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Impact of Feeding Lemon Juice-Enriched Mulberry Leaves on The Larval Growth, Protein Profiles and Economic Traits in the Silkworm, *Bombyx mori*

KEYWORDS: *Bombyx mori*, Larval growth, Lemon juice, Proteins, Economic traits

Thulasi, N
Department of Zoology, Smt. N.P. S. Government College for Women, Chittoor (517 002). A.P. INDIA.

*** Sivaprasad, S**
Department of Zoology, Smt. N.P. S. Government College for Women, Chittoor (517 002). A.P. INDIA.
* Correspondent author:

ABSTRACT *Minimum effective concentration of lemon juice (LJ) was determined and its positive impact demonstrated on Bombyx mori, with reference to larval growth, protein profiles and economic parameters of sericulture. LJ works well at a concentration of 3% in distilled water and stimulates growth rates in silkworm larvae during fourth and fifth instar developmental stages, but more effectively during fifth instar. It stimulates fibroin (core silk protein) synthesis and retards floss protein synthesis in silk gland, more particularly in its anterior region. Further, lemon juice promotes protein mobilization from fat body to silk gland via haemolymph. In doing so, it contributes to sericulture industry by causing improvements in profit making economic traits such as gland-body ratio, cocoon weight, shell weight, raw silk weight, denier and renditta and by reducing the production of floss, which contributes to loss in the sericulture industry. Lemon juice is suggested as a profitable supplementary diet for silkworm.*

INRODUCTION

Nutrition is the single most factor that influences the growth and development of *B. mori* (Laskar and Datta, 2000; Kanafi *et al.,* 2007). Enriching the silkworm diet (i.e., mulberry leaves) with exogenous nutrients such as proteins, carbohydrates, amino acids, vitamins, minerals, hormones, antibiotics and assessing their impact on larval growth, metabolism and silk production has become the order of traditional research in sericulture (Sanappa *et al.,* 2002; Etebari *et al.,* 2004; Bhattacharya and Kaliwal, 2004, 2005a, 2005b, 2005c, 2005d; Chakrabarty and Kaliwal, 2011). Of all, the role of vitamins in sericulture has been extensively investigated (Mossallenejad, 2002; Etebari *et al.,* 2004; Faruki, 2005; Rajabi *et al.,* 2006a, 2006b).The vitamin enrichment studies largely focused on all commercially available vitamins such as B_9-folic acid (Nirwani and Kaliwal, 1996; Khan and Saha,1996), B_{12}-cyanocobalamine (Das and Medda, 1998), ascorbic acid (Javed and Gondal, 2002; Hussain and Javed, 2002; Cui *et al.,*2003), B_1- thiamine (Khan and Saha, 2003), B_3-niacin (Etebari and Matindoost, 2004), B_2-riboflavin and B_6-pyridoxine (Faruki, 2005; Rajabi et al., 2006 a, 2006 b,), multi-vitamin compounds (Etebari and Matindoost, 2005) and a variety of fat soluble vitamins (Mosallanejad, 2002), often with positive impact on silkworm growth and economic parameters of sericulture. The mineral enrichment studies concentrated on the application of salts of cobalt, potassium, magnesium, calcium and zinc (Bhattacharya and Kaliwal, 2005 a, 2005 b, 2005 c, 2005d; Kavitha *et al.,* 2012), often with mixed results on economic traits of silkworm.

Review of literature reveals that the nutritional-enrichment studies were based largely on the application of expensive commercial sugars, proteins, vitamins and minerals. The combined effects of most of these exogenous nutrients could be achieved through a single natural nutrient, called lemon juice (LJ), which is a rich source of multiple vitamins and minerals (Markus and Sass, 2003;Albertini *et al.,* 2006). Further, lemons are perennial and inexpensive fruits, readily available in all seasons. Despite its high nutritional value, the impact of lemon juice has not been examined with reference to silkworm growth and economic parameters of sericulture. The present study intends to determine the minimum effective concentration (MEC) of LJ and to test its efficacy on the silkworm growth, protein synthesis and economic traits of sericulture.

MATERIAL AND METHODS

The present investigation was carried out on Pure Mysore x CSR_2 hybrid strain of *Bombyx mori*, reared under standard environmental conditions of 28°C, 85% RH as per Krishnaswami, 1986. After hatching, the worms were fed with M_5 variety of mulberry leaves, five times a day at 6 AM, 10 AM, 2 PM, 6 PM and10 PM, under normal 12 hr light and 12 hr dark conditions. The experimental design was divided into five phases namely lemon juice feeding pattern, determination of minimum effective concentration (MEC), study of growth patterns, the assay of tissue proteins and analysis of economic traits of sericulture.

Lemon juice feeding pattern: After the third moult, the fourth instar larvae were divided into five batches, one control and four experimental with 100 worms each. The control batch was given 5 feedings as scheduled, while in respect of experimental batches one feeding at 6.00PM was replaced by mulberry leaves dipped in lemon juice. The LJ-fortified mulberry leaves were prepared by first dipping them in different concentrations of lemon juice (viz., 5%, 3%, 2% and 1%) and subsequently drying them under cool weather conditions.

Determination of minimum effective concentration: The minimum effective concentration (MEC) of lemon juice was determined by a step-down process starting from a higher concentration (5%) to a lower concentration (1%), while running a parallel zero- dose control as given by Williams, 1971 and modified by Li Jan, 2005 and Kavitha *et al.,* 2011. Accordingly, each of the four experimental batches was fed with mulberry leaves dipped in one of the four concentrations (5% / 3%/ 2% / 1%) of LJ. The MEC of LJ was determined separately for larval growth, tissue proteins and economic traits.

Silkworm growth: Studies under this head included the impact analysis of LJ on the growth of larva and silk gland (SG). While, the larval growth rate was determined by recording the body weight during the fourth and fifth instars, the SG growth rate was similarly determined during the fifth instar. For experimental convenience, the larval growth rates were measured in two batches, each comprising a control and two treatment conditions (Table 1). However, SG growth rates were ascertained under single treatment condition, i.e., after feeding the larvae with mulberry leaves fortified with minimum effective concentration (3%) of LJ. Further, the larval growth rates were examined every day throughout the fourth

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(4 days) and fifth instars (7 days), the SG growth rates were analyzed on alternative days (i.e., 1st, 3rd, 5th, 7th) during fifth instar development. Since, the body weight is an index of animal growth, the mean body weight of 25 randomly selected silkworm larvae and the mean weight of 5 silk glands were measured in an electronic balance (ELICO: Model BL-22 0H) and the same was expressed in grams .

