ASIAN J. EXP. BIOL. SCI. VOL 3(2) 2012: 642-653

ISSN 0975-5845
AJEBS
http://www.ajebs.com

ORIGINAL ARTICLE

Correlation Studies on Haemolymph and Midgut Tissue Proteins with Commercial Characters of Silkworm *Bombyx mori* L.

Farshid Ghasemi Kasmaei* and H. B. Mahesha
Department of Sericulture, Yuvaraja's College, University of Mysore, Mysore-570 005, India
*Department of Veterinary, Sahneh Branch, Islamic Azad University, Sahneh, Iran.

ABSTRACT

Four mulberry silkworm races viz., Pure Mysore, Nistari, NB_4D_2 & CSR_2 and two hybrid (Pure Mysore × CSR_2 and Nistari × NB_4D_2) silkworms were selected for the present study. The total soluble protein present in the haemolymph as well as midgut tissue was estimated. The qualitative analysis of total protein was carried out by SDS-PAGE. The commercial characters viz., fecundity, larval weight, larval duration, single cocoon weight, single shell weight, shell ratio, filament length, denier and renditta were selected. They were subjected for statistical analysis to know the level of correlation between the biomolecules and commercial characters. The results clearly showed that haemolymph protein has positive correlation with selected commercial characters except larval duration and renditta. Also the midgut protein indicated positive correlation except fecundity, larval weight, shell ratio and renditta. The SDS-PAGE analysis also revealed the variations in protein profiles of the experimental sets.

Keywords: *Bombyx mori, haemolymph, midgut, protein, SDS-PAGE, commercial characters.*

INTRODUCTION

Mulberry silkworm, *Bombyx mori* L. is the most important insect being used for commercial production of silk in sericulture industry. To enhance the productivity and quality of silk fibers, many attempts are being made to improve the silkworm stocks through genetic manipulation. The conventional breeding programmes have contributed substantially by the introduction of improved silkworm breeds and more than 2000 races of silkworm are maintained in the germplasm banks of several countries [1]. In conventional breeding, the parental selection and performance prediction is on the basis of either their performance [2] or performance of the progeny [3-4]. However, the traditional breeding approaches have to overcome some limitations like actual genetic basis of yield improvement, polygenic inheritance *etc*. Recent advances in genetics and molecular biology have offered a number of alternative strategies to overcome the above limitations [5]. Silkworm breeders mainly concentrating on protein polymorphism in different races so as to provide insight into genetic variability between races to step up further hybridization programme to breed the best [6-7] reported that the protein polymorphism gives a clue on the heterosis expression for selected traits and can be used as an index in silkworm breeding. Thus, the study of polymorphic proteins of *Bombyx mori* is significantly important for selection and hybridization [8]. The correlation between yield and biochemical parameters [9], genetic variability for egg characters [10]; pupal size to fecundity and silk yield [11]; larval silk gland and shell weight [12] were reported. However, correlation studies combining biomolecules like proteins with commercial characters of silkworm *Bombyx mori* are rather scarce. Hence, the present investigation was undertaken.

MATERIALS AND METHODS

Four mulberry silkworm races *viz.,* Pure Mysore (PM), Nistari, NB_4D_2 & CSR_2 and two hybrid (Pure Mysore × CSR_2 and Nistari × NB_4D_2) silkworms were used for the present investigation. The silkworm rearing was conducted in the laboratory following the method described by Krishnaswamy [13, 14]. The economic traits selected for present study

included fecundity, weight of fifth instar larva, larval duration, single cocoon weight, single shell weight, shell ratio, filament length, denier and renditta. In each replication 500 larvae were kept after third moult.

The larvae from first day of fifth instar were collected daily with a regular interval of 24h till the end of fifth instar. The abdominal legs were punctured and haemolymph was collected in a pre cooled eppendorf tubes containing 1 mM thiourea [15], centrifuged at 3000 rpm for 5 minutes in a cooling centrifuge at 5°C and preserved in a deep freezer at -20°C as stock and it was used whenever required. The midgut tissue was obtained from five larvae of fifth instar by dissecting the larvae in ice cold water and the gut contents were removed. The tissue was thoroughly washed in distilled water. A 10 % (w/v) homogenate of the midgut tissue was prepared in pre cooled distilled water using mortar and pestle. The homogenate was centrifuged at 3000 rpm for 10 minutes in a cooling centrifuge at 5°C. The clear supernatant was used for the analysis proteins.

The total soluble protein present in the haemolymph as well as midgut tissue was estimated by following the method of Lowry *et al.* [16]. Bovine Serum Albumin was used as standard protein. The results were expressed as µg of protein/µl and µg of protein/mg for haemolymph and midgut tissue respectively.

The experimental data were statistically analyzed through SPSS by two way ANOVA [17], Scheffe's post hoc test [18] and linear regression analysis using the formula $Y = b X + a$ [19] wherever they were applicable.

The qualitative analysis of total protein was carried out according to Laemmli [20] by 12 % sodium dodesil sulphate poly acrylamide gel electrophoresis with slight modifications [15, 21]. A uniform quantity of protein (100 g) from each batch was loaded to each slot of the gel. Molecular weight markers from Genei were also used in a slot to compare the molecular weight of the proteins separated from that of the sample. After appropriate destaining, the gels were scanned, analyzed and photographed in a gel scanner (Vilber Laurmat Bioprofil image analysis system).

