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SYNERGISTIC EFFECT OF HONEY AND LEMON JUICE-ENRICHED MULBERRY DIETS ON THE GROWTH OF SILKWORM, *BOMBYX MORI* DURING METAMORPHOSIS

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ABSTRACT Synergistic impact of honey and lemon juice-enriched mulberry diets on the growth of *Bombyx mori* was studied. The body size progressively increased during larval stage, but declined during pupal and adult stages. The nutrient diets promoted larval growth and positively modulated pupal and adult growth. The larval growth curves are typical Gompertz trajectories that reflected the growth promoting nature of honey and lemon juice. The log-based growth curves were used to derive critical larval body size determinants that control molting and metamorphosis. The nutrient diets improved critical body size determinants without affecting their time schedules. The compound periodical growth rates showed instar-specific and stage-specific variations. The size specific growth rates in body mass, length and perimeter dimensions indicated the prevalence of an effective mass management mechanism as defined in the Hutchinson's investment principle. The silkworm recorded either higher or lower growth ratios indicating deviation from the Dyar's constancy rule.

KEYWORDS : *Bombyx mori*, critical size, growth, honey, lemon juice, threshold size.

INTRODUCTION

Insects acquire appropriate body size through multi-dimensional body growth by actively mobilizing of energy and resources from surroundings (Maino and Kearney, 2015). The genetical and physiological mechanisms underlying this feature has been extensively studied in several lepidopteran insects (eg. Shingleton, 2005; Nijhout *et al*, 2014). Amongst all, the mulberry silkworm, *Bombyx mori* has become a model insect for such studies in view of its economic importance in sericulture. The rationale is that the silkworm accumulates 72% of body mass during the fifth and final instar and the larval growth kinetics change from day-to-day and from instar-to-instar with a systematic violation of Dyar's constancy rule (Siva Prasad, 2022). Further, the silkworm growth is characterized by the existence of inconsistent growth rates obviously due to its complex life cycle involving distinct larval instars, moulting cycles, pupal and adult stages that are sensitive to environmental conditions (Gotthard, 2004; Esperk & Tammaru, 2004). As such, it is difficult to arrive at reliable estimates of the acquisition of appropriate body size at maturity. Therefore, a comprehensive technique becomes inevitable for fitting silkworm growth curves in relative magnitudes (proportionate increases) rather than absolute magnitudes (actual increases), as proposed by Woodruff (1939) and ratified by D'Amico *et al*. (2001) and Siva Prasad (2022) in their reports.

Further, such growth studies carried out under the impact of nutrient-enriched mulberry diets would yield helpful findings for profitability of sericulture. The technique of food-fortification with exogenous nutrients and feeding silkworms with such nutrient-enriched diets have become the order of current research in sericulture (Thulasi and Sivaprasad,2013, 2014; Bhatti *et al*., 2019; Tamilselvi *et al*., 2020; Madhavi and Siva Prasad, 2022). This technique was further improved by the use of mulberry-based and nutritionally-enriched artificial diets with promising insights into the regulation of silkworm metabolism and silk production (Nikolova, 2020).

The report of Baci *et al*., (2021) highlighted the valuable effects of honey and vitamin C (L-ascorbic acid: the chief constituent of lemon juice) and suggested further research on the applicability of these two natural products in sericulture. Against this backdrop, it was considered necessary to gain an in-depth knowledge of the quantitative profiles of silkworm growth under the nutritional impact of honey and lemon juice-enriched mulberry diets as it could have positive implications for evolving appropriate strategies for rearing silkworms.

MATERIAL AND METHODS

Test Species & Experimental Design

The present investigation was carried out on the Fc_1 x Fc_2 hybrid silkworm, *Bombyx mori L (Lepidoptera: Bombycidae). T*he experiment was designed to quantitatively analyze the growth of ***B. mori***, with particular reference to the impact of honey and lemon juice-enriched mulberry diets. The silkworms were reared in separate wooden trays on a standard diet (100%) of V_1 variety of mulberry leaves with 3 feeds per day at 6AM, 12 PM and 6PM, under standard environmental conditions of 28°C, 85% RH, 12h light and 12 h dark conditions. After the second molt, the third instar larvae were divided into four nutrient batches of 100 worms each and labelled them as zero dose control (ZDC), honey-fed experimental (HFE), lemon juice-fed experimental (LFE) and honey+lemon juice-fed experimental groups. While, the ZDC larvae were fed with normal mulberry leaf, the HFE, LFE and HLFE larvae were fed with the mulberry leaves that were enriched either with honey (HFE) or lemon juice (LFE) or with both the nutrients (HLFE) at their 12.00 pm diet. The honey and lemon juice-enriched diets were prepared by soaking mulberry leaves, separately in 2% honey and 3% lemon juice solutions in distilled water and in their mixed solution as per the standard minimum effective concentrations (Thulasi and Siva Prasad, 2014; Madhavi *et al*., 2018). Growth-related studies were carried out on the larvae of both control and experimental batches simultaneously by measuring the body weight, length and perimeter during the larval (3^{rd}, 4^{th} & 5^{th} instars), pupal and adult stages. The mean body weight was determined by measuring the weight of 25 randomly selected worms in an electric balance (ELICO; Model LBL-22OH), and the same was expressed in grams. The mean body length and mean body perimeter were measured by thread and scale method and the same were expressed in centimeters.

Statistical Analysis

The numerical 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). In addition, the following statistical tools were also applied in order to draw meaningful conclusions on silkworm growth.

Growth Index (GI):

The term GI refers to the actual growth capacity of insect larva during metamorphosis. Within each instar, it correlates the final biomass data with initial data. Accordingly, it was computed by the formula $W_f - W_i / W_i$ where, W_i and W_f represent the initial and final body weights of silkworm larva in each instar.

Overall Growth Rate (OGR):
The OGR refers to percentage change in larval growth from the beginning to the end of an instar and it was calculated using an online Percent Change Flatform (https://www.percent change com / index php). In a similar fashion growth indices were calculated for body length and perimeter variables.

Compound Periodical Growth Rate (CPGR):
The CPGR is an innovative growth parameter that explains day-to-day larval growth changes within each instar. It was computed online using the Investopedia flatform (https://www.investopedia.com), as given by Siva Prasad (2012).

Critical Body Size Determinants:
The critical and threshold sizes are considered as the critical body size determinants. The former represents the optimal larval body size within an instar that must be passed in order to trigger commitment to subsequent moulting, while the latter represents the size threshold of silkworm final larval instar that must be passed in order to trigger commitment to pupation. The corresponding values were identified at the inflection points of log curves on the exponential phase of the growth trajectories as demonstrated by Sharma (2005).

Dyar's coefficient:
The Dyar's coefficient (also called Growth ratio) is a calculated value arrived at by dividing the value of a growth variable of one instar by the same value of preceding instar as given by Thakur (2016).

RESULTS AND DISCUSSION

The life cycle of the mulberry silkworm *Bombyx mori* is completed in ~50 days, which includes an embryonic egg stage, five larval instars, pre-pupal, pupal and adult stages. The larval instars, are behaviorally and morphologically separated from each other by ecdysis or molting. They feed and grow constantly, accumulate body mass, provide energy resource and prepares the silkworm for metamorphosis. At the end of the pre-pupal stage, the larva spins a silk cocoon secretes a hard outer cuticle and undergoes a complex process of metamorphosis that includes hyperplastic and hypertrophic growth as well as apoptosis and histolysis of specific larval tissues. After 9 or 10 days, the adult moth emerges from the cocoon by a process called eclosion. Adults have no functional mouth parts and do not feed. Soon after eclosion the female and male moths copulate and the female lays eggs. Both the moths die in four or five days of eclosion and mating (Ganga 2003). Metamorphosis-induced growth changes were measured in larval pupal and adult stages, in terms of body mass, length and perimeter, under the nutritional impact of honey and lemon-juice-enriched mulberry diets , both individually and synergistically and the numerical growth data of zero dose control (ZDC), honey-fed experimental (HFE), lemon juice-fed experimental (LFE) and honey+ lemon juice-fed experimental (HLFE) groups are presented in Tables 1 to 11 and Figures 1 to 8. The log-based larval growth curves vis-à-vis the growth kinetics of larval, pupal and adult stages are discussed with reference to the Hutchinson's investment principle (HIP), Dyar's coefficients and the attainment of critical and threshold body size determinants.

Nutrient-enriched mulberry diets promote larval growth
Larval Body Weight (LBW):
The larval body mass, measured as LBW, showed elevated growth trends from third to fifth instar in ZDC, HFE, LFE and HLFE groups. In third instar, the LBW increased from an initial value of about 0.07g to 0.20 g, representing a growth index (GI) of 1.86 and an overall growth rate (OGR) of ~186% in a span of three days in ZDC. At the same time, the LBW increased to 0.25 g in HFE, to 0.22 g in LFE and to 0.29 g in HLFE, showing a GI of 2.57 and an OGR of ~257% in HFE, a GI of 2.14 and an OGR of ~214% in LFE and a GI of 3.14 and an OGR of ~314% in HLFE (Table 1 A). In fourth instar, the body mass increased from 0.26 g to 0.63 g in ZDC and registered a GI of 1.42 and OGR of ~142% in four days. At the same time, its value increased to 0.75 g with a GI of 1.88 and an OGR of ~188% in HFE, to 0.70 g, with a GI of 1.69 and an OGR of ~169% in LFE and to 0.83 g with a GI of 2.19 and an OGR of ~219% in HLFE (Table 1 B). In fifth instar, the increase in body mass was more significant than that in earlier instars, wherein the LBW scaled from an initial value of 0.78 g to 2.31 g, registering a GI of 1.96 and OGR of ~196% in ZDC. At the same time, its value increased to 2.41 g in HFE, with a GI of 2.09 and an OGR of ~209%, to 2.38 g in LFE with a GI of 2.05 and an OGR of ~205% and to 2.45 g in HLFE, with a GI of 2.14 and an OGR of ~214% (Table 2).

Evidently, the honey and lemon juice-enriched diets showed growth stimulating effect on the body mass, both individually and synergistically. In a span of 14 days (i.e. from third to fifth instar), the silkworm gained a total mass of 2.24 g, (2.31 – 0.07g) in ZDC, 2.34 g, (2.41 – 0.07g) in HFE, 2.31 g, (2.38 – 0.07g) in LFE and 2.38 g, (2.45 – 0.07g) in HLFE. The increase in body mass was equal to ~3200% in ZDC, ~3343% in HFE, ~3300% in LFE and ~3400% in HLFE. This was made possible by appropriately modulating growth rates from instar to instar. In third instar, the OGR was enhanced by 71 percentage points (257-186) in HFE, 28 percentage points (214-186) in LFE and by 128 percentage points (314-186) in HLFE. In fourth instar, it was enhanced by 46 percentage points (188-142) in HFE, 27 percentage points (169-142) in LFE and by 77 percentage points (219-142) in HLFE (Table 1B). In fifth instar, the OGR was enhanced by 13 percentage points (209-196) in HFE, 9 percentage points (205-196) in LFE and by 18 percentage points (214-196) in HLFE (Table 2).

Table 1: The Synergistic Effect Of Honey And Lemon Juice-enriched Mulberry Diets On The Larval Body Weight Of *Bombyx Mori*, During Third And Fourth Instar Development.

A: THIRD INSTAR					
Day	Statistical Tool	ZDC	HFE	LFE	HLFE
1	Mean SD (±)	0.07 0.00	0.07 0.001	0.07 0.001	0.07 0.001
2	Mean PC (%) SD (±)	0.12 71.43 0.001*	0.14 100.00 0.00*	0.13 85.71 0.001*	0.15 114.23 0.001*
3	Mean PC (%) SD (±)	0.20 66.67 0.001*	0.25 78.51 0.001*	0.22 69.23 0.001*	0.29 93.3 0.001*
GI		1.86	2.57	2.14	3.14
OGR (%)		185.71	257.14	214.29	314.29
CPGR(%)		69.03	88.98	77.28	103.54
B: FOURTH INSTAR					
Day	Statistical Tool	ZDC	HFE	LFE	HLFE
1	Mean SD (±)	0.26 0.001	0.26 0.001	0.26 0.001	0.26 0.001
2	Mean PC (%) SD (±)	0.43 64.38 0.001*	0.49 88.46 0.005*	0.47 80.77 0.006*	0.51 96.15 0.005*
3	Mean PC (%) SD (±)	0.53 23.26 0.001*	0.62 26.53 0.004*	0.59 25.53 0.005*	0.68 33.33 0.004*
4	Mean PC (%) SD (±)	0.63 18.87 0.003*	0.75 20.97 0.004*	0.70 18.64 0.006*	0.83 22.05 0.002*
GI		1.42	1.88	1.69	2.19

OGR (%)	142.30	188.46	169.23	219.23
CPGR(%)	34.31	42.35	39.12	47.24

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

Each value is a mean, ± standard deviation of four individual observations on a larval group comprising 25 worms in ZDC (zero dose control), HFE (honey-fed experimental), lemon juice-fed experimental and HLFE (honey+lemon juice fed experimental) batches. Growth trends were analyzed in terms of percent changes (PC), growth index (GI), overall growth rate (OGR) and compound periodical growth rate (CPGR).

