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Research Article

A COMPARATIVE STUDY ON THE BIOCHEMICAL COMPOSITION OF HAEMOLYMPH OF THE FIFTH INSTAR LARVAE OF A MULTIVOLTINE BREED AND A BIVOLTINE BREED OF THE MULBERRY SILKWORM, *BOMBYX MORI* L.

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ABSTRACT

Changes in the composition of haemolymph reflect the physiological and biochemical transformations taking place in the insect tissues. In this work, the biochemical composition of haemolymph of the fifth instarlarvae of a multivoltine breed (L×CSR2) was compared with that of a bivoltine breed (CSR6×CSR12) of the mulberry silkworm, *Bombyx mori*. The larval period of the fifth instar larvae of both breeds lasted for 6 days. Increased trend of biochemical properties from the 1st day to the 6thday was noticed in both the breeds. The protein concentration of the fifth instar larvae of bivoltine breed was significantly lower than that of the multivoltine breed on all days. But the concentration of amino acid and carbohydrate were greater in the haemolymph of the bivoltine breed than that of the haemolymph of the multivoltine breed on all days. Lipid concentration was lower in the multivoltine breed than that of the bivoltine breed on the first four days whereas on the later days reverse trend was observed. The 't' values indicate that the differences in the biochemical parameters between the two breeds were significant on all days.

Keywords: Protein, Amino acids, Carbohydrate, Lipid.

INTRODUCTION

Insect haemolymph is a complex mixture of proteins, lipids, carbohydrates, amino acid, nucleic acids, hormones and their degradation products. It is primarily responsible for supplying nutrients, transferring metabolic wastes to maintain normal growth and development. It serves important roles in the immune system and in transport of hormones, nutrients, and metabolites. The silkworm has an open circulatory system containing haemolymph, which delivers nutrients and oxygen to all parts of the body. It is also an important repository for nutrition and energy. Major biomolecules such as proteins, carbohydrates and lipids play an important role in biochemical process underlying the growth and development of the silkworm (Ito and Horie, 1959). Haemolymph is the only extracellular fluid containing the products required for every physiological activity of the insect body. Thus changes in the composition of haemolymph reflect the physiological and biochemical transformations taking place in the insect tissues. Lauffer (1960) was the first ever observed haemolymph proteins in silkworm *Bombyx mori*. Haemolymph protein content increases throughout the fifth instar and reaches maximum at the end of the fifth instar in the silkworm races. Murthy *et al.* (2014) reported that the total proteins in the whole body rapidly increases from the 1st instar and reaches maximum at the end of the 4thinstar in multivoltine (Pure Mysore PM), crossbreed (PM×CSR2) and bivoltine (CSR2) breeds of the silkworm. Haemolymph proteins and carbohydrates rapidly increase from the 1st instar and reach maximum at the end of the 5th instar. Only a very little information pertaining to variations in the composition of the haemolymph of different races of silkworm is available. Thus a comparative analysis on changes in the major biochemical constituents such as carbohydrates, proteins, amino acids and lipids in the haemolymph of the fifth instar larvae of two breeds of the

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mulberry silkworm, *Bombyx mori* i.e. L×CSR2 and CSR6×CSR12 was carried out in this work.

MATERIALS AND METHODS

Silkworm breeds used for study

In the present study two races of the commercially exploited multivoltine crossbreedL×CSR2 and Bivoltine breed CSR6×CSR12 silkworm *B. mori* were selected. The eggs of this race were procured from Silkworm Rearing Department, Rayanur, Karur district. They were kept under lab conditions and allowed to hatch. The emerged first instar larvae were fed with young leaves of mulberry variety MR2. The larvae were acclimatized to the lab conditions by rearing them during the month of November, 2016 till fifth instar in the laboratory with the relative humidity of 80-90% and the temperature of 27-30˚C.

Haemolymph collection

Haemolymph was collected in a pre-chilled eppendorf tube containing a few crystals of thiourea by cutting the first proleg of fifth instar larva. Haemolymph was collected at an interval of 24h for six days from day one. Haemolymph was centrifuged at 3000 rpm for 3 min and supernatant was used for estimation of total haemolymph ptoteins, amino acids, carbohydrates and lipids.