RESULTS

The summary of the studied commercial characters are presented in the table 1. From the table it is clear that the two bivoltine races are superior for productivity traits whereas multivoltines are superior for viability traits. The hybrids showed average values of their parents. The results of Two way ANOVA revealed that the variation in all commercial characters among the experimental batches are all significant at 0.1 % ($P<0.001$). The concentration of total protein in haemolymph and midgut tissue samples is shown in the tables 2 & 3 respectively. The concentration of total proteins showed significant increase in their levels at every 24 hours till the end of fifth instar. Same trend was observed in both the tissues of all the experimental batches.

Table 1: Mean values ± SD of nine commercial characters in six races of silkworm, *Bombyx mori*

Silkworm Races/ Breeds	Fecundity	Larval Weight (g)	Larval Duration (h)	Single Cocoon Weight (g)	Single Shell Weight (g)	Shell Ratio (%)	Filament Length (m)	Denier	Renditta
PURE MYSORE	467.22±10.96	2.01±0.06	660±10.39	1.02±0.75	0.12±0.01	12.57±0.49	426.44±19.83	1.77±0.09	11.77±0.82
NISTARI	485.11±5.30	2.83±0.06	564.88±10.01	1.14±0.71	0.15±0.01	13.41±0.87	435.66±17.21	1.78±0.07	13.26±0.24
CSR_2	509.10±16.58	4.07±0.05	578.88±6.45	1.81±0.47	0.43±0.01	24.02±0.18	1011.99±12.34	2.93±0.22	5.78±0.23
NB_4D_2	520.55±16.65	4.16±0.05	576.67±11.08	1.76±0.30	0.35±0.01	20.27±0.15	1020±29.96	2.48±0.06	8.34±0.47
PM× CSR_2	466.66±11.52	2.68±0.07	610±11.10	1.67±0.23	0.28±0.01	17.29±0.21	910±18.74	2.75±0.06	7.64±0.12
NISTARI X NB_4D_2	490.77±6.81	3.46±0.04	557±10.21	1.47±0.22	0.23±0.01	16.06±0.85	805.99±12.36	1.83±0.02	9.22±0.85
F	89.775	4210.79	853.92	3570.99	3898.36	1484.63	65.17	311.48	28230.47

Values are the mean± SD of Pre monsoon, Monsoon and post monsoon observations.
The variation between the races is statistically significant at 0.1 % ($P<0.001$).

The highest concentration haemolymph protein was observed in $PMXCSR_2$ (45.62 µg/µl was the average during fifth instar) followed by CSR_2 (44.03 µg/µl), Nistari X NB_4D_2 (43.28 µg/µl), NB_4D_2 (42.8 µg/µl), Nistari (39.93µg/µl) and Pure Mysore (36.85 µg/µl). The results of Two way ANOVA revealed that the variation among the experimental batches are all significant at 0.1 % ($P<0.001$). In midgut tissue, the highest concentration protein was observed in $PMXCSR_2$ (33.82 µg/µl) followed by Nistari X NB_4D_2 (29.99 µg/µl), Pure Mysore (23.99 µg/µl), CSR_2 (23.24 µg/µl), NB_4D_2 (21.64 µg/µl) and Nistari (20.39 µg/µl). The results of Two way ANOVA revealed that the variation among the experimental batches are all significant at 0.1 % ($P<0.001$). The results of regression analysis between haemolymph proteins and commercial characters are presented in figures 1-9. From the results of statistical analysis it is very clear that the haemolymph protein with cocoon weight ($R^2=0.784$), shell weight ($R^2=0.549$), filament length ($R^2=0.721$) and denier ($R^2=0.563$) ,larval weight ($R^2=0.345$), larval duration ($R^2=0.263$) and shell ratio ($R^2=0.469$) revealed strong positive relationships; where as, fecundity ($R^2=0.092$) and renditta (Y= -0.695X+38.6) showed weak and negative relationships respectively. In case of midgut tissue protein and commercial characters, the results are presented in figures 10-18. From the results of regression analysis it is clearly showed that the larval duration ($R^2=0.015$), single cocoon weight ($R^2=0.063$), filament length ($R^2=0.066$) and denier ($R^2=0.046$) showed weak positive relationships, whereas fecundity (Y= -2.11X+543), larval weight (Y= -0.037X+4.154), shell ratio (Y= -0.037X+18.22) and renditta (Y= -0.192X+14.23) showed strong negative relationship with midgut proteins. However, shell ratio ($R^2=0.000$) exhibited neutral status with midgut tissue proteins, as it indicates weak relationship.

A number of qualitative and quantitative variations in the protein bands were observed in haemolymph and midgut tissue proteins (figures 1-6). In the case multivoltines, the haemolymph protein profiles exhibited almost same pattern of banding pattern. However, in the case of Pure Mysore a protein band with 84.8 kDa showed almost same intensity during the entire 5th instar, whereas in Nistari same band is very paler. In the case of Nistari, two protein bands with 35.84 and 34.12 kDa appeared from 4th to 6th days only. Among the bivoltines, a protein band of 105.81 kDa in NB_4D_2 worms is paler during the entire 5th instar, whereas in the case of CSR_2 race same band is darker. Three protein bands with 44.21, 42.16 and 41.10 kDa are very paler in NB_4D_2 silkworms, but in the case of CSR_2 silkworms same bands are prominent. Also, two protein fractions of 26.17 and 25.46 kDa are present only in CSR_2 silkworms. In case of hybrids, a protein band of 59.22 kDa showed gradual decrease in its intensity as the age advances. Two protein bands of 81.43 and 66.94 kDa are prominent in Nistari X NB_4D_2 worms, whereas in the case of $PMXCSR_2$ silkworms same bands are paler in their intensities.

