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Research Article

Biochemical Studies Of Amino Acid Content In The Haemolymph, Fat Body And Silk Gland Of Iv And V Instars Of Bombyx Mori Larvae Fed With Control And Morus Indica Copper Nanoparticles (Micunps) Treated V-1 Mulberry Leaves

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ABSTRACT
Generally, silkworm *Bombyx mori* L. is monophagous in character and feeds solely on mulberry leaves. Silkworm larva obtains different amino acids from the mulberry leaves and uses them to synthesize silk proteins secreted during spinning. The present study was carried out to analyze the variations in the biochemical constituents of amino acid in different body tissues viz., haemolymph, silk gland and fat body in the healthy and bacterial infect fouth and fifth instar larvae of the *Bombyx mori* silkworm. The feed efficacy of mulberry leaves such as V-1 and leaves treated with Copper nanoparticles on the amino acid contents in the haemolymph, fat body and silk gland of silkworm, *Bombyx mori* are fragmentary. The *Bombyx mori* larvae fed with different concentrations (25%, 50%, 75% and 100%) of Copper nanoparticles treated V-1 Mulberry leaves. The 25% is effective's dose of silk worm. Hence, it has been programmed in the present study to find out the feed efficacy of mulberry leaf variety (V-1) and leaves treated with Copper nanoparticles on amino acid content in the silkworm *Bombyx mori*. It may be concluded that V-1 leaf variety treated with copper nanoparticles elicits some favourable responses in silkworms with regard to the commercial improvement as evidenced from the results of the present study.

Key words: *Bombyx mori*, Copper nanoparticles, V-1 leaf variety

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1. INTRODUCTION

Nutrition is the single most factors that influence the growth, biochemical constituents and development of *B. mori. Ganoderma lucidum* known as medicinal mushroom and traditional Chinese medicine, which has been used for the prevention and treatment of many disorders. The fortify mulberry leaves with *G. lucidum* so as to improve the mulberry leaf quality and feed efficiency of silkworm, which in turn help to increase silk quality. In the present study different concentrations of *G.lucidium* extract were used on the growth and biochemical constituent such as carbohydrates, lipids and protein of the silk glands, fat body, muscles and haemolymph of *B.mori* were enhanced (Dr.C. Dyana Selin, *et al.*, 2021).

The silkworm larvae feed only on mulberry leaves; all the amino acids required for fibroin and sericin and body proteins of silkworm are derived from mulberry leaf proteins. Necessary amino acids are derived from the amino acids present in body fluid in a free state and from the amino acids formed in the posterior silk gland cells. Silk worm requires all the ten essential amino acids for growth and development (Ito, 1978).

Silk worm requires all the ten essential amino acids for growth and development of *Bombyx mori* L. *Bombyx mori* (race: nistari) is a resistant variety of multivoltine mulberry silkworm in the Northern Belt of India (Ullal SR, Narasimhanna MN, 1981). The ultimate aim of developing sericulture industry is heavy production of standard quality of raw silk which can sustain in the world market. Being an animal protein the synthesis of silk in the world market (Machida J 1926). As animal protein the synthesis of silk in the fat body of *Bombyx mori* is directly influenced by the amino acids content in the haemolymph and silk gland (Barsagade DD). Silkworm faces a wide range of temperature variation which may bring certain changes in the biochemical constituent like change in lipid level with the change in temperature (Bheemanna C, 1964) and the growth and survival of organisms (Bheemanna C, *et al.*, 1990).

