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Variation in Metal Ion Concentrations in the Haemolymph of the Silkworm, *Bombyx mori* During Development

[1]*Firdose Ahmad Malik and* [2]*M.A. Malik*

[1]Division of Sericulture, Sher-e-Kashmir University of Agricultural Sciences and Technology, Mirgund- Kashmir, 190 001, India
[2]Department of Studies in Sericultural Science, University of Mysore, Manasagangotri, Mysore - 570 006, India

Abstract: Changes in the levels of haemolymph metal ions of two binoltine races of the silkworm, *Bombyx mori* (NB4D2 and CSR2) and the multivoltine race (Pure Mysore) was studied at different stages of larval and pupal development. The haemolymph metal ions showed significant changes during different stages of larval development like moult, post-moult, feeding and spinning and pupal stages like pre-pupa, pupa and pharate adult, in selected silkworm races. The larval haemolymph was characterized by low manganese and very high zinc and iron concentrations and moderately high copper ions. Haemolymph metal ions were relatively higher in NB4D2 and CSR2 than multivoltine Pure Mysore due to higher levels of ingestion in the bivoltine races. The haemolymph metal ions of bivoltine races were about 30% higher than the multivoltine Pure Mysore. Haemolymph zinc of pre-pupae showed a significant increase from that of spinning larvae. The zinc levels improved significantly during different stages of pupal development. Higher osmolar concentration of haemolymph of bivoltine races provides a favourable passive absorption gradient of digested nutrients in the lumen to haemolymph across midgut epithelium. The impact of races was significantly more than that of ontogeny in upward changes in haemolymph metal ions.

Key words: *Bombyx mori* · Haemolymph · Iron · Manganese · Copper · Zinc

INTRODUCTION

Insect haemolymph provides and distributes metabolic sources and products throughout the insect body. The maintenance of homeostasis is essential for insect development. It is well known that in the larval haemolymph of phytophagous Lepidoptera, the cation composition is characterized by high magnesium, potassium and low sodium concentration [1]. However, the haemolymph cation pattern in insects is significantly modified with dietary condition and metamorphosis [2]. Furthermore, the change in the haemolymph cation level during larval-pupal development is of interest in connection with changes in behaviour and metamorphosis accompanying histolysis and organogenesis [3].

Calcium, iron, magnesium, manganese, phosphorus, potassium and zinc are essential for normal growth and development of the mulberry silkworm [4]. Minerals account 10 % of dried mulberry leaves, but 28 % of the silkworm [5]. Absorption of nutrients depends on the composition of the diet and midgut enzyme activity and permeability [6, 7]. Traces of copper, iron, aluminum, zinc, manganese and a number of other metallic compounds have been identified in insect blood [8]. Manganese and iron are involved in chlorophyll production in plants and, along with copper and zinc, function as enzyme cofactors in plants and animals [9]. Dietary micronutrient intake can affect the growth and development of vertebrates [10, 11]. The essentiality of these elements in insect nutrition is well established [12]; however, the optimal micronutrient requirements of insects are largely unknown. Primary emphasis has been placed on elements which are required in considerable quantity (e.g., Na^+, K^+, Ca^{2+} and Mg^{2+}) and are therefore more easily measured [13]. No information is available on the dynamics of trace elements such as iron, copper, manganese and zinc in the silkworm, *Bombyx mori*. It is therefore important to know the variation of trace elements in the haemolymph of silkworm races during different stages of larval and pupal development.

Corresponding Author: Dr. Firdose Ahmad Malik, Achabal, District Anantnag, Kashmir 192201.

MATERIALS AND METHODS

Animals: The silkworm breeds namely, Pure Mysore (PM), NB_4D_2 and CSR $_2$ were utilized in the present investigation to represent a tropical multivoltine, less productive bivoltine and highly productive bivoltine, respectively. Disease free layings (DFLs) of the three races were brushed and reared as per the standard rearing technique suggested by Dandin *et al*. [14]. Fresh mulberry leaves of V-1 variety were used for feeding the larvae.

Haemolymph Collection: Haemolymph was collected in a pre-chilled test tube containing a few crystals of thiourea by cutting the first proleg of larva. Haemolymph from pupae was obtained by piercing a sharp sterilized syringe needle into the first abdominal segment and applying gentle pressure on the thorax and abdomen. The haemolymph was collected from the larvae in 4_{th} moult, after moult, feeding and spinning stages and pupae in different stages of development in control and temperature treated batches of the three races. The haemolymph was centrifuged at 3000 g for 10 min at 4°C and the supernatant used in the metal ion estimations.