Table 2: The Synergistic Effect Of Honey And Lemon Juice-enriched Mulberry Diets On The Larval Body Weight Of *Bombyx Mori* During Fifth Instar Development

Day	Statistical tool	ZDC	HFE	LFE	HLFE
1	Mean SD (±)	0.78 0.01	0.78 0.01	0.78 0.01	0.78 0.01
2	Mean PC (%) SD (±)	1.0 28.2 0.003*	1.30 66.6 0.01*	1.30 66.6 0.01*	1.35 73.1 0.01*
3	Mean PC (%) SD (±)	1.52 52.0 0.002*	1.75 34.6 0.002*	1.71 31.5 0.002*	1.79 32.6 0.002*
4	Mean PC (%) SD (±)	1.92 26.3 0.002*	2.19 25.1 0.004*	2.15 25.7 0.002*	2.23 24.6 0.004*
5	Mean PC (%) SD (±)	2.38 19.6 0.001*	2.55 16.4 0.002*	2.45 13.9 0.003*	2.59 16.1 0.02*
6	Mean PC (%) SD (±)	2.70 13.4 0.001*	2.86 12.2 0.002*	2.81 14.7 0.00*	2.92 12.7 0.002*
7	Mean PC (%) SD (±)	2.31 -14.4 0.003*	2.41 -15.7 0.003*	2.38 -15.3 0.004*	2.45 -16.1 0.003*
GI		1.96	2.09	2.05	2.14
OGR (%)		196.2	208.97	205.13	214.10
CPGR(%)		16.60	20.69	17.47	21.02

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

**The remaining notations are the same as those given under table 1.*

Larval Body Length (LBL):

The growth trends of the larval body in its length dimension were more or less similar to those in the body mass. In third instar, the LBL increased from an initial value of 1.7 cm to 2.0 cm, showing a GI of 0.17 and an OGR of ~18% in in ZDC. At the same time, its value increased to 2.2 cm in both HFE and LFE and to 2.48 cm in HLFE. Thus, the body length recorded a GI of 0.29 and an OGR of ~29% in HFE and LFE and a GI of 0.46 and an OGR of ~46% in the HLFE (Table 3 A). In fourth instar, this growth variable increased from 2.1 cm to 3.4 cm, depicting a GI of 0.62 and OGR of ~ 62% in ZDC. At the same time, its value increased to 4.1 cm with a GI of 0.95 and an OGR of ~95% in HFE, to 4.0 cm, with a GI of 0.90 and an OGR of ~90% in LFE and to 4.4 cm with a GI of 1.09 and an OGR of ~109% in HLFE (Table 3 B). In fifth instar, the increase was more significant. Its value raised from 4.43 cm to 5.85 cm, thus showing a GI of 0.32 and an OGR of ~32% in ZDC. At the same time, its value increased to 6.85 cm in both HFE and LFE, with a GI of 0.54 and an OGR of ~54% and to 7.05 cm in HLFE, with a GI of 0.59 and an OGR of 59% (Table 4). Thus, the body growth in the longitudinal axis was significantly impacted by nutrient diets. In a span of 14 days (i.e. from third to fifth instar), the silkworm gained a total body length of 4.15 cm, (5.85 – 1.7 cm) in ZDC, 5.15 cm, (6.85 – 1.7 cm), both in HFE and LFE and 5.35 cm, (7.05 – 1.7 cm) in HLFE. Thus, the body length grew by ~244% in ZDC, ~301% both in HFE and LFE and by ~315% in HLFE. This was made possible by appropriately modulating OGRs. In third instar, the OGR were enhanced by 11 percentage points (29-18%) each in HFE and LFE and by 26 percentage points (46-18%) in HLFE (Table 3B). In fifth instar, it was enhanced by 23 percentage points (55-32%), each in HFE and LFE and by 27 percentage points (59-32%) in HLFE (Table 4).

Table 3: The Synergistic Effect Of Honey And Lemon Juice-enriched Mulberry Diets On The Larval Body Length Of *Bombyx Mori* During Third And Fourth Instar Development.

A: THIRD INSTAR					
Day	Statistical Tool	ZDC	HFE	LFE	HLFE
1	Mean SD (±)	1.7 0.00	1.7 0.00	1.7 0.00	1.7 0.00
2	Mean PC (%) SD (±)	1.8 5.9 0.00	1.9 11.8 0.00	1.9 11.8 0.00	2.00 17.6 0.00
3	Mean PC (%) SD (±)	2.00 11.1 0.00	2.2 15.8 0.00	2.2 15.8 0.00	2.48 24.0 0.05*
GI		0.17	0.29	0.29	0.46
OGR (%)		17.65	29.41	29.41	45.88
CPGR(%)		8.47	13.76	13.76	20.78
B: FOURTH INSTAR					
Day	Statistical Tool	ZDC	HFE	LFE	HLFE
1	Mean SD (±)	2.1 0.05	2.1 0.05	2.1 0.05	2.1 0.05
2	Mean PC (%) SD (±)	2.4 14.3 0.06*	2.6 23.8 0.05*	2.6 23.8 0.06*	2.8 33.3 0.06*
3	Mean PC (%) SD (±)	2.6 8.3 0.05*	3.2 23.1 0.05*	3.1 19.2 0.05*	3.3 17.9 0.05*
4	Mean PC (%) SD (±)	3.4 30.8 0.05*	4.1 28.1 0.05*	4.0 48.1 0.08*	4.4 33.3 0.09*
GI		0.62	0.95	0.90	1.09
OGR (%)		61.90	95.23	90.47	109.52
CPGR(%)		17.42	24.98	23.96	27.96

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

*The remaining notations are the same as those given under table 1.

Table 4: The Synergistic Effect Of Honey And Lemon Juice-enriched Mulberry Diets On The Larval Body Length Of *Bombyx Mori* During Fifth Instar Development

Day	Statistical tool	ZDC	HFE	LFE	HLFE
1	Mean SD (±)	4.43 0.05	4.43 0.05	4.43 0.05	4.43 0.05
2	Mean PC (%) SD (±)	4.75 7.2 0.06*	5.35 20.8 0.06*	5.05 13.9 0.06*	5.40 21.9 0.05*
3	Mean PC (%) SD (±)	5.02 5.7 0.06*	5.75 7.5 0.06*	5.68 12.5 0.06*	6.07 12.4 0.06*
4	Mean PC (%) SD (±)	5.74 14.3 0.05*	6.55 13.9 0.06*	6.45 13.6 0.06*	6.95 14.5 0.06*
5	Mean PC (%) SD (±)	6.35 10.6 0.06*	7.46 13.9 0.05*	7.38 14.4 0.05*	7.75 5.4 0.06*
6	Mean PC (%) SD (±)	6.65 4.7 0.06*	7.63 2.3 0.05*	7.63 3.4 0.06*	8.15 5.2 0.06*

7	Mean PC (%) SD (±)	5.85 -19.3 0.06*	6.85 -10.2 0.06*	6.85 -10.2 0.06*	7.05 -13.5 0.06*
GI		0.32	0.54	0.54	0.59
OGR (%)		32.05	54.62	54.62	59.14
CPGR(%)		4.74	7.53	7.53	8.05

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

*The remaining notations are the same as those given under table 1.

Larval Body Perimeter (LBPM):

In third instar, the LBPM increased from an initial value of about 1.2 cm to 1.4 cm, showing a GI of 0.17 and an OGR of ~17% in ZDC. At the same time, its value increased to 1.55 cm in both HFE and LFE and to 1.75 cm in the HLFE. Thus, the body length recorded a GI of 0.29 and an OGR of ~29% in HFE and LFE and a GI of 0.46 and an OGR of ~46% in HLFE (Table 5 A). In fourth instar, this growth variable increased from 1.5 cm to 2.5 cm in ZDC, projecting a GI of 0.67 and an OGR of ~67% in four days. At the same time, its value increased to 2.8 cm with a GI of 0.87 and an OGR of ~87% in HFE, to 2.7 cm, with a GI of 0.80 and an OGR of ~80% in LFE and to 2.9 cm with a GI of 0.93 and an OGR of ~93% in HLFE (Table 5 B). In fifth instar, the increase in body length was more pronounced. In this final instar, its value was elevated from 2.85 cm to 3.65 cm, thus showing a GI of 0.28 and an OGR of ~28% in ZDC, to 3.85 cm, with a GI of 0.35 and an OGR of ~35% in HFE, to 3.79 cm with a GI of 0.33 and an OGR of ~33% in LFE and to 4.15 cm with a GI of 0.46 and an OGR of ~46% in HLFE (Table 6).

Table 5: The Synergistic Effect Of Honey And Lemon Juice-enriched Mulberry Diets On The Larval Body Perimeter Of *Bombyx Mori* During Third And Fourth Instar Development.

A: THIRD INSTAR					
Day	Statistical Tool	ZDC	HFE	LFE	HLFE
1	Mean SD (±)	1.2 0.00	1.2 0.00	1.2 0.00	1.2 0.00
2	Mean PC (%) SD (±)	1.3 8.3 0.00	1.4 16.7 0.00	1.4 16.7 0.00	1.45 20.8 0.06**
3	Mean PC (%) SD (±)	1.4 7.7 0.00	1.55 10.7 0.06*	1.55 10.7 0.06*	1.75 20.7 0.06*
GI		0.17	0.29	0.29	0.46
OGR (%)		16.66	29.17	29.17	45.83
CPGR(%)		8.01	13.65	13.65	20.76
B: FOURTH INSTAR					
Day	Statistical Tool	ZDC	HFE	LFE	HLFE
1	Mean SD (±)	1.5 0.05	1.5 0.05	1.5 0.05	1.5 0.05
2	Mean PC (%) SD (±)	1.7 13.3 0.05*	2.0 33.3 0.05*	1.8 20.0 0.05*	2.0 33.3 0.06*
3	Mean PC (%) SD (±)	1.9 11.8 0.06*	2.3 14.9 0.05*	2.2 22.2 0.05*	2.4 19.9 0.06*
4	Mean PC (%) SD (±)	2.5 31.6 0.05*	2.8 21.7 0.05*	2.7 22.7 0.06*	2.9 20.8 0.06**
GI		0.67	0.87	0.80	0.93
OGR (%)		66.67	86.7	80.00	93.3
CPGR(%)		18.56	23.13	21.64	24.58

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

*The remaining notations are the same as those given under table 1.

Thus, during larval growth, the silkworm gained a total perimeter of 2.45 cm (3.65 –1.2 cm) in ZDC, 2.65 cm, (3.85 –1.2 cm) in HFE, 2.09 cm, (3.79 –1.2 cm) in LFE and 2.45 cm (4.15–1.7 cm) in HLFE. In all, the larval body recorded an increase of ~204% in ZDC, ~221% in HFE, ~216% in LFE and ~346% in HLFE in its horizontal plane. This was made possible by appropriate modulation of OGRs by the three nutrient diets. In third instar, the OGR was enhanced by 12 percentage points (29-17%), each in HFE and LFE and by 29% percentage points (46-17%) in HLFE (Table 5 B). In fourth instar, it was enhanced by 20 percentage points (87-67%) in HFE, 13 percentage points (80-67%) in LFE and by 26 percentage points (93-67%) in HLFE and in fifth instar, by 7 percentage points (35-28%) in HFE, 5 percentage points (33-28%) in LFE and by 18 percentage points (46-28%) in HLFE (Table 6).

Table 6: The Synergistic Effect Of Honey And Lemon Juice-enriched Mulberry Diets On The Larval Body Perimeter In *Bombyx Mori* During Fifth Instar Development.

Day	Statistical tool	ZDC	HFE	LFE	HLFE
1	Mean SD (±)	2.85 0.06	2.85 0.06	2.85 0.06	2.85 0.06
2	Mean PC (%) SD (±)	2.95 3.50 0.06*	3.26 14.4 0.06*	3.20 12.3 0.06*	3.36 17.9 0.06*
3	Mean PC (%) SD (±)	3.19 8.1 0.1**	3.59 10.1 0.1*	3.54 10.6 0.06*	3.72 10.7 0.06*
4	Mean PC (%) SD (±)	3.62 13.5 0.06*	4.02 11.9 0.06*	3.85 8.8 0.05*	4.07 9.4 0.06*
5	Mean PC (%) SD (±)	4.05 11.9 0.06*	4.43 10.2 0.05*	4.33 12.5 0.1*	4.62 13.5 0.05**
6	Mean PC (%) SD (±)	4.28 5.7 0.05*	4.75 7.2 0.06*	4.68 8.1 0.06*	4.87 5.4 0.06*
7	Mean PC (%) SD (±)	3.65 -14.7 0.06*	3.85 -18.9 0.06*	3.79 -19.0 0.06*	4.15 -14.7 0.06*
GI		0.28	0.35	0.33	0.46
OGR (%)		28.07	35.09	32.98	45.61
CPGR(%)		4.21	5.14	4.87	6.46

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

*The remaining notations are the same as those given under table 1.