Estimation of Protein, Carbohydrate, Amino acid and Lipid

Total haemolymph protein was estimated according to Lowry *et al.* (1951) using Bovine Serum Albumin (BSA) as standard. The quantitative estimation of total carbohydrate in haemolymph was done by anthrone method of Dubois *et al.* (1956) using glucose as standard. Total haemolymph amino acid was estimated by Ninhydrin method of Moore and Stein, (1954) using aspartic acid and leucine as standard. Total haemolymph lipids was estimated by the method of Zoellner and Kirsch (1962) using cholesterol as standard. The results were statistically analyzed and discussed.

RESULTS AND DISCUSSION

Haemolymph brings about functional homeostasis of insect organs by transporting chemical substances into and out of the cells of the tissues and thus serves as a medium of chemical communication of distant and distinct organ systems of insect body. It serves as the transport milieu for the exchange of essential materials between cells, tissues and organs (Mullins, 1985; Karpells *et al.,* 1990). Transport of biochemicals from the different tissues may be required to meet the higher physiological activities in silkworm such as increased body growth and cocoon formation. So the biochemical parameters such as, proteins, amino acids, carbohydrates, lipids, nucleic acids etc., vary significantly during the life cycle of all living organisms. The quantitative variation of these biomolecules in insects during growth and metamorphosis have been reported by Nagata and Yashitake (1989). Similarly quantitative variations in the concentration of protein, amino acids and carbohydrates of the haemolymph have been observed on all days of the fifth instar larvae of both breeds of the mulberry silkworm in the present study (Tables 1, 2, 3 & 4). The results of the present work were in line with the report of Yogananda Murthy (2015) that is the bivoltine breed was superior to multivoltine with respect to amino acid and carbohydrate contents of the haemolymph. But higher content of protein was noticed on all days in the haemolyph of the multivoltine breed compared to that of the bivoltine breed. Quantity of lipid was greater in the haemolyph of the bivoltine than that of the multivoltine breed till 4^{th} day and this condition reversed on the 5^{th} and 6^{th} days. 't' values of all parameters on all days were significant at $p < 0.05$. This indicated that the differences between the biochemical compositions of haemolymph of two breeds were greater and significant. Such significant differences between the two breeds might be correlated with the different in the inherent utilization ability. The utilization capacity of bivoltine must be greater as the haemolymph protein concentration in bivoltine was always lower than that of the multivoltine larvae.

Proteins

In this study, the larval period of the fifth instar of the larvae of both breeds lasted for 6 days. In multivoltine, protein content of haemolymph was found to be increasing during the larval period of the fifth instar from day one to the sixth day. On the first day, haemolymph contained 167.5 mg/ml of protein. It increased to 205.6 mg/ml on the sixth day. In bivoltine breed also, an increase in protein was observed from 63.6 mg/ ml on the first day to 149.8 mg/ml on the last day. Comparatively, the protein concentration of the haemolymph of the fifth instar larvae of bivoltine breed was significantly lower than that of the multivoltine breed on all days.

Proteins play an important role in the growth and development of *B. mori* and synthesis of silk proteins in silk gland during larval development (Seo *et al.,* 1985). During the larval development high molecular weight proteins are synthesized in large amount by the larval fat body and secreted into the haemolymph. Such proteins are known as major larval haemolymph proteins or storage proteins. Synthesis of such proteins is dependent on the nutritional status of silkworm during the larval development (Ramesh Babu *et al.,* 2009) and environmental conditions (Benchamin and Anantharaman, 1990; Ramesha *et al.,* 2010). Proteins are important for the development, metamorphosis and to maintain a number of physiological functions (Murthy *et al.,* 2014).Variations in haemolymph proteins reflect the balance between the synthesis, storage, transport and degradation of structural and functional proteins during ontogeny as well as response to particular ecological and physiological conditions (Florkin and Jeuniaux, 1974).

