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SYNERGETIC EFFECT OF ASCORBIC ACID AND LEMON JUICE ON THE GROWTH AND PROTEIN SYNTHESIS IN THE SILKWORM, *BOMBYX MORI* AND ITS INFLUENCE ON ECONOMIC TRAITS OF SERICULTURE

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ABSTRACT

The effect of ascorbic acid and lemon juice on the silkworm, *Bombyx mori* has been studied with reference to larval growth, protein synthesis and silk production. Both these exogenous factors, when supplemented with mulberry diet, showed positive impact on larval growth during the fourth and fifth instar developmental stages. The analysis of growth trends in terms of compound periodical growth rates showed that, the larval growth was more pronounced in fourth instar compared to fifth instar. Day-wise analysis of total protein profiles showed that 1% ascorbic acid caused an elevation in their levels in silk gland and haemolymph, but lemon juice showed no significant impact on protein synthesis. Synergetically, both ascorbic acid and lemon juice are quite effective against protein profiles of fat body, but silk protein levels were not much influenced by them. Ascorbic acid yielded significant gains in economic parameters such as the cocoon weight, pupal weight, raw silk weight and denier and the lemon juice in shell weight, shell protein and denier. Synergistically, they caused an elevation in floss protein content, which is a sericultural wastage. Though, the nutritional importance of ascorbic acid is well substantiated, the role of lemon juice needs further investigations at higher concentrations.

Keywords: *Ascorbic acid, Bombyx mori, Economic parameters, Growth, Lemon juice, Sericulture.*

Number of Tables: 3	**Number of Figures: 2**	**Number of References: 41**

INTRODUCTION

The output in the sericulture industry i.e., silk production, depends on the quality and nutritional status of the mulberry leaf. Of late, the enrichment of mulberry leaf with a multitude of exogenous factors, such as vitamins, minerals, antibiotics, hormones and study their impact on silk production, has become the order of traditional research in sericulture (Sannapa *et al.*, 2002; Etebari *et al.*, 2004; Bhattacharya *et al.*, 2004, 2005 a, 2005 b, 2005 c, 2005 d ; Chakrabarty and Kaliwal, 2011). One important nutrient that attracted the attention of researchers in this field is vitamin C or L- ascorbic acid. This vitamin is present in the mulberry leaf and plays many vital roles in *B.mori* (Lombardi, 1964). It has been identified as a powerful anti- oxidant, potential phago-stimulant, efficient growth promoter and booster of silk production in the silkworm (Ito, 1961; Javed and Gondal ,2002; Hussain and Javed, 2002; Prasad, 2004; Etebari *et al.*, 2004). The silkworm and many other insects are incapable of synthesizing this vital vitamin in their body but harness it from their dietary sources (Ito and Arai, 1965). If, the mulberry diet is reinforced with ascorbic acid, it is possible to show improvement in the sericulture industry. Since, the use of ascorbic acid is not cost effective, and since, it is not available to farmers of sericulture, it is imperative to substitute this vitamin with the chief and easily available lemon juice, which is a rich source of not only vitamin C and many more nutrients (Markus and Sass, 2003: Albertini *et al.*, 2006). The present study explores this possibility, by analyzing the impact of ascorbic acid vis-à-vis lemon juice on the growth, protein profiles and economic parameters of sericulture.

MATERIAL AND METHODS

The present investigation was carried out on Pure Mysore x CSR_2 hybrid variety of the silkworm *Bombyx mori*, reared under standard environmental conditions of 28^{o}C, 85% RH as per Krishnaswami, 1986. After hatching, the worms were reared on M_5 mulberry variety with 5 feeds per 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 four phases namely feeding pattern, study of larval growth, assay of proteins and analysis of economic parameters.

