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IMPACT OF SEASONAL VARIATION ON PHYSIOLOGICAL TRAITS AND BIOCHEMICAL CONSTITUENTS IN SILK GLANDS OF *Bombyx mori* L.

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ABSTRACT

The study was conducted during spring and summer seasons of 2010-2011 to examine the changes in biochemical constituents in silk gland contents and to identify the races with better growth of silk glands in various seasons. Larval weight and silk gland weight and size were significantly high in 'SK-1' and 'SK-6' and low in 'CSR-2' during both the seasons. The values were marginally high in spring than summer season. Biochemical contents in silk glands decreased more in summer than spring. There was no substantial variation in the expression of molecular weight of silk gland proteins in all the genotypes tested. Carbohydrate and protein contents were significantly high in race 'SK-1' during both the seasons but low values in race 'CSR-2'. There was no substantial difference in the molecular weight of silk gland proteins in different breeds evaluated.

Key words: *Bombyx mori*, carbohydrates, proteins, seasons, SDS-PAGE, silk gland

INTRODUCTION

The domesticated silkworm *Bombyx mori* L. is the target of intensive scientific study since silkworm is used as a source of silk for producing exquisite textiles and dress materials. India is the second largest producer of raw silk in the world. The demand for quality silk produced by bivoltine silkworms is growing both in national and international markets. Jammu and Kashmir State is the only traditional bivoltine/univoltine silk belt in India owing to its salubrious climatic conditions for silkworm rearing and quality bivoltine silk production. However, lack of productive bivoltine silkworm breeds/hybrids, suited to temperate agro-climatic conditions, is one of the major constraints in boosting cocoon and raw silk production in J&K (Trag *et al.,* 1992).

The growth of silk gland in silkworm is of paramount importance as it is the site for the synthesis of silk proteins, the basic raw materials of silk cocoon (Sutherland *et al*., 2010). Prominently, the silk gland grows during 4^{th} and 5^{th} larval instars, the rate of which is modulated by environmental factors such as light, diet, temperature and relative humidity (Shimizu, 1982). Among the various environmental factors, temperature and nutrition play a vital role in silkworm growth and productivity (Sen *et al*., 1993). The late age silkworm performance is relatively lower than the young age ones (Krishnaswamy, 1994) and the fluctuations in temperature during different stages of larval development has been found more favourable for growth and development of larvae than constant temperature (Anonymous, 1975). Proteins are necessary for various biological activities during development, metamorphosis and maintenance of various physiological functions in different tissues

(Kumar *et al*., 2011). Carbohydrates serve as protecting constituents for silkworms during adverse conditions and are essential energy source for various biological activities. Temperature is considered highly significant to ascertain state of activity, longevity, productivity of an organism, physiological and biochemical adaptation carried out by silkworm to survive stress conditions (Singh *et al*., 2013). Keeping this in view, an attempt was made to analyze some biochemical constituents in silk glands of commercial silkworm breeds of Kashmir.

MATERIALS AND METHODS

The present study was conducted at Temperate Sericulture Research Institute, SKUAST-Kashmir, Mirgund, J&K (India) during spring and summer seasons of 2010-2011. Two breed types *viz.*, plain (races: SK-6, SK-24, SK-34, SK-30 and CSR-2) and marked (races: SK-1, SK-13, SK-28, SK-31 and Dun-22) each comprising of 250 silkworms, were used as experimental material. The experiment was conducted in a completely randomized design with each treatment replicated three times. The temperature and relative humidity were maintained at 28^{o}C and 70-80%, respectively, during whole period of experimentation. The data on the growth of larvae and rearing performance in terms of larval weight, silk gland weight and size were recorded from ten randomly selected 6th day old 5th instar larvae from each treatment. These larvae were dissected to calculate the size and weight of silk glands. The silk glands were solubilized in 10 mm tris buffer (pH 7.4) and SDS page carried out on 8% gel. The protein and carbohydrate contents in silk glands were estimated.