Estimation of Metal Ions: To 1 ml of haemolymph, 10 ml of diacid 9:4 (nitric acid and perchloric acid) was added and digested using digestion chamber (Digester 1009) until a clear solution was obtained. The solution was then cooled and the volume was made upto 20ml with double distilled water. The solution was filtered through Whatman No.1 filter paper. The cations were estimated from the aliquots of filtrate. Traces of metal ions (Iron, copper, manganese and zinc) were estimated using atomic absorption spectrophotometer (GBC 932 Plus).

Statistical Analysis: Analysis of variance (ANOVA) was used to test the significance of difference between the mean values of six independent observations of iron, copper, manganese and zinc levels of the haemolymph of silkworm larvae and pupae. Tukey's [15] multiple comparison tests were used to find the significance of difference between the races and different developmental stages. Differences were considered significant at $P < 0.05$.

RESULTS

The levels of iron, copper, manganese and zinc in the haemolymph showed significant changes at different stages of larval and pupal development in all the three races of the silkworm (Table 1 & Figs. 1-2).

Haemolymph Iron Concentration: Haemolymph iron concentrations were relatively higher in the bivoltine races of NB_4D_2 and CSR_2 than the multivoltine PM (Table 1). Iron concentration in larval haemolymph was relatively low during 4^{th} moult and showed significant decrease in post moult larvae before commencement of the feeding (Fig. 1a). The iron concentration showed a significant increase in the larval haemolymph during feeding period. A significant drop in the iron levels was observed in spinning larvae. The iron concentrations in the haemolymph of pupae were relatively less than those in the haemolymph of spinning larvae in all the three races (Fig. 1a).

Haemolymph Manganese Concentration: The haemolymph manganese levels of 5^{th} instar larvae were relatively lower than iron levels (Table 1). Manganese levels in larval haemolymph were relatively low during 4^{th} moult and showed further drop in post moult larvae (Fig. 1b). The levels of manganese showed a significant increase in the larval haemolymph during feeding larvae and decrease in spinning larvae. Manganese levels were also seen to be higher in bivoltine races than the multivoltine PM. The haemolymph manganese was relatively higher in pharate-adult development stage in all the three races (Fig. 1b).

Haemolymph Copper Concentration: Haemolymph copper levels in moulting larvae were relatively higher in the bivoltine races of NB_4D_2 and CSR_2 than the multivoltine PM (Table 1). A significant drop in copper levels was observed in moulted larvae in all the three races. The haemolymph copper levels increased significantly in feeding and spinning larvae and an acute drop in haemolymph copper levels was observed in pre-pupae in all the three races (Fig. 2c). But the levels of copper improved significantly during the pupal stages of development.

Haemolymph Zinc Concentration: The haemolymph zinc concentration of 5^{th} instar larvae was relatively higher than iron levels (Table 1). Haemolymph zinc showed a significant drop in post moult larvae whereas increase during feeding period (Fig. 2d). A significant decrease in haemolymph zinc levels was noticed in spinning larvae. The levels of haemolymph zinc were relatively higher in pupae than larvae. Haemolymph zinc of pre-pupae showed a significant increase from that of spinning larvae. The zinc levels improved significantly through different stages of pupal development namely pupae and pharate adult in all the three races.