Feeding pattern: After the third moult, the fourth instar larvae were divided into four batches of 100 worms each. The first batch was given normal feedings 5 times a day and treated as control. The second, third and fourth batches were treated as experimental samples and the larvae reared under these categories were fed with mulberry leaves fortified with 1%, ascorbic acid, 1% lemon juice and 1% ascorbic acid + 1% lemon juice respectively. Before feeding, the mulberry leaves were dipped in solutions containing 1% ascorbic acid or 1% lemon juice or both ascorbic acid and lemon juice in distilled water and shade dried under cool dry weather conditions. Such feedings were given to the larvae of both fourth and fifth instars, once in a day at 6 PM, while continuing normal feeding pattern throughout.

Larval growth: The larval growth was studied by analyzing the changes in the body weight of silkworm during fourth and fifth instar developmental stages. The body weights was determined in an electronic balance by taking randomly selected 25 worms each from the control and experimental batches and the mean weight of a single worm is expressed in grams.

Assay of proteins: The total protein profiles of silk gland, fat body and haemolymph were determined in *B. mori* on alternative days (Day 1, day 3, day 5 and day 7) during fifth instar development by the method of Lowry *et al.,* (1951) in 1% homogenates of tissues and 1:9 diluted haemolymph and the values were expressed in mg/g wet weight of tissue or mg/ml of haemolymph. The silk gland and fat body were isolated from fifth instar larval body by making mid-dorsal dissection, in the Silkworm Ringer (Yamaoka *et al.,* 1971).The haemolymph was extracted by cutting the telson and pro-legs of the larval body. Similarly, the protein content of the silk 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.

Analyses of economic parameters: Economic parameters of the sericulture such as green cocoon weight, shell weight, pupal weight, shell protein, floss protein, denier, renditta, raw silk weight were analyzed as per the methods given by Bohidar *et al.*,2007; Rahmathulla *et al.,* 2007; Sailaja and Sivaprasad, 2010; Kavitha *et al.*, 2012).

Statistical analysis of data: The data obtained from the present investigation were presented with suitable statistical interpretation such as Mean, Standard Deviation and test of significance. While the Mean and SD were computed using M.S Excel, the test of significance and percent changes was calculated online by using graph pad and percent change software, (www.graphpad.com/quick calcs/ index cfm/ and www.percent – change com/index php). As against non time practice computing percent changes the growth trends in the levels of biochemical constituents were interpreted in terms of an innovative statistical parameter called compound periodical growth rate (CPGR) whereever possible, as given by Sivaprasad (2012), in order to draw meaningful conclusions.

RESULTS AND DISCUSSION

The individual and synergetic effects of ascorbic acid and lemon juice on *B.mori* were analyzed in two ways. Firstly, by day-wise impact analysis taking previous day's value as control and secondly by the overall impact analysis at the end of fourth/fifth instar, taking first- day value as zero-dose control (Tables 1 to 3; Figures 1 and 2).

Effect on larval growth: Both ascorbic acid and lemon juice showed positive impact on larval growth during fourth and fifth instar developmental stages (Table 1: Fig 1). During fourth instar, the larval body weight of control larvae increased by ~160% on day 2, ~35% on day 3 and by~37% on day 4, with a CPGR of 68.39%. The larvae of the first experimental batch, fed with ascorbic acid fortified mulberry leaves, grew

by~160% on day 2, ~31% on day 3 and by 44% on day 4, with a CPGR of 69.85%. At the same time, the larvae fed with lemon juice grew by ~150% on day 2, 40% on day 3 and by 40% on day 4, with a CPGR of 69.85%. On the other hand when the larvae were fed with both ascorbic acid and lemon juice, they grew by 150%, 40% and ~49% on 2nd, 3rd and 4th days of fourth instar, but recorded a CPGR of 73.25% (Table 1:Fig 1). During fifth instar, the larval growth showed lower growth trends compared to those of fourth instar. The daily growth trends ranged from ~ 9 to 63%, with a CPGR of 25.78% in the control batch. At the same time, the larvae fed with ascorbic acid showed a growth range of 13.6 to ~48% with a CPGR of 29.68% and those fed with lemon juice showed a growth range of ~20 to 46% in fifth instar with a CPGR of 28.92%. On the other hand, the larvae fed with both ascorbic acid and lemon juice showed a growth range of ~8 to 92%, with a CPGR of 29.30% during fifth instar development (Table 1: Fig 1).