In the case of midgut protein profiles multivoltines there is no significant differences observed. In the case of bivoltines, two protein bands with 176.58 and 171.01 kDa showed gradual increase in their intensities as age advances, whereas same bands are paler in NB_4D_2 silkworms. Among the hybrids, three protein bands with 182.64, 179.48 and 148.96 kDa are paler in $PMXCSR_2$ silkworms when compared to Nistaru X NB_4D_2 silkworms.

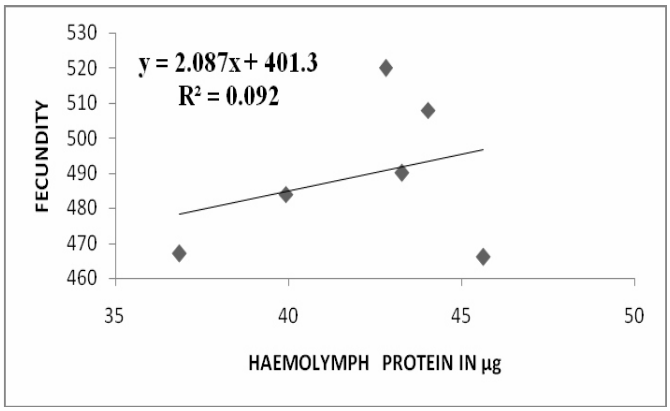

Figure 1: Correlation between haemolymph protein & fecundity

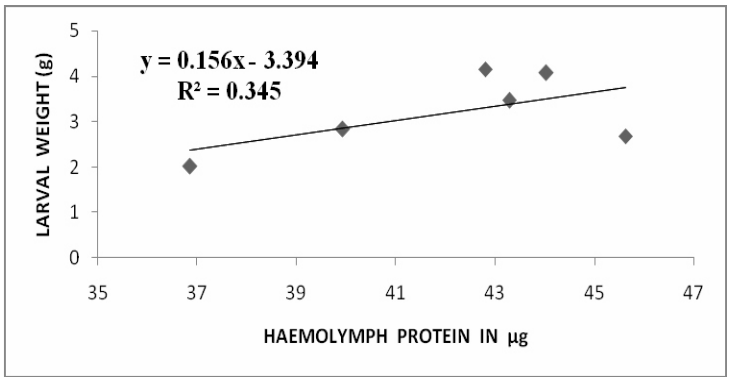

Figure 2: Correlation between amount of haemolymph protein & larval weight

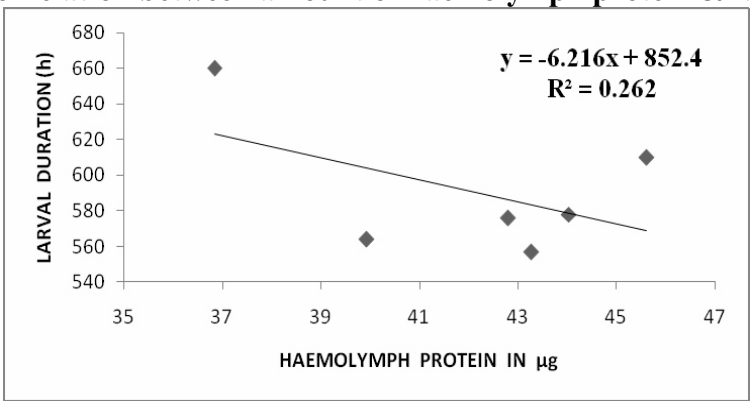

Figure 3: Correlation between amount of haemolymph protein & larval duration

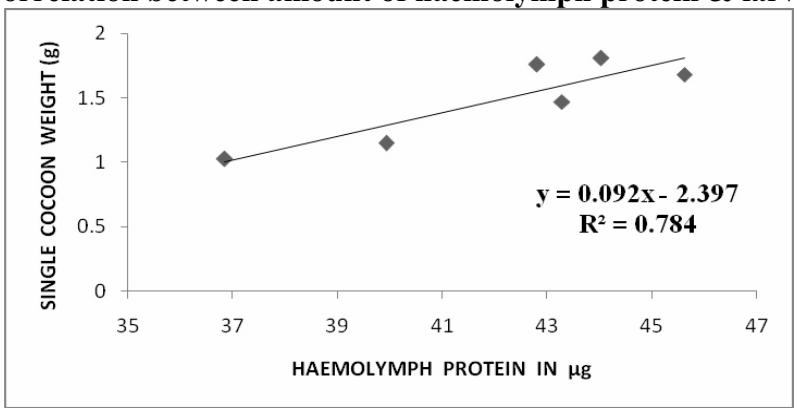

Figure 4: Correlation between amount of haemolymph protein & cocoon weight

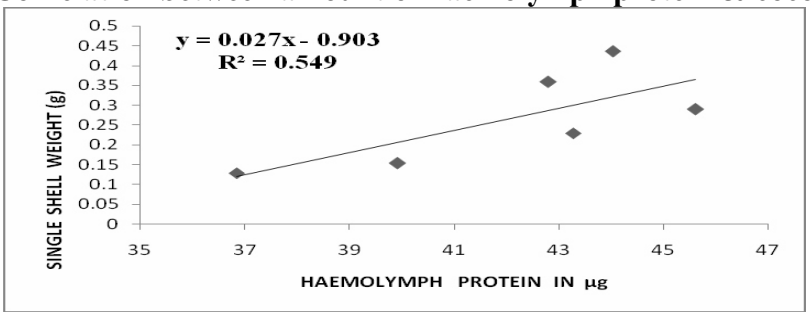

Figure 5: Correlation between amount of haemolymph protein & single shell weight

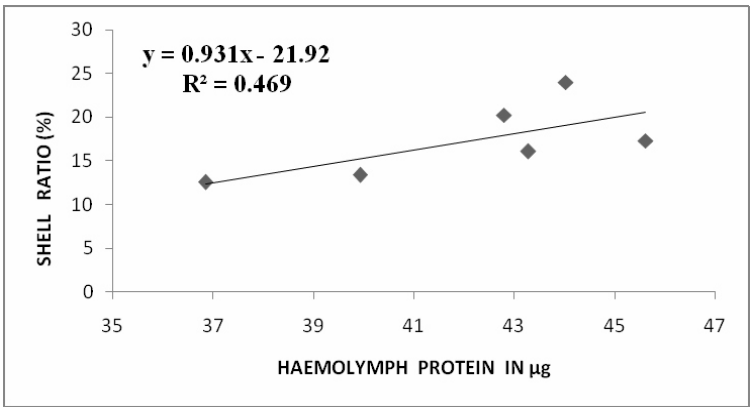

Figure 6: Correlation between amount of haemolymph protein & shell ratio

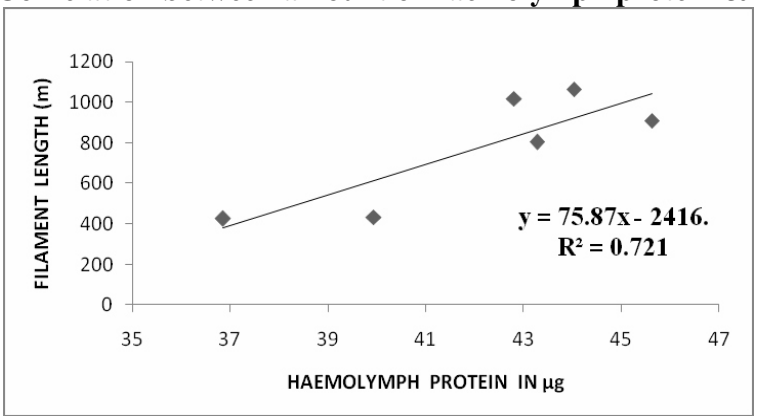

Figure 7: Correlation between amount of haemolymph protein & filament length