Nutrient-enriched Diets Modulate Growth In Pupal And Adult Stages

The growth in *B. mori* is complex process due to complex life cycle. The pre-pupal stage is the final stage for food consumption because the adults, lacking functional mouth parts exist only briefly to reproduce and its body size is determined by an ecdysone pulse at the pre-pupal size (Grunet *et al.*, 2015). Once its larvae enter metamorphosis, energy stored during the pre-pupal stage will be the sole source available for the remainder of the life cycle. Not surprisingly, then, the body mass depletes, length decreases and perimeter increases with age during 9-day pupation and 3-day adulthood.

Table 7: The Synergistic Effect Of Honey And Lemon Juice-enriched Mulberry Diets On The Pupal And Adult Body Weight Of *Bombyx Mori*.

Day	Statistical tool	Pupal Body Weight (g)				Adult Body Weight (g)			
		ZDC	HFE	LJFE	LJ+HFE	ZDC	HFE	LJFE	LJ+HFE
1	Mean SD (±)	1.28 0.01	1.28 0.01	1.28 0.01	1.28 0.01	0.53 0.03	0.53 0.03	0.53 0.03	0.53 0.03

2	Mean	0.91	1.09	1.07	1.15	0.50	0.48	0.51	0.52
	PC (%)	-28.9	-14.8	-16.4	-10.2	-5.7	-19.9	-3.8	-1.9
	SD (±)	0.005*	0.005**	0.001*	0.004*	0.14*	0.18**	0.17*	0.17**
3	Mean	0.90	1.08	1.04	1.12	0.47	0.46	0.51	0.52
	PC (%)	-1.1	-0.9	-2.9	-2.6	-6.00	-4.2	0.00	0.00
	SD (±)	0.15**	0.06*	0.09**	0.12**	0.19**	0.2**	0.19**	0.28**
4	Mean	0.88	1.07	1.01	1.09	-	-	-	-
	PC (%)	-2.2	-0.9	-2.9	-2.7				
	SD (±)	0.01*	0.01*	0.02*	0.18**				
5	Mean	0.86	0.89	0.95	1.01	-	-	-	-
	PC (%)	-2.3	-16.8	-5.9	-7.3				
	SD (±)	0.01*	0.01*	0.04*	0.003*				
6	Mean	0.80	0.82	0.92	0.96	-	-	-	-
	PC (%)	-6.9	-7.9	-3.2	-4.9				
	SD (±)	0.001*	0.11*	0.13**	0.06**				
7	Mean	0.79	0.80	0.81	0.87	-	-	-	-
	PC (%)	-1.3	-2.4	-11.9	-9.4				
	SD (±)	0.11**	0.003*	0.001*	0.01*				
8	Mean	0.76	0.78	0.78	0.83	-	-	-	-
	PC (%)	-3.8	-2.5	-3.7	-4.6				
	SD (±)	0.03*	0.01*	0.01*	0.08*				
9	Mean	0.65	0.72	0.74	0.80	-	-	-	-
	PC (%)	-14.5	-7.7	-5.1	-3.6				
	SD (±)	0.004*	0.02**	0.03*	0.1*				
GI		-0.49	-0.43	-0.42	-0.37	-0.11	-0.13	-0.04	-0.02
OGR (%)		-49.22	-43.75	-42.19	-37.50	-11.32	-13.20	-3.77	-1.89
CPGR(%)		-8.12	-6.94	-6.62	-5.71	-5.83	-6.84	-1.90	-0.95

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

Each value is a mean, ± standard deviation of four individual observations on a larval group comprising 25 worms in ZDC (zero dose control), HFE (honey-fed experimental), lemon juice-fed experimental and HLFE (honey+lemon juice fed experimental) batches. Growth trends were analyzed in terms of percent changes (PC), growth index (GI), overall growth rate (OGR) and compound periodical growth rate (CPGR).

Pupal Body Weight (PBW):

The PBW values recorded negative growth in ZDC and nutrient groups. From an initial value of 1.28 g, the PBW declined to 0.65 g in ZDC, 0.72 g in HFE, 0.74 g in LFE and 0.80 g in HLFE. Thus, the pupal body mass recorded a GI of -0.49 and an OGR of -49% in ZDC, a GI of -0.43 and an OGR of -44% in HFE, a GI of -0.42 and an OGR of -42% in LFE and a GI of -0.37 and an OGR of -37% in HLFE. As shown in Table 7, the depletion rate of body mass was minimized in three nutrient groups. It was reduced by 5 percentage points (49-44%) in HFE, 7 percentage points (49-42%) in LFE and by 12 percentage points (49-37%) in HLFE.

Adult Body Weight:

During the 3-day moth regime, the adult body weight (ABW) declined from an initial value of 0.53 g to 0.47 g in ZDC, to 0.46 g in HFE, 0.51g in LFE and 0.52 g in HLFE. Thus, the body mass recorded a GI of -0.11 and an OGR of -11% in ZDC, a GI of -0.13 and an OGR of -13% in HFE, a GI of -0.04 and an OGR of -4% in LFE and a GI of -0.02 and an OGR of about -2% in HLFE. The depletion rate in adult body mass was minimized by 2 percentage points (-13-11%) in HFE, 7 percentage points (11-4%) in LFE and by 9 percentage points (11-2%) in HLFE (Table 7).

Pupal Body Length (PBL):

From an initial value of 4.33 cm, the PBL declined to 2.05 cm in ZDC, 2.55 cm in HFE, 2.50 cm in LFE and 2.60 cm in HLFE. Thus, the body length recorded a GI of -0.53 and an OGR of -53% in ZDC, a GI of 0.42 and an OGR of -42% both in HFE and LFE and a GI of -0.40 and an OGR of -40% in HLFE. Similar to that in body mass, the reduction in PBL was minimized 11 percentage points (53-42%) in both HFE and LFE groups and by 13 percentage points (53-40%) in HLFE. (Table 8).

Adult Body Length:

The ABL increased considerably during adult phase. From an initial value of 2.08 cm, its value was elevated to 2.23 cm in ZDC, 2.31 cm in HFE, 2.35 cm in LFE and 2.65 cm in HLFE. Thus, the body length recorded a GI of 0.07 and an OGR of about 7% in the control group, a GI of 0.11 and an OGR of 11% in HFE, a GI of 0.13 and an OGR of 13% in LFE and a GI of 0.27 and an OGR of 27% in HLFE (Table 3.8). In all, the ABL was enhanced by 4 percentage points (11-7%) in HFE, 6 percentage points (13-7%) in LFE and by 20 percentage points (27-7%) in HLFE (Table 8). Thus, all the nutrient diets marginally enhanced the already depleted levels in body length during pupal-adult transition.

Table 8: The Synergistic Effect Of Honey And Lemon Juice-enriched Mulberry Diets On The Pupal And Adult Body Length Of *Bombyx Mori*.

Day	Statistical tool	Pupal Body length(cm)				Adult Body length (cm)			
		ZDC	HFE	LFE	HLFE	ZDC	HFE	LFE	HLFE
1	Mean	4.33	4.33	4.33	4.33	2.08	2.08	2.08	2.08
	SD (±)	0.06	0.06	0.06	0.06	0.05	0.05	0.05	0.05
2	Mean	3.30	3.45	3.43	3.72	2.20	2.25	2.30	2.45
	PC (%)	-23.8	-20.3	-20.8	-14.1	5.8	8.2	10.6	17.8
	SD (±)	0.05*	0.06*	0.05*	0.05*	0.08**	0.21*	0.12**	0.24**
3	Mean	3.10	3.35	3.30	3.55	2.23	2.31	2.35	2.65
	PC (%)	-6.1	-2.9	-3.8	-4.6	1.4	2.7	2.2	8.2
	SD (±)	0.00	0.06*	0.00	0.06*	0.1*	0.14**	0.06*	0.17**
4	Mean	3.00	3.30	3.28	3.50				
	PC (%)	-3.2	-1.5	-0.6	-1.4				
	SD (±)	0.0*	0.0*	0.05*	0.0*				
5	Mean	2.88	3.15	3.15	3.35		-	-	-
	PC (%)	-4.0	-4.5	-3.9	-3.9				
	SD (±)	0.05*	0.00	0.06*	0.06*				
6	Mean	2.85	3.15	3.05	3.35		-	-	-
	PC (%)	-1.0	0.00	-3.2	0.00				
	SD (±)	0.17**	0.12**	0.06**	0.1**				
7	Mean	2.35	3.05	3.03	3.35				
	PC (%)	-17.5	-3.2	-0.65	-0.7				
	SD (±)	0.06*	0.06*	0.21**	0.06*				
8	Mean	2.28	3.00	3.00	3.20	-	-	-	-
	PC (%)	-2.9	-1.6	-0.9	-4.5				
	SD (±)	0.00	0.0	0.00	0.00				
9	Mean	2.05	2.55	2.50	2.60	-	-	-	-
	PC (%)	-10.1	-15.0	-16.7	-18.8				
	SD (±)	0.06**	0.06*	0.0*	0.0*				

GI	-0.53	-0.42	-0.42	-0.40	0.07	0.11	0.13	0.27
OGR (%)	-52.66	-42.11	-42.26	-39.95	7.21	11.06	12.98	27.40
CPGR(%)	-8.92	-6.40	-6.64	-6.18	3.54	5.38	6.29	12.87

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

*The remaining notations are the same as those given under table 7.

Pupal Body Perimeter (PBPM):

Contrary to those in body mass and length, the pupal body perimeter showed growth increments that are closely associated with enlargement of body size in its horizontal plane. In ZDC, the LBPM increased from an initial value of about 2.85 cm to 3.35 cm, showing a GI of 0.17 and an OGR of ~17% in a span of nine days. At the same time, its value increased to 3.45 cm both in HFE and LFE and to 3.5 cm in HLFE. Thus, the body length recorded a GI of 0.21 and an OGR of ~21% in HFE and LFE and a GI of 0.23 and an OGR of ~23% in the HLFE (Table 9). The nutrient-enriched diets positively impacted growth rates in the dimension. Under their influence, its OGR was enhanced by 4 percentage points (21-17%), each in HFE and LFE and by 6 percentage points (23-17) in HLFE (Table 9).

Table 9: The Synergistic Effect Of Honey And Lemon Juice-enriched Mulberry Diets On The Pupal And Adult Body Perimeter Of *Bombyx Mori*.

Day	Statistical tool	Pupal Body Perimeter(cm)				Adult Body Perimeter (cm)			
		ZDC	HFE	LJFE	HLFE	ZDC	HFE	LJFE	HLFE
1	Mean SD (±)	2.85 0.06	2.85 0.06	2.85 0.06	2.85 0.06	2.75 0.29	2.75 0.29	2.75 0.29	2.75 0.29
2	Mean PC (%) SD (±)	2.95 3.5 0.06**	3.15 10.5 0.06*	3.10 8.8 0.0*	3.08 -8.1 0.05*	2.80 1.8 0.23**	2.83 2.9 0.72**	2.83 2.9 0.67**	2.85 3.6 0.69**
3	Mean PC (%) SD (±)	3.00 1.7 0.0*	3.25 3.2 0.06**	3.20 3.2 0.05**	3.18 3.2 0.05**	2.93 4.6 0.1**	3.05 3.5 0.21**	3.10 9.5 0.29**	3.38 18.6 0.05
4	Mean PC (%) SD (±)	3.03 1.0 0.05*	3.30 1.5 0.0**	3.28 2.5 0.05*	3.20 0.6 0.00*				
5	Mean PC (%) SD (±)	3.10 2.3 0.0*	3.35 1.5 0.06*	3.30 0.6 0.08*	3.38 5.6 0.05*	-	-	-	-
6	Mean PC (%) SD (±)	3.15 1.6 0.06*	3.35 0.00 0.06*	3.38 2.4 0.05*	3.40 0.6 0.0**	-	-	-	-
7	Mean PC (%) SD (±)	3.20 1.6 0.00	3.35 0.00 0.06**	3.40 0.6 0.00	3.40 0.00 0.00	-	-	-	-
8	Mean PC (%) SD (±)	3.28 2.5 0.21**	3.40 1.5 0.00	3.40 0.00 0.0*	3.45 1.5 0.06**	-	-	-	-
9	Mean PC (%) SD (±)	3.35 2.1 0.06**	3.45 1.5 0.06**	3.45 1.5 0.06*	3.50 0.00 0.00*	-	-	-	-
GI		0.17	0.21	0.21	0.23	0.06	0.11	0.13	0.24
OGR (%)		17.54	21.05	21.05	22.81	6.54	10.91	12.73	23.91
CPGR(%)		2.04	2.42	2.42	2.60	3.22	5.31	6.17	10.86

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

*The remaining notations are the same as those given under table 7.

Adult Body Perimeter (ABPM):

The ABPM values also increased in silk moth projecting enlargements of body size in its horizontal plane in all the four groups examined. In ZDC, it increased from an initial value of about 2.75 cm to 2.93 cm, showing a GI of 0.06 and an OGR of ~6% in a span of three days. At the same time, its value increased to 3.05 cm in HFE to 3.10 cm in LFE and to 3.38 cm in HLFE. Thus, the body length reached a GI of 0.11 and an OGR of ~11% in HFE, a GI of 0.13 and an OGR of ~13% in LFE and a GI of 0.24 and an OGR of ~24% in the HLFE (Table 9). The nutrient-enriched diets positively impacted growth rates in the ABPM. Under their influence, its OGR was enhanced by 5 percentage points (11-6%) in HFE, 7 percentage points (13-6%) in LFE and by 18 percentage points (24-6) in HLFE (Table 9). Thus, the impact of nutrient diets on ABW, ABL and ABPM was generally positive and the positivity was more pronounced in the HLFE. Thus, the all the nutrient diets saved body mass and body length depletion and by doing so, they also saved the energy requirement during pupal and adult stages.