In this study, we observed larval body weight, length and weight of the silk gland, weights of pupae and shells, total hemocyte count, total hemolymph protein concentration in larvae, total lipid -amylase activity in the gut of eri-silkworms reared on cassavaαconcentration in pupae, and leaves or Silkmate L4M. Understanding the nutritional physiology of eri-silkworms when reared on an artificial diet and determining the factors affecting its life history could help in planning effective rearing options for this insect (Jatuporn Tungjitwitayakul and Nujira Tatun, 2017). The aim of this study was to investigate the mechanisms responsible for the poor development and low silk protein synthesis efficiency of silkworms fed artificial diets. After multigenerational selection of the ingestive behavior of silkworms to artificial diets, we obtained two strains, one of which developed well and another in which almost all its larvae starved to death on the artificial diets. Subsequently, we analyzed the metabolomics of larval haemolymph by gas chromatography/liquid chromatography–mass spectrometry, and the results showed that vitamins were in critically short supply, whereas the nitrogen metabolic end product of urea and uric acid were enriched substantially, in the haemolymph of the silkworms reared on the artificial diets. Mean while, amino acid metabolic disorders, as well as down regulation of carbohydrate metabolism, energy metabolism, and lipid metabolism, co-occurred. Furthermore, 10 male-dominant metabolites and 27 diet-related metabolites that differed between male and female silkworms were identified. These findings provide important insights into the regulation of silkworm metabolism and silk protein synthesis when silkworms adapt to an artificial diet (Hui-Ling Dong *et al.*, 2017). Changes in the levels of total protein, free amino acids, carbohydrate and lipids in haemolymph, midgut and silk gland of the silkworm, *Bombyx mori* reared on Goshoerami. Ichinose and Kokksu mulberry varieties were investigated during three different seasons. The mean total protein levels were found to be significantly higher in the haemolymph followed by silk gland and midgut, whereas the total free amino acid levels were significantly higher in silk gland and haemolymph than midgut. Among the varieties, there was a significant increase in the mean protein and free amino acid levels of the larvae fed leaves of Goshoerami followed by Ichinose and Kokuso. Similarly, the carbohydrate and lipid levels also showed a significant increase in all the tissues when they were fed leaves of Goshoerami and Ichinose. The increase in the biochemical constituents in the tissues fed with Goshoerami and Ichinose could be attributed to their relatively higher levels of biochemical constituents (Awquib Sabhat, M. A. *et al.*, 2010).

Silkworm (*Bombyx mori*) is a lepidopteran-holometabolic model organism. To understand its developmental biochemistry, we characterized the larval hemolymph metabonome from the third instar to prepupa stage using 1H NMR spectroscopy whilst hemolymph fatty acid composition using GC-FID/MS. We unambiguously assigned more than 60 metabolites, among which tyrosineo-β-glucuronide, mesaconate, homocarnosine, and picolinate were reported for the first time from the silkworm hemolymph. Phosphorylcholine was the most abundant metabolite in all developmental stages with exception for the periods before the third and fourth molting. We also found obvious developmental dependence for the hemolymph metabonome involving multiple pathways including protein biosyntheses, glycolysis, TCA cycle, the metabolisms of choline amino acids, fatty acids, purines, and pyrimidines. Most hemolymph amino acids had two elevations during the feeding period of the fourth instar and prepupa stage. Trehalose was the major blood sugar before day 8 of the fifth instar, whereas glucose became the major blood sugar after spinning. C16:0, C18:0 and its unsaturated forms were dominant fatty acids in hemolymph (Lihong Zhou *et al.*, 2015).

Shimura, (1978) reported that, haemolymph acts an amino acid reservoir between midgut and silk gland, supplied amino acids to silk gland for silk synthesis. Lauffer, (1960) was the first ever observed haemolymph proteins in Silkworm *B. mori* L. Haemolymph protein content increases throughout the 5th instar and reaches the maximum level at the end of 5th instar in all the three silkworm races examined. Nagata and Kobayashi, (1990) reported a quantitative change in storage proteins during silkworm larval development. Silkworms depend on the biomolecules viz., protein, carbohydrate, lipid etc. for the growth and development. During the larval feeding stages, energy reserves are accumulated in the fat body, later to be utilized for their growth and sequence of metamorphic events as well as to provide energy for the developing adult moth to drive copulation

and egg laying (Sivaprasad and Bhuvaneswari, 2018). In the life cycle of silkworm, the fifth larval instar is considered as the silk synthesizing phase, where the silk gland grows tremendously and the insects prepare its body with the reserved metabolic compounds for its future metamorphic events. The amount of nutrients stored in the form of biochemical reserve during the larval stages accommodates stage specific morphogenetic changes as well as pupal and adult metamorphic events due to its non-feeding nature. The health status of the larva determines the spinning behaviour, size of the cocoon, survivability of pupa and moth. If the larvae are exposed to unfavourable condition, it results in non-spinning or partial spinning and even if spun, the cocoon size will be smaller. In such conditions, silkworms adopt for survival rather spinning cocoon by depending on accumulated reserves, such as carbohydrates and amino acids. Hence, the present study enlightens the comparative analysis of the alteration of a few biochemical events associated with the energy metabolism of healthy spinning and unspun larvae (E. Bhuvaneswari, *et al.,* 2022).