The impact as of ascorbic acid on *B.mori* seems to be dose- dependent. While lower concentrations (1 or 2%) yielded positive results, higher concentrations (>3%) showed the opposite effect, obviously due to hyper-vitaminosis (Gothif and Beck, 1967; Karaksy and Idriss, 1990; Eteberi *et al.*, 2004). Though the minimum effective concentration of lemon juice was not determined in the present case, 1% solutions of both ascorbic acid and lemon juice were used, keeping in view the previous successful studies on vitamin C at this concentration (Sengupta *et al.*, 1972; Thilsath et *al.*, 2008; Singh and Bandey, 2012). In growth studies involving cause and effect relationship, as in the present case, the analysis of compound periodical growth rates (CPGR) provides meaningful insights(Sivaprasad,2012). Such an analysis revealed that the growth of silkworm is more conspicuous in the control of fourth instar (CPGR: 68.39%) compared to that in fifth instar (CPGR: 25.78%). In experimental batches, both ascorbic acid and lemon juice caused slight elevations in larval growth, both in fourth (CPGR: 69.85% each) and fifth (CPGR: about 29% each) instars. On the other hand, when the both nutrients were combined, their synergetic effect was more preponderant in fourth instar (CPGR: 73.25%) compared to fifth instar (CPGR: 29.30%). Furthermore, the day- wise analysis indicates that maximal growth takes place on day 2 in fourth instar (160%) and on day 3 in fifth instar (~63%). Both the nutrients showed similar increasing trends in larval growth during the corresponding days of fourth and fifth instar larval stages (Fig: 1).

Effect on protein synthesis: The analysis of total protein profiles in the silkworm tissues is considered as an index of protein synthesis. The analysis of trends in their levels in terms of CPGR, presented in Table 2 and Fig 2 indicate the mixed impact of exogenous nutrients on protein synthesis in different tissues of silkworm. While in the controls, the protein synthesis in the silk gland grew by over 64% during fifth instar development, it recorded a higher CPGR of ~77.42% when fed with ascorbic acid, but showed lower CPGR when fed with lemon juice (76.8%) alone and with ascorbic acid+ lemon juice (73.3%) together (Table 2; Fig

2).In the fat body the impact is totally positive in respect of all nutrients administered. While, the protein synthesis recorded a CPGR of 17.3% in control, it showed a higher CPGR's when the larvae were fed with ascorbic acid (18.61%), lemon juice (21.01%) separately and synergistically (23.30%). The haemolymph protein recorded mixed trends under the influence of two nutrients. While the protein pool grew by 13.93% in control, it recorded a significantly higher CPGR (31.16%) under the influence of ascorbic acid. Though, lemon juice has not caused any significant elevation in its protein profiles, together with ascorbic acid it caused an elevation of 21.02% at the end of fifth instar (Table 2; Fig.2).

All the three tissues, viz , the silk gland, fat body and haemolymph have their own proteins and functions during larval growth and metamorphosis in *B. mori*. The silk gland is the major site of silk protein synthesis and consists of over 93 proteins including fibroin and sericin (Jin *et al.*, 2004; Zhang *et al.*, 2006; Hou *et al.*, 2007 a). The fat body is the major metabolic organ and is known to contain over 177 proteins that involved in various functions (Scott *et al.*, 2004; Hou *et al.*, 2007 b). The haemolymph, on the other hand, is the chief circulating fluid and flowing reservoir of over 298 proteins that are mostly synthesized in other tissues of silkworm (Lix *et al.*, 2006; Chai *et al.*, 2008; Nakahara *et al.*, 2009). The data presented in table 2 and figure 2, showed differential effects on protein synthesis. While the ascorbic acid caused an elevation in the levels of silk proteins (fibroin and sericin) in the silk gland, metabolic proteins in the fat body and circulating proteins in the haemolymph. This indicates that ascorbic acid is a potential stimulator of protein synthesis in the silkworm tissues (Thilsath *et al.*, 2008) and hence it could be used at lower concentration for the benefit of sericulture industry. The lemon juice, which is a major source of vitamin C, showed similar positive impact on protein synthesis in silk gland and fat body but inhibited the release of tissue proteins into the haemolymph. Though, its impact on silk protein synthesis is not so effective at 1% concentration, its use could be explored at higher concentrations for benefits in sericulture. Synergistically, both ascorbic acid and lemon juice showed similar effect on protein synthesis, but their impact was more pronounced on the fat body compared to silk gland and haemolymph. Obviously, vitamin C, works as a potent modulator of metabolism in silkworm and influences its larval growth and development during metamorphosis.