Assay of tissue proteins: Total protein levels of the silk gland (SG), fat body (FB) and haemolymph (HL) were analyzed on day 1, day 3, day 5 and day 7 during fifth instar larval development. While the SG and FB were isolated by mid-dorsal dissection of the larval body in the Silkworm Ringer (Yamaoka *et al.,* 1997), the HL was extracted by cutting its telson and pro-legs. The total protein content was estimated in 1% homogenates of SG and FB and 1:9 diluted HL (1:9 haemolymph and distilled water) by the method of (Lowry *et al.,* 1951) and the same was expressed in mg protein/ gram wet weight of tissue (or) mg/ml of haemolymph. Similarly the protein content of cocoon was estimated in 1% homogenate in distilled water. Since the silk cocoon is not soluble in distilled water, it was first soaked in diluted sodium hydroxide solution before homogenized in distilled water.

Analysis of economic parameters: Some important economic parameters of sericulture, such as the larval weight, silk gland weight, gland- body ratio, cocoon weight, shell weight, renditta, denier, silk weight, shell protein, floss protein were analyzed as per the methods given by (Bohidar *et al*., 2007; Rahmathulla *et al*., 2007; Sailaja and Sivaprasad , 2010; Chakrabarty and Kaliwal, 2011; Kavitha *et al*., 2012).

Statistical analysis: The data obtained from the present investigation were analyzed by statistical tools such as mean, standard deviation (SD), percent changes and test of significance. While the mean and SD were computed using M.S Excel platform, the test of significance and percent changes was calculated online by using the Graph Pad (*www.graph-pad.com/quick calcs/ index cfm/)*and Percent Change (*www. percent – change com/index php*) tools respectively. Further, in order to ensure uniformity in data analysis and interpretation and to draw meaningful conclusions thereof, the growth trends in the larval body weight and those of proteins were interpreted in terms of an innovative statistical parameter called compound periodical growth rate (CPGR) as given by Sivaprasad (2012).

RESULTS

The findings of the present investigation on the larval growth, gland growth, protein profiles and economic traits of sericulture are presented in Tables 1 to 7 and in Figures 1 to 5.

Larval body growth: The growth trends of the fourth and fifth instar silkworm larvae are presented in the table 1 and figure 1. During the fourth instar development, the larval body weight of the control recorded growth rates ranging from 150 to 160% on 2nd day, 16 to 35% on 3rd day and 37 to 52% on 4th day. At the same time the CPGR of control batch ranged from 63.86% to 68.69% during the fourth instar (Table 1; Cols. 3, 6). When the larvae were fed with 5% LJ fortified mulberry leaves, the larvae grew by 140% on 2nd day, 4% on 3rd day ~56% on 4th day, with a CPGR of 57.41% representing an overall ~11 decline from the zero-dose control (Col.4). At 3% concentration of LJ, the body weight grew maximally by 160% on 2nd day, 44% on 3rd day and by ~39% on 4th day with a CPGR of 71%, representing 4% net increase in growth compared to zero-dose control (Col. 7). At 2% concentration, LJ enhanced the growth rate by 140% on day 2, just by 8% on day 3 and by ~54% on day 4, with a CPGR of 58.74% and a deviation of about -9% from the zero-dose control (Col. 5). At 1% concentration of LJ, the larval growth recorded an elevation of 150% on 2nd day, 40% on 3rd day and ~44% on 4th day with a CPGR of 69.85% and a 2% deviation from zero-dose control (Col.8).

Table 1: Effect of different concentrations (5%, 3%, 2% and 1%) of lemon juice on the larval growth in *Bombyx mori*, during fourth and fifth instar developmental stages.

Day	Statistical tools	Fourth instar						Fifth instar					
		Batch-I			Batch-II			Batch-I			Batch-II		
		Control	Experimental		Control	Experimental		Control	Experimental		Control	Experimental	
			5% LJ	2% LJ		3% LJ	1% LJ		5% LJ	2% LJ		3%LJ	1% LJ
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)	(14)
1	Mean	0.10	0.10	0.10	0.10	0.10	0.10	0.58	0.58	0.58	0.49	0.49	0.49
	S.D	±0.001	±0.001	±0.001	±0.001	±0.001	±0.001	±0.01	±0.01	±0.01	±0.01	±0.01	±0.01
2	Mean	0.25	0.24	0.24	0.26	0.25	0.25	0.75	0.71	0.73	0.62	0.69	0.65
	P.C	150.0	140.0	140.0	160.0	160.0	150.0	29.31	22.41	25.86	26.53	40.81	32.65
	S.D	±0.01*	±0.009*	±0.04*	±0.01*	±0.003*	±0.007*	±0.001*	±0.001*	±0.001*	±0.01*	±0.003*	±0.020*
3	Mean	0.29	0.25	0.26	0.35	0.36	0.35	0.96	0.90	0.91	1.01	1.04	0.95
	P.C	16.0	4.16	8.33	34.61	44.0	40.0	20.0	26.76	24.65	62.90	50.72	46.15
	S.D	±0.05**	±0.003*	±0.02**	±0.007*	±0.009**	±0.003**	±0.005*	±0.0005*	±0.0009*	±0.04*	±0.01*	±0.04*
4	Mean	0.44	0.39	0.40	0.48	0.50	0.49	1.13	1.03	1.11	1.20	1.51	1.25
	P.C	51.72	56.0	53.8	37.14	38.88	44.11	25.55	14.44	21.97	18.81	45.19	31.57
	S.D	±0.001*	±0.001*	±0.0005*	±0.005*	±0.005*	±0.009*	±0.001*	±0.001*	±0.001*	±0.04*	±0.07*	±0.04*
5	Mean							1.16	1.11	1.23	1.57	1.86	1.54
	P.C	-	-	-	-	-	-	2.65	7.76	10.81	30.83	23.17	23.2
	S.D							±0.02*	±0.0005*	±0.006*	±0.04*	±0.07*	±0.01*
6	Mean							1.32	1.29	1.39	1.78	2.05	1.88
	P.C	-	-	-	-	-	-	13.79	16.21	13.00	13.37	10.21	22.07
	S.D							±0.007*	±0.006*	±0.03*	±0.07*	±0.03*	±0.06*
7	Mean							1.40	1.36	1.42	1.94	2.39	2.25
	P.C	-	-	-	-	-	-	6.06	5.42	2.15	8.98	12.19	19.68
	S.D							± 0.009*	±0.007*	±0.008*	±0.001*	±0.04*	±0.01*
% change from zero – dose control		(0.0)	-11.36	-9.09	(0.0)	4.16	2.08	(0.0)	-2.85	1.42	(0.0)	23.19	15.97
CPGR		63.86%	57.41%	58.74%	68.69%	71.0%	69.85%	15.82%	15.26%	16.19%	25.78%	30.23%	28.92%

*** Statistically significant. **Statistically not significant.**

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Each value is a mean, ± standard deviation of four individual observations (P value < 0.001). Each mean represents the average weight of 25 worms, expressed in grams. The percent changes were calculated taking control as the base value and the compound periodical growth rates (CPGR), were computed on the basis of duration of larval stages (4 days in fourth instar and 7 days in fifth instar) as per Sivaprasad, 2012.