Silkworm eats only mulberry leaves to make its cocoon, producing the silk. Mulberry leaves are rich in proteins and amino acids; therefore, an increase in proteins level of mulberry leaves may lead to improvements in silk productivity (Eman M. Hassan, *et al.,* 2014). The production of high quality and quantity of natural silk depends mainly on larval feeding, suitable environmental conditions and protection from diseases (Machii and Katagiri, 1991; Parra,1991; Saha *et al.,* 1995 and Dechu *et al.,* 1997). The nutritional value of the mulberry leaves can be improved by enriching them with extra nutrients like glucose, glycine, egg albumin, molasses, etc., was found to increase the larval growth and improve cocoon characteristics (Sengupta *et al.,* 1992).

Silkworm larvae converts mulberry leaf proteins into its constituent amino acids on one hand and utilizes these amino acids for its growth and synthesis of fibroin and sericin proteins on the other. So, silkworm larvae feed on mulberry leaves for three purposes,

1) Development of body
2) Synthesis of fibroin protein and
3) Synthesis of sericin protein

Insects in addition to sugar and lipids, use free amino acids as the readily available source of respiratory fuels (Candy and Kilby, 1975). From the above cited literature, the works in relation to feed efficacy of mulberry leaves such as V-1 and leaves treated with Copper nanoparticles on the amino acids contents in the haemolymph, fat body and silk gland of silkworm, *Bombyx mori* are fragmentary. Hence, it has been programmed in the present study to find out the feed efficacy of mulberry leaf variety (V-1) and leaves treated with Copper nanoparticles on amino acid content in the silkworm *Bombyx mori*.

2. MATERIALS AND METHODS

Silkworm V instar of popular Indian bivoltine hybrid (CSR_2 x CSR_4) silkworm *Bombyx mori* (Local Bivoltine) race were collected from silkworm culture centre at Vatharayanthethu, Melbhuvanagiri, Cuddalore distict in Tamilnadu, and they were maintained up to cocoon.

The larvae transported from Vatharayanthethu, Melbhuvanagiri, were transferred to bamboo baskets of size 26 cm diameter and 5 cm height as described by Govindan *et al.,* (1981). The bamboo baskets were covered with paraffin paper and placed in an iron stand with ant wells. The larvae were reared simultaneously both in control and experimental groups separately on mulberry leaves dipped in copper nanoparticles solution in the laboratory. The V instar larvae placed at ambient temperature of 25+ 27℃ and relative humidity of 70 to 80%. The larvae were reared in card board boxes measuring 22×15×5 cms covered with nylon net and placed in an iron stand with ant wells. The control and copper nanoparticles treated V-1 mulberry (*Morus indica)* leaves were fed to silkworm, *Bombyx mori.*

Preparation of *Morus indica* leaf extract

Fresh leaves of *Morus indica* were collected from faculty of Agriculture, Vatharanthethu, Melbhuvanagiri, Tamilnadu, India.

Thoroughly cleaned *Morus indica* leaves were then dried in the sun for 5-7 days. Fine powder was made by grinding dried leaves. 30 gm of leaf powder and 300 ml of distilled water were refluxed in a soxhlet device at 100°C for five hours to create the aqueous extract. The extracts were then gathered and stored for later use in a deep freeze in an airtight bottle (G.Valli and S.Geetha, 2016).

Green synthesis of Copper nanoparticles from *Morus indica* leaf extract

A 40 ml aqueous solution of 10 mM Copper sulphate was combined with 10 ml of *Morus indica* leaf extract for reduction into Cu+ ions, which was then stored at room temperature for incubation (in the dark). For 10 mM of $CuSo_4$, the filtrate here serves as a reducing and stabilize agent. Throughout the studies, appropriate controls (40 ml of distilled water plus 10 ml of plant extract in a different test tube) were kept. The extract's color changed from bright yellow to dark brown after the reduction of copper sulphate to copper ions. Additionally, a control setup was kept in place without copper sulphate being added to the plant extract. Further research was done on the creation of copper nanoparticles (G.Valli and S.Geetha, 2016).

Experimental group

The *Bombyx mori* larvae were divided into four experimental groups, those are (i) Control, (ii) Treated, (iii) Disease Control and (iv) Disease Treated.

Table. 1. Experimental groups of *Bombyx mori* larvae

Group 1	**Control**	*Bombyx mori* larvae fed with normal V-1 Mulberry leaves.
Group 2	**Treated Subgroups (T_1, T_2, T_3 & T_4)**	*Bombyx mori* larvae fed with different concentrations (25%, 50%, 75% and 100%) of Copper nanoparticles treated V-1 Mulberry leaves (Select the effectives dose).
Group 3	**Diseased Control**	Bacterial infected *Bombyx mori* larvae fed with control V-1 Mulberry leaves.
Group 4	**Diseased Treated**	Bacterial infected *Bombyx mori* larvae fed with MiCuNps (effective dose) treated V-1 Mulberry leaves

Each groups contains 50 larvae except group 2 (200 larvae) fed with V-1 Mulberry (*Morus indica*) leaves, five feedings / per day.