Haemolymph proteins undergo radical changes both in quality and quantity during development. In the present study the concentration of protein in haemolymph

increased progressively during the larval development and reached maximum in the late fifth instar larvae of both races as recorded by Lauffer (1960) in three races, multivoltine (Pure Mysore PM), crossbreed (PM×CSR2) and bivoltine (CSR2). It was found that during the development of the fifth instar larvae of *Samiaricini*, there is a rapid increase in the haemolymph protein concentration which attains its peak at the end of larval life irrespective of the season. Similar observations have been recorded where protein concentration was found to be higher at the end of larval stage in the silkworm (Ito and Arai, 1963; Banno *et al.*, 1993; Murthy *et al.*, 2014). The increase in feeding and growth as reported by Hurlimann and Chen, (1974) and active secretion of proteins and carbohydrate by other tissues like fat bodies as proved by Nagata and Kobayashi, (1990) and Chen (1978) could be cited as reasons. The increasing trend was to meet the higher requirement of these macromolecules during metamorphosis. High concentration of haemolymph proteins could be correlated with high consumption of mulberry leaves and subsequently higher rate of conversion and their accumulation in haemolymph (Banno *et al.*, 1993; Aruga, 1994).

In the present study, a steady increase in the haemolymph protein concentration during the development of last larval instar indicates the higher utilization of dietary proteins. The increase in protein could be attributed to the regular feeding, initiation of silk protein synthesis in silk gland at end of the fifth instar and the development of reproductive organs, and the increased rate of metabolism (Sinha and Sinha, 1994). Such increased proteins could be the compensatory replacement of the proteins which are utilized for the formation of puparium (Malik and Malik, 2009). The most abundant proteins in larval haemolymph belongs to a class known as storage or hexamerins which are synthesized by the fat body and reach extremely high concentrations in the last instar (Sumino *et al.*, 1980; Kanost *et al.*, 1990). These results were in confirmation with the earlier works of Satish (1998) that, haemolymph protein in sericigenous insects is responsible for the formation of silk proteins in silk glands. High protein concentration in fifth instar larval was an indication of a greater metabolic activity and immune response. The haemolymph of the multivoltine breed was found to have higher protein level than that of the bivoltine breed and thus it was considered as a better strain in terms of cocoon weight and shell weight as reported by Chakravorty and Neog (2006). On contrary Banno *et al.* (1993) reported that the bivoltine showed a higher protein content in the haemolymph followed by cross breed and multivoltine during fifth instar. High protein concentration is an indication of greater metabolic activity. Synthesis and utilization of haemolymph proteins are conditioned by genetic and hormonal control (Hurliman and Chen, 1974).

Amino acids

The quantity of amino acid ranged from 44.0 mg/ml on the first day to 140.0 mg/ml on the sixth day in the multivoltine breed. In Bivoltine breed (CSR6×CSR12), fifth instar larvae recorded an increase in the amino acid level from 122.9 mg/ml on the first day to 176.6 mg / ml on the first day to 149.8mg/ ml on the last day. Though the increasing trend was noticed in the amino acid content of the haemolymph of both breeds, the increase was only gradual in the bivoltine breed whereas it was highly significant in the multivoltine breed. Amino acids are the essential components of all living cells. They are the building blocks of proteins. Many insects including the silkworm are known to contain usually large amount of free amino acids (Ramsay, 1958). Amino acids are essential for growth and survival of the silkworm. Shimura (1978) reported that, haemolymph acts an amino acid reservoir between midgut and silk gland and supplies amino acids to silk gland for silk synthesis. The amino acid content of the haemolymph was found to be gradually increasing from the first day to last day. Highest level of amino acid was recorded on the fifth day and the increase in amino acid could be attributed to the initiation of protein balance in the silkworm at end of the fifth instar and the proteolysis. The amino acid level of bivoltine race is higher than the multivoltine race silkworm *B. mori*. In the present investigation variations in the amino acid levels of two breeds were the reflections of the variations in the protein metabolism of two breeds which are ultimately the result of genetic variations. The total free amino acid amino acid levels declined from the early-fourth instar to the mid-fifth instar and were elevated during the late-fifth instar in the metamorphosing silkworm, *B. mori* (Sivaprasad and Muralimohan, 1990). The differences in the amino acid composition between two breeds reflected in the quality of silk they produced. Such difference was ultimately due to genetic variation.