In the present study, *B. mori*, reached the pre-pupal stage on the seventh day of fifth instar. Not surprisingly, this is the final stage for food consumption and growth and upon reaching the maximum size in this stage, the body mass decreased with age during pupation and brief adulthood. Similar growth trends were observed in *B. mori* during the larval development and it is further substantiated by growth studies made on the silkworm from our laboratories (Madhavi and Siva Prasad, 2022). Once its larvae enter metamorphosis, energy stored during this stage will be the sole source available for the remainder of the life cycle in pupal and adult stages. A recent report on *B. mori* (Blossman Myer and Burggren , 2010) provides an illustrative account on growth, metabolism and body mass management in silkworm during metamorphosis and also provides a clear explanation for the findings of present investigation. It highlights in importance of growth in three specific tissues-midgut, silk glands and cuticle. According to them the gut is the major dominant organ in larval silkworm and its growth contributes significantly to the mass accumulation. The midgut has the lowest body proportion grows phenomenally during larval growth and attains the highest proportion in fifth instar and its digestive activity increases from a low to high during larval growth from third to fifth instar. As a result, the thickness of midgut wall increases and generates the peristaltic mobilization of gut contents and absorptive surface area facing the gut lumen. Likewise, the silk glands rapidly reach their peak mass by the end of fifth instar and the larva attains the highest proportion of silk gland mass to total body mass. During their growth, the silk glands synthesize and secrete silk proteins that are used to spin the cocoon at pupation and later they are catabolized during larval-pupal metamorphosis. The third factor that might contribute to the accumulation of body mass is the hyperplastic growth in the epidermal layers underlying the cuticle during ecdysis growth and development of cuticle, which contributes minutely to the body mass growth during larval development as it frequently shed during molts. obviously due to the ongoing process of histolysis in the larval nervous tissue, epidermis, prothoracic glands and parts of the digestive tract. Thus, in *B. mori*, the body mass secondarily generated by metamorphic-induced histolysis in different organs like the gut, musculature, nervous tissue, epidermis, silk gland and other parts are effectively utilized during the adult stage for the simultaneous production of morphological structures like the tracheal muscles, flight muscles, eyes, wings, legs, genitalia and the adult nervous system (Heyland and Moroz 2006). The pattern development in the adult moth vis-à-vis the arrangement of newly formed organs must have been caused by biomass consolidation in longitudinal and horizontal planes of the body. Perhaps, because of this reason the adult silk moth registered increasing growth trends in its body length as well as body perimeter by extension of the abdomen as a result of the unfolding of intersegmental membranes.

Growth Promoting Effect Of Nutrient Diets Reflected In Growth Curves

The larval growth curve is a typical Gompertz trajectory. In each instar it is truncated with an initial exponential phase and a final declining phase that finally assumes an overall sigmoidal shape. According to Grunert (2015), the Gompertz equation implies that there is a decline in the growth exponent during the instar. It gives an idea as to the acquisition of instar specific body size and thus helps deciphering silkworm growth at a glance for descriptive and predictive purposes (Fitzhugh (1976). Such a truncated growth is explained with reference to body mass vis-à-vis developmental changes in water composition. The larval *B. mori* receives water largely through its food and hence, the water content becomes the highest in mid-larval developmental stages, but declines slowly as the larva prepares for a molt at the end of an instar and the fall in water level becomes more pronounced at the end of larval stage with the commencement of pupation and metamorphosis (Heyland and Moroz 2006). Thus, the larval growth stops well before the sigmoidal asymptote is reached, as a result of ecdysone pulse (Nijhout *et al*., 2006). This ecdysone pulse occurs well before a larva reaches its maximum potential size and causes the larva to stop feeding and initiate next molt or in the last larval instar, its entry into the wandering stage in preparation for pupation. The larval growth gradually tapers off during pre-pupal period although it continues to live and feed for a long time before it starts metamorphosis (Truman and Riddiford, 1974). As observed in the present investigation, the larval growth slowes down on the seventh day of fifth instar indicating the commencement of the pre-pupal stage (Tables 1, 2 & 3).

We evaluated the hypothesis of exponential growth by preparing a dual purpose charts with exponential and logarithmic functions, using iterative software algorithms available on MS Excel platform. Accordingly, the log function was added to the exponential trajectory in the form of a logarithmic trend line. It apparently, gives an idea of how many data points fall within the regression equation and thereby facilitates the identification of coefficient of determination (R^2) that could be used as an indicator of better goodness of fit. In such, growth curves, the direction or slope or curvature of log-based trend line was used as an indicator of growth pattern. Accordingly, if the curve bends upwards (towards left) it was assumed that the growth rate is increasing and if it bends downwards (towards right) the growth rate is decreasing (Woodruff, 1939; Siva Prasad, 2022). Thus, its left deflection reflects growth increment, while its right deflection indicates growth decrement, the proportion of which is directly related to the degree of deflection. The greater the degree of deflection, the greater would be the growth change in that direction. Within an instar, the log curve showed left deflection initially and finally tilted to right crossing the growth trajectory before it reaches sigmoidal asymptote. The intersection point of log curve with the Gompetz trajectory was considered as the optimal growth required by an insect larva to attain critical body size determinants, which differed from one variable to the other as a function of the compound periodical growth rate (CPGR).

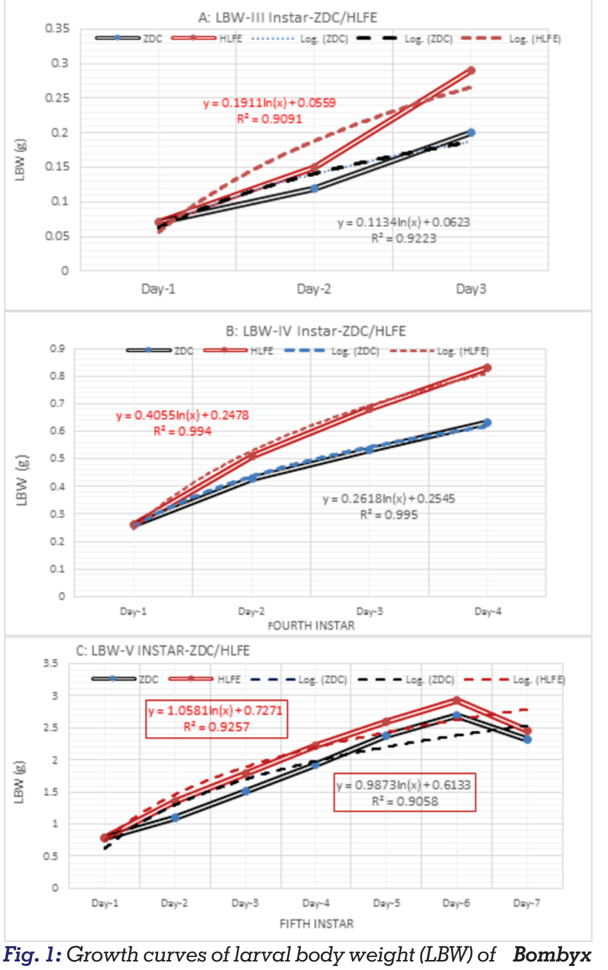

Fig. 1: *Growth curves of larval body weight (LBW) of* ***Bombyx mori*** *during third (A) , fourth (B) and fifth (C) instar developmental stages in zero dose control (ZDC) and honey-fed experimental (HEF) batches (Source: Tables 1 & 2).*

Body Mass Growth Curves:

With regard to LBW, the degree of its left deflection was inversely related to the number of instar and declined from third to fourth and from fourth to fifth instar. Accordingly, the degree of its left deflection was more predominant in third instar and its proportion was reduced significantly in fourth instar and further narrowed down in fifth instar (Fig. 1 A, B, C). Concurrently, the LBW recorded a higher CPGR (69.03%) in third instar, moderate CPGR (34.31%) in fourth instar and a low CPGR (16.60%) in fifth instar (Table 1).

The honey and lemon juice-enriched mulberry diets have significantly tilted the log-curves further left showing higher CPGRs and more biomass accumulation. The synergistic impact of honey and lemon juice on the growth of larval body mass was more pronounced than their individual effects (Fig. 4). As for instance, both the growth trajectory and its log-curve of HLFE showed higher degree of left deflections indicating higher CPGRs in third (103.54%), fourth (47.24%) and fifth instar (21.02%). Further, the log curve of fifth instar showed a left deflection in the first half and right deflection in the second half, indicating an exponential body growth during the first four days and a fall in growth during the last three days of final instar and hence recorded lower CPGRs compared to those in third and fourth instar.

Body Length Growth Curves:

Contrary to the body mass, the larval body length (LBL) showed rapid growth in fourth instar compared to that in third and fifth instars. Accordingly, the degree of its left deflection was more pronounced in fourth instar and its proportion was reduced significantly in third and fifth instars (Fig. 2 A, B, C). Evidently, the LBL recorded a higher CPGR (17.42%) in fourth instar and it was followed by a moderate CPGR (8.47%) in third instar and a very low CPGR (4.74%) in fifth instar, despite a large growth in body length and associated mass deposition in the longitudinal axis of the larval body (Table 3 & 4).

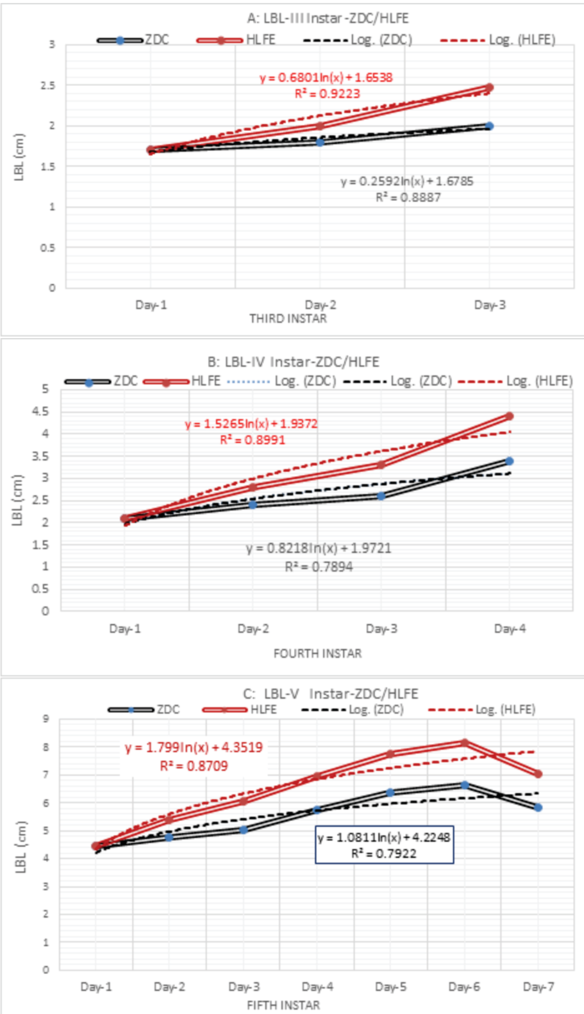

Fig. 2: Growth curves of larval body length (LBL) of ***Bombyx mori*** *during third, fourth and fifth instar developmental stages in zero dose control (ZDC) and honey-fed experimental (HEF) batches (Source: Tables 3 & 4).*

Like those in body mass, the nutrient (honey and lemon juice) enriched mulberry diets have significantly tilted the log-curves further left showing higher CPGRs and more biomass deposition in the length-wise plane of the larval body. Importantly, the synergistic impact of honey and lemon juice on the growth of larval body length was more pronounced than their individual effects. As shown in Fig.5, both the growth trajectory and its log-curve of HLFE showed higher degree of left deflections in a declining order of CPGRs in fourth (27.96%), third (20.78%) and fifth instar (8.05%). As in body mass, the log curve of fifth instar showed a low degree of left deflection at the instar beginning and small right deflection at the end of the instar and hence recorded lower CPGRs compared to those in earlier instars.

Body Perimeter Growth Curves:

The growth curves of larval body perimeter (LBPM) are similar to those of body length. The LBPM showed more vigorous growth in fourth instar followed by third and fifth instars. Accordingly, the degree of its left deflection was more pronounced in fourth instar and its proportion was reduced significantly in third instar and further narrowed down in fifth instar (Fig. 3 A, B, C). Evidently, the LBPM recorded a higher CPGR (18.56%) in fourth instar and it was followed by a moderate CPGR (8.01%) in third instar and a very low CPGR (4.21%) in fifth instar (Tables 5 & 6). Like those in body mass and body length, the nutrient-enriched mulberry diets have significantly tilted the log-curves further left showing higher CPGRs and more biomass deposition in the perimeter-wise plane of the larval body. Significantly, the synergistic impact of honey and lemon juice on the growth of larval body perimeter was more pronounced than their individual effects. In HLFE, both the growth trajectory and its log-curve of HLFE displayed higher degree of left deflections indicating higher CPGRs in fourth (24.58%), third (20.76%) and fifth instar (6.46%), in the same decreasing order (Fig.6; Tables 5 & 6). As in body mass and body length the log curve of body perimeter of fifth instar showed a low degree of left deflection at the instar beginning and small right deflection at the end of the instar and hence recorded lower CPGRs compared to those in earlier instars.