Estimation of total free amino acids

Colorimetric micro method of Moore and Stein, (1954) was adopted for the quantitative estimation of total free amino acids in the fat body, haemolymph and silk gland. Known amount of tissues (25 mg) were homogenized in 10 per cent deproteinizing agent (TCA) and centrifuged at 3000 rpm for 15 minutes. The supernatant was used for the estimation of total free amino acids.

To 0.25 ml supernatant, 0.25 ml of distilled water and 2 ml of ninhydrin reagents were added and the colour was developed by heating gently in a water bath (100°C) for 6.5 minutes. The pale transparent pink colour was read at 570 nm against a reagent blank in a Grating Linear Readout Spectrophotometer (CECIL Model, CE373). The optical density (OD) values were compared with that of standard graph. The amount of total free amino acids present in the tissue was expressed on µg / mg wet weight of tissue.

3.RESULT

(i). Quantitative analysis of free amino acid (control and MiCuNPS (25%) treated groups)

The free amino acid content of haemolymph, fat body and silk gland of IV and V instar larvae fed with control V-1 mulberry leaves and MiCuNps (25%) treated V-1 mulberry leaves are presented in Table 1.

The free amino acid content of the haemolymph seems to be increased steadily from 0.173 ± 0.0028 µl/ml to 0.320 ± 0.0026 µl/ml in IV and V instar larvae fed with control V-1 mulberry leaves. Similarly, free amino acid content of haemolymph also found to be steadily increased in IV and V instar larvae fed with MiCuNps (25%) treated V-1 mulberry leaves such as 0.363 ± 0.0070 µl/ml and 0.443 ± 0.0091 µl/ml, respectively. In these two observations, MiCuNps (25%) treated IV and V instar larvae free amino acid content of haemolymph was highly significant at 1% level (P<0.01) than control. Significant was indicated by the t-value such as 62.415 and 35.706 of IV and V instar larvae (control and MiCuNps (25%)), respectively.

The free amino acid content of the fat body seems to be increased steadily from 0.156 ± 0.0057 µg/mg to 0.304 ± 0.0026 µg/mg in IV and V instar larvae fed with control V-1 mulberry leaves. Similarly, free amino acid content of fat body also found to be steadily increased in IV and V instar larvae fed with MiCuNps (25%) treated V-1 mulberry leaves such as 0.380 ± 0.0029 µg/mg and 0.470 ± 0.0064 µg/mg, respectively. In these two observations, MiCuNps (25%) treated IV and V instar larvae free amino acid content of fat body was highly significant at 1% level (P<0.01) than control. Significant was indicated by the t-value such as 83.096 and 53.027 of IV and V instar larvae (control and MiCuNps (25%)), respectively.

The free amino acid content of the anterior silk gland (ASG) seems to be increased steadily from 0.078 ± 0.0074 µg/mg to 0.403 ± 0.0060 µg/mg in IV and V instar larvae fed with control V-1 mulberry leaves. Similarly, free amino acid content of anterior silk gland (ASG) also found to be steadily increased in IV and V instar larvae fed with MiCuNps (25%) treated V-1 mulberry leaves such as 0.100 ± 0.0092 µg/mg and 0.802 ± 0.0102µg/mg, respectively. In these two observations, MiCuNps (25%) treated IV and V instar larvae free amino acid content of anterior silk gland (ASG) was highly significant at 1% level (P<0.01) than control. Significant was indicated by the t-value such as 6.779 and 210.291 of IV and V instar larvae (control and MiCuNps (25%)) respectively.

The free amino acid content of the middle silk gland (MSG) seems to be increased steadily from 0.117 ± 0.0086 µg/mg to 0.150 ± 0.0073 µg/mg in IV and V instar larvae fed with control V-1 mulberry leaves. Similarly, free amino acid content of middle silk gland (MSG) also found to be steadily increased in IV and V instar larvae fed with MiCuNps (25%) treated V-1 mulberry leaves such as 0.181 ± 0.0056 µg/mg and 0.396 ± 0.0106 µg/mg, respectively. In these two observations, MiCuNps (25%) treated IV and V instar larvae free amino acid content of middle silk gland (MSG) was highly significant at 1% level (P<0.01) than control. Significant was indicated by the t-value such as 36.950 and 49.069 of IV and V instar larvae (control and MiCuNps (25%)) respectively.