During fifth instar development, the growth rate of control batches recorded an increasing range of ~27 to 29% on 2nd day, ~20 to 63% on 3rd day, 19 to 26% on 4th day, 3 to 31% on 5th day, ~13 to 14% on 6th day and ~6 to 9% on the 7th day, with a CPGR of ~16 to 26% (Cols. 9 and 12). Under treatment conditions, the growth rates projected different trends at different concentrations. At 5% concentration, the larval body weights recorded an increase of ~22% on 2nd day, ~27% on 3rd day, ~14% on 4th day, ~8% on 5th day, ~16% on 6th day and ~5% on the last day, with CPGR of 15.26% and a negative deviation of about 3% from the zero-dose control (Col. 10). At 3% concentration, the larval growth recorded an elevation of ~41% on 2nd day, ~51% on 3rd day, ~45% on 4th day, ~23% on 5th day, ~10% on 6th day and ~12% on the last day, with a maximal CPGR of 30.23% and a positive impact to the extent of about 23% from the zero-dose control (Col. 13). At 2% concentration, the larval growth showed a rise of ~26% on 2nd day, ~25% on 3rd day, ~22% on 4th day, ~11% on 5th day, ~13% on 6th day and ~2% on the last day, with a low CPGR of 16.19% and a minimal positive impact (1.4%) compared to zero-dose control (Col. 11). At 1% concentration, the larval growth depicted an increase of ~33% on 2nd day, ~46% on 3rd day, ~32% on 4th day, ~23% on 5th day, ~22% on 6th day and ~20% on the last day, with a higher CPGR of 28.92% and a positive deviation of ~16% from the zero-dose control (Col. 14).

Body versus gland growth: Under natural conditions, the larval weight grew maximally by 133% on day-3, ~64% on day-5 and minimally by ~12% on day-7 with an overall increase of ~329% and a CPGR of 62.50% (Table 2 and Fig. 2), while that of SG grew maximally by 413% on day-3, ~102% on day-5 and minimally by 87.5% on day-7, representing an overall increase of ~1775% during fifth instar and a CPGR of 210.72% (Col.4). Thus, the computed gland body ratio showed an increase of 121% on day-3, 23% on day-5 and ~61% on day-7 with an overall increase of 338% and a CPGR of ~63.65% in the control batch. Under the influence of 3% LJ, the larval body weight recorded an increase of ~140% on day-3, 71% on day-5 and 11.5% on day-7, resulting in an overall increase of ~358% during fifth instar with a CPGR of 66.09% (Col. 6). At the same time, the SG grew by ~525% on day-3, ~130% on day-5 and by just 9% on day-7, resulting in an overall growth of 2150% with a CPGR of 230.19%. Concomitant with gland growth, the gland-body ratio recorded an increase of ~161% on day-3, ~34% on day-5 and ~40% on day-7, representing an overall growth of ~392% with a CPGR of 70.13% (Col. 8; Table 2).

Table 2: Effect of 3% lemon juice on body weight, silk gland (SG) weight and gland-body ratio (GBR) in *Bombyx mori* during fifth instar larval development.

Day	Statistical tools	Control			Experimental (3% L J)		
		Body weight	SG weight	GBR	Body weight	SG weight	GBR
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
1	Mean P.C S.D	0.55 - ±0.0009	0.016 - ±0.0008	2.90 - ±0.009	0.55 - ±0.0009	0.016 - ±0.0008	2.90 - ±0.009
3	Mean P.C S.D	1.28 132.72 ±0.005*	0.082 412.5 ±0.0008*	6.40 120.68 ±0.009*	1.32 140 ±0.008*	0.100 525 ±0.001*	7.57 161.03 ±0.005*
5	Mean P.C S.D	2.10 64.06 ±0.0001*	0.166 102.43 ±0.0008*	7.90 23.43 ±0.009*	2.26 71.21 ±0.008*	0.23 130 ±0.008*	10.17 34.34 ±0.008*
7	Mean P.C S.D	2.36 12.38 ±0.0001*	0.300 87.5 ±0.0009*	12.71 60.88 ±0.005*	2.52 11.50 ±0.008*	0.36 9.09 ±0.008*	14.28 40.41 ±0.008*
Overall change from base value		329.09%	1775%	338.27%	358.18%	2150%	392.41%
CPGR		62.50%	210.72%	63.65%	66.09%	230.19%	70.13%

*** Statistically significant **Statistically not significant**

Each value is a mean, ± standard deviation of four individual observations. (P value < 0.001). The percent changes were calculated taking the control as the base value and the compound periodical growth rates (CPGR) were computed on the basis of first and seventh day values as per Sivaprasad, 2012.

Protein profiles

Silk gland proteins (SGP): In general, the SGP levels recorded elevatory trends during fifth instar development. In the control batch, the increase ranged from 122 to 123% on day-2, just ~8% on day-5 and 79 to 83% on day-7, with a CPGR of 63 to 64% during the period (Table 3; Col. 3, 6). When the larvae were fed with 5% LJ- fortified mulberry leaves, SGP levels showed an increase of ~103% on day-3, ~36% on day-5 and 50% on day-7 with a CPGR of 60.70% and a 4% decline from the zero-dose control (Col.4; Table 3). At 3% concentration of LJ, the SGP recorded an elevation of ~137% on day-3, ~25% on day-5 and by 95% on day-7 with a CPGR of 79.50% and a net increase of ~31% over zero-dose control (Col.7; Table 3). At 2% level, the LJ caused lower elevations in SGP levels. It was ~103% on day-3, ~36% on day-5 and by 93% on day-7, with a CPGR of 74.96% and ~24% increase over zero-dose control (Col.5; Table 3). However, at 1% concentration, the LJ caused a hike of ~112% on day-3, ~38% on day-5 and ~89% on day-7, with a CPGR of 76.8% and ~25% increase over zero-dose control (Col. 8; Table 3).

Table 3: Effect of different concentrations (5%, 3%, 2% and 1%) of lemon juice on the protein profiles of whole silk gland in *Bombyx mori* during fifth instar larval development.