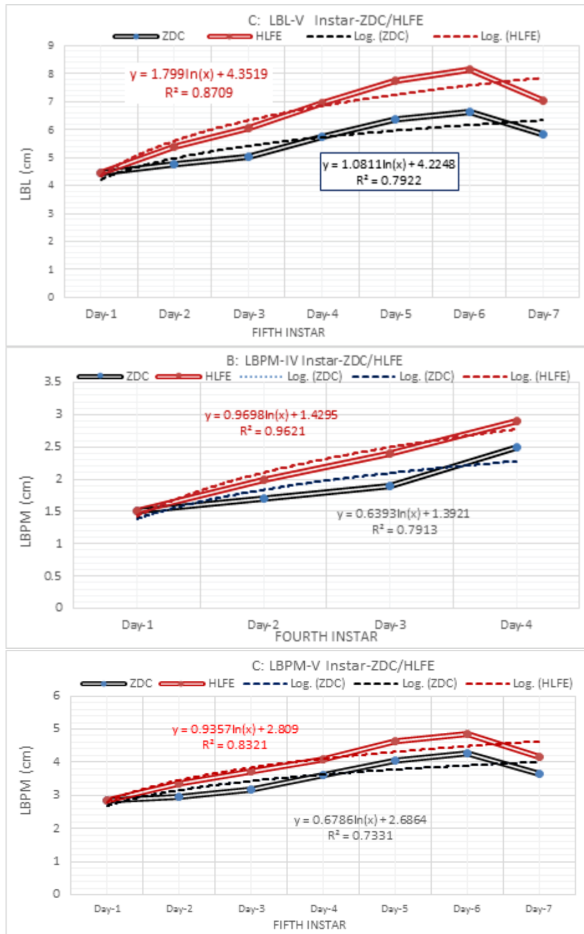

Fig. 3: Growth curves of larval body perimeter (LBPM) of ***Bombyx mori*** *during third, fourth and fifth instar developmental stages in zero dose control (ZDC) and honey-fed experimental (HEF) batches (Source: Tables 5 &6).*

Compound Periodical Growth Rates Show Instar-specific And Stage Specific Variations

The silkworm growth shows day-specific, instar-specific and stage-specific variations. The instar-specific variations are seen in larval growth, while the stage-specific variations occur in pupal and adult stages. As such it is difficult to define a characteristic 'rate' either within an instar or across the entire growth trajectory. This problem could be resolved by applying an innovative statistical tool called compound periodical growth rate (CPGR) as suggested by Siva Prasad (2012). The CPGR describes growth trends more accurately than the traditional method of arithmetic mean return (AMR), which is based on the calculation of periodical percent changes and thereby facilitates meaningful, reliable and accurate interpretations of growth-related studies. More importantly, it is best suited for the comparative analysis of growth rates of groups of insects and their larval forms reared under different nutritional or treatment conditions. The comparative analysis of growth trends in body weight (LBW, PBW, ABW), body length (LBL, PBL, ABL) and body perimeter (LBPM, PBPM, ABPM) vis-à-vis CPGR reveals instar-specific and stage-specific variations in all the four groups examined.

Body Mass CPGRs:

As shown in Tables 1 and 2, the larval body weight (LBW) in ZDC increased from 1st day of third instar (0.07g) to 6th day of fifth instar (2.70g), registering a massive increase of about 3757%. The silkworm achieved this, by recording bigger a CPGR of 69. 03% in third instar, a moderate CPGR of 34.12% in fourth instar and the lowest CPGR of 16.60% in fifth instar. The honey and lemon juice-enriched diets suitably enhanced body mass accumulation with relatively higher CPGRs. As seen in HFE, the honey-enriched diet boosted the body mass level to 2.86g (~3986% rise), with relatively higher CPGRs in the third (88.98%), fourth (42.35%) and fifth (20.69%) instars. In LFE, the LBW grew from 0.07 to 2.81g (3914% rise), registering a CPGR of 77.2% in third instar, 39.12% in fourth instar and 17.47% in fifth instar. Similarly, the LBW of HLFE scaled to a peak of 2.92g (4071% elevation) with higher CPGRs in third (103.54%), fourth (47.24%) and fifth (21.02%) instars. The study indicated that the CPGRs of larval body mass are inversely related to the number of instar, the lower the former, the higher would be the latter. Obviously, the scaling in larval body mass was accompanied by falling CPGRs during larval development from third to fifth instar. Thus, the larval body mass grows by higher CPGRs in third instar, moderate CPGRs in fourth instar and by smaller CPGRs in fifth instar.

Contrary to that in larval instars, the CPGR of body mass declined in pupal and adult stages. In ZDC, the PBW of the pupa decreased from 1.28g to 0.65g (~49% drop) and registered a negative CPGR of -8.12% during the 9-day quiescent period. In HFE, the pupal body mass depleted to 0.72g (~44% loss) with a CPGR of -6.94% per day and in LFE, it dropped to 0.74 (~42% loss), with a steady CPGR of 6.62% per day. Synergistically, both honey and lemon juice reduced the loss in body mass. In HLFE, the PBW slumped to 0.80g (~38% loss) with a steady depletion rate of 5.71% per day during the pupal period. Thus, the pupa lost about 40% of the accumulated body mass during pupal stage with decreasing CPGRs in the control and experimental groups. In adult, the body mass depletion followed suit. The ABW of adult moth dropped from an initial value of 0.53g in ZDC and three experimental groups. Its value dropped to 0.47g (~11% loss) at a CPGR of 5.83% in ZDC, to 0.46g (~13% loss) at a CPGR of -6.84% in HFE, to 0.51g (~4% loss) at a CPGR of 1.90% in LFE and to 0.52g (~2% loss) with a CPGR of 0.95% in HLFE. Thus, during pupal-adult transformation, the pupa loses about 10% of body mass (2 to 4% in nutrient groups) by registering low negative CPGRs during the 4-day adult regime.

Body Length CPGRs:

In ZDC, the LBL of silkworm increased from 1.7 cm on 1st day of third instar to 6.65 cm on 6th day of fifth instar, registering an elevation of about 291% during the last three instars. It resulted from a moderate CPGR of 8.47% in third instar, larger 17.42% in fourth instar and a low CPGR of just 4.74% in fifth instar. In both HFE and LFE, the body length increased to 6.85 cm (~349% rise) each, with a moderate CPGR of 13.76%% in the third instar, a higher CPGR of 24.98% (LFE:23.96%) in fourth instar and a low CPGR of 7.53% in fifth instar. In HLFE, the LBW increased to 8.15 cm (~317% rise), with a CPGR of 20.78% in third instar, 27.96% in fourth instar and a low CPGR of just 8.05% in fifth instar. Thus, the larval body length grows more rapidly in third and fourth in stars and slowly in the fifth and final instar.

The pupal body recorded negative CPGRs and the adult body positive CPGRs in their growth in the longitudinal axis. In ZDC, the PBL dropped from 4.33 cm to 2.05 cm (~53% drop), losing half of its body at steady CPGR of -8.92% during the 9-day quiescent period. In HFE, the PBL dropped to 2.55 cm (~42%% drop) at a steady CPGR of -6.40% per day. In LFE, the body length dropped to 2.50 cm (~42% loss), with a steady CPGR of 6.64% per day. Synergistically, both honey and lemon juice further slowed down the body shortening rate. In HLFE, the PBL was shortened down to 2.60g (~40% loss) at a CPGR of -6.18% per day during the pupal period. Thus, the pupa loses 42 to 48% of its body length during the pupal stage by registering smaller negative CPGRs all through its development. The body shortening process continued in adult stage as well. Contrary to that in the pupal stage, the ABL of adult moth increased slightly from 2.08 cm to 2.23 (~7% rise) at a CPGR of 3.54% in ZDC. The same positive trend continued in nutrient groups. In HFE, the ABL increased to 2.31 cm (~11% rise) at a CPGR of -5.38% in HFE, to 2.35 (~13% rise) with a CPGR of 6.29% in LFE and to 2.65 cm (~27% rise) at a CPGR of 12.87% in HLFE. Thus, the body length of silk moth registered positive growth trends with low CPGRs (3.54 to 12.7%) in different nutritional groups.

Body Perimeter CPGRs:

The body perimeter projected similar instar-specific and stage-specific trends. From a small value of 1.20 cm on the 1st day of third instar, the LBPM scaled to a maximal value of 4.28 cm on the 6th day of fifth instar, achieving about 257% rise in three larval instars. While doing so, it registered a CPGR of 8.01% in third instar, 18.56% in fourth instar and 4.21% in fifth instar. Thus, the LBPM grows more rapidly in fourth instar compared to the third and fifth instars. All the nutrient diets, boosted the growth of perimeter throughout the larval regime. In HFE, the LBPM scaled to 4.75 cm (~296% rise) at a CPGR of 13.65% in third instar, 23.13% in fourth instar and 5.14% in fifth instar. Similarly, in LFE, the LBPM raised to the level of 4.68 cm (~190% rise) at a CPGR of 13.65% in third instar, 21.64% in fourth instar and 4.87% in fifth instar. In HLFE it rose to a further higher level of 4.87 (~306%) at a CPGR of 20.76% in third instar, 24.58% in fourth instar and 6.46% in fifth instar.

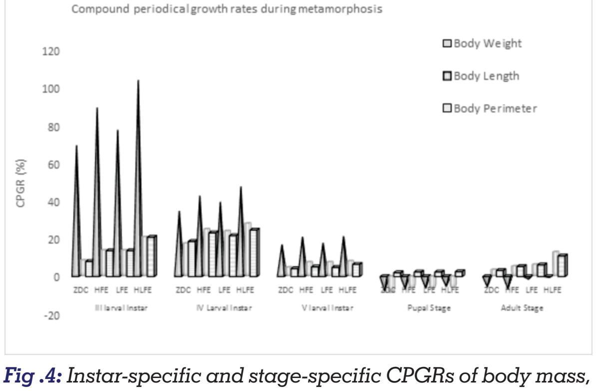

***Fig .4:** Instar-specific and stage-specific CPGRs of body mass, length and perimeter in B. mori under the influence of honey and lemon juice-enriched mulberry diets (Source: Tables 1 - 9).*

Interestingly, the pupal and adult bodies recorded positive growth and positive CPGRs in their horizontal axis. In ZDC, the PBPM was elevated from 2.85 cm to 3.35 cm (~17% rise) at a steady CPGR of 2.04% during the 9-day quiescent period. In

HFE and LFE, the PBPM was elevated to 3.45 cm (~21% rise) each at a steady CPGR of 2.42% per day. Synergistically, both honey and lemon juice further boosted the body perimeter size. Accordingly, in HLFE the PBPM scaled to 3.50g (~23% up) at a CPGR of 2.60% per day during the pupal period. Thus, the pupal body gained size increment (17 to 22%) in its perimeter during the pupal stage by registering smaller CPGRs. The body widening process continued in adult stage as well. In ZDC, the ABPM increased slightly from 2.75 cm to 2.93cm (~7% rise) at a CPGR of 3.22% I during the adult regime. The same positive trend continued in nutrient groups. The ABPM increased to 3.05 cm (~11% rise) at a CPGR of -5.31% in HFE, to 3.10 (~13% rise) at a CPGR of 6.17% in LFE and to 3.38 cm (~24% rise) at a CPGR of 10.86% in HLFE. Thus, the body perimeter of the silk moth showed positive growth trends with low CPGRs in different nutritional groups.

Thus, our study clearly demonstrated that there is a decline in CPGR at the end of an instar, especially during molts and pupal and adult transformations. As suggested by Grunert (2015), this could be attributed to three independent processes that constrain pure exponential growth and cause the decline in the growth rates. Firstly, the exoskeleton has limited capacity to expand and the gradually increasing tension exerted by the epicuticle slows the growth rate. Secondly, the limited capacity of the tracheal system to supply oxygen to a growing body during the intermolt period and thirdly the decrease in the relative capacity of the gut to transport nutrients due to hypo-allometric growth with respect to body mass would limit the growth during transitional periods. It is not known, whether one or two of these processes or all the three are jointly constrain further growth as the larva becomes larger. Further, the falling CPGRs from third to fifth instar apparently imply that the growth in silkworm is associated with increased accumulation of low-metabolizing tissues like fat bodies or gut content or cuticle or probably a combination of all the three. Nonetheless, the instar-specific and stage-specific growth trends indicate the following four features.

I. Larval stage represents growth enhancement phase. During this phase the body grows phenomenally in all the three size dimensions of mass, length and perimeter and attains appropriate body size that triggers moulting and metamorphosis. Silkworm accomplishes this task by rapid CPGRs throughout the larval regime, more particularly during the third and fourth instars. These growth rates were instar-specific in the sense, the CPGRs are larger in third and fourth instars and smaller in fifth instar.