The free amino acid content of the posterior silk gland (PSG) seems to be increased steadily from 0.081 ± 0.0014 µg/mg to 0.097 ± 0.0028 µg/mg in IV and V instar larvae fed with control V-1 mulberry leaves. Similarly, free amino acid content of posterior silk gland (PSG) also found to be steadily increased in IV and V instar larvae fed with MiCuNps (25%) treated V-1 mulberry leaves such as 0.126 ± 0.0040 µg/mg and 0.194 ± 0.0068 µg/mg, respectively. In these two observations, MiCuNps (25%) treated IV and V instar larvae free amino acid content of posterior silk gland (PSG) was highly significant at 1% level (P<0.01) than control.

Significant was indicated by the t-value such as 42.270 and 47.331 of IV and V instar larvae (control and MiCuNps (25%)) respectively.

(ii). Quantitative analysis of free amino acids (diseased and diseased treated groups)

The free amino acid content of haemolymph, fat body and silk gland of IV and V instars of diseased *B. mori* larvae fed with control and MiCuNps (25%) treated V-1 mulberry leaves are presented in Table 2.

The free amino acid content of the haemolymph seems to be increased steadily from 0.043 ± 0.0026 µl/ml to 0.064 ± 0.0018 µl/ml in IV and V instar of diseased larvae fed with control V-1 mulberry leaves, respectively. Similarly, free amino acid content of haemolymph also found to be steadily increased in IV and V instar of diseased larvae fed with MiCuNps (25%) treated V-1 mulberry leaves, such as 0.077 ± 0.0067 µl/ml and 0.108 ± 0.0020 µl/ml, respectively. In these two observations, MiCuNps (25%) treated IV and V instar of diseased larvae free amino acid content of haemolymph was highly significant at 1% level (P<0.01) than diseased larvae (fed by control leaves). Significant was indicated by the t-value such as 18.075 and 64.409 of IV and V instar larvae (diseased and treated diseased) respectively.

The free amino acid content of the fat body seems to be increased steadily from 0.086 ± 0.0013 µg/mg to 0.116 ± 0.0023 µg/mg in IV and V instar of diseased larvae fed with control V-1 mulberry leaves, respectively. Similarly, free amino acid content of fat body also found to be steadily increased in IV and V instar of diseased larvae fed with MiCuNps (25%) treated V-1 mulberry leaves, such as 0.146 ± 0.0017 µg/mg and 0.201 ± 0.0048 µg/mg, respectively. In these two observations, MiCuNps (25%) treated IV and V instar larvae free amino acid content of fat body was highly significant at 1% level (P<0.01) than diseased larvae (fed by control leaves). Significant was indicated by the t-value such as 77.460 and 67.949 of IV and V instar larvae (diseased and treated diseased), respectively.

The free amino acid content of the anterior silk gland (ASG) seems to be increased steadily from 0.046 ± 0.0018 µg/mg to 0.145 ± 0.0015 µg/mg in IV and V instar of diseased larvae fed with control V-1 mulberry leaves, respectively. Similarly, free amino acid content of anterior silk gland (ASG) also found to be steadily increased in IV and V instar of diseased larvae fed with MiCuNps (25%) treated V-1 mulberry leaves such as 0.107 ± 0.0024 µg/mg and 0.298 ± 0.0039 µg/mg, respectively. In these two observations, MiCuNps (25%) treated IV and V instar of diseased larvae free amino acid content of anterior silk gland (ASG) was highly significant at 1% level (P<0.01) than diseased larvae (fed by control leaves). Significant was indicated by the t-value such as 105.655 and 91.435 of IV and V instar larvae (diseased and treated diseased) respectively.

The free amino acid content of the middle silk gland (MSG) seems to be increased steadily from 0.061 ± 0.0109 µg/mg to 0.080 ± 0.0011 µg/mg in IV and V instar of diseased larvae fed with control V-1 mulberry leaves, respectively. Similarly, free amino acid content of middle silk gland (MSG) also found to be steadily increased in IV and V instar of diseased larvae fed with MiCuNps (25%) treated V-1 mulberry leaves, such as 0.109 ± 0.0148 µg/mg and 0.140 ± 0.0013 µg/mg, respectively. In these two observations, MiCuNps (25%) treated IV and V instar of diseased larvae free amino acid content of middle silk gland (MSG) was highly significant at 1% level (P<0.01) than diseased larvae (fed by control leaves). Significant was indicated by the t-value such as 5.032 and 67.082 of IV and V instar larvae (diseased and treated diseased), respectively.