Day	Statistical tools	Batch - I			Batch-II		
		Control	Experimental			Experimental	
			5% LJ	2% LJ	Control	3% LJ	1% LJ
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
1	Mean P.C S.D	17.57 - 0.43	17.57 - 0.43	17.57 - 0.43	16.46 - 2.10	16.46 - 2.10	16.46 - 2.10
3	Mean P.C S.D	39.08 (122.42) ±0.25*	35.63 (102.78) ±0.27*	35.74 (103.41) ±1.17*	36.73 (123.14) ±3.47*	39.01 (136.99) ±3.46*	34.90 (112.02) ±1.66*
5	Mean P.C S.D	42.36 (8.39) ±0.35*	48.53 (36.20) ±0.75*	48.68 (36.20) ±1.08*	39.8 (8.35) ±0.88**	48.83 (25.17) ±5.42*	48.17 (38.02) ±0.90*
7	Mean P.C S.D	76.00 (79.41) ±0.80*	72.91 (50.23) ±1.51*	94.10 (93.30) ±0.96*	72.91 (83.19) ±1.51*	95.20 (94.96) ±2.53*	90.97 (88.85) ±2.82*
% change from Zero-based control		(0.0)	-4.06	23.81	(0.0)	30.57	24.77
CPGR		62.93%	60.70%	74.96%	64.23%	79.50%	76.80%

*** Statistically significant **Statistically not significant**

Each value is a mean, ± standard deviation of four individual observations. (P value < 0.001). The percent changes were calculated taking the control as the base value and the compound periodical growth rates (CPGR) were computed on the basis of first and seventh day values as per Sivaprasad, 2012.

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Table 4: Effect of 3% lemon juice on total protein profiles of different regions of the silk gland in the fifth instar larva of the silkworm, *Bombyx mori***.**

Day	Statistical tools	ASG		MSG		PSG	
		Control	Experimental (3%LJ)	Control	Experimental (3%LJ)	Control	Experimental (3%LJ)
1	2	3	4	5	6	7	8
1	Mean P.C S.D	9.44 - ±0.30	9.44 - ±0.30	15.47 - ±0.70	15.47 - ±0.70	17.17 - ±3.66	17.17 - ±3.66
3	Mean P.C S.D	10.62 (12.5) ±0.13*	20.03 (112.18) ±0.68*	25.40 (64.18) ±0.94*	40.56 (162.18) ±1.42*	24.41 (42.16) ±1.28*	36.05 (109.95) ±4.1*
5	Mean P.C S.D	12.24 (15.25) ±1.06*	22.28 (11.23) ±1.39*	39.08 (53.85) ±0.77*	43.39 (6.97) ±1.30*	31.69 (29.82) ±1.94*	41.11 (14.03) ±0.87**
7	Mean P.C S.D	12.75 (4.06) ±0.47**	26.95 (20.96) ±0.55*	47.13 (20.59) ±0.81*	53.17 (22.53) ±1.16*	35.59 (12.30) ±1.04*	49.38 (20.11) ±1.00*
Overall change from base value		35.06%	185.48%	204.65%	243.69%	107.28%	187.59%
CPGR		10.54%	169.84%	44.97%	50.91%	27.50%	42.21%

*** Statistically significant: **Statistically not significant**

Each value is a mean, ± standard deviation of four individual observations. (P value < 0.001). The percent changes were calculated taking the control as the base value and the compound periodical growth rates (CPGR) were computed on the basis of first and seventh day values as per Sivaprasad, 2012.

ASG: Anterior silk gland; MSG: Middle silk gland; PSG: Posterior silk gland

Since, the LJ caused significant improvements in whole SGP, it was felt necessary to find as to which region of SG responds more effectively to this exogenous factor. Hence, protein levels were estimated separately in its three regions; anterior silk gland (ASG), middle silk gland (MSG) and posterior silk gland (PSG) and the relevant data are presented in table 4. The protein profiles of ASG in the control and LJ treated batches were elevated respectively by ~13 and 112% on day-3, ~15 and 11% on day-5 and by just ~4 and 21% on day-7 (Cols. 3, 4). In the MSG, the corresponding elevations were ~64 and 162% on day-3, ~54 and 7% on day-5 and ~21 and 22% on day-7 (Cols. 5, 6), while those in the PSG were ~42 and 110% on day-3, ~30 and 14% on day-5 and ~12 and 20% on day-7 (Cols.7, 8). During the entire period of fifth instar, the overall growth trends in the total protein levels of control and experimental batches were ~35 and 185% in ASG, ~205 and 244% in MSG and ~107 and 188% in PSG. Similarly, the corresponding CPGRs were 10.54 and 169.84% in ASG, 44.97 and 50.91 in MSG and 27.50 and 42.21% in PSG (Table 4).

Fat body proteins (FBP): The impact of LJ on FBP was more significant at lower concentrations than those at higher concentrations (Table 5; Fig.4). The daily growth trends in FBP levels in the control batches during fifth instar ranged from ~15 to 30% on 3rd day, ~5 to 16% on 5th day and ~3 to 21% on 7th day and that of CPGR for entire fifth instar ranged from 12.25 to 17.13% (Cols. 3, 6). In the larvae fed with 5% LJ treated mulberry leaves, the FBP levels decreased by ~13% on day-3 but increased by 8% on day-5 and by ~37% on day-7, with a negative CPGR ~4%, representing an overall decrease of ~9% during the fifth instar (Col. 4). On the other hand, when the larvae were fed with 3% LJ, the FBP levels showed significant gains throughout the fifth instar. At this concentration, the protein levels were elevated by ~20% on day-3, ~12% on day-5 and by ~23% on day-7 with a net positive impact of 3.5% and a CPGR of 18.51% (Col. 7). At 2% concentration of LJ, the FBP levels declined initially by ~20%on 3rd day by, but recorded elevations ranging from ~43% on day-5 and ~17% day-7, with an overall negative impact of 4.6% and a CPGR of 2.26% (Col.5). Finally, at 1% concentration the FBP levels recorded an increase of ~15% on day-3, ~35% on day-5 and ~14% on day-7, with an overall positive impact of 10% and a CPGR of 21.01% during the same period (Col. 8; Table 5).