Carbohydrates

The quantity of carbohydrate ranged from 8.2 mg/ml on the first day to 35.0 mg/ml on the sixth day in the multivoltine breed. In Bivoltine breed (CSR6×CSR12), fifth instar larvae recorded an increase in the carbohydrate level from 34.1mg/ml on the first day to 117.0 mg/ ml on the last day. The percentage of increase in carbohydrate was not uniform in multivoltine breed whereas the increase in bivoltine breed was high and gradual till the fourth day and then the increase was very low on later days (6.35 and 12.7%). Carbohydrates also play an important role as energy source and protecting silkworm during adverse condition. The late age silkworm larvae accumulate higher carbohydrates compared to young age worms. Simex and Kodrik (1986) have reported that the free carbohydrate in the haemolymph changed significantly during last larval instar of the silkworms. The level of carbohydrates during larval development reveals the degree of utilization of carbohydrates, which are the major sources of energy in the body, for growth and development of the larva that might ultimately determine the difference in the quality and quantity of silk production. The carbohydrate content of the haemolymph was found to be gradually increasing from the first day to last day. The total sugar content in the haemolymph increased constantly from the first to sixth day in bivoltine race. Glycogen and trehalose are main

constituents of haemolymph and they play an important role during growth, metamorphosis and diapause (Jo and Kim, 2001). It has been reported that high concentration of carbohydrates in hemolymph are maintained during larval development as energy reserve to be utilized later during metamorphosis, pupal and adult stage (Simex and Kodrik, 1986; Mishra *et al.,* 2010). Higher carbohydrate in the bivoltine breed revealed that it had the higher capacity accumulate carbohydrates than the multivoltine breed. Carbohydrates increased with the advancement of age of larva and reached at its peak on the last day of fifth instar larva of both breeds. Similar observation has been recorded by Simex and Kodrik, (1986), Mishra *et al.* (2010) and Murthy *et al.*(2014). Higher concentration of carbohydrates in haemolymph of later stages of development could be utilized for the energy required for the formation of cocoon, pupal and adult cuticle and other developmental processes (Simex and Kodrik, 1986; Misra *et al.,* 2010; Chippandale, 1978).

Lipids

The quantity of lipids ranged from 6.66 mg/ml on the first day of the fifth instar to 44.44 mg/ml on the sixth day in the multivoltine breed. In Bivoltine breed (CSR6×CSR12), fifth instar larvae recorded an increase in the lipid level from 30.24 mg/ml on the first day to 35.57 mg/ml on the last day. Gradual increase was noticed in the carbohydrate content of bivoltine breed whereas about 133% and 60% increase was noticed on the second and fifth day respectively of the multivoltine breed. The total lipid respectively of the multivoltine breed. The total lipidcontent was found to be increasing as the day advanced in the haemolymph of fifth instar larvae of both breeds of the silkworm. However lower level of lipids was observed in multivoltine variety than the bivoltine variety on the first four days and later reverse trend was observed on the 5th and 6th days. There was a significant difference between the two breeds in the lipid level of haemolymph on all days. Lipids are used as a source of energy required for growth and metamorphosis of insects. The lower level of lipid in the multivoltine breed might be due to the over utilization of lipid and fatty acids for growth, moulting and metamorphosis (Mallikarjuna *et al.,* 2016). Mobilization of lipid to the fat body might also be and increased lipase activity the reasons for low level of lipids in multivoltine breed (Streit, 1978).

The 't' values indicate that the differences in the biochemical parameters between the two breeds were significant on all days. The differences between the multivoltine and bivoltine breeds of silkworm in terms of major biochemical composition in the haemolymph might be attributed to the differences in feeding efficiency, utilization efficiency, conversion efficiency, hormones level, metabolic rates etc. Though many physiological reasons were cited, the differences are breed specific and their different adaptability are attributed to the genetic characteristics gained from their parental stock. Thus biochemical profile of haemolymph can be used as an index to screen germplasm stock for developing a breed with higher survival rate.

Table 1. Quantity of Protein in the haemolymph of the fifth instar larvae of a multivoltine breed (L×CSR2) and a bivoltine breed (CSR6×CSR12) of *Bombyx mori*.

Days	Multivoltine L×CSR2 (mg/ml)	Bivoltine CSR6×CSR12 (mg/ml)	't'- test value Level of Significance at $p < 0.05$
1st day	167.57±1.67	63.71± 1.27	110.18 Significant
2nd day	171.02 ±1.57 (2.0%)	66.29 ±1.29 (4.0%)	114.87 Significant
3rd day	180.59 ±1.14 (5.5%)	82.75 ± 1.10 (24.9%)	137.30 Significant
4th day	189.14 ±1.01 (4.7%)	105.19 ± 1.0 (27.0%)	131.45 Significant
5th day	199.11 ±1.21 (5.2%)	121. 60 ±0.89 (15.6%)	115.44 Significant
6th day	205.64 ±1.29 (3.2%)	149.84 ±1.82 (23.1%)	55.86 Significant

Values inside the parentheses indicate the percentage of increase in biochemical content over the previous day.