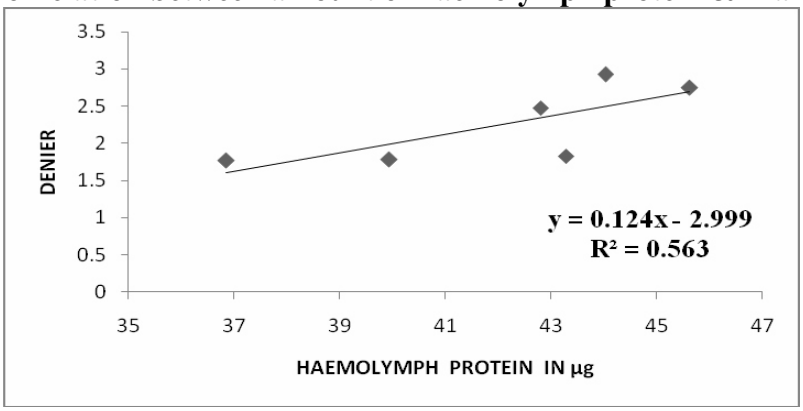

Figure 8: Correlation between amount of haemolymph protein & denier

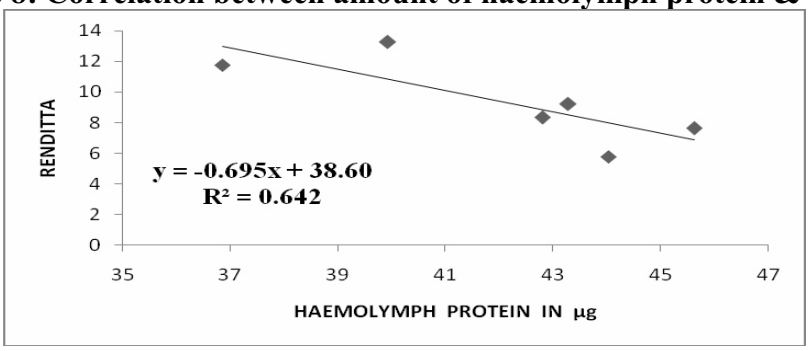

Figure 9: Correlation between amount of haemolymph protein & renditta

Table 2: Concentration of total proteins (µg/µl) in haemolymph during fifth instar development

Silkworm Races/ Breeds	1st Day	2nd Day	3rd Day	4th Day	5th Day	6th Day	7th Day	8th Day	AVERAGE
PM	21.86	27.20 (+24.42)	31.20 (+14.70)	33.86 (+8.52)	37.46 (+10.63)	43.20 (+15.32)	48.26 (+11.71)	52.00 (+7.74)	36.85
NISTARI	26.13	28.26 (+8.15)	32.00 (+13.23)	43.73 (+36.65)	53.60 (+22.57)	56.93 (+6.21)	-	-	39.93
CSR_2	24.53	32.00 (+30.45)	36.00 (+12.50)	46.66 (+29.61)	61.33 (+31.44)	64.00 (+4.35)	-	-	44.03
NB_4D_2	21.60	30.40 (+40.74)	34.13 (+12.26)	47.46 (+39.05)	60.00 (+26.42)	63.46 (+5.76)	-	-	42.80
PM× CSR_2	28.33	29.60 (+4.48)	32.80 (+10.81)	48.53 (+47.95)	54.40 (+12.09)	59.46 (+9.30)	66.40 (+11.67)	-	45.62
NISTARI× NB_4D_2	32.00	36.00 (+12.50)	42.40 (+17.77)	46.13 (+8.79)	47.20 (+2.31)	56.00 (+18.64)	-	-	43.28

The variation between the races is statistically significant at 0.1 % (P<0.001).
Values within parentheses represent per cent change over previous day.