Table 5: Effect of different concentrations (5%, 3%, 2% and 1%) of lemon juice on the protein profiles of fat body in *Bombyx mori* **during fifth instar larval development.**

Day	Statistical tools	Batch - I			Batch - II		
		Control	Experimental		Control	Experimental	
			5%LJ	2%LJ		3%LJ	1%LJ
1	2	3	4	5	6	7	8
1	Mean P.C S.D	32.65 - 0.73	32.65 - 0.73	32.65 - 0.73	26.95 - 1.07	26.95 - 1.07	26.95 - 1.07
3	Mean P.C S.D	42.37 (29.77) ±1.66*	28.35 (-13.16) ±4.43*	26.18 (-19.81) ±0.86*	30.92 (14.73) ±0.60*	32.46 (20.44) ±1.64*	30.95 (14.81) ±0.68*
5	Mean P.C S.D	44.67 5.42 ±0.52*	30.73 (8.39) ±0.87*	37.50 (43.23) ±1.53*	35.77 (15.68) ±1.22*	36.36 (12.01) ±2.90**	41.92 (35.44) ±1.62
7	Mean P.C S.D	46.18 3.38 ±0.46*	42.21 (37.35) ±1.23*	44.05 (17.46) ±1.03*	43.34 (21.07) ±1.58*	44.86 (23.37) ±3.75*	47.76 (13.93) ±4.29*
% change from Zero-based control		(0.0)	-8.59	-4.61	(0.0)	3.50	10.19
CPGR		12.25%	-3.57%	2.26%	17.13%	18.51%	21.01%

*** Statistically significant. **Statistically not significant.**

Each value is a mean, ± standard deviation of four individual observations. (P value < 0.001). The percent changes were calculated taking the control as the base value, while the compound periodical growth rates (CPGR) were computed on the basis of first and seventh day values as per Sivaprasad, 2012.

Haemolymph proteins (HLP): The circulating protein pool of the haemolymph projected growth trends similar to those of the silk gland both in the control and experimental batches (Table 6; Fig. 5). The HLP levels of control batches ranged from ~4 to 13% on 3rd day and ~3 to 8% on 5th day and about 21% on 7th day and recorded CPGRs ranging from 9.4 to 13.93% (Cols. 3, 6). At 5% concentration, LJ caused an elevation of ~5% in HLP levels on day-3, 2% on day-5 and 6% on day-7, representing an overall negative impact of ~14% and a CPGR of ~4.24% (Col. 4). At 3% level of LJ, HLP levels recorded an increase of ~22% on day-3, ~5% on day-5 and ~27% on day-7 with a net positive impact of 10% and a CPGR of 17.46% (Col. 7). At 2% level, the LJ caused an increase of ~ 7% on day-3, ~3% on day-5 and ~1% on day-7, with an overall negative impact of ~15% and a low CPGR of 3.73% (Col.5). However, under the influence of 1% LJ, HLP levels shot-up by ~20% on day-3, ~3% on day-5 and ~18% on day-7, with an overall positive impact of 2% and a CPGR of 13.44% (Col. 8; Table 6).

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Table 6: Effect of different concentrations (5%, 3%, 2% and 1%) of lemon juice on the total protein profiles of haemolymph in *Bombyx mori* during fifth instar larval development.

Day	Statistical tools	Batch - I			Batch - II		
		Control	Experimental		Control	Experimental	
			5% LJ	2% LJ		3% LJ	1% LJ
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
1	Mean P.C S.D	6.63 - ±0.07	6.63 - ±0.07	6.63 - ±0.07	5.85 - 0.32	5.85 - 0.32	5.85 - 0.32
3	Mean P.C S.D	6.92 (4.37) ±0.15*	6.96 (4.97) ±0.16*	7.09 (6.93) ±0.03*	6.60 (12.82) ±0.43*	7.13 (21.88) ±0.22*	7.04 (20.34) ±0.29*
5	Mean P.C S.D	7.16 3.46 ±00.05*	7.09 (1.86) ±00.03**	7.30 (2.96) ±00.05*	7.14 (8.18) ±00.16**	7.51 (5.32) ±00.10	7.23 (2.69) ±0.04**
7	Mean P.C S.D	8.70 21.50 ±0.14*	7.51 (5.92) ±00.10*	7.40 (1.36) ±0.04*	8.65 (21.14) ±0.65*	9.48 (26.63) ±0.13*	8.54 (18.11) ±1.63*
% change from Zero-based control		(0.0)	-13.67	-14.94	(0.0)	9.59	2.19
	CPGR	9.4%	4.24%	3.73%	13.93%	17.46%	13.44%

*** Statistically significant. **Statistically not significant.**

Each value is a mean, ± standard deviation of four individual observations. (P value < 0.001). The percent changes were calculated taking the control as the base value and the compound periodical growth rates (CPGR) were computed on the basis of first and seventh day values as per Sivaprasad, 2012.

Economic traits

The positive impact of 3% LJ has been extended to the economic parameters of sericulture such as the cocoon weight, shell weight, floss weight, pupal weight, shell protein, floss protein, raw silk weight, denier and renditta (Table 7). The impact yielded significant gains in profitable traits and reductions in loss-making ones. Under its influence, the green cocoon weight increased by ~20%, shell weight by 60%, pupal weight by ~23%, shell protein weight by ~9%, raw silk weight by ~4% and the denier by ~13%. The positive impact of LJ is further reinforced by reduction in floss weight by ~25%, floss protein weight by ~22% and renditta by ~3% (Table 7).

Table 7: Effect of 3% lemon juice on the economic traits of *Bombyx mori*.

S. No.	Parameter	Statistical tools	Control	Experimental (3%LJ)
1	Weight of single cocoon (g)	Mean P.C S.D	0.986 - ±0.05	1.18 (19.67) ±0.006*
2	Weight of single shell (g)	Mean P.C S.D	0.15 - ±0.07	0.24 (60.0) ±0.07*
3	Weight of single floss (g)	Mean P.C S.D	0.016 - ±0.002	0.012 (-25.0) ±0.0005*
4	Weight of single pupa (g)	Mean P.C S.D	0.793 - ±0.04	0.974 (22.82) ±0.002*
6	Shell protein (mg/g)	Mean P.C S.D	11.91 - ±0.008	12.94 (8.64) ±0.01*
7	Floss protein (mg/g)	Mean P.C S.D	8.09 - ±0.008	6.32 (-21.87) ±0.008*
8	Raw silk weight(g)	Mean P.C S.D	12.88 - ±0.01	13.37 (3.80) ±0.001*
9	Denier	Mean P.C S.D	10.72 - ±0.009	12.08 (12.68) ±0.008*
10	Renditta	Mean P.C S.D	7.02 - ±0.01	6.86 (-2.27) ±0.008*

***Statistically significant: **Statistically not significant**

Each value is a mean ± standard deviation of four individual observations. (P value < 0.001). The weights of the cocoon, shell and floss represent the mean of 25 individual cocoons. The values in parentheses represent the percent changes from the control.