Protein estimation

The quantitative estimation of protein in silk gland was carried out as per the standard method of Lowery *et al.* (1951) using crystalline bovine serum albumin (BSA) as standard. The values were expressed in terms of mg protein g^{-1} silk gland. For preparation of a standard curve, 0.5 mL sample was taken, 0.5 mL protein reagent added and incubated for 5 min. Then 0.5 mL diluted Follin's reagent was added with thorough mixing and the mixture allowed to stand for 30 min. at room temperature for colour development against a blank. The absorbance was measured at 660 nm using UV/VIS spectrophotometer. The quantity of proteins was calculated by plotting a standard curve by taking different concentrations of BSA as a standard. The values were expressed in mg g^{-1} silk gland.

Carbohydrate estimation

The quantitative estimation of total sugar present in silk glands was estimated by anthrone method (Dubois *et al*., 1956) using glucose as standard. The values were expressed in terms of mg carbohydrate g^{-1} silk gland. For preparation of standard curve, standard dextrose solution of 0.0, 0.2, 0.4, 0.6, 0.8, 1.0 mL and silk gland extract solution of 0.5 mL was pippetted into a series of test tubes. Distilled water was added to each test tube to make final volume of 1 mL. Then 5 mL anthrone reagent was added to each test tube, kept in boiling water bath for 15 min and cooled. The optical density measured at 620 nm against blank. The standard graph of concentration verses optical density of the samples was prepared. The concentration of carbohydrates (mg g^{-1} sample) was calculated using by the formula:

$$\text{Concentration of sample} = \frac{\text{Optical density of sample x Concentration of standard}}{\text{Optical density of standard}}$$

Qualitative analysis of total proteins

The qualitative analysis of total soluble protein in silk gland was done by using sodium dodecyle sulphate polyacrylamide gel electrophoresis (Laemmuli *et al.*, 1970). A uniform quantity of protein from each breed of larvae was loaded to each slot of the gel. Two silk proteins *viz.*, sericin and

fibroin were isolated. For sericin, the middle silk gland (1 g from each larva) from ten 6 day old 5th instar silkworm larvae was suspended in 50 mL 0.025 N NaOH, and stirred for 12 h at 4°C (Rajan *et al.*, 1992). The protein solution was dialyzed against double distilled water, concentrated under vacuum and stored at -20°C. For fibroin, ten 6th day old 5th instar silkworm larvae were selected, their posterior silk glands discriminated and dissolved in 60% lithum thiocyanate solution and incubated overnight at 4°C with gentle stirring (Rajan *et al.*, 1992). The soluble protein was collected and dialyzed against tris HCl (pH 8.0). The solution was dialyzed against double distilled water. The dialyzed samples were centrifuged at 8000 rpm and the supernatant stored at -20°C.

Protein samples were resolved in their components by sodium dodecyle sulphate polyacrylamide gel electrophoresis as per the method of Laemmuli *et al.* (1970). The separating gel was polymerized in a mini-gel apparatus for 30-45 min. and the stacking gel polymerized for 15 to 20 min. The thickness of the gel was 1.5 mm. The sample was mixed with an equal volume of 2X sample buffer and incubated in a boiling water bath for 2 to 5 min. It was then applied to the sample walls and electrophoresis carried out at a constant current of 20 to 30 mA. The gel was stained with Coomassie blue for 1 to 2 h and destained with destaining solution over a period of 24 h.

The analysis of variance (ANOVA) was done to test the significance of differences between the mean values of protein and carbohydrate levels of silkworm races reared during different seasons. Least significant difference (LSD) was used to find significance of differences between the means in races, seasons and days (Gomez and Gomez, 1984).