Table 2. Quantity of Amino acids in the haemolymph of the fifth instar larvae of a multivoltine breed (L×CSR2) and a bivoltine breed (CSR6×CSR12) of *Bombyx mori*.

Days	Multivoltine L×CSR2 (mg / ml)	Bivoltine CSR6×CSR12 (mg / ml)	't'- test value Level of Significance at $p < 0.05$
1st day	44 ±1.22	122.90 ±1.54	-89.33 Significant
2nd day	80 ±1.58 (81.8%)	127.27 ±1.63 (3.4%)	-46.53 Significant
3rd day	94 ±1.58 (17.5%)	135.63 ± 1.74 (6.6%)	-38.75 Significant
4th day	110 ±1.58 (17.0%)	157.09 ± 0.88 (11.2%)	-58.12 Significant
5th day	122 ±1.58 (10.9%)	172.90 ±3.21 (14.5%)	-31.68 Significant
6th day	140 ±1.58 (14.7%)	176.67 ±1.74 (2.1%)	-34.82 Significant

Values inside the parentheses indicate the percentage of increase in biochemical content over the previous day.

Table 3. Quantity of Carbohydrate in the haemolymph of the fifth instar larvae of a multivoltine breed (L×CSR2) and a bivoltine breed (CSR6×CSR12) of *Bomby xmori*.

Days	Multivoltine L×CSR2 (mg / ml)	Bivoltine CSR6×CSR12 (mg / ml)	't'- test value Level of Significance at $p < 0.05$
1st day	8.21±0.54	34.17± 0.64	-66.04 Significant
2nd day	11.78±0.73 (42.6%)	47.01±1.58 (37.8%)	-44.99 Significant
3rd day	13.57±1.47 (15.3%)	65.22± 2.28 (38.72%)	-42.39 Significant
4th day	20.71±0.72 (53.3%)	97.61± 1.56 (49.6%)	-99.81 Significant
5th day	22.50±1.17 (8.6%)	103.88±1.60 (6.35%)	-91.31 Significant
6th day	35±1.58 (55.5%)	117.01±1.22 (12.7%)	-91.67 Significant

Values inside the parentheses indicate the percentage of increase in biochemical content over the previous day.

Table 4. Quantity of Lipids in the haemolymph of the fifth instar larvae of a multivoltine breed (L×CSR2) and a bivoltine breed (CSR6×CSR12) of *Bombyxmori*.

	Multivoltine	Bivoltine	't'- test value
1st day	6.66 ± 0.47	30.24 ± 0.90	-51.45 Significant
2nd day	15.55 ± 0.15 (133.4%)	31.57 ± 0.72 (4.3%)	-48.04 Significant
3rd day	17.77 ± 0.10 (14.2%)	32.09 ± 0.52 (1.6%)	-59.79 Significant
4th day	22.22 ± 1.10 (25.0%)	32.24 ± 0.81 (0.46%)	-16.34 Significant
5th day	35.55 ± 0.67 (59.9%)	33.19 ± 1.07 (2.9%)	4.15 Significant
6th day	44.44 ± 0.67 (25.0%)	35.57 ± 0.85 (7.17%)	15.26 Significant

Values inside the parentheses indicate the percentage of increase in biochemical content over the previous day.

CONCLUSIONS

Quantitative variations in the concentration of protein, amino acids and carbohydrates in the haemolymph have been observed on all days of the fifth instar larvae of both breeds of the mulberry silkworm. The concentration of protein in haemolymph increased progressively during the larval development and reached maximum in the late fifth instar larvae of both races. The multivoltine breed was found to have higher protein level in the haemolymph of the fifth instar. The concentration amino acid and carbohydrate were greater in the haemolymph of the bivoltine breed than that of the haemolymph of the multivoltine breed on all days. Lipid concentration was lower in the multivoltine breed than that of the bivoltine breed on the first four days whereas on the later days reverse trend was observed. The differences between the multivoltine and bivoltine breeds of silkworm in terms of major biochemical composition in the haemolymph are breed specific and are attributed to their genetic characters.

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