DISCUSSION

Lemon juice (LJ), the extract of the yellow fruit of ***Citrus limen*** (Family: Rutaceae), is a natural and most popular multi-factorial nutrient. It is a rich source of vitamin-C (64%) and B- complex vitamins such as pantothenic acid (4%) as folic acid (3%), thiamine (3%), riboflavin (2%) and niacin (1%). Additionally, it includes carbohydrates, sugars, lipids, proteins, minerals (Ca, Fe, Mg, P, K, Zn etc) and some organic acids (Markus and Sass, 2003; Albertini *et al.*, 2006). Needless to say, it plays a vital role in the growth and metabolism of organisms, a fact that has been amply demonstrated in ***B. mori*** in its larval growth, protein profiles and economic traits of sericulture. Its impact seems to be concentration-dependent and tissue-specific and hence, it is imperative to determine its minimum effective concentration for different parameters under study.

Minimum effective concentration

Minimum effective concentration (MEC), the lowest concentration level with a response greater than that of the zero-dose control, is a powerful tool for tracing the concentration-response relationship in growth related studies (Stewart and Ruberg, 2000; Li Jan, 2005; Amalarani *et al.*, 2011; Kavitha *et al.*, 2011). The present investigation, involving a step-down process with four different concentrations (viz., 5%, 3%, 2% and 1%) and a zero- dose control has revealed that the impact of LJ was more pronounced at a concentration of 3% in distilled water than those at three other concentrations. This was clearly reflected in silkworm larval growth, silk gland growth and tissue proteins. The MEC of lemon juice was assessed by two methodological approaches. The first one by the evaluation of compound periodical growth rates (CPGR) that describes the steady growth rates during larval duration and second one by the analysis of percent changes, through which deviations from the zero-dose control were ascertained (Fig.1to 5).

Fig.1: Effect of different concentrations (5%, 3%, 2% and 1%) of lemon juice on the growth of *Bombyx mori* during fourth and fifth instar larval stages, reflected as deviations from the zero-dose control.

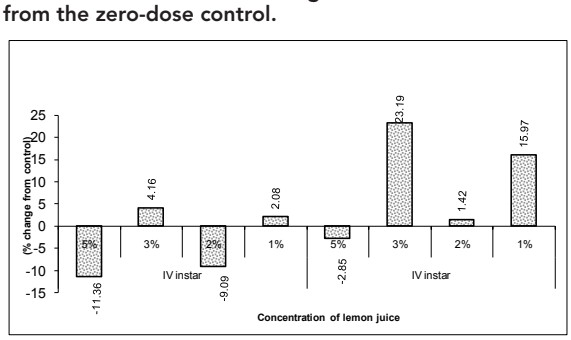

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Fig.2: Effect of different concentrations (5%, 3%, 2% and 1%) of lemon juice on silk gland proteins in the fifth instar larva of *Bombyx mori, reflected as deviations from zero-dose control***.**

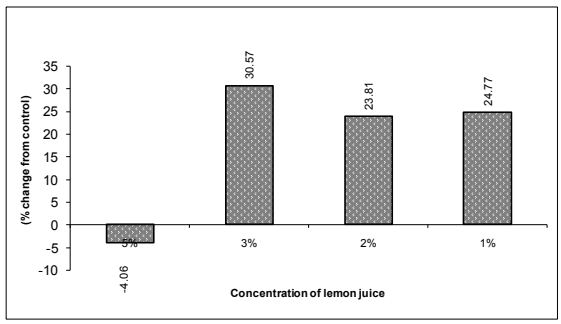


Fig. 3: Overall effect of 3% lemon juice on the total protein profiles of different segments of the silk gland in *Bombyx mori* **during fifth instar development.**

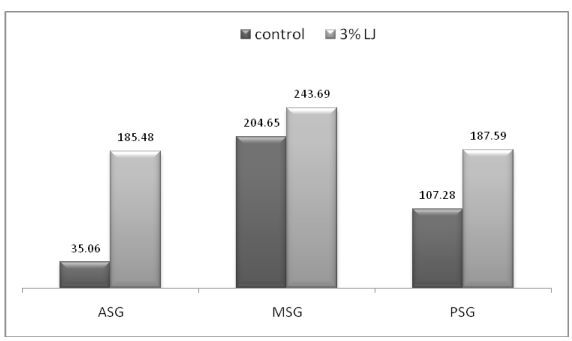
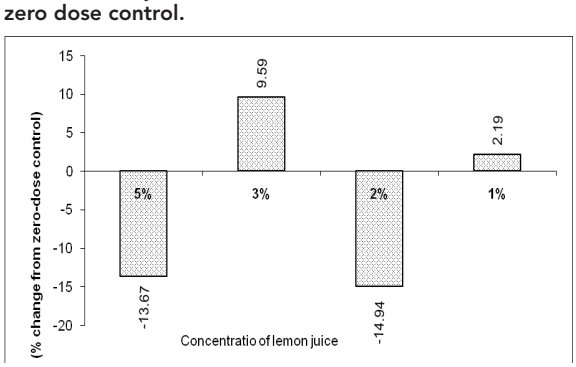


Fig.4: Effect of different concentrations (5%, 3%, 2% and 1%) of lemon juice on the fat body proteins in the fifth instar larva of Bombyx *mori,* as reflected in deviations from zero-dose control.

Fig.5: Effect of different concentrations (5%, 3%, 2% and 1%) of lemon juice haemolymph proteins in the fifth instar larva of *Bombyx mori***, as reflected in deviations from the zero dose control.**



The CPGRs in the larval body weight under treatment conditions ranged from ~57 to 71% during fourth instar and ~16 to 30% during fifth instar and it seems to vary as a function of concentration of lemon juice. Significantly, 3% LJ caused greater elevations in larval growth during fourth (71%) and fifth (~30%) instar developmental stages. When compared with the zero-dose control, 3% LJ enhanced the larval growth rate by 4% in fourth instar and by 23% in fifth instar (Table 1; Cols.7, 13). At other concentrations, LJ showed either negative or low positive impact. For instance, at 5% concentration, it retarded the larval growth rate by ~11% in fourth instar and by ~3% in fifth instar. At 2% level, it retarded the growth rate by ~9% in fourth instar, but enhanced it just by ~1% in fifth instar (Cols. 5 and 11). Similarly, at 1% concentration, it caused a low elevation in growth rate (~2%) in fourth instar, but maximal elevation (~16%) in fifth instar. Both the approaches revealed that the LJ works well at 3% concentration level (i.e., 3 ml of LJ in 100 ml/100 worms) and this is strongly recommended as the minimum effective concentration (MEC) for larval growth.