The free amino acid content of the posterior silk gland (PSG) seems to be increased steadily from 0.038 ± 0.0015 µg/mg to 0.011 ± 0.0018 µg/mg in IV and V instar of diseased larvae fed with control V-1 mulberry leaves, respectively. Similarly, free amino acid content of posterior silk gland (PSG) also found to be steadily increased in IV and V instar of diseased larvae fed with MiCuNps (25%) treated V-1 mulberry leaves, such as 0.061 ± 0.0039 µg/mg and 0.040 ± 0.0017 µg/mg, respectively. In these two observations, MiCuNps (25%) treated IV and V instar larvae free amino acid content of posterior silk gland (PSG) was highly significant at 1% level (P<0.01) than diseased larvae (fed by control leaves). Significant was indicated by the t-value such as 10.724 and 23.946 of IV and V instar larvae (diseased and diseased treated), respectively.

Table. 2. Quantitative analysis of free amino acids content in the haemolymph, fat body and silk gland of IV and V instars of *Bombyx mori* larvae fed with control and MiCuNps treated V-1 mulberry leaves

Larval stage	Groups	Haemolymph (µl/ml)	Fat body (µg/mg)	ASG (µg/mg)	MSG (µg/mg)	PSG (µg/mg)
IV instar	**Control**	0.173±0.0028	0.156±0.0057	0.078±0.0074	0.117±0.0086	0.081±0.0014
	MiCuNps (25%)	0.363±0.0070	0.380±0.0029	0.100±0.0092	0.181±0.0056	0.126±0.0040
V instar	**Control**	0.320±0.0026	0.304±0.0026	0.403±0.0060	0.150±0.0073	0.097±0.0028
	MiCuNps (25%)	0.443±0.0091	0.470±0.0064	0.802±0.0102	0.396±0.0106	0.194±0.0068

Values are Mean ± Standard Deviation.

Table. 2 (a). 't' - test

Larval stage	Haemolymph (µl/ml)	Fat body (µg/mg)	ASG (µg/mg)	MSG (µg/mg)	PSG (µg/mg)
IV instar	62.415**	83.096**	6.779**	36.950**	42.270**
V instar	35.706**	53.027**	210.291**	49.069**	47.331**

**Highly Significant at 1% level (P<0.01).

Table. 3. Quantitative analysis of free amino acids content in the haemolymph, fat body and silk gland of IV and V instars of diseased *Bombyx mori* larvae fed with control and MiCuNps treated V-1 mulberry leaves

Larval stage	Groups	Haemolymph (µl/ml)	Fat body (µg/mg)	ASG (µg/mg)	MSG (µg/mg)	PSG (µg/mg)
IV instar	**Diseased Control**	0.043±0.0026	0.086±0.0013	0.046±0.0018	0.061±0.0109	0.038±0.0015
	MiCuNps (25%)	0.076±0.0067	0.146±0.0017	0.107±0.0024	0.109±0.0148	0.061±0.0039
V instar	**Diseased Control**	0.064±0.0018	0.116±0.0023	0.145±0.0015	0.080±0.0011	0.011±0.0018
	MiCuNps (25%)	0.108±0.0020	0.201±0.0048	0.298±0.0039	0.140±0.0013	0.040±0.0017

Values are Mean ± Standard Deviation.

Table. 3 (a). 't' - test

Larval stage	Haemolymph (µl/ml)	Fat body (µg/mg)	ASG (µg/mg)	MSG (µg/mg)	PSG (µg/mg)
IV instar	18.075**	77.460**	105.655**	5.032**	10.724**
V instar	64.409**	67.949**	91.435**	67.082**	23.946**

**Highly Significant at 1% level (P<0.01).

4. DISCUSSION

Biochemical studies on fat body and silk gland of (Free Amino acids) *Bombyx mori* Larvae

Biochemical studies was carried out to analyze the variations in the biochemical constituents like protein, carbohydrates and amino acids in different body tissues viz., haemolymph, silk gland and fat body in the healthy and Tiger band infect fifth instar larvae of the oak tasar silkworm A. proylei Jolly. The larvae were reared during the late spring season (2015-17) at Munsyari, Uttarakhand under poly houses and were fed with *Q. semecarpifolia*. A significant decline in the concentration of the protein, carbohydrates and amino acids was recorded in different body tissues mainly due to the incidence of a deadly viral disease commonly called as Tiger band disease. Maximum decline was recorded in the silk gland protein. As a result of this disease, the larva becomes lethargic, stops feeding and finally leads to the death. The variations in temperature and humidity were main contributing factors responsible for the incidence of the disease in the larvae (Dalip K. Mansotra and P. C. Joshi, 2020).