Table 3: Concentration of total proteins (µg/mg) in midgut tissue during fifth instar development

Silkworm Races/ Breeds	1st Day	2nd Day	3rd Day	4th Day	5th Day	6th Day	7th Day	8th Day	AVERAGE
PM	13.60	16.80 (+23.52)	19.46 (+15.83)	22.66 (+16.44)	24.80 (+9.44)	26.93 (+8.58)	31.20 (+15.85)	36.53 (+17.08)	23.99
NISTARI	13.60	14.66 (+7.79)	18.13 (+23.66)	20.80 (+14.72)	24.26 (+16.63)	30.93 (+27.49)	-	-	20.39
CSR_2	15.20	16.80 (+10.52)	20.26 (+20.59)	24.26 (+19.74)	29.33 (+20.89)	33.60 (+14.55)	-	-	23.24
NB_4D_2	14.40	16.26 (+12.91)	18.93 (+16.42)	22.40 (+18.33)	25.86 (+15.44)	32.00 (+23.74)	-	-	21.64
PM× CSR_2	20.80	25.33 (+21.77)	30.40 (+20.01)	35.20 (+15.78)	39.70 (+12.78)	41.33 (+4.10)	44.00 (+6.46)	-	33.82
NISTARI× NB_4D_2	19.20	24.26 (+26.35)	28.26 (+16.48)	33.33 (+17.94)	35.20 (+5.61)	39.73 (+12.86)	-	-	29.99

The variation between the races is statistically significant at 0.1 % (P<0.001).
Values within parentheses represent per cent change over previous day.