Effect on economic parameters: In the present study the impact of ascorbic acid and lemon juice was analyzed with reference to nine economic traits of sericulture, viz, cocoon weight, shell weight, floss weight, pupal weight, shell protein content, floss protein content, raw silk weight, denier and renditta (Table 3). In general, both the nutrients showed positive impact on all economic parameters, but differed in the degree of influence. While, ascorbic acid yielded significant gains in cocoon weight (~17%), pupal weight (~20%), raw silk weight (~ 3.5%), and denier (11.6%), the lemon juice yielded gain in shell weight (~20%), shell protein content (~7%), and the

denier (~7.8%). Synergetically, they showed positive influence on all economic factors, but significantly contributed to elevations in the floss protein content of the cocoon, which is considered the sericultural wastage. The economic impact of lower concentration of ascorbic acid on sericulture has been widely acknowledged (Eg.; Babu *et al.*, 1992; Chauhan and Singh, 1992; Etebari, 2004; Hussain and Javed, 2002; Thilsath et *al.*, 2008; Singh and Bandey, 2012; Ganesh Prabhu *et al* ., 2013). The current findings, while substantiating the positive impact of synthetic vitamin C suggests ways and means for substituting it with naturally available sources such as lemon juice, which is not only a major source of vitamin C, but also a multitude of minerals and other nutrients (Albertini *et al.*, 2006). Obviously, the possibility of the use of lemon juice could be explored in sericulture as it has potential to stimulate the synthesis of silk proteins (fibroin and sericin) and contributes to the thickness of silk fiber, as evidenced by elevations in the levels of shell and floss proteins and the denier under the synergetic influence of ascorbic acid and lemon juice.

Conclusion: The present study on silkworm growth and nutritional effects of ascorbic acid and lemon juice emphasizes three points. Firstly, the larval growth is more rapid in fourth instar than in fifth instar, and that in each instar, much of growth occurs in the early phases (day 2 or day 3) both in control and those fed with exogenous nutrients (lemon juice and ascorbic acid). Secondly, the synthetic vitamin C (Ascorbic acid) is quite effective against protein synthesis, more particularly that of silk at a minimum effective dose of one percent. Thirdly, the lemon juice, which is a natural source of vitamin C, shows positive impact, but its effectiveness could be realized only at higher concentrations.

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Table 1: *Effect of ascorbic acid (AA) and lemon juice (L J) on the larval growth in the silkworm, Bombyx mori during the fourth and fifth instar developmental stages.*