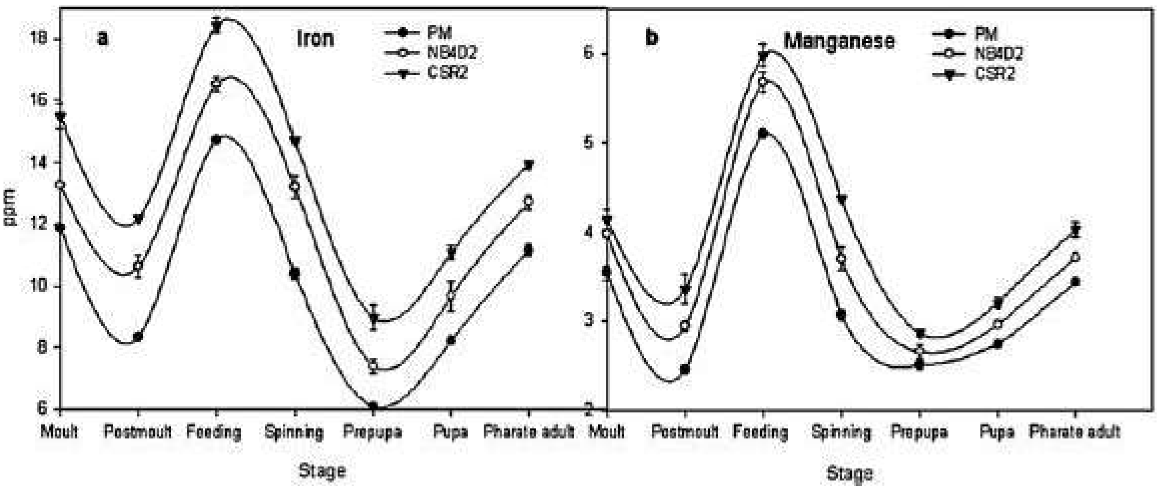

Fig. 1: Changes in the levels of a) iron and b) manganese ions in the haemolymph at different stages of larval and pupal development in three selected silkworm races (Each value is the mean±SD of three separate observations)

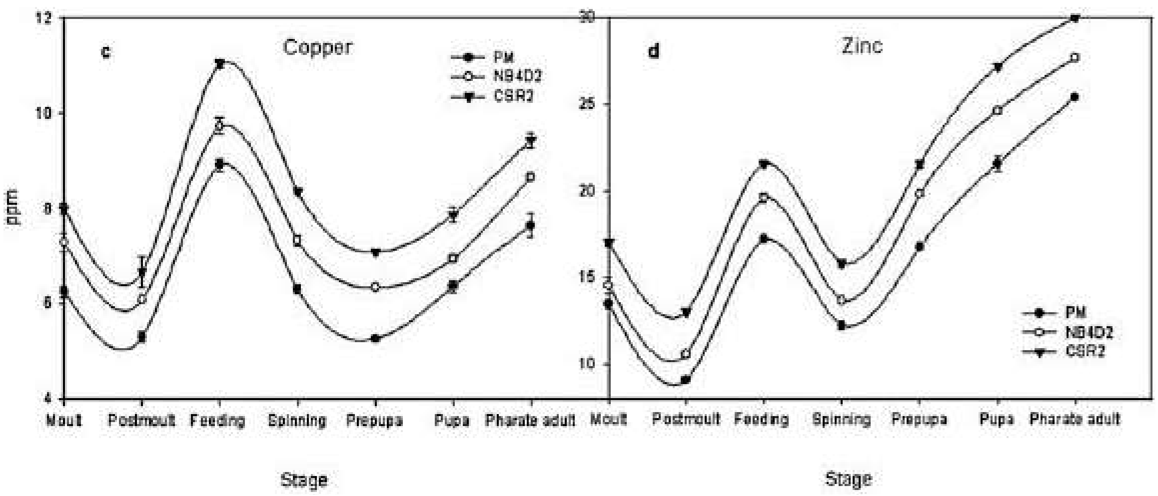

Fig. 2: Changes in the levels of c) copper and d) zinc ions in the haemolymph at different stages of larval and pupal development in three selected silkworm races (Each value is the mean±SD of three separate observations)

Table 1: Summary of ANOVA showing the interaction effect of race on haemolymph metal ions on different stages of *Bombyx mori*

		Haemolymph metal ions (ppm)		
Treatment	Iron	Manganese	Copper	Zinc
Race				
PM	10.2±2.69^{x}	2.88±0.96^{x}	6.59±1.12^{x}	16.9±2.42^{x}
NB4D2	11.9±2.85^{y}	3.66±0.96^{y}	7.34±1.25^{y}	18.6±2.56^{y}
CSR2	13.4±3.17^{z}	3.99±0.99^{z}	8.19±1.34^{z}	19.7±4.04^{z}
F-test	**	*	**	**
Stage				
Moult	13.5±1.68^{e}	3.88±0.27^{d}	7.18±0.71^{d}	14.6±2.83^{c}
Post moult	10.4±1.59^{c}	2.92±0.45^{b}	6.02±0.25^{a}	10.5±0.37^{a}
Feeding	16.6±1.61^{f}	5.58±0.39^{e}	9.89±0.82^{g}	19.0±1.56^{d}
Spinning	12.8±1.90^{d}	3.72±0.58^{c}	7.33±0.67^{e}	13.5±1.50^{b}
Pre pupa	7.38±0.86^{a}	2.68±0.16^{a}	6.24±0.57^{b}	19.6±1.34^{e}
Pupa	9.45±0.57^{b}	2.77±0.49^{a}	6.96±0.56^{c}	24.4±1.09^{f}
Pharate adult	12.9±0.81^{d}	3.73±0.30^{c}	8.01±0.40^{f}	27.3±1.55^{g}
F-test	**	*	**	**