RESULTS AND DISCUSSION

The pooled analysis of variance revealed significant differences among the races and their interaction with the environment for all the characters studied during both the seasons (Table 1), thereby indicated the existence of a good amount of genetic variability. The larvae weight in different races of silkworm varied significantly during spring and summer seasons. During spring, races SK-1, SK-31 and SK-6 were at par with each other but showed significantly higher larval weight than other races tested. The lowest larval weight of 39.0 g was observed in race SK-2. Similarly, in summer season race SK-1 had significantly highest larval weight (45.23 g) while race CSR-2 had lowest weight (36.66 g). The larval weight, irrespective of races, was higher in spring than summer. The significant variation in larval weights indicated that under stress conditions the races seem to have interacted well for favourable growth, and genetic consistency invoked more on expression of post-cocoon traits for survival. The insignificant variation indicated that the environment might have played a major role in the expression of phenotypes as these races/ genotypes were acclimatized to survive under variable stress environments, thus it maybe predicted that these races behaved uniformly for survivability in new environment. Race x environment inter-

Table 1: Pooled analysis of variance (ANOVA) for silk gland and associated parameters

Source of variation	d.f.	Mean sum of squares		
		Larval weight (g^{-10})	Silk gland weight (g^{-10})	Silk gland size/larvae (cm)
Environment	1	438.539**	72.820**	0.496
Races	9	77.365**	7.580**	0.954
Races x environment	9	9.082*	0.451*	0.718
Error	40	2.567	0.977	0.621

*Significant at ($p<0.05$); **Significant at ($p<0.01$)

Table 2: Performance of silkgland and associated parameters in silkworm (*Bombyx mori* L.)

Seasons	Races	Larval weight (g^{-10})	Silk gland	
			Weight (g^{-10})	size (cm)
Spring	SK-30 (P)	41.43	13.20	23.93
	SK-34 (P)	40.93	14.50	24.17
	SK-24 (P)	44.26	14.33	23.97
	SK-6 (P)	49.53	14.13	23.83
	CSR-2 (P)	39.00	11.40	23.50
	SK-1 (M)	52.46	15.37	24.13
	SK-13 (M)	48.40	12.33	24.33
	SK-28 (M)	49.20	14.23	23.70
	SK-31 (M)	49.56	13.37	26.03
	Dun22 (M)	44.26	12.77	23.47
$CD_{0.05}$		2.930	1.751	ns
Summer	SK-30 (P)	39.36	11.27	23.60
	SK-34 (P)	37.46	12.27	23.87
	SK-24 (P)	41.23	12.17	23.97
	SK-6 (P)	42.70	12.67	23.97
	CSR-2 (P)	36.66	9.30	23.77
	SK-1 (M)	45.23	12.27	24.03
	SK-13 (M)	39.20	10.10	24.00
	SK-28 (M)	42.30	11.37	23.97
	SK-31 (M)	43.33	12.00	24.07
	Dun22 (M)	37.50	10.20	24.00
$CD_{0.05}$		2.522	1.613	ns
Pooled mean	SK-30 (P)	40.40	12.23	23.77
	SK-34 (P)	39.20	13.38	24.02
	SK-24 (P)	42.75	13.25	23.97
	SK-6 (P)	46.12	13.40	23.90
	CSR-2 (P)	37.84	10.35	23.63
	SK-1 (M)	48.85	13.82	24.08
	SK-13 (M)	43.80	11.22	24.17
	SK-28 (M)	45.75	12.80	23.83
	SK-31 (M)	46.45	12.68	25.05
	Dun22 (M)	40.88	11.48	23.73
$CD_{0.05}$		2.641	1.633	ns

(P) – Plain race; (M) – Marked race; ns – Non-significant

actions are extremely important in the development and evaluation of varieties because they reduce genotypic stability values under diverse environments (Hebert *et al.*, 1995). Our findings are in agreement with Nair *et al.* (2006), Kumar and Elangovan (2010), Amala *et al.* (2011) and Sabhat *et al*. (2011).

Silk gland weight was marginally higher in spring season than summer in all the races tested. During spring season significantly highest silk gland weight of 15.37 g was observed in race SK-1. During summer season, silk gland weight was significantly high in race SK-6 (12.67 g) than race CSR-2 (9.30 g). Similarly, the pooled mean of silk gland size over two seasons as well as in spring and summer seasons were at par in all the races tested (Table 2). The mean size of silk gland during spring and summer seasons was higher in race SK-31 (26.03 and 24.07 cm, respectively) while it was lower in races Dun22 (23.47 cm) and SK-30 (23.6 cm) during spring and summer seasons, respectively. However, the pooled data showed highest silk gland size in race SK-31 (25.05 cm) and lowest in race CSR-2 (23.63 cm).

