily investment rates, illustrated in terms of CPGRs were also improved by 3.9(28.68 -24.78%), 7.02 (16.77-9.75%) and 7.44 (17.24-9.80%) percentage points under the impact of honey-rich diet (Table 1). Simultaneously during this larval period, the over all investment rates of proteins and FAA were improved by 13(85-72%) and 128 (2163-2035%) percentage points and the daily investment rates by 1.27 (10.75-9.48%) and 1.63 (68.19-66.56%) percentage points respectively under the impact of honey-enriched diet. As predicted by Hutchinson *et al.,* (1997), the larval growth and body mass accumulation in silkworm, depend on three factors; larval duration, diet volume and feeding time. The greater the larval duration, the greater the feeding time available and greater would be the feeding volume. Since, the silkworm has longer duration of 7 days in fifth instar with longer feeding time, it accumulated more mass during this stage (Tables 1, 2).In the mid-pupal stage, the incorporation rates of glycogen (104%), trehalose (43%), glucose 18%), proteins (141%) and FAA (1125%) registered significant

elevations under normal dietary conditions. but were not significantly affected by the hone-enriched diet. Their investment pattern denotes three interesting features of energy metabolism in silkworm; 1) the fat body synthesizes and stores glycogen and proteins continuously during larval and pupal stages, 2) glucose and FAA levels are continuously replenished in both the stages, by way of dietary supplies during larval stage and through increased rates of proteolysis and tissue disintegration during pupal stage and 3) increased turnover of free amino acids that might originate from dietary sources during actively feeding larval stage, proteolytic sources during non-feeding pupal stage and de novo generation from the glucose during both larval and pupal stages (Garrido *et al*., 2015; Sivaprasad, 2015). As observed in other lepidopterans (Matsuda *et al.,* 2015), our study showed that glycogen has faster incorporation kinetics than trehalose and glucose and in contrast, glucose has slower incorporation kinetics than trehalose and glycogen in silkworm. Obviously, the glycogen investment policy aims to maintain glucose homeostasis by allowing interconversion between these two sugars, probably under the control of hormones like adepokinetic hormone (Akh) and octopamine, which regulates the mobilization of glycogen under starved conditions similar to that of glucagon in mammals (Yamada *et al*., 2018). In terms of mass retention trehalose accounts for about 28% carbohydrate reserves in silkworm. Though, its role has not been examined in the present investigation, it is either hydrolyzed and used as a carbon source of energy or synthesized and used as a stabilizer of cellular membranes, stress manager and cryoprotectant in the cellular medium. But many reports pointed out that the primary function of trehalose is not as an energy reserve, but as a cryoprotectant of cell membranes and proteins under extreme stress conditions of dehydration and freezing that deplete the activity of intracellular water in body tissues (Bolat, 2008; Han *et al*., 2008). This is perhaps, what happens in the fat body of silkworm during the larval- pupal- adult metamorphosis. Presumably, the trehalose prevents glycolysis by diverting glucose molecules carrying phosphate groups into trehalose synthetic process that results in massive accumulation of trehalose in its body tissues throughout the larval and mid-pupal stages. Probably, the depletion of glucose levels vis-à-vis elevations in trehalose levels would create favourable conditions for the operation of transdeamination process that meets the major energy demands of metamorphosis.

Utilization of carbohydrate reserves in energy generation: The storage sugars are utilized as energy sources for the survival during fasting and non-feeding stages (Yamada *et al*., 2018). In silkworm, this occurred during larval-pupal and pupal-adult transitions. During larval-pupal transition, about 40% each of trehalose and glycogen and 14% of glucose are used-up and their utilization slightly improved in HFE. In the subsequent period of pupal-adult transition these reserves were utilized in a sex sex-specific fashion; more in males and less in females, without much noticeable distinction between ZDC and HFE. As the silkmoth doesn't feed during adult stage, it substantially mobilizes its energy reserves, probably in preparation for maturation of eggs, insect flight, mating and reproduction (Shukla *et al.,* 2015). In the whole process, glycogen and trehalose act as a backup energy reserves that make available adequate levels glucose, which facilitates energy production through two different channels depending on the availability/non-availability of SDH activity. In SDH active cells it ensures energy release through glycolytic oxidative phosphorylation and in SDH deficient cells it does so indirectly through a non-glucogenic energy pathway called transdeamination (Lussey-Lepoutre*et al.,* 2015).