The analysis of tissue-based protein profiles under treatment conditions is used as an index of metabolic rate in *B. mori* (Kavitha *et al.*, 2011). Obviously, the MEC of lemon juice that could positively impact the larval metabolism in the silkworm can be determined by analyzing the protein profiles of SG, FB and HL. The analysis of CPGRs of protein profiles of these three tissues during fifth instar has revealed that 5% LJ caused a steady positive growth of 60.7% in the SG (Col. 4; Table 3), negative growth of 3.57% in FB (Col.4; Table 5) and a positive growth of just 4.24% in HL (Col. 4; Table 6). This culminated in an overall reduction of protein levels by ~4% in SG and ~9% in FB and ~14% in HL. At 3% level, it caused a steady positive growth of 79.5% in SG (Col. 7; Table 3), 18.51% in FB (Col.7; Table 5) and 17.46% in HL (Col. 7; Table 6). This effect caused a net positive impact of ~31% on SGP, ~3.5% on FBP and ~10% on HLP. At 2% level, it caused a steady positive growth of 74.96% in SG (Col. 5; Table 3), just 2.26% in FB (Col.5; Table 5) and 3.73% in HL (Col. 5; Table 6) and the same resulted as an elevation of SGP levels by 23.81% but reduced FBP levels by 4.6% and HLP levels by ~15%. At 1% level, the LJ caused a steady positive growth of 76.8% in SG (Col. 8; Table 3), 21.01% in FB (Col.8; Table 5) and 13.44% in HL (Col. 8; Table 6) and this effect manifested as an elevation in SGP by ~25%, FBP by ~10% and HLP by just 2%. Obviously, the MEC of LJ differs from tissue to tissue. While, 3% LJ caused greater response with regard to SGP and HLP, it is not so with regard to FBP. Surprisingly positive impact of LJ on fat body was achieved at 1% level, a concentration much lower than that works well with SG and HL. Necessarily, the FB responds to lower concentrations of LJ, while SG and HL do so at higher concentrations. Hence, 3% LJ is recommended as MEC for modulating metabolism in respect of SG and HL and 1% LJ for FB. Though, the present study suffers from the paucity of relevant literature, the one involving the zinc salt and its effect on silkworm metabolism substantiates that the two tissues; SG and FB of silkworm respond to opposing concentrations/doses of exogenous factors provided through the mulberry diet. While FB responds well to higher doses of zinc, SG and HL respond at low concentrations (Kavitha et al., 2011). The opposite is true with regard to lemon juice, wherein the SG and HL showed maximal response to 3% LJ and FB to 1% LJ. Apparently, the silk output in *Bombyx mori* could be enhanced at 3% concentration of LJ, while the metabolic rate in the fat body could be stimulated at 1% level.

Effect of lemon juice on silkworm growth

Body growth: The larval growth rate in silkworm show instar specificity. The observed CPGRs were higher in fourth instar, but lower in fifth instar. In control batches, they ranged from 63.86 to 69.85% in fourth instar and 15.82 to 25.78% in fifth instar. When the larvae were fed with 3% LJ supplemented mulberry leaves, the corresponding figures were elevated to 71.0% in fourth instar and 30.23% in fifth instar.

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Thus, LJ positively reinforces the day-to-day larval growth rates by 2.31percentile points (71.0 - 68.69%) in fourth instar and by 4.45 percentile points (30.23 - 25.78%) in fifth instar. Consequently, the overall larval growth rates were enhanced by 3 to 4 times during fourth and fifth instar developmental stages (Table 1; Fig. 1). Another noteworthy feature of silkworm development is that the larvae grow exponentially in early phases of instars but slowly at their later phases. For instance, during fourth instar development, the early growth rates ranged from 150 to 160% and latter growth rates from 37 to 52% (Cols 3 and 6). The corresponding figures for fifth instar ranged from ~27 to 29% in the early phase and 6 to 9% in the latter phase (Cols 9 and 12). Notwithstanding some minor day-to-day fluctuations, the positive impact of 3% lemon juice continued throughout the larval life, but its marginal (~4%) in fourth instar and maximal (~23%) in fifth instar (Cols 7 and 13). Obviously, the LJ stimulates growth rate in the silkworm larvae at a time when it is slower as in fifth instar rather than when it is faster as in the fourth instar. The additional growth in the larval body is attributable to the phagostimulant action of vitamins C, the chief constituent of lemon juice, the positive role which was widely acknowledged (Sarker *et al*., 1995).

Body growth versus silk gland growth: The determination of gland-body ratio (GBR) by comparatively analyzing the changes in the weight of the larval body and silk gland during fifth instar development was viewed as an important economic parameter of sericulture (Sailaja and Sivaprasad, 2010). Higher GBR could be achieved by enriching mulberry leaves with a variety of exogenous modulators and nutrients. The present study demonstrates that lemon juice, at its minimum effective concentration (MEC) of 3%, could be used as a powerful modulator of GBR in silkworm. As shown in table 2, this exogenous factor has shown profound effect on larval body weight and silk gland weight vis-à-vis GBR. The analysis of data in terms of CPGR and overall percent change reflect day-wise and instar-wise growth trends respectively. Under the influence of 3% LJ, the CPGR grew by 3.59 additional percentile points (66.09 – 62.50%) in the body weight, by 19.47 additional percentile points (230.19 – 210.72%) in SG weight and by 6.48 additional percentile points (70.13 - 63.65%) in GBR. This resulted in an overall enhancement of 29.06 percentile points (358.18 – 329.09%) in body weight, 375 percentile points (2150 – 1775%) in SG weight and 54.14 percentile points (392.41 – 338.27%) in the GBR (Table 2). Though, the present findings are not adequately represented in previous studies, the impact of lemon juice on larval growth is attributable to its multiple vitamins such as the ascorbic acid, thiamine, riboflavin, niacin, folic acid, biotin and pyridoxine and mineral nutrients, such as Ca, P, Mg and Zn (Bhattacharya and Kaliwal, 2005a, 2005b, 2005c; Rajabi *et al*., 2006a; Kavitha *et al*., 2012).