Day of Instar	Statistical Parameters	IV Instar				V Instar			
		Control	AA	L J	AA+L J	Control	AA	L J	AA+L J
Day 1	Mean	0.10	0.10	0.10	0.10	0.49	0.49	0.49	0.49
	S.D	±0.001	±0.001	±0.001	±0.001	±0.01	±0.01	±0.01	±0.01
Day 2	Mean	0.26	0.26	0.25	0.25	0.62	0.69	0.65	0.53
	P.C	(160.0)	(160.0)	(150.0)	(150.0)	(26.53)	(40.81)	(32.65)	(8.16)
	S.D	±0.01*	±0.003*	±0.007*	±0.009*	±0.01*	±0.001*	± 0.020*	±0.002*
Day 3	Mean	0.35	0.34	0.35	0.35	1.01	1.02	0.95	1.02
	P.C	(34.61)	(30.76)	(40.0)	(40.0)	(62.9)	(47.82)	(46.15)	(92.45)
	S.D	±0.007*	±0.02*	±0.003*	±0.007*	±0.04*	±0.009*	±0.04*	±0.04*
Day 4	Mean	0.48	0.49	0.49	0.52	1.2	1.44	1.25	1.5
	P.C	(37.14)	(44.11)	(40.0)	(48.57)	(18.81)	(41.17)	(31.57)	(47.05)
	S.D	±0.005*	±0.009*	±0.009*	±0.01*	±0.04*	±0.05*	±0.04*	±0.005*
Day 5	Mean					1.57	1.77	1.54	1.78
	P.C	-	-	-	-	(30.83)	(22.91)	(23.2)	(18.66)
	S.D					±0.04*	±0.03*	±0.01*	±0.04*
Day 6	Mean					1.78	2.01	1.88	2.05
	P.C	-	-	-	-	(13.37)	(13.55)	(22.07)	(15.16)
	S.D					±0.07*	±0.01*	±0.06*	±0.04*

Day 7	Mean P.C S.D	-	-	-	-	1.94 (8.98) ±0.001*	2.33 (15.92) ±0.01*	2.25 (19.68) ±0.01*	2.29 (11.7) ±0.02*
	CPGR	68.39%	69.85%	69.85%	73.25%	25.78%	29.68%	28.92%	29.30%

* Statistically significant:

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 from the control as the base value and the compound periodical growth rates (CPGR), were computed on the basis of initial (control) and final values (Values of 4th day in case of IV instar and 7th day in case of V instar) as per Sivaprasad, 2012.

Table 2: *Effect of ascorbic acid (AA) and lemon juice (L J) on the protein profiles of the silk gland, fat body and haemolymph in the silkworm, Bombyx mori during fifth instar development.*

V Instar	Statistical parameters	Silk gland				Fat body				Haemolymph			
		Control	AA	L J	AA+L J	Control	AA	LJ	AA+L J	Control	AA	L J	AA+LJ
Day-1	Mean S.D	16.46 ±2.1	16.46 ±2.1	16.46 ±2.1	16.46 ±2.1	26.95 ±1.07	26.95 ±1.07	26.95 ±1.07	26.95 ±1.07	5.85 ±0.32	5.85 ±0.32	5.85 ±0.32	5.85 ±0.32
Day-3	Mean P.C S.D	36.73 (123.14) ±3.47*	35.3 (114.45) ±1.31*	34.9 (112.02) ±1.66*	28.01 (70.17) ±2.51*	30.92 (14.73) ±0.60*	37.58 (39.44) ±4.18*	30.95 (14.81) ±0.68*	39.69 (47.27) ±3.19*	6.6 (12.82) ±0.43*	6.21 (6.15) ±1.48**	7.04 (20.34) ±0.29*	7.63 (30.42) ±0.34*
Day-5	Mean P.C S.D	39.8 (8.35) ±0.88**	45.19 (28.01) ±1.08*	48.17 (38.02) ±0.90*	44.45 (58.69) ± 2.42*	35.77 (15.68) ±1.22*	40.85 (8.7) ±2.20**	41.92 (35.44) ±1.62	42.95 (8.21) ±0.95*	7.14 (8.1) ±0.016**	8.87 (42.83) ±0.14*	7.23 (2.69) ±0.04**	8.24 (7.99) ±0.33*

Day-7	Mean P.C S.D	72.91 (83.19) ±1.51*	91.93 (103.42) ±0.88*	90.97 (88.85) ±2.82*	85.68 (92.75) ±1.2*	43.34 (21.07) ±1.58*	44.97 (10.08) ±1.61*	47.76 (13.93) ±4.29*	50.52 (17.62) 4.54*	8.65 (21.14) ±0.65*	13.2 (48.81) ±0.52*	8.54 (18.11) ±1.63*	10.37 (25.84) ±1.12*
	CPGR	64.23%	77.42%	76.80%	73.31%	17.13%	18.61%	21.01%	23.30%	13.93%	31.16%	13.44%	21.02%

* 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 initial (control) and final values (7th day values) as per Sivaprasad, 2012.