II. The pupal stage represents the quiescent phase characterized by growth retardation and body size realignment through steep mass depletion, length reduction and abdominal enlargement. The silkworm achieves this through low negative CPGRs in PBW and PBL and low positive rates in PBPM.

III. The adult stage represents reproductive phase and this involves origin and development of head, eyes, sensory organs, adult nervous system, wings, genital organs and associated musculature and abdominal enlargement. The silk moth achieves this through adult-specific negative CPGRs in body mass and body length dimensions and positive CPGRs in body perimeter dimension, particularly in the abdominal region.

IV. The nutrient-enriched mulberry diets either positively stimulated or appropriately modulated CPGRs in all body size dimensions, both individually as in HFE and LFE groups and synergistically as in HLFE group in all stages of silkworm metamorphosis.

Size-specific growth rates support Hutchinson's investment principle

In silkworm, the overall body size growth trajectories follow an upward trend up to the sixth day of fifth instar and a downward trend thereafter during pupal and adult stages (Figs. 1, 2 & 3). Obviously, they reflect the growth of body mass accumulation and its utilization in all the three body dimensions up to pre-pupal stage. Hutchinson *et al.*, (1997) explained biomass accumulation in insects in terms of an investment principle, which became popular as the Hutchinson's investment principle (HIP). It assumes that the molt-dependent growth of insect larval soft bodies as investment of energy resources for future use. It believes that insects, whose feeding rate depends on the size of structures that grow by molt, monotonously display declining trends in size increments and increasing trends in instar durations. The HIP, as illustrated by Maino and Kearney (2015) in their mechanistic dynamic energy budget model, delineates insect biomass into components of structure (size) and energy reserve (nutritional condition) and predicts that the growth in the former is always accompanied by growth in the latter leading to higher production efficiency in later developmental stages that manifests as the tracheal expansions and fat body depositions required for structural components of growth and reproduction (Miranda *et al.*, 2002; Kooijman, 2010; Greenlee *et al.*, 2013). Recently, the concept of reserve and structure has been extended to larval growth and biomass accumulation in silkworm (Siva Prasad, 2022). The findings of present study throw light on the energy reserve and structure concept and emphasizes the role of HIP in *B. mori* metamorphosis. The mass-specific, length-specific and perimeter-specific growth rates observed in the present study, illustrate the basic tenets of silkworm growth with particular reference to HIP.

Mass-Specific Growth Rates:

The growth trends in LBW, PBW and ABW bear strong testimony to mass accumulation and utilization in silkworm. Evidently, the body mass accumulates phenomenally during larval regime, especially from the 1^{st} day of third instar to 6^{th} day of fifth instar, notwithstanding minor drops during the intervening molts and declines subsequently in pupal and adult stages. As shown in Tables 1 and 2, the LBW increased from 0.07g to 2.70g registering an overall increase of 3757% in body mass in larval tissues. Further analysis of mass specific growth rates (MSGRs) show that the body mass accumulation occurred in day-specific and instar-specific manners. For instance, higher MSGRs were recorded on day-2 (~71%) and day-3 (~67%) in third instar, on day-2 (~64%) of fourth instar and on day-2 (~28%) and day-3 (~52%) of fifth instar (Tables 1 & 2). The instar-wise trends indicated that the actual growth increments were ~186% (increase from 0.07 g to 0.20g) in third instar, ~215% (increase from 0.26 g to 0.63g) in fourth instar and ~329% (increase from 0.78g to 2.70g) in fifth instar. The body mass, so accumulated up to 6^{th} day of fifth instar (pre-pupal stage) has been subsequently utilized during pupal and adult stages as evident from the mass decrements and negative MSGRs observed in its growth. Clearly, the body mass declined from a higher level of 2.7g in fifth instar to 0.47g on the fourth day of adult stage, thus recording ~83% loss in body mass (~2.24g utilization) jointly in pupal and adult stages.

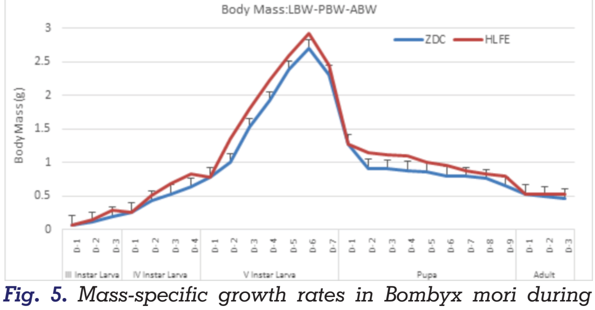

Fig. 5. *Mass-specific growth rates in Bombyx mori during metamorphosis under the synergistic impact of honey+lemon juice-enriched mulberry diet (Source: Tables 1,2 & 7).*

The nutrient diets stimulated larval MSGRs, individually and synergistically and positively modulated biomass utilization during pupal and adult regimes. By and large, identical growth trends were recorded in all the experimental groups. For instance, as seen in HLFE, the honey and lemon juice caused a synergistic effect of about 4071% increase (0.07 to 2.92g) in body mass accumulation in three growth increments of about 314% in third instar, 315% in fourth instar and 363% in fifth instar. This positive impact extended to pupal and adult stages wherein about 2.45g of body mass (2.92-0.47g), equivalent to 82% was utilized. Thus, the HLFE silkworm accumulated 0. 22g (2.92-2.70g) of additional biomass that provides extra energy for metabolism, growth, silk production and metamorphosis.

Length-Specific Growth Rates (LSGRs):

The biomass accumulation was accompanied by enhanced growth rates in body length during larval stage up to the 6th day of fifth instar that dropped subsequently during pupal and adult stages. The growth trends in LBL, PBL and ABL bear strong testimony to length elevation and biomass orientation in longitudinal axis. In larval *B. mori*, the body length grew phenomenally from the 1st day of third instar to 6th day of fifth instar, notwithstanding minor drops during the intervening molts. During this growth period, the LBL grew from 1.70 cm to 6.65 cm, registering an impressive elevation of 291% in three instars. Further analysis of LSGRs show that the body lengthening process vis-à-vis mass accumulation in longitudinal axis follows day-specific and instar-specific approach. For instance, higher LSGRs were recorded on day-3 (~11%) in third instar, on day-4 (~31%) of fourth instar and on day-2 (~7%) and day-4 (~14%) and day-5 (~11%) in fifth instar (Tables 3 & 4). The instar-wise trends indicated that the growth increment was ~18% (increase from 1.7 cm to 2.0 cm) in third instar, ~62% (increase from 2.1 cm to 3.4 cm) in fourth instar and ~50% (increase from 4.43 cm to 6.65 cm) in fifth instar. The body length, so gained up to 6th day of fifth instar has been subsequently reduced during pupal and adult stages as evident from the length decrements and negative LSGRs observed in its growth. Clearly, the body length declined from a higher level of 6.65 cm in fifth instar to 2.23 cm on the fourth day of adult stage, showing a negative OGR of about 66%, jointly during pupal-adult metamorphosis.

The nutrient quality of mulberry diet positively influenced the LSGRs. The nutrient diets stimulated body length prolongation during larval regime and positively modulated its orientation during pupal and adult regimes. For example, as seen in HLFE, the honey and lemon juice caused a synergistic effect of about ~379% increase (1.7 to 8.15 cm) in body length in three growth increments of ~21% in third instar, ~28% in fourth instar and ~59% in fifth instar. This positive impact continued during pupal-adult metamorphosis wherein about 5.92 cm of body length (8.15-2.23 cm), equivalent to 73% got shortened, but an additional length of 1.50 cm (8.15-6.65 cm) generated which probably provides extra mass and extra energy that gives new impetus to silkworm growth.

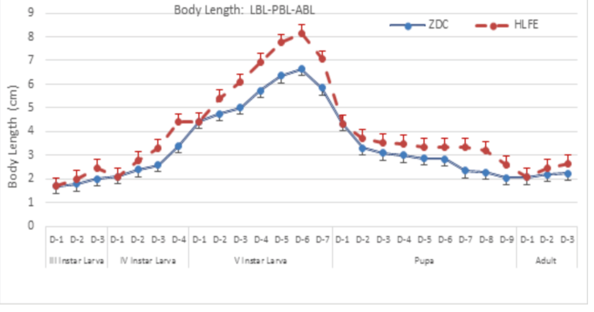

Fig. 6. Length-specific growth rates in Bombyx mori during metamorphosis under the synergistic impact of honey+lemon juice-enriched mulberry diet (Source: Tables 3, 4 & 8).

Perimeter-Specific Growth Rates (PSGR):

The body perimeter grown exponentially during larval stage, reaching its peak on the 6th day of fifth instar and fell subsequently during pupal and adult stages. The growth trends in LBPM, PBPM and ABPM bear strong testimony to body widening process in silkworm. From the 1st day of third instar to 6th day of fifth instar, the LBPM grew from 1.2 cm to 4.28 cm, registering an overall growth rate (OGR) of 257% in horizontal plane notwithstanding minor drops during the intervening molts. Further analysis of PSGRs showed that the body widening process vis-à-vis mass accumulation in horizontal axis follows day-specific and instar-specific approaches. Higher PSGRs were recorded on day-2 (~8%) in third instar, on day-4 (~32%) in fourth instar and on day-3 (~8%) and day-4 (~14%) in fifth instar (Tables 3.3 & 3.4). The instar-wise trends indicated that the growth increment was ~17% (increase from 1.2 cm to 1.4 cm) in third instar, ~67% (increase from 1.5 to 2.5 cm) in fourth instar and ~28% (increase from 2.85 cm to 3.65 cm) in fifth instar. The body length, so gained up to 6th day of fifth instar has been subsequently reduced during pupal and adult stages as evident from the length decrements and negative PSGRs observed in its growth. Clearly, the body perimeter declined from a higher level of 4.28 cm in fifth instar to 2.93 cm on the fourth day of adult stage, recording a loss of ~32%, jointly during pupal-adult metamorphosis. At the same time, the LBPM scaled by about 257% (increase from 1.2 to 4.28 cm) in ZDC, 296% (increase from 0.12 to 4.75 cm) in HFE, 290% (increase from 1.2 to 4.68 cm) in LFE and by about 306% (increase from 0.12 to 4.87 cm) in HLFE during the last three instars. The honey and lemon-juice-enriched diets stimulated body widening process during larval regime and positively modulated its re-orientation during pupal and adult regimes. For example, as seen in HLFE, the honey and lemon juice caused a synergistic effect of about ~306% (increase from 1.2 to 4.87 cm) in body enlargement in three growth increments of about 46% in third instar, 93% in fourth instar and 46% in fifth instar. Thus, in HLFE, the silkworm gained an additional perimeter of 0.59 cm (4.87-4.28 cm) and lost ~1.49 cm (4.87-3.38 cm), equivalent to 31% in shortening process during pupal-adult metamorphosis at no extra cost. Probably, this extra perimeter provides extra mass and extra energy that gives new impetus to silkworm growth.

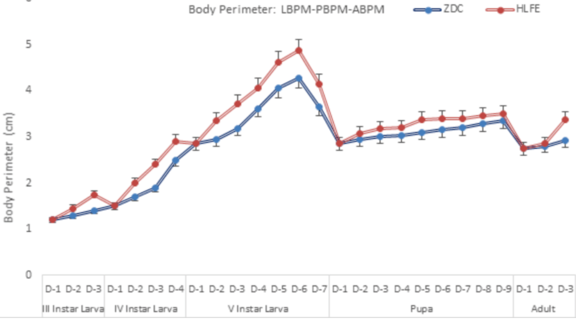

Fig. 7. Perimeter-specific growth rates in Bombyx mori during metamorphosis under the synergistic impact of honey+lemon juice-enriched mulberry diet (Source: Tables 5, 6 & 9).

After all, the growth is considered the accumulation of biomass as a result of the complicated biological balance of absorption, assimilation and dissimilation. Though, the apparent growth exponent declines at the end of each instar, the growth of body mass increases from instar to instar reaching its peak in fifth instar, probably in tune with its voracious feeding habit coupled with high power of digestibility (Hou *et al.*, 2010). The size increase in three larval body dimensions (weight, length & perimeter) is indispensable for a variety of reasons. Recently, Llandres *et al.*, (2015) observed 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. Similarly, the size-specific decline in the growth rates indicate the utilization of body mass during pupal-adult metamorphosis (Venugopal Reddy *et al.*, 2015), probably due to the fact that the reproductive efficiency of silkworm is constrained by body mass and energy resources accumulated during the larval stages that cannot be offset by compensatory feeding in the adult stage (Chown and Nicolson, 2004; Boggs and Freeman, 2005). The size-specific variations in growth rates are in consonance with those in *Manduca sexta* (Nijhout *et al.,* 2006; Sears *et al.,* 2012) and accordingly portrays three important events of biomass management; mass accumulation, mass consolidation and mass utilization in *B. mori*. While the first process solely occurs in the larval stage, the second and third processes are represented in all the three stages (larval, pupal and adult) as detailed below.