The analysis of variance for protein and carbohydrate contents in individual and pooled over environments revealed significant mean squares for environments (Table 3). Highly significant variations were seen in races and their interaction with environment, thereby indicating the existence of good amount of genetic variability in the races tested.

Quantitative analysis of biochemical constituents

The protein content in larvae, irrespective of races, decreased in summer season compared to the spring season (Table 4). During spring season significantly higher protein content (114.50 mg g^{-1}) was observed in race SK-1, while low protein content (94.64 mg g^{-1}) was noticed in race CSR-2. A. Similar trend in protein content was observed in summer season with highest value (103.47 mg g^{-1}) in SK-1 and lowest value (92.5 mg g^{-1}) in race CSR-2. Proteins are essential macro-molecules that are required for growth and development of silkworm and for silk biosynthesis. Any deficiency in essential amino acids or total nitrogen is reflected in growth, development and silk biosynthesis. The deficiency could arise either due to the absence of specific nutrients in required quantity or failure to utilize nutrients present in leaf by larvae (Nair *et al.*, 2006). Rahmathulla *et al*. (2011) reported that nearly all the biological processes including the rates of bio-chemical and physiological reactions are affected by temperature and humidity which ultimately affect the quality

Table 3: Pooled analysis of variance (ANOVA) of some biochemical traits of silkworm (*Bombyx mori* L.)

Source of variation	d.f.	Mean sum of squares	
		Protein content (mg g^{-1})	Carbohydrate content (mg g^{-1})
Environment	1	990.625**	61.468**
Race	9	94.784**	18.411**
Race x environment	9	11.069*	0.100*
Error	40	4.0531	0.475

*Significant at ($p<0.05$); **Significant at ($p<0.01$)

and quantity of cocoon. In both the seasons, higher protein content was noticed in race SK-1 which may be due to the ability of race to utilize the available resources for biomass production. The finding is in conformity with Nagalakshmamma and Naga Jyothi *et al*. (2010), Sabhat *et al*. (2011), Sangamithirai *et al*. (2014) and Yogananda Murthy *et al*. (2014).

Table 4: Performance of some biochemical traits of silkworm (*Bombyx mori* L.)

Season	Races	Protein content (mg g^{-1})	Carbohydrate content (mg g^{-1})
Spring	SK-30 (P)	102.59	9.53
	SK-34 (P)	107.87	11.47
	SK-24 (P)	106.18	11.57
	SK-6 (P)	108.87	12.93
	CSR-2 (P)	96.77	8.50
	SK-1 (M)	114.50	12.97
	SK-13 (M)	107.43	9.67
	SK-28 (M)	109.80	12.17
	SK-31 (M)	109.47	12.07
	Dun22 (M)	100.17	8.70
$CD_{0.05}$		3.627	1.316
Summer	SK-30 (P)	98.25	7.30
	SK-34 (P)	98.17	9.43
	SK-24 (P)	97.17	9.17
	SK-6 (P)	100.50	11.50
	CSR-2 (P)	92.50	6.60
	SK-1 (M)	103.47	10.90
	SK-13 (M)	98.47	7.50
	SK-28 (M)	99.70	10.30
	SK-31 (M)	98.52	10.03
	Dun22 (M)	95.63	6.60
$CD_{0.05}$		3.218	1.014
Pooled mean	SK-30 (P)	100.42	8.42
	SK-34 (P)	103.02	10.45
	SK-24 (P)	101.67	10.37
	SK-6 (P)	104.69	12.22
	CSR-2 (P)	94.64	7.55
	SK-1 (M)	108.98	11.93
	SK-13 (M)	102.95	8.58
	SK-28 (M)	104.75	11.23
	SK-31 (M)	104.00	11.05
	Dun22 (M)	97.90	7.65
$CD_{0.05}$		3.320	1.138