***Table 3**: Effect of ascorbic acid (AA) and lemon juice (L J) on the economic parameters of the silkworm, Bombyx mori.*

S. No	Economic Parameter	Statistical parameters	Control	AA	LJ	AA+LJ
1.	Cocoon weight (g)	Mean P.C S.D	0.986 - ±0.05	1.15 (17.34) ±0.02*	1.08 (10.20) ±0.01*	1.09 (13.26) ±0.01*
2.	Shell weight (g)	Mean P.C S.D	0.15 - ±0.07	0.17 (13.33) ±0.006*	0.18 (20.0) ±0.01*	0.18 (20.0) ±0.002*
3.	Floss weight (g)	Mean P.C S.D	0.016 - ±0.002	0.018 (125.0) ±0.002**	0.016 (0.0) ±0.0005*	0.018 (12.5) ±0.002**

4.	Pupal weight	Mean P.C S.D	0.793 - ±0.04	0.950 (19.79) ±0.048*	0.835 (5.29) ±0.002**	0.865 (9.07) ±0.019*
5.	Shell Protein (mg/g)	Mean P.C S.D	11.91 - ±0.008	12.64 (6.12) ±0.009*	12.78 (7.30) ±0.01*	12.76 (7.13) ±0.02*
6.	Floss Protein (mg/g)	Mean P.C S.D	8.09 -- ±0.008	7.63 (-5.68) ±0.01*	8.35 (3.58) ±0.01*	9.54 (17.92) ±0.01*
7.	Raw silk weight (g)	Mean P.C S.D	12.88 - ±0.01	13.33 (3.49) ±0.007*	12.00 (-6.83) ±0.005*	13.33 (3.49) ±0.0005*
8.	Denier	Mean P.C S.D	10.72 - ±0.009	11.6 (8.20) ±0.16*	10.00 (-6.71) ±0.008*	11.04 (2.98) ±0.01*
9.	Renditta	Mean P.C S.D	7.02 - ±0.01	7.005 (-0.28) ±0.0008*	7.82 (11.39) ±0.01*	7.08 (0.854) ±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.