1. *B. mori* accumulates energy reserves in a phased manner during larval stage and deposits it in body tissues, to be used in the ensuing non-feeding pupal and adult stages. The intent of biomass accumulation is to acquire appropriate body size, which is more problematic owing to the fact the delivery of oxygen becomes a limiting factor late in the larval growth (Shingleton *et al*., 2008; Callier and Nijhout, 2011; Sears *et al*., 2012). Apparently, the silkworm overcomes this problem by consolidating larval body mass in three dimensions of weight, length and perimeter. By doing so, the silkworm achieves the much required physiological plasticity in organ size; as for instance the increase in gut size and silk gland under nutrient stress. The physiological plasticity in organ size vis-à-vis increase in the size of gut and silk gland confers adaptive significance for silkworm, which does not eat during pupation and even lack functional mouthparts during the adult phase (Blossmam-Myer and Burggren, 2010; Yeoh *et al.,* 2012). As predicted by Hutchinson *et al.,* (1997) and ratified by Siva Prasad (2022), its larval growth vis-a-vis body mass accumulation depends 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, compared to those of 3-day period in third instar and 4-day period in fourth instar.

2. The body mass so acquired is consolidated in in the length and perimeter dimensions as evidenced by increased growth rates in LBL and LBPM throughout the larval regime. Accordingly, the silkworm accumulates biomass and attains optimal larval body size and develops the tracheal system and ventilatory structures that could ensure free oxygen delivery and meet its energy needs of aerobic metabolism and overall organismal functions (Harrison and Haddad , 2011; Heinrich *et al*., 2011; Greenlee *et al*., 2013). Obviously, such growth mechanisms, becomes indispensable during the final larval instar period during which the fat body grows tremendously and makes heavy demands on the tracheal system to supply more oxygen.

3. Body mass utilization continues through larval, pupal and adult stages for maintenance and metabolism. The highest growth rates observed in LBW, LBL and LBPM of silkworm during larval instars are indicative of increased tracheal expansion that meets the increased energy demands during larval-larval transformation as observed in many other lepidopteran insects (Centanin *et al*., 2010; Helm and Davidowitz, 2013; Nijhout and Callier, 2015). Further, the body perimeter is the only growth variable that shows positive growth uniformly in all the three stages (larval, pupal and adult) indicating increased mass accumulation in the horizontal plane of the body. The pupal body shows negative growth trends in body mass and length dimensions and positive trend in body perimeter dimension. The adult body projects negative growth trend in body mass and positive trends in body length and perimeter dimensions.

Thus, in *B. mori*, the Hutchinson's investment principle works by biomass accumulation in larval tissues, biomass consolidation by the formation of need-based new organs or tissues and biomass utilization in histolysis, energy production, metabolism and maintenance during metamorphosis. Moreover, the positive impact of honey and lemon juice on silkworm growth is substantiated by related studies (Thulasi and Sivaprasad, 2015; Madhavi *et al*., 2018, Madhavi and Siva Prasad, 2022). The study of Helm and Davidowitz (2013) demonstrated that insect structural growth in the tracheal system is positively influenced by larval nutrition and that the caterpillars reared on a quality nutrient diet invested relatively more mass into their tracheal systems and that the expansion in tracheation occurs in a size-dependent manner. It is also known that the high quality diet can lead to an optimization pattern in growth in tracheal system vis-à-vis growth in oxygen supply and increased metabolic demand (Harrison and Haddad, 2011). It has been strongly established that steroid hormone ecdysone and insulin-like growth factors promote normal tissue growth at low concentrations under the impact of nutrient manipulation and that the timing of PTTH secretion was presumably regulated by the same (yet unknown) physiological processes that control PTTH secretion in larvae reared under normal diet (Davidowitz and Nijhout, 2004; Nijhout *et al.,* 2014). In tune with these observations, the silkworms reared on honey and lemon juice-enriched mulberry diets, grew with higher size increments compared to those reared on normal leaf. The reserve concept advocated in HIP is motivated by the observation that nutritional history qualitatively affects biomass, which in turn has metabolic consequences. Obviously, the higher growth rates in the body size parameters observed under the impact of nutrient diets could have caused raised metabolic demands and created a physiologically optimal respiratory environment with greater tracheal expansion that could have led to increased oxygen supply to the growing tissues all through the metamorphosis.

Nutrient Diets Improve Critical Larval Body Size Determinants Without Affecting Their Time Schedules

Quite often, the larval growth in insects is analyzed with reference to two body size determinants, namely the critical size and threshold size. The realistic method for critical size determination is not available. Nevertheless, it is presumed that the intersection point of log curve with the Gompertz trajectory indicates the level of optimal growth required by an insect larva to attain critical body size determinants (Sharma, 2005; Grunert *et al.,* 2015). The present investigation adopted the same methodology, which has already been successfully illustrated with reference to larval growth in silkworm (Siva Prasad, 2022; Madhavi and Siva Prasad, 2022).

Critical Size:

The critical size is defined as the minimal larval body size limit that must be passed in order to trigger commitment to moulting or ecdysis (Davidowitz *et al*., 2003). It is equivalent to 55% of larval body mass and is considered as an 'immediate cause' that triggers moulting and larval-larval (instar-to-instar) transformation through optimal somatic growth. After the attainment of this size limit, an interval timer sets in and simulates terminal larval growth phase leading to molting. After a molt, this size becomes initial size for the next instar and growth resumes. Aptly, the CS could be used as an indicator of silkworm growth that helps predict its moulting and metamorphosis.

This concept has been examined with reference to larval body size in three dimensions of critical weight (CW), critical length

(CL) and critical perimeter (CPM) in third, fourth and fifth larval instars, both in control (ZDC) nutrient groups (HFE, LFE & HLFE) and presented in Table 3.10. The values and the time of acquisition of CS differed from instar to instar and from one nutritional group to the other. In third instar, the silkworm attained a CW of 0.17g, (R^2=0.9223), a CL of 1.9 cm (R^2 = 0.8887) and a CPM of 1.45 cm (R^2 = 0.9777) in ZDC, a CW of 0.21g (R^2=0.9249), a CL of 2.1 cm (R^2 = 0.9314) and a CPM of 1.45 cm (R^2 = 0.9954) in HFE, a CW of 0.19g (R^2=0.9314), a CL of 2.3 cm (R^2 = 0.9223) and a CPM of 1.61 cm (R^2 = 0.9596) in LFE and a CW of 0.24g (R^2=0.9091), a CL of 2.3 cm (R^2 = 0.9223) and a CPM of 1.61 cm (R^2 = 0.9596) in HLFE, all on 2.7th day or approximately after 65 hours of exponential growth. In fourth instar, it attained a CW of 0.58g, (R^2=0.9950), a CL of 3.0 cm (R^2 = 0.7894) and a CPM of 2.1 cm (R^2 = 0.7913) in ZDC, a CW of 0.68g (R^2=0.9961), a CL of 3.6 cm (R^2 = 0.8946) and a CPM of 2.52 cm (R^2 = 0.9589) in HFE, a CW of 0.64g (R^2=0.9979), a CL of 3.5 cm (R^2 = 0.8888) and a CPM of 2.4 cm (R^2 = 0.9072) in LFE and a CW of 0.76g (R^2=0.9940), a CL of 3.8 cm (R^2 = 0.8991) and a CPM of 3.7 cm (R^2 = 0.9621) in HLFE, all on 3.7th day or approximately after 88 hours of exponential growth. In fifth instar, the larva attained a CW of 2.1g, (R^2=0.9058), a CL of 5.6 cm (R^2 = 0.7922) and a CPM of 3.6 cm (R^2 = 0.7331) in ZDC, a CW of 2.2g (R^2=0.9243), a CL of 6.6 cm (R^2 = 0.8803) and a CPM of 4.0 cm (R^2 = 0.7467) in HFE, a CW of 2.2g (R^2=0.9297), a CL of 6.4 cm (R^2 = 0.8769) and a CPM of 3.8 cm (R^2 = 0.7369) in LFE and a CW of 2.2g (R^2=0.9257), a CL of 6.8 cm (R^2 = 0.8709) and a CPM of 4.1 cm (R^2 = 0.8321) in HLFE, all on 4th day or approximately after 96 hours of exponential growth.

Thus, the honey and lemon juice--enriched mulberry diets have significantly enhanced critical size in three dimensions of body mass, length and perimeter without altering their time lines. The study further indicated that the timing of acquisition of critical size by the silkworm is crucial and that all those involved in silkworm rearing should know this in order to ensure right feeding for right growth of its larvae. Though, the physiological basis of critical size attainment is not examined in the present investigation, it is presumed that the attainment of critical body size dimensions, vis-à-vis larval-larval molt are triggered prothoracicotrophic hormone (PTTH) and ecdysteroids that are secreted into the haemolymph from time to time (Browder *et al.,* 2001). Obviously, in silkworm, these changes occur after 65 h growth in third instar, 88 h growth in fourth instar and 96 h growth in fifth instar. Probably, the silkworm attains critical size dimensions by appropriately modulating the day-to-day growth rates within an instar (Siva Prasad, 2022). The higher growth rates in larval body mass, recorded on day-2 (71 to 114%) and day-3 (66 to 93%) in third instar, day-2 (64 to 96%) in fourth instar and day-3 (31 to 52%) in fifth instar could have contributed to the acquisition of critical size dimensions by the silkworm.

Table 10: The Synergistic Effect Of Honey And Lemon Juice-enriched Mulberry Diets On The Critical And Threshold Body Size Determinants Of *Bombyx Mori* During Larval Growth.

Size Parameter	ZDC Batch Value	Day	R2 Value	HFE Batch Value	Day	R2 Value	LFE Batch Value	Day	R2 Value	HLFE Batch Value	Day	R2 Value
A. THIRD INSTAR												
CW (g)	0.17	2.7	0.9223	0.21	2.7	0.9249	0.19	2.7	0.9314	0.24	2.7	0.9091
CL (cm)	1.90	2.7	0.8887	2.10	2.7	0.9314	2.30	2.7	0.9223	2.30	2.7	0.9223
CPM (cm)	1.45	2.7	0.9777	1.45	2.7	0.9954	1.61	2.7	0.9596	1.61	2.7	0.9596
B. FOURTH INSTAR												
CW (g)	0.58	3.7	0.9950	0.68	3.7	0.9961	0.64	3.7	0.9979	0.76	3.7	0.9940
CL (cm)	3.00	3.7	0.7894	3.60	3.7	0.8946	3.50	3.7	0.8888	3.80	3.7	0.8991
CPM (cm)	2.10	3.7	0.7913	2.52	3.7	0.9589	2.40	3.7	0.9072	2.60	3.7	0.9621
C. FIFTH INSTAR												
CW (g)	2.10	4.0	0.9058	2.20	4.0	0.9243	2.20	4.0	0.9297	2.20	4.0	0.9257
CL (cm)	5.60	4.0	0.7922	6.60	4.0	0.8803	6.40	4.0	0.8769	6.80	4.0	0.8709
CPM (cm)	3.60	4.0	0.7331	4.00	4.0	0.7467	3.80	4.0	0.7369	4.10	4.0	0.8321
TW (g)	1.60	3.0	0.6781	1.70	3.0	0.7009	1.60	3.0	0.6781	1.80	3.0	0.7076
TL (cm)	5.00	3.0	0.7735	5.90	3.0	0.7847	5.90	3.0	0.7741	5.90	3.0	0.7795
TPM (cm)	3.20	3.0	0.7932	3.40	3.0	0.8094	3.30	3.0	0.7979	3.60	3.0	0.8115

Source: *Figures 1 to 30. The critical and threshold values and the timing of their occurrence were identified from the intersection points of corresponding log curves and exponential growth curves.* R^2 *values above 0.5000 are considered significant for the goodness of fit.*

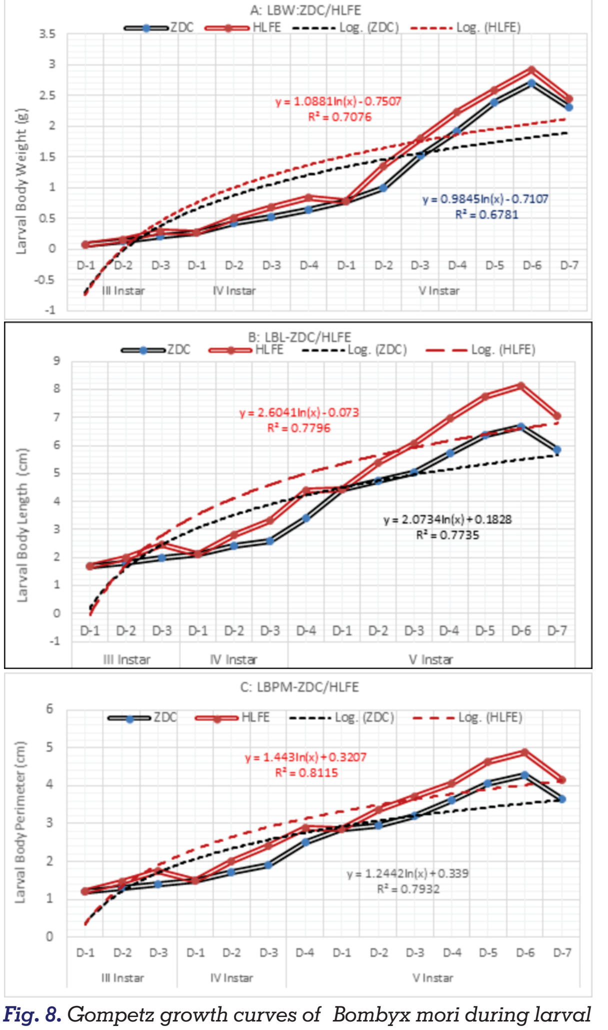

Fig. 8. *Gompetz growth curves of Bombyx mori during larval development under the synergistic impact of honey+lemon juice-enriched mulberry diet (Source: Tables 1 to9).*