(P) – Plain race, (M) – Marked race

Carbohydrates are the major source of energy for silkworm which they procure from mulberry leaves. Horie (1973) found that the gross energy ingested by silkworm in 4^{th} and 5^{th} instars is 1.84 and 21.0 kcal, respectively. From this, 45% energy is utilized for growth and the rest is stored in the form of carbohydrates for utilization during pupal and adult stages in which 12% of energy stored is utilized for oogenesis. The results in present study revealed that the carbohydrate content in spring season was higher than summer in all the breeds tested (Table 2). The mean carbohydrate content (12.97 mg g^{-1}) in spring season was significantly higher in race SK-1 than most of the other races but was at par with races SK-6 (12.93 mg g^{-1}), SK-28 (12.17 mg g^{-1}) and SK-31 (12.07 mg g^{-1}). The lowest carbohydrate content of 8.50 mg g^{-1} was recorded in CSR-2. However, in summer season significantly higher carbohydrate content was observed in race SK-6 (11.5 mg g^{-1}) but was at par with races SK-1 (10.9 mg g^{-1}). Races CSR-2 and Dun22 had low carbohydrate content (6.6 mg g^{-1}). It appears that the races SK-1 and SK-6 have greater exposure to temperate climate and have evolved alternate means for adaptability in both the seasons. The low level of biochemical contents in other breeds may be attributed to environmental stress and utilization of carbohydrates as an energy source for maintaining the normal physiology of the insect. Our results

are in agreement with earlier reports of Yogananda Murthy *et al*. (2014) and Mishra *et al*. (2010).

Qualitative analysis of total proteins

During qualitative analysis of silk gland proteins of 350, 66 and 25 kDa were observed on the gel when compared to the known protein marker (Fig. 1. . Though the protein content was more in spring than summer season, there was no substantial variation in the expression of molecular weight of silk gland proteins of all the races studied. This suggests that the expression of protein is developmentally regulated (Dash *et al*., 2006).However, in spring season, the intensity of bands was high which may be attributed to the variation in protein concentration. Zhang *et al*. (2005) carried out analysis of whole silk gland proteins in silkworm through SDS-PAGE and peptide mass fingerprinting method, and identified eight P25 isoforms and silk gland proteins. Similarly, Dash *et al*. (2006) reported that a single band of about 200 kDa was detected both in reducing and non-reducing conditions. The heavy chain proteins from these breeds were of 350 kDa and the light chain proteins were of 25 kDa on the gel against known molecular marker. Shimura *et al*. (1982) reported that silk thread is composed of two major proteins fibroin and sericin. Physiological strategy adopted by silkworm at low temperature revealed that exposure to low temperature leads to accumulation of high concentration of biomolecules as compared to control which significantly influences the isozyme activity (Singh *et al*., 2010). The qualitative analysis of amylase indicated six types of changes *i.e*. the intensity of the bands either more or less besides, some of the bands either present or absent. Some of the bands increased or decreased in their intensity as the age advances in addition to altered RF value.

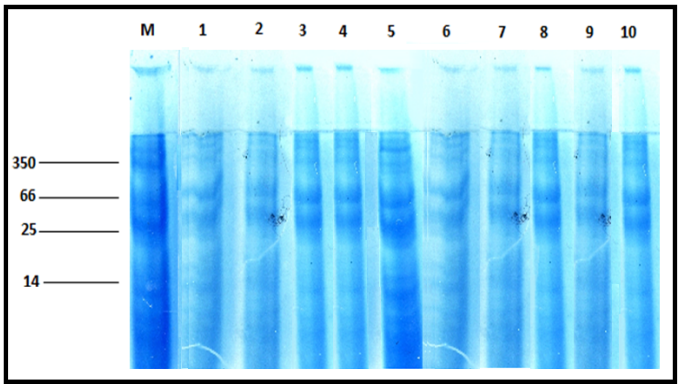

Fig. 1: SDS-PAGE of silk protein during spring season; where, M-Marker, 1: SK-30, 2: SK-34, 3:SK-24, 4: SK-6, 5: CSR-2, 6: SK-1, 7: SK-13, 8: SK-28, 9: SK-31, 10: DUN-2

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