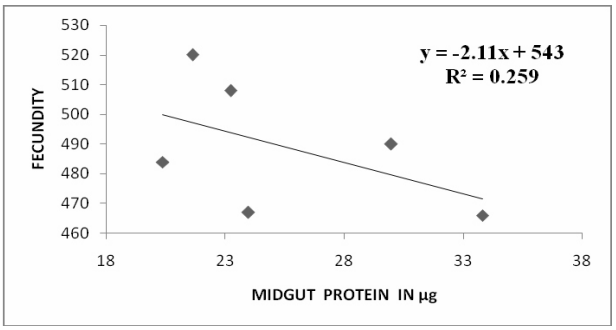

Figure 10: Correlation between midgut protein & fecundity

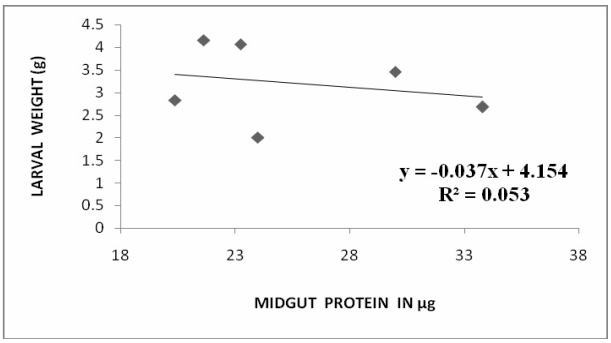

Figure 11: Correlation between midgut protein & larval weight

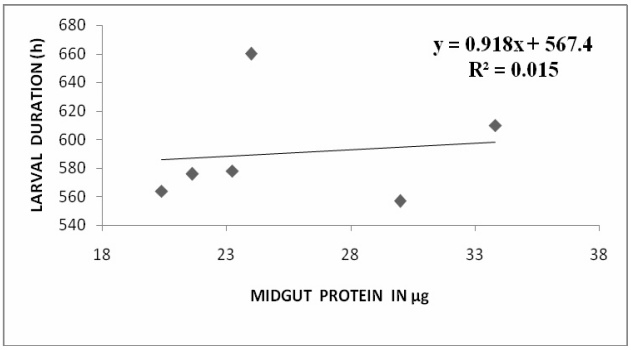

Figure 12: Correlation between midgut protein & larval duration

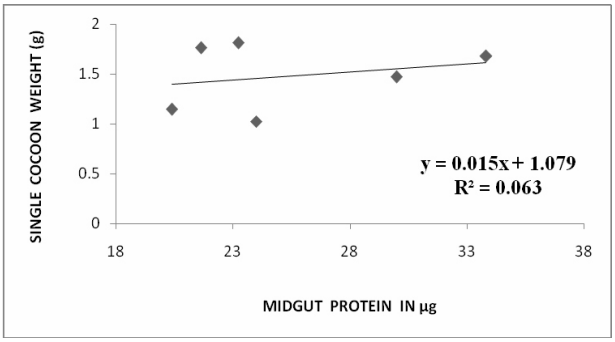

Figure 13: Correlation between midgut protein & cocoon weight

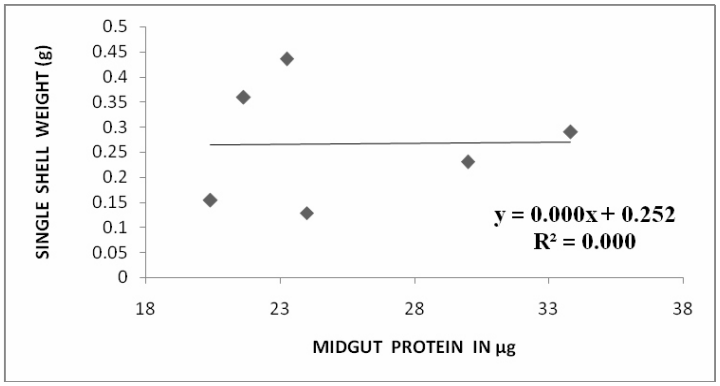

Figure 14: Correlation between midgut protein & shell weight

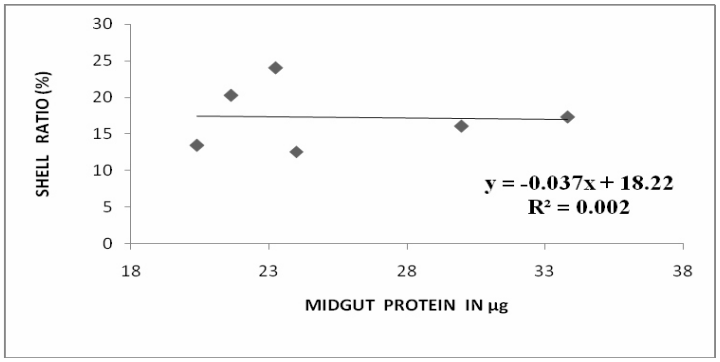

Figure 15: Correlation between midgut protein & shell ratio

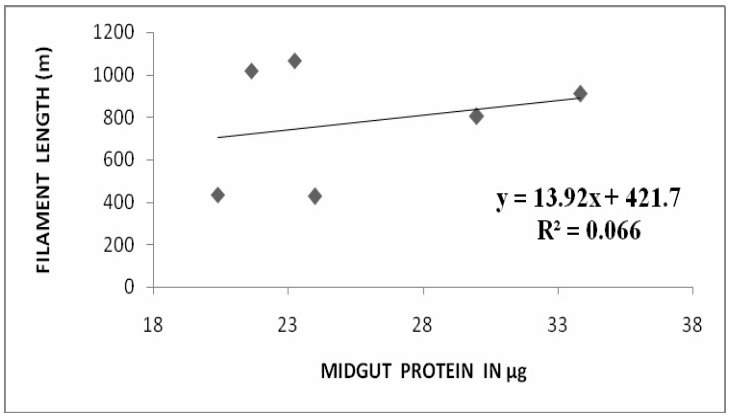

Figure 16: Correlation between midgut protein & filament length

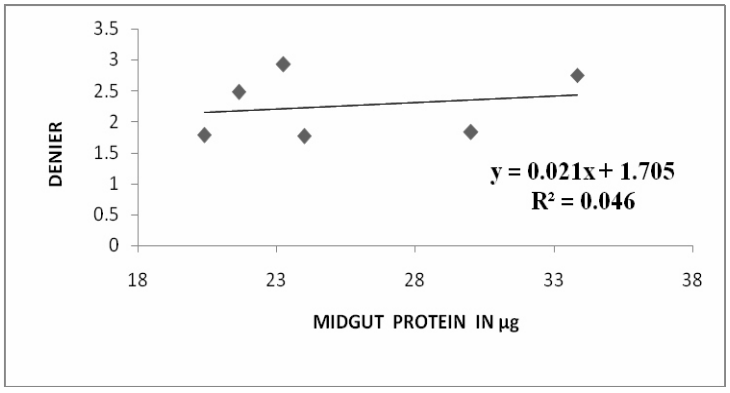

Figure 17: Correlation between midgut protein & denier

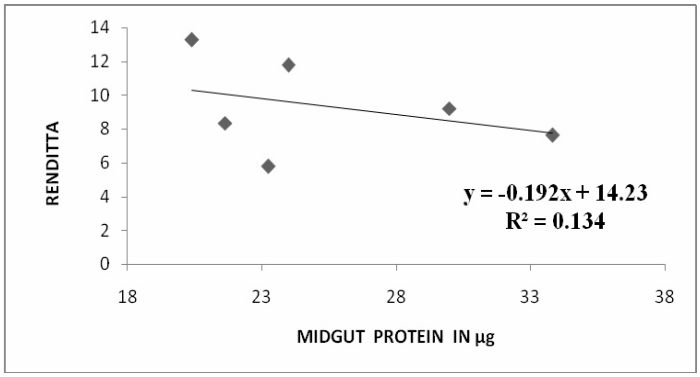

Figure 18: Correlation between midgut protein & renditta

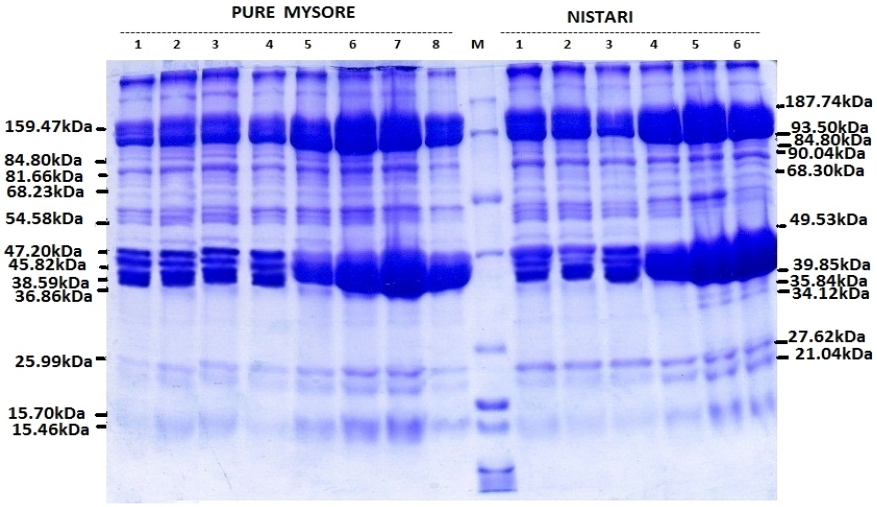

Figure 19: SDS-PAGE analysis of haemolymph proteins of Pure Mysore and Nistari silkworms. Lanes: 1-7 days in fifth instar. M- Molecular Weight Marker