Effect of lemon juice on tissue proteins

Silk gland proteins: The silk gland is the major site of silk protein synthesis. Apart from two silk proteins (fibroin and sericin), it synthesizes and stores 91 other proteins involved in metabolism, immunity, heat-shock mechanism, cytoskeleton formation, protease inhibition, transport and transcription (Nirmala *et al*., 2001; Jin *et al*., 2004; Takasu *et al*., 2005; Kyung *et al*., 2006; Zang *et al*., 2006; Hou *et al*., 2007a). Under the impact of 3% LJ, the protein base of SG grew additionally by 15.27 percentile points (79.50 – 64.23%) during fifth instar development (Table 3; Fig. 2). Within the silk gland, ASG is relatively inert and shows low protein profiles, while MSG and PSG act as protein repositories (Shimura, 1993). The study yielded interesting results and demonstrated that LJ stimulates protein synthesis more predominantly in the ASG than in MSG and PSG. Evidently, the analysis of trends in CPGRs showed that LJ enhanced day-to-day protein synthetic rate maximally by 159.30 (169.84 – 10.54%) percentile points in the ASG, minimally by 5.94 (50.91- 44.97%) percentile points in MSG and moderately by 14.71 (42.21 – 27.50%) percentile points in PSG. In terms of overall change during the entire period of fifth instar, the protein levels were enhanced by 150. 42 (185.48-35.06%) percentile points in ASG, by 39.04 (243.69-204.65%) percentile points in MSG and by 80.31 (187.59-107.28%) percentile points in PSG (Fig.3). Needless to say, the LJ stimulates silk protein synthesis in the most inert region of the silk gland (i. e., ASG), though its impact is generally positive in other two regions (MSG and PSG). Though the conclusive proof for the positive impact of lemon juice is not in sight, it is presumed that its constituent minerals and vitamins could have significantly influenced the protein synthesis in the silk gland as supposed by earlier workers (Thilsath *et al*., 2008; Etebari and Matindoost, 2004; Hussain and Javed, 2002;Thulasi and Siva prasad,2013).

Fat body proteins (FBP): The insect fat body represents the chief site of proteins synthesis and amino acid metabolism and mediates all metabolic activities like those of liver and adipose tissue of higher vertebrates (Wigglesworth, 1972; Scott *et al*., 2004). In silkworm, the FB synthesizes and stores over 177 proteins implicated in larval growth and development (Hak *et al*., 2005; Hou *et al*., 2007b). The present study highlights the positive impact of LJ on FBP at its MEC (i. e., 1%). Under its influence, the CPGR of protein profiles were elevated by 3.88 percentile points (21.01-17.13%) on day-to-day basis, resulting in a 10% hike over zero-dose control (Table 5; Fig. 4). Implicitly, the mineral salts of potassium and magnesium present in LJ could have significantly contributed to the rise in the levels of FBP (Bhattacharya and Kaliwal, 2005).

Haemolymph proteins (HLP): The HL of *B. mori* plays a dual role of transportation and storage. It stores and transports about 241 to 298 proteins involved in a multitude of functions such as larval growth, metamorphosis, ecdysis, chitin and haemocyte formation, growth of silk gland and reproductive organs (Lix *et al*., 2006; Chai *et al*., 2008; Nakahara *et al*., 2009). The HLP levels are potentially modulated by 3% LJ, like those of SG. At this concentration, the CPGR of FBP was elevated maximally by 3. 53 additional percentile points (17.46-13.93%) resulting in an overall hike of ~10% over and above the zero-dose control during fifth instar development (Table 6; Fig. 5). The study highlights that LJ-enriched mulberry leaves significantly alter the biochemical composition of HL and thereby contributes to the somatic growth in the larval body during metamorphosis, probably by modulating the levels of bombyxin hormone (Satake *et al*., 2000; Nijhout and Grunert, 2002; Etebari and Matindoost, 2004). This requires further elucidation.

The study demonstrates two points. Firstly, the MEC of lemon juice differs from tissue to tissue, being 3% for SG and HL and 1% for FB. Secondly, its tissue-based difference indicates that LJ modulates the tissue protein profiles either by de novo synthesis or by mobilization from other tissues. Both these mechanisms are likely in *B. mori*. The fact that LJ works well at 3% level on SG and HL and at 1% level on FB, indicates that the major effect of lemon juice on SG seems to enhance silk protein synthesis. This, it does so, both by de novo protein synthesis at source and by mobilizing additional proteins from FB. The prevalence of higher levels of proteins in the SG, more particularly in its anterior segment (ASG) and HL, coupled with their decline in FB, at 3%concentration of LJ, indicates that proteins are not only synthesized in SG, but also mobilized from the reserve pool of FB. More importantly, the elevation in the levels of haemolymph proteins at this concentration further indicates that the protein reserves are mobilized from FB and transported to SG through the circulating medium of haemolymph as pre-supposed (Kiran kumar *etal.,* 1998).

Effect of lemon juice on economic traits of sericulture

The impact of LJ on the economic parameters of sericulture is generally positive at its minimum effective concentration of 3% in distilled water. Its effectiveness is attributable to its stimulatory role on growth and silk protein synthesis during

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larval and pupal stages. Evidently, the positive impact of LJ on the larval growth, observed during the active fourth (~4%) and fifth instar (~23%) stages has been extended to the quiescent pupal stage, with 23% improvement in body weight (Table 7; Fig. 1). Similarly, its 31% elevatory effect on silk protein synthesis (Col. 7; Table 3) has culminated in the rise of green cocoon weight by ~20% and the shell weight by ~60% with a concomitant reduction in the floss weight by 25% (Table 7). The profitable impact of lemon juice on economic traits appears to be achieved by its two-fold impact on protein synthesis. Firstly, it acts as a potential stimulator of protein synthesis in all the three segments (ASG, MSG and PSG) of silk gland, but more predominantly in ASG. Secondly, its impact is positive (+9%) on shell protein and negative (-22%) on floss protein (Table 7). Because of this reason the raw silk weight rose by 3.8% and that of floss (the chief wastage of sericulture industry) fell by 22%. Probably, this dual effect of lemon juice is responsible for the marginal reduction (~2%) in the renditta (i.e., the number of kilograms of green cocoons required for production of 1 kg raw silk) and considerable increase (~12%) in the denier. The study represents an extension of our previous investigation, wherein we have tested the synergistic effect of lemon juice and ascorbic acid on the silkworm biology and economics of sericulture (Thulasi and Sivaprasad, 2013). Though the economic impact of LJ on sericulture has not been elucidated so far, the nutritional importance of ascorbic acid, one of its chief constituents has been studied (Etebari *et al*., 2004; Hussain and Javed, 2002; Thilsath et *al*., 2008; Singh and Bandey, 2012; Ganesh Prabhu *et al* ., 2013). Since, the lemon juice is a natural repository of vitamins and minerals with a potential to stimulate fibroin synthesis in SG with concomitant reduction in sericin synthesis and its ability to enhance growth rate and metabolism in silkworm, the possibility of its application in sericulture could be explored. The current findings, while substantiating the positive impact of synthetic vitamin C suggests that the lemon juice could be made an integral both mulberry and artificial diets in order to reach economies of scale in the sericulture industry. Needless to say, the application of lemon juice acts as an additional dietary input for silkworms that creates both tangible (increase in silk output) and intangible (reduction in sericultural wastage) benefits to the farmers of sericulture.

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