The comparative analysis of trends in the activity levels of succinate dehydrogenase (SDH), vis-à-vis the quantum levels of glucose are true indicators of oxidative energy metabolism. The SDH has been recognized as the key enzyme that participates in the Krebs cycle and electron transport chain and thus links the former with oxidative phosphorylation (Oyedotun and Lemire 2004;Kasmaei and Mahesha, 2012). In the Krebs cycle, it catalyzes the reaction succinate + Q □ fumarate + QH_2 and the derived electrons are fed into the respiratory chain complex III to reduce oxygen and form water. In silkworm, the SDH activity runs on a low note with different rates of expression in different stages (Fig.1). Its activity is closely associated with changed levels of glucose that are channelized through glycolytic oxidative phosphorylation. Obviously, it showed elevatory trends in larval and adult stages and declining trends in pupal stage. In the honey-fed larvae, its activity was additionally boosted by 18.5 (37.5-19.0%) percentage points. But, surprisingly, the levels of both glucose and SDH activity slumped remarkably during pupal stage, indicating reduced inflow of glucose through oxidative phosphorylation in the backdrop of truncated SDH activity. But surprisingly, when glucose levels are replenished through enhanced glycogenolysis, the SDH activity revived during pupal-adult transition, indicating the resurgence of glycolytic oxidative phosphorylation in the adult stage. During this transitional phase, its activity was enhanced additionally by 17 (50-33%) percentage points in males and -25 (75-100%) percentage points in females under the impact of honey-enriched mulberry diet (Table 1 and Fig.1).

Utilization of non-carbohydrate reserves in energy generation: In compelling situations like

the shortage of glucose and loss of SDH activity, the cells are forced to obtain energy from non-carbohydrate energy reserves through transdeamination (Ravichandran and Ramesh Kumar., 2014; Siva Prasad and Bhuvaneswari, 2018). It is a complex energy generating process, accomplished with the active participation of amino acids like aspartate, alanine and glutamate and enzymes like aspartate aminotransferase (AAT) and alanine aminotransferase (ALAT) and glutamate dehydrogenase (GDH) and this involves two sequential reactions (Scott *et al*., 2004). The first one is a transamination reaction mediated by aminotransferases (AAT and ALAT), resulting in the transfer of amino group of one amino acid to keto acid to generate another amino acid. Subsequently, such amino groups are collected in the form of L-glutamate, which in the presence of GDH is converted to alpha-keto glutarate and ammonia, which in turn connects protein metabolism with citric acid metabolism and meets the energy demands of metamorphosis (Yaginuma and Ushzima, 2005; Ravichandran and Ramesh Kumar., 2014).

The comparative analysis of growth trends in the levels of FAA and the activity levels of AAT, ALAT and GDH clearly demonstrate transdeamination kinetics (Fig. 2). Evidently, the spurt in FAA levels during fifth instar (ZDC: 2035%; HFE: 2163%), with concomitant outbursts in the activity levels of AAT (~1050%), ALAT (~567%) and GDH (~210%) reflect the prevalence of transdeamination pathway during the entire period of larval life and its further continuation through larval-pupal transition with similar ups and downs. In the honey-fed larvae, the activity levels of AAT, ALAT and GDH were further boosted by 150 (1200-1050%), 16 (583-567%) and 53 (263-210%) percentage points respectively. One noteworthy finding of the present investigation is that AAT took lead role in sustaining the transdeamination process during the course of larval-pupal transition. This is true because the activity levels of ALAT, GDH and SDH dropped significantly (11 to 38%) and that of AAT alone enhanced (46%) during this phase. Needless to say a continuous supply of aspartate is necessary for sustained AAT activity and this has been successfully accomplished in silkworm during this crucial period of metamorphic transition. There could be a valid reason for this assumption and this exactly comes from a recent report on SDH activity in mammalian tumor cells (Lussey-Lepoutre*et al.,* 2015), which states that the SDH deficient cells lose metabolic plasticity and display significantly increased synthesis of aspartate from glucose by activating pyruvate carboxylase apart from collecting exogenous aspartate from neighbouring disintegrating cells. More or less, a similar mechanism operates in the silkworm during pupation, which is associated with low SDH activity coupled with fresh replenishments of amino acids from disintegrating tissues like the gut, muscle and silk gland. Nevertheless, all the three enzymes dropped their activities significantly during the rest of the quiescent pupal stage, reflecting the low rate of transdeamination. But surprisingly, the activity of all transdeamination enzymes revived in a great spurt during the grand finale of the pupal-adult transformation, with a bias towards the male sex. Nevertheless, what is surprising is that the GDH assumed greater responsibility of energy supply during this phase, particularly with reference to male sex as evidenced by its peak activity in males (380%), compared to that (104%) in females. The honey-enriched mulberry diet has virtually no significant effect on AAT and ALAT, but reduced the activity of GDH by 109 percentage points in adult males (Fig.6.9). Such a sex-biased explosion in GDH activity substantively confirms that the energy demands of male sex and its expression are met exclusively through enhanced levels of GDH and that the alpha ketoglutarate generated in transamination reaction is used as a substrate for sperm production, sperm motility and successful mating that stimulates fecundity and productivity of in *B. mori* (Hemavathi, 2001; Siva Prasad and Bhuvaneswari, 2018). Further, a close look at the kinetics of transdeamination enzymes indicate that they work in cohesion with singular objective of generating energy from the available free amino acids and in doing so they share their responsibilities with changed preferences between AAT and GDH, in which the former takes lead role in the larval and early pupal stages, while the latter plays vital role in adults (Fig.2).