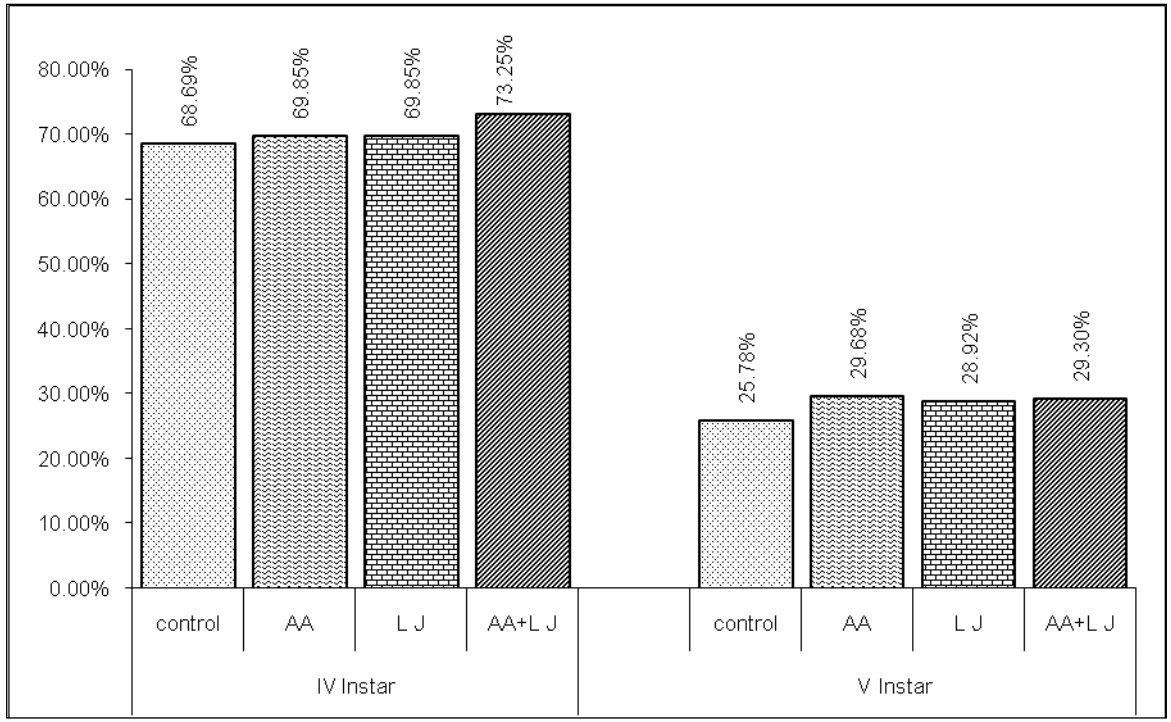

***Fig.1**: Compound periodical growth rates in the larval body weights in the silkworm, Bombyx mori during the fourth and fifth instar developmental stages, under the influence of ascorbic acid (AA) and lemon juice (L J). Source: Table 1.*

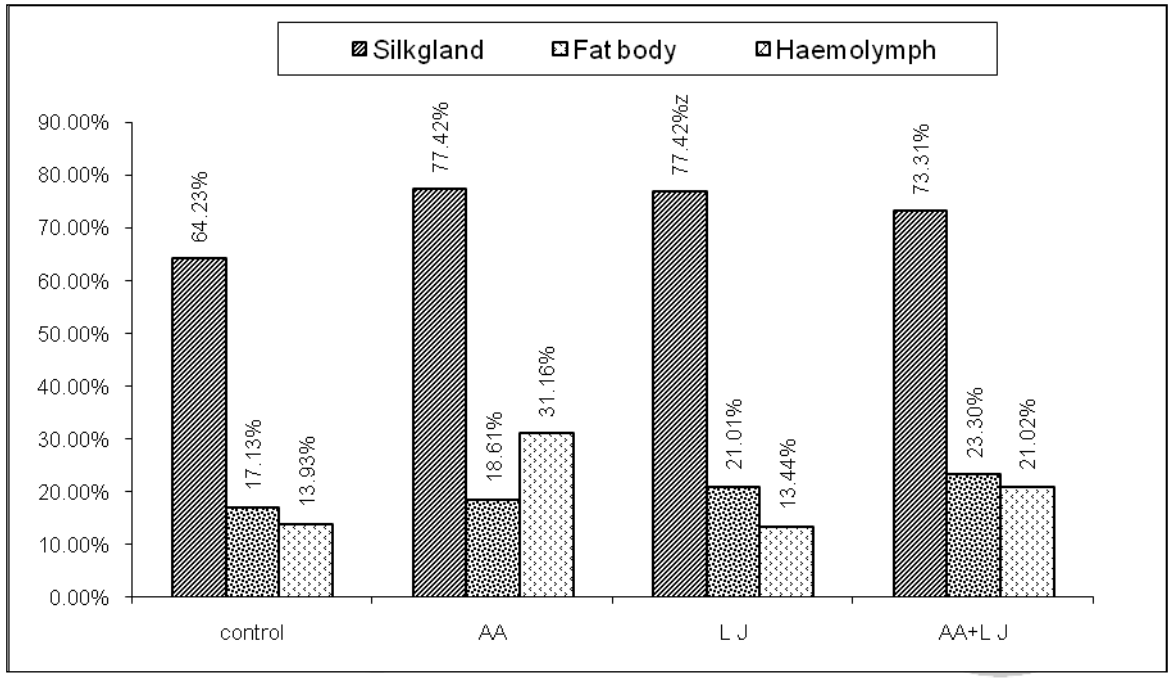

***Fig.2**: Compound periodical growth rates in the total protein profiles of silk gland, fat body and haemolymph in the silkworm, Bombyx mori during the fifth instar developmental stage, under the influence of ascorbic acid (AA) and lemon juice (L J). Source: Table 2.*

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