Silk is made up of two proteins such as fibroin and sericin. Fibroin forms the core and is surrounded by sericin. These two proteins differ in their characteristics and secreted from different parts of silk gland. Fibroin is secreted from the posterior part and sericin is secreted from middle part of silk gland. Fibroin is formed from the amino acids of posterior silk gland cells. Sericin quality is one of the important features of cocoon. Sericin is classified in various ways, but generally as α-sericin and β-sericin. α-sericin being presents in the inner layer of cocoon and differs from βsericin present in the outer layer (SK Gupta, *et al.,* 2018).

Bombyx mori, which produces a great amount of sericin to the end of fifth larval instar and together with the fibroin, form the silk thread used in the production of the cocoon, structure that provides the ideal conditions fourth occurrence of larval metamorphosis to adults (Singh A, *et al.,* 2010) working on *Philosamia ricini* have reported the decrease in amino acid content in the fat body of the larvae under cold stress conditions. Decrease in the free amino acid content of fat body may indicate the possibility of active feeding of amino acid in Kreb's cycle and glycolytic pathway to meet the emergent energy needs as well as their utilization in the production of some new proteins synthesized to cope with the low temperature stress.

Published studies of silks focus on processed fibres or the optimum conditions for their production consequently the effect of temperature on the biochemical properties of cocoon are either poorly understood or kept as closely guarded industrial secrets. Present investigation inferred that the total free amino acids content in the fat body has been influenced significantly by varying temperature. The maximum level of free amino acids was noticed in the fat body obtained from the pupae reared at 26 °C while minimum levels was recorded in the fat body of adult *Bombyx mori* at 14 °C (SK Gupta, *et al.,* 2018).

For the production of a large amount of silk protein during a brief period in 5th instar, it is essential to supply sufficient amino acids. Among the amino acids, glycine is the key amino acid for controlling the synthesis of silk proteins. About 70% of glycine is synthesized in the silkworm tissues, presumably in the fat body and silk-gland (Aparupa Borgohain, 2015).

Silkworm, *B. mori* is a suitable insect for the study of plant extractives influence. The plant extracts G. lucidum are common and medicinally important was used in this study. The plant extractives through the food fortify the digestion of the larvae, which may reflect into the quality of the silk worm growth, silk gland ratio, biochemical contents protein, carbohydrate and lipid content of silkgland, haemolymph, fatbody and muscles were enchanced by *G. lucidum* extract (Dr.C. Dyana Selin, *et al.,* 2021).

A significant difference in the larval lipid content exists among different mulberry varieties highlighting the importance of mulberry varieties in providing appropriate nutrition to support and attain target growth levels at each instar stage of the silkworm. The present study reveals that the most desirable of the mulberry varieties examined for good lipid levels were Goshoerami and the most congenial season for higher accumulation of biochemical constituents in all the tissues is spring (Awquib Sabhat, M. A. *et al.*, 2010).

Rearing the eri-silkworm using an artificial diet containing mulberry leaves may not be a suitable choice, and the development of an artificial diet containing their natural host plants, such as cassava leaves or castor leaves, that does not have an effect on the biological and biochemical properties of the eri-silkworm is needed for best silkworm quality (Jatuporn Tungjitwitayakul and Nujira Tatun, 2017).

Exposure of silkworm eggs to appropriate doses of ultrasound in terms of intensity and duration of exposure would lead to acceleration of protein metabolism with reference to protein and free amino acid levels and the activity levels of protease, aspartate and alanine aminotransferases and glutamate dehydrogenase. The enhancement in protein metabolism would in turn result in the improvement of economic characters of the silkworm, having a positive impact on the profitability of Sericulture (Murali Mohan, P *et al.,* 2014).