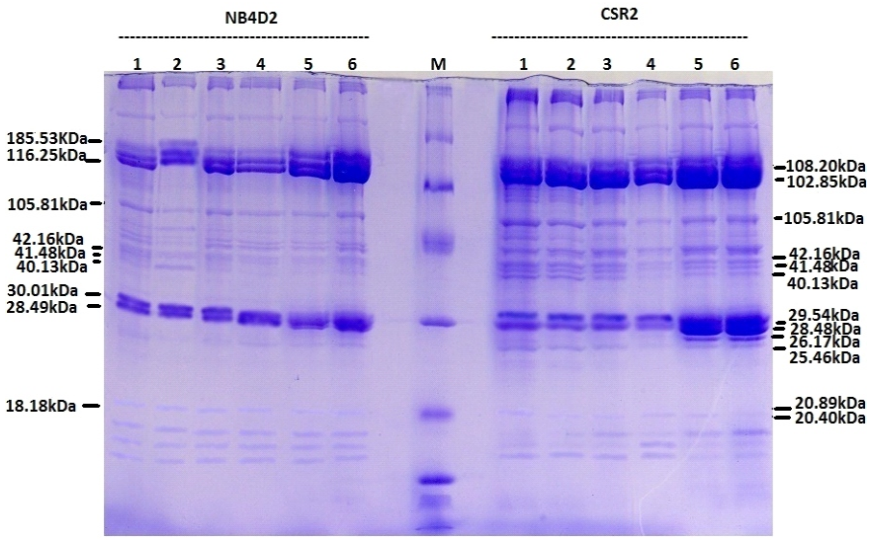

Figure 20: SDS-PAGE analysis of haemolymph proteins of NB_4D_2 and CSR_2 silkworms Lanes: 1-6 days in fifth instar. M- Molecular Weight Marker

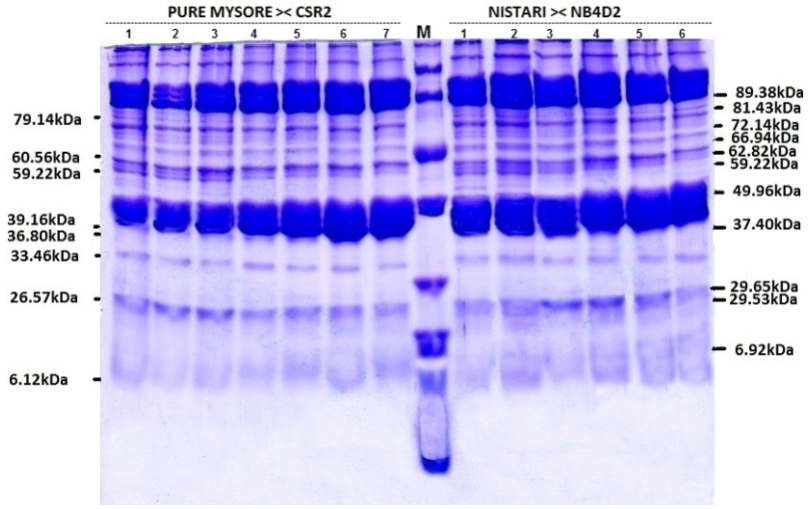

Figure 21: SDS-PAGE analysis of haemolymph proteins of Pure Mysore × CSR_2 and Nistari × NB_4D_2 silkworms.
Lanes: 1-7 days in fifth instar. M- Molecular Weight Marker

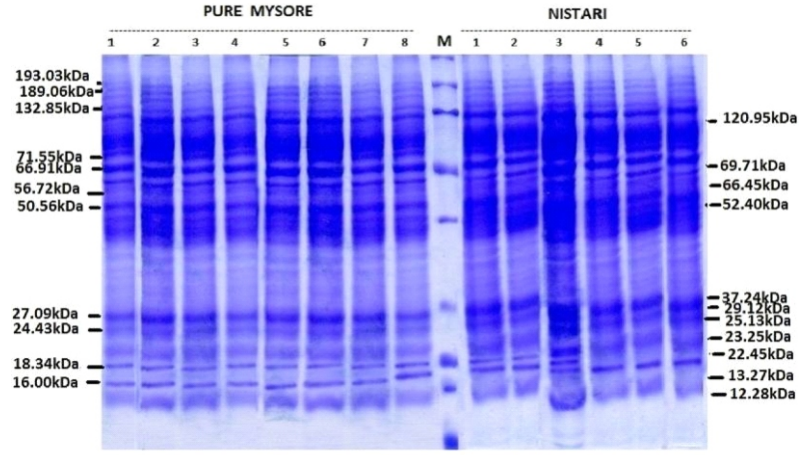

Figure 22: SDS-PAGE analysis of midgut proteins of Pure Mysore and Nistari silkworms
Lanes: 1-7 days in fifth instar. M- Molecular Weight Marker