Relative importance of glycolytic oxidative phosphorylation and transdeamination: The present study amply demonstrates that the silkworm metamorphosis is powered by the energy derived from intermediary metabolism. Though, the relative importance of glycolytic and non-glucogenic energy pathways is not clear, it could be deduced from the CPGR data of all the four key enzymes that explains their day-to-day growth rates in energy metabolism (Fig.3).In fifth instar, the AAT recorded the highest CPGR (50.24%) and it was followed by ALAT (37.19%), GDH (20.69%) and SDH (2.91%). However, during pupal stage all the four enzymes recorded negative CPGRs. Of all, the SDH recorded the greatest decline in its activity (-18.22%) and it was followed by GDH (-16.42%), AAT (-9.87%) and ALAT (-2.29%). The availability of carbohydrate and non-carbohydrate reserves on one hand and preferential changes in the activity levels of key enzymes of energy metabolism indicate that the fat body successfully accomplishes energy production through glycolytic and non-glycolytic oxidative phosphorylation pathways, either alternatively or

together. The choice of pathway obviously, depends on energy needs and energy reserves of metamorphosis. Accordingly, our study reflects three facts about the energy metabolism in *B. mori.* Firstly, the transdeamination profoundly expresses in all stages (larval, pupal and adult), indicating the fact the non-carbohydrate energy reserves are used for energy generation during larval-pupal and pupal-adult metamorphosis. Secondly, carbohydrate-based glycolytic oxidative phosphorylation expresses moderately in larval and adult stages and probably it meets the energy requirements of mechanical and behavioural aspects of metamorphosis. Thirdly, the transdeamination activity is the most preferred pathway in energy metabolism and it takes preponderance in males compared to that in females. These assumptions are based on the fact that the silkworm is metabolically active in all stages, behaviourally inert in pupal stage, but regains its behavioural activeness again in adult stage. Interestingly, the SDH activity is closely associated with larval mechanical behavioural responses like locomotion, feeding and cocoon spinning and adult behavioural responses like wing beat, copulation and mating, whereas it is not so with physiological and metabolic events of pupal stage. Such versatility in energy generating mechanisms confirms additional advantage for holometabolous insects like *B. mori*, which do not eat during pupation and even lack functional mouthparts during adulthood.

Conclusion

In accordance with the Hutchinson's investment principle, the silkworm invested carbohydrate and non-carbohydrate energy reserves in its fat body and metabolizes them for energy release during subsequent pupal and adult stages. Its fat body showed metabolic plasticity in energy production in response to energy demands of metamorphosis vis-a-vis the availability of energy reserves. Owing to the shortage of glucose and loss of SDH activity and excessive availability of amino acids, coupled with higher activities of aminotransferases and GDH, the silkworm reduces its exclusive energy-dependency on glycolytic oxidative phosphorylation and switches over to an alternative energy generation mechanism called transdeamination. The honey-enriched mulberry diet showed mixed impact on energy metabolism in the silkworm. Its impact was positive during actively feeding larval stage and either neutral or negative during non-feeding pupal and adult stages. Importantly, the honey-enriched diet significantly influenced the parameters of glycolytic phosphorylation and non-glycolytic transdeamination during larval life. However, its detectable impact on these parameters has not been noticed during pupal and adult stages, owing to the fact that these two stages represent the non-feeding stages and have absolutely no chance to get dietary supplements of honey. Our work is the first comprehensive preliminary work on energy metabolism in *B. mori.* It not only dispelled the miscomprehensions on energy generation mechanisms during metamorphosis, but also demonstrated that the sericultural productivity could be improved by enriching the mulberry leaf with sugar-rich exogenous nutrients that stimulate glycolytic oxidative phosphorylation.

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