This study showed that dynamic metabonomic changes in silkworm hemolymph were closely associated with the silkworm larva development involving multiple metabolic pathways. Such changes were dominated by degradation of the old skins and synthesis of new skins during moulting processes (i.e., ecdyses) and metamorphosis. This was accompanied by biosynthesis of pigments by using tyrosine and tryptophan metabolites. In the fifth instar, biosyntheses of silk protein and body proteins were also some outstanding metabolic events. The other dominant changes were related to trehalose metabolism including its biosynthesis and conversion into glucose for energy generation involving glycolysis and TCA cycle. The main “blood sugar” for larvae, trehalose, had a sharp level decrease during wondering period, and then glucose became an abundant blood sugar after spinning period. Hemolymph quinate generated by mulberry leaves (i.e., silkworm's sole diet) for biosynthesis of aromatic amino acids can be a good marker for dietary intakes and probably silkworm health. The hemolymph levels of both fatty acids and choline were directly affected by diet intakes facilitating biosynthesis of phosphorylcholine for cell membrane. C18:0 and its unsaturated forms together with C16:0 accounted for 95% of hemolymph fatty acids, among which C18:3n3 accounted for more than 40% of all fatty acids; n3-to-n6 ratios for fatty acids were about 3:1 and 4:1. These provided detailed information on silkworm hemolymph metabonome and its dynamic changes at various stages of larval development from the third instar to prepupal period. Future studies are necessary to obtain metabolic information for silkworm before the third instar and its adulthood (Lihong Zhou *et al.,* 2015).

Amino acids serve as the precursors for many biologically essential compounds (Dawber and Moore, 1980). Many functions like protein synthesis, cocoon construction and energy production for flight are attributed to amino acids (Sacktor, 1953). Shiegmatsu , (1958 & 1960a) has identified the blood globulin and three different protein components in the haemolymph of silkworm has shown further that some new proteins, in addition to these are synthesized in the fat body and released into the blood during development.

Further, it has been shown that the amount of amino acid contents in the haemolymph of IV instar larvae were comparatively less than V instar larvae, suggested that the occurrence of this decrement may be due to the mobilization of less amounts of protein component from the fat body via haemolymph into the silk gland. Similar trends have also been observed in the fat body, suggested that the less amount of amino acid comparatively in IV and V instar larvae of *B. mori* when fed with V-1 and copper nanoparticles treated mulberry leaves. These changes may be attributed due to the synthesis and sequestration of more quantity of both amino acid and protein for the enhancement of breakdown and synthesis of proteins which are required for silk production

The productivity of silkworm interms of cocoon crop depends on several factors that operate within and outside the body of silkworm (Benchamin and Jolly, 1986). The high levels of free amino acid in haemolymph indicate the synthesis of proteins. Padmaja, (1995) has reported that this will be received by the haemolymph after digestion of the leaf. Proteins are probably absorbed after digestion by proteases and then amino acids are intracellulary liberated into the gut epithelium by aminopeptidases as shown by Horie *et al.*, (1982) in *Bombyx mori* as in other insects (Law *et al.*, 1977).

In the present study, the amount of free amino acid content have been found to increase concomitantly more in the fat body, haemolymph and silk gland of *Bombyx mori* from III, IV and V instar larvae fed with copper nanoparticles treated mulberry leaves than V-1 leaf variety. From this result, it has been suggested that the active proteolytic and protein synthesis have been taken place for the elaboration of protein for the synthesis of silk fiber as well as immediate formation of amino acid have been found to be increased for the synthesis of proteins. These results are in conformity with the works

of Sundaramurthy and Ahmed, (1978) and Kajiura and Yamashita (1989). Davis *et al.,* (1990) and Centhilnayaki (2004) for *Spodoptera litura, Bombyx mori, Cymantia dispar* and *Bombyx mori* respectively, when these insects were treated with methoprene and V-1 mulberry variety induced increase in the protein content along with feed.

It has been revealed from the present study that the contents of protein and amino acids were comparatively more and also concomitantly increased in all the target tissues and haemolymph of III to V instar larvae may be attributed due to the contents in V-1 leaf variety treated with copper nanoparticles probably be activated the fat body glycogen phosphorylase and promotes oxygen consumption by the larvae. These metabolic adjustments add upto the development of the silkworm manifesting an increased silk production. Similar results have been observed by Gade, (1981) in *Bombyx mori* fat body during development and also as reported by Prasad and Mohan, (1990) that the total free amino acid level in the V instar larvae were elevated during the metamorphosis of *Bombyx mori*. These results were correlated with active protein synthesis and silk production in developing *Bombyx mori* in the present study, when the silkworms fed with -1 leaf variety treated with copper nanoparticles than V-1 leaf variety.

It may be concluded that V-1 leaf variety treated with copper nanoparticles elicits some favourable responses in silkworms with regard to the commercial improvement as evidenced from the results of the present study.

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