Threshold Size:

The commitment to metamorphosis is determined by a property called the threshold size (TS), which is operationally measured as the mass or head capsule width of the larva at the time of the molt (Kingsolver, 2007). Also called minimal variable size (MVS), the TS, represents the minimal body mass required for larval-pupal metamorphosis (De Moed *et al.,* 1999). Generally, the final instar larva acquires this property and upon reaching TS, no additional instars are formed and the larva prepares for pupation (Grunert *et al.,* 2015). In fact, the TS is considered as the long-term cause that initiates a cascade of developmental events leading to larval-pupal metamorphosis and acquisition of adult body size. Similar to that of critical size, the concept of TS is explained in terms of threshold weight (TW), threshold length (TL) and threshold perimeter (TPM) and the relevant values, corresponding to the inflection points of log curve on the Gompertz trajectory at its peak level were identified and presented in (Table 10). Evidently, in fifth instar the silkworm attained a TW of 1.6g, (R^2=0.6781), a TL of 5.0 cm (R^2 = 0.7735) and a TPM of 3.2 cm (R^2 = 0.7932) in ZDC and a higher TW of 1.7g (R^2 = 0.7009), a TL of 5.9 cm (R^2 = 0.7847) and a TPM of 3.4 cm (R^2 = 0.8094) in the HFE, a TW of 1.6g (R^2 = 0.6781), a TL of 5.9 cm (R^2 = 0.7741) and a TPM of 3.3 cm (R^2 = 0.7979) in LFE and TW of 1.8g (R^2 = 0.7076), a TL of 5.9 cm (R^2 = 0.7795) and a TPM of 3.6 cm (R^2 = 0.8115) in HLFE all on day-3, exactly after 72 h of exponential growth (Table 10; Fig. 8). Similar to critical size dimensions, the threshold size dimensions are enhanced under the dietary influence of honey and lemon juice, but the timing of their occurrence remained unaltered. The threshold growth dimensions are substantially acquired through instar-specific CPGRs, more particularly those in third and fourth instars. Though the larval size is minute in third instar, the speed of larval growth was extremely higher as evident from the CPGRs recorded in in larval body mass (69.03 to 103.54%) in different nutritional groups. Further, the higher CPGRs recorded in the length dimension of the larval body in third (8.47 to 20.78%) and fourth (17.42 to 27.96%) instars and in perimeter dimensions in third (8.01 to 20.76%) and fourth (18.56%) instar also would have contributed to the acquisition of appropriate threshold body size in silkworm. The studies on larval growth in *Manduca sexta* (Kingsolver, 2007), lend support to our investigation. The physiological mechanism of the critical and threshold sizes of silkworm has not been examined in the present investigation. It is believed that the growth in insects is controlled by the coordinated action of juvenile hormone (JH), prothoracicotropic hormone (PTTH) and ecdysteroids. While, the former promotes larval growth, the other two hormones trigger molting or pupation (Nijhout, 1981). Perhaps, this is what happens in ***B. mori*** during metamorphosis. Recent findings of Miranda *et al*., (2002) indicated that the prevalence of JH titers in haemolymph sustains larval growth and silk gland growth, provides clear proof of endocrine regulation of silkworm metamorphosis. However, this needs further elucidation in silkworm.

Growth ratios deviate from Dyar's constancy rule

Any report on insect growth, without reference to Dyar's constancy rule (DCR) is incomplete.

It has been widely acknowledged that the insect growth is accompanied by concurrent growth trends in its exoskeleton vis-a-vis body mass by a constant factor at each molt. This results in a geometric progression of size from instar to instar. This constancy of the size increment is explained in terms of DCR, which asserts that the pre-molt / post-molt growth ratios remain constant from instar to instar during the ontogeny in insects and other arthropods (Dyar and Rhinebeck, 1890; Hutchinson, 1997). The DCR further assumes that there is a constant and identical increase in growth dimensions at each molt, and this view is reinforced by the apparent excellent fit of exponential regressions, illustrated in Figs.1, 2, 3 and 8 and the high r^2 values for the regressions would generally be taken as evidence for a good fit. Aptly, the DCR is based on the computation of growth ratio or Dyar's coefficient, which is obtained by dividing the size of one instar by the size of the preceding instar. Accordingly, growth ratios of silkworm larvae of successive instars were determined by dividing the mean weight/length/perimeter of respective instar stage by that of the previous instar (Thakur, 2016; Siva Prasad, 2022).

The values of Dyar's coefficients during transitional stages, together with mean body size dimensions for *Bombyx* larvae, grown in different nutrient conditions are presented in Table 11. But, the silkworm recorded higher growth ratios in all the three growth variables examined. Under normal nutritional conditions as seen in ZDC, the mean body weight (MBW) recorded a growth ratio of 3.31 between, third and fourth instar, 4.19 between fourth and fifth instar, 0.48 between larva and pupa stage and 0.57 between pupa and adult. The honey and lemon-juice-enriched nutrient diets of HFE, LFE and HLFE groups caused both ups and downs in growth ratios. In these groups, the Dyar's coefficients ranged from 3.53 to 3.80 in larval stage, 0.48 to 0.50 in pupal stage and from 0.51 to 0.57 in adult stage. The second growth variable, the mean body length recorded a growth ratio of 1.43 between third and fourth instar, 2.11 between fourth and fifth instar, 0.52 between larval and pupal stage and 0.74 between pupal and adult stages. In the three nutrient groups the coefficients ranged from 1.53 to 2.11 in larval stage, equally 0.52 in pupal stage and from 0.68 to 0.74 in adult stage. The mean body perimeter (MBPM) on the other hand recorded a growth ratios ranging from 1.46 to 1.85 in larval stage, 0.83 to 0.87 in pupal stage and 0.88 to 0.91 in adult stage.

Thus, in silkworm the Dyar's coefficients are not constant, but increased from instar to instar in larval stages, but significantly declined in pupal and adult stages. The inconsistencies in Dyar's coefficients indicate that the growth increments or decrements are not constant during metamorphosis. While, the insects that follow DCR, show Dyar's coefficients in the range of 1.199 and 1.222 or 1.255 and 1.277 in between two successive instars or stages (Hutchinson *et al*., 1997; Thakur, 2016), the silkworm recorded higher values (1.43 to 4.19) during larval-larval transitions and lower values during larval-pupal (0.48 to 0.91) and pupal-adult transitions. Thus, the observed Dyar's coeffients are either larger or smaller than the expected values of DCR. Thus, the log-transformed exponential growth trajectory (exponential regression) of *Bombyx mori* systematically violated DCR throughout its growth during metamorphosis, a common feature of lepidopteran insects that have five larval instars, which show growth ratios in the range of 1.6 -1.8 (Grunert *et al.,* 2015).

Table 11: The Synergistic Effect Of Honey And Lemon Juice-enriched Mulberry Diets On The Mean Body Size Parameters And Dyar's Coefficients Of *Bombyx Mori* During Larval-pupal-adult Metamorphosis.

A. MEAN BODY WEIGHT (g)								
	ZDC Batch		HFE Batch		LFE Batch		HLFE Batch	
	Mean	Dyar's Coefficient	Mean	Dyar's Coefficient	Mean	Dyar's Coefficient	Mean	Dyar's Coefficient
III Instar	0.13	-	0.15	-	0.14	-	0.17	-
IV Instar	0.43 (231)	3.31	0.53 (274)	3.53	0.51 (264)	3.64	0.55 (224)	3.24
V Instar	1.80 (319)	4.19	1.98 (274)	3.74	1.94 (280)	3.80	2.02 (267)	3.67
Pupa	0.87 (-52)	0.48	0.95 (-52)	0.48	0.96 (-51)	0.49	1.01 (-50)	0.50
Adult	0.50 (-43)	0.57	0.49 (-48)	0.52	0.52 (-46)	0.54	0.52 (-49)	0.51
B. MEAN BODY LENGTH (C.M)								
III Instar	1.83	-	1.93	-	1.93	-	2.06	-

IV Instar	2.62 (43)	1.43	3.0 (55)	1.70	2.95 (53)	1.53	3.15 (53)	1.53
V Instar	5.54 (111)	2.11	6.21 (107)	2.07	6.21 (111)	2.11	6.54 (107)	2.08
Pupa	2.90 (-47)	0.52	3.26 (-47)	0.52	3.23 (-48)	0.52	3.43 (-48)	0.52
Adult	2.17 (-25)	0.74	2.21 (-32)	0.68	2.24 (-31)	0.68	2.39 (-30)	0.69
C. MEAN BODY PERIMETER (C.M)								
III Instar	1.30	-	1.38	-	1.38	-	1.47	-
IV Instar	1.90 (46)	1.46	2.15 (56)	1.56	2.05 (49)	1.52	2.20 (50)	1.49
V Instar	3.51 (85)	1.85	3.82 (78)	1.78	3.75 (83)	1.83	3.93 (79)	1.49
Pupa	3.10 (-12)	0.85	3.29 (-14)	0.86	3.26 _(-	0.87	3.27 (-17)	0.83
Adult	2.87 (-7)	0.90	2.88 (-12)	0.88	2.89 (-11)	0.89	2.99 (-9)	0.91

The mean body weight (MBW, mean body length (MBL) and mean body perimeter (MBP) were calculated from the corresponding values of tables 1 to 9. Figures in parentheses represent the percent changes (%) from the preceding values. Dyar's coefficients or growth ratios were calculated by dividing the values of MBW, MBL and MBPM of one instar by the corresponding values of the preceding instar.

The constancy principle embodied in DCR holds good for the linear measure of exoskeleton (not examined in the present study), but not for the other growth dimensions of soft tissues of the larval body as observed in the present case. This view is reinforced by apparent excellent fit of exponential regressions such as those illustrated in figures 1, 2, 3 & 8. The higher range of R^2 values (0.7076 - 0.9961) for regression, observed in the present investigation could be generally taken as an evidence for a good fit. Thus, the present study demonstrates that important features of silkworm growth. First, it systematically deviates from the apparent constancy of size increment embodied in DCR. Second, it meticulously obeys the concept of energy and structures envisaged in the Hutchinson's investment principle (Hutchinson *et al.,* (1997).

CONCLUSION

The current study on the growth kinetics of ***Bombyx mori*** highlighted several interesting features. Barring minor declines during molts, the growth showed profound increase in three body dimensions of mass, length and perimeter during larval regime from third to fifth instar, but declined later during pupal and adult regimes. The study raises two important questions. First, what causes the progressive increase in the size increment at each molt? and second, what causes its decline during pupal and adult stages? The answers lie in the Hutchinson's investment principle, which emphasizes an adaptive explanation stating that the molt-dependent growth of larval soft bodies vary as a function of investment of energy reserves and structures for future use during metamorphosis. The acquisition of appropriate body size at maturity was explained in terms of critical and threshold sizes that sets-in the time lines for molting and metamorphosis. The silkworm attained these two size determinants on a particular day and at particular time in each instar. Though, the physiological mechanism underlying larval body size was not examined in silkworm, it is presumed to have been controlled by time-depended secretion of juvenile hormone, prothoracicotropic hormone and ecdysone. Nevertheless, the timing of attainment of critical size in third and fourth larval instars and threshold size in fifth instar are not significantly affected by the honey-enriched mulberry diet. Another notable feature of the silkworm growth is that it recorded higher growth ratios during larval-larval transition and lower growth ratios during larval-pupal and pupal-adult transitions and by doing so, it deviates from the Dyar's constancy rule. The honey and lemon juice-enriched mulberry diets stimulated the accumulation of biomass during larval growth in all the three dimensions of body weight, body length and body perimeter and positively modulated the its utilization during pupal-adult metamorphosis.

The honey and lemon juice are natural products. The honey consists of wide range of curative properties due to the presence of more than 200 bioactive compounds. Moreover, honey represents an important alternative agent against antibiotic resistant bacteria and hence, it has been used since ancient times in various forms to sweeten and flavor different types of foods (Dezmirean *et al.*,2015). More importantly, it has been applied in sericulture to prepare silk fibroin-based biomaterials (Baci *et al*., 2021). On the other hand, lemon juice, the extract of the yellow fruit of *Citrus limen* (Family: Rutaceae), has been widely used in nutritional studies obviously due to its richness in ascorbic acid. Both these nutrients, when fortified with the mulberry leaf and fed to *B. mori* showed promising results for sericulture. Both individually and synergistically they stimulated silkworm growth significantly in three body dimensions of mass, length and perimeter. Hence, further research in this area should explore the possibility of including these two natural products in the recipe of the silkworm diet.

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