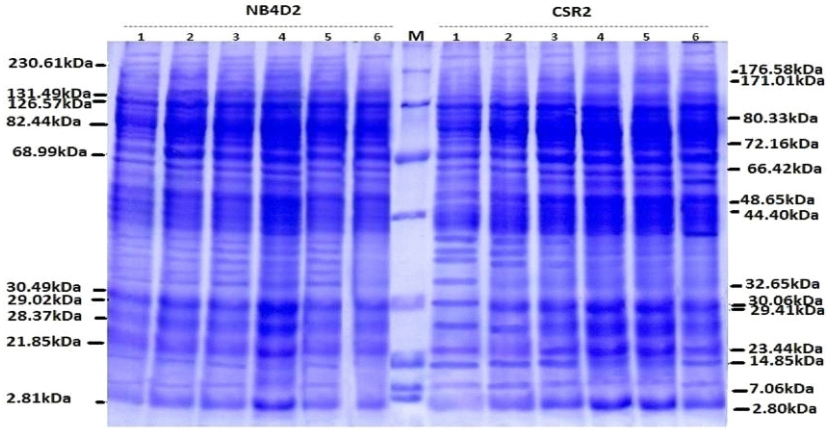

Figure 22: SDS-PAGE analysis of midgut proteins of NB_4D_2 and CSR_2 silkworms.
Lanes: 1-6 days in fifth instar. M- Molecular Weight Marker

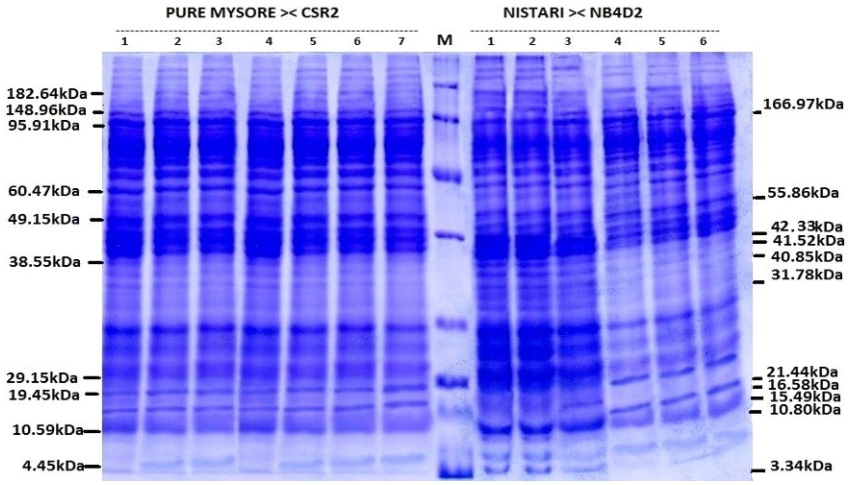

Figure 25: SDS-PAGE analysis of midgut proteins of Pure Mysore × CSR_2 and Nistari × NB_4D_2 silkworms.
Lanes: 1-7 days in fifth instar. M- Molecular Weight Marker.

DISCUSSION

Quantitative analysis of proteins clearly indicated that there is either positive or negative correlation between haemolymph and midgut proteins with commercial characters. The studies on proteins are of paramount importance in the growth and development of organisms. As the haemolymph composition of insects reflects the nature and degree of metabolism of the tissues bathed in this fluid, changes in the protein of the haemolymph may show the level of modification in the organism [15]. Also, as the silkworm alimentary canal plays an important role in digestion and assimilation of food, it is possible to have a clear picture of protein metabolism by studying haemolymph and midgut proteins. Saranghi [22], Nagata and Yashitake [23] in their investigations utilizing haemolymph of silkworm *Bombyx mori*, demonstrated that haemolymph proteins which functions as a specific transport media plays a vital role in the growth and development of larvae and it is variable among different breeds. Our present results also correlates with the results of the above workers, wherein the amount of soluble protein content in the haemolymph and midgut in the four pure races and two hybrids are differently expressed. The quantitative haemolymph proteins may be considered as a marker molecule for cocoon weight (R^2=0.784), shell weight (R^2=0.549), filament length (R^2=0.721) and denier (R^2=0.563) as their correlation coefficient is at higher side.

Observations on the protein fractions in fifth instar have revealed that the banding pattern differs between pure races, between hybrids and between pure races and hybrids. The qualitative analysis of proteins indicated five types of changes *viz.,* the intensity of the protein bands either more or less between the silkworm varieties. Besides, some of the protein fractions either present or absent. Some of the protein bands increased in their intensity only in some varieties. Presence or absence of proteins bands indicates either the non production or utilization or degradation of blood proteins [15] when the studies are restricted within a particular race/breed. However, when the studies are concentrated between the races it directly targets the genetic material as they are directly determined by the DNA. Therefore, by studying the silkworm protein with commercial characters, it is possible to have a clear picture of the correlation between them. An understanding of such correlations will help us to identify and exploit the marker molecule during the evolution of new races of silkworm *Bombyx mori.*

CONCLUSION

The present results clearly indicated that the total haemolymph protein has positive correlation with selected commercial characters except larval duration and renditta. Also, the midgut protein indicated positive correlation except fecundity, larval weight, shell ratio and renditta. Hence, by studying the silkworm proteins with commercial characters, it is possible to have a clear picture of the correlation between them. An understanding of such correlations will help us to identify and exploit the marker molecule during the evolution of new races of silkworm *Bombyx mori* with improved traits.

ACKNOWLEDGMENTS

Authors wish to thank University of Mysore for extending the facilities to carry out this work.

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Corresponding Author: Farshid Ghasemi Kasmaei, Department of Veterinary, Sahneh Branch, Islamic Azad University, Sahneh, Iran. E-mail: f641g@yahoo.com