** Significant at 1% (P<0.01); * Significant at 5% (P<0.05)

Means with different superscripts are significantly different from each other (as indicated by Turkey's HSD)

DISCUSSION

Knowledge of changes in haemolymph constituents, such as the metal ions, is of paramount importance to the developmental studies of the silkworm, *Bombyx mori*, since the haemolymph is the direct extracellular environment, from which cell can acquire substances and receive signals needed to function. Changes in haemolymph ion concentration might thus represent a source of information to alter the physiological function of many tissues and organs surrounded by the environment. Metals are essential for the activity of several enzymes. It has been reported that mineral salts are essential for growth and development of the silkworm, *B.mori* [16].

Haemolymph metal ion composition showed significant changes at the five physiologically distinct stages of growth and development *viz*., moult, post-moult (before first feeding) and feeding, spinning and pupal stages. Haemolymph metal ions were relatively higher in NB4D2 and CSR2 than multivoltine PM (Table 1) due to higher levels of ingestion in the bivoltine races. The majority of studies using insects have focused upon the toxic effects of environmental pollution with heavy metals [17]. However, several species of plants have evolved metal hyperaccumulation strategies, assimilation of toxic concentrations of the metals Cd, Co, Cr, Cu, Mn, Pb, Se and Zn, which are thought to discourage herbivory [18]. Although in general the metals content of herbivorous insects reflect the foliage upon which they have fed, elimination of high or toxic levels of selected metals such as Zn may occur [19]. The results of the present study indicate that silkworm haemolymph plasma was characterized by low manganese and very high Zinc and iron concentrations and moderately high copper (Table 1). Zinc is an important cofactor in many enzymes such as NA, RNA polymerases, alkaline phosphatases, alcohol dehydrogenases [10]. Zinc plays an important role in protein synthesis, carbohydrate, nucleic acid and lipid metabolism [20]. Severe zinc deficiency is reported to cause retardation [21]. House crickets, *Acheta domesticus*, have dietary requirements of Cu and Zn [22]. Nutrients or phytochemicals in foliar tissues that may alter the immunocompetence of insect larvae include sterols [23], tannins, chlorogenic acids [24], Fe [25], Zn [26] and Se [27].

The haemolymph iron levels increased significantly in feeding and spinning larvae and showed an acute drop in pre-pupae in all the three races. Structural studies show that many insects have iron-rich cells in the midgut [28]. Iron must be absorbed from the diet into gut cells, shuttled from the apical to the basal membrane of the gut epithelium and transferred to the haemolymph. These studies have not shown whether the regions are for uptake or excretion or both. Iron must be acquired to provide catalysis for oxidative metabolism, but it must be controlled to avoid destructive oxidative reactions [29]. In *Drosophila melanogaster,* three transporters actively accumulate Cu from the midgut lumen [30]. Cu is a cofactor in a number of important enzymes, including tyrosinase and phenoloxidase; and a deficiency leads to disruption of cuticular melanization [30]. Inclusion of Cu chelator in *H. virescens* larval diet reduced survival following challenge with AcMNPV [31], perhaps by reducing the activity of plasma phenoloxidase [32].

The haemolymph metal ions were relatively high during moult and less during post moult. An increase in haemolymph cation concentration was observed during IV moult in the larvae of the giant silk moth, *Hyalophora cecropia* [33]. Ziegler *et al*. [34] observed that the sudden increase in the haemolymph volume between late pre-moult and intra-moult served to expand the cuticle during moult in the isopod, *Ligia pallasii*. The drop in the metal ions from moult to post moult stage can be attributed to swift changes occurring in the fluid levels of haemolymph during moult cycle. The haemolymph metal ions in spinning stage drop to the levels observed during moult and post moult by increased rates of elimination of haemolymph metal ions. The spinning larvae shrink in size and store large quantities of amorphous silk in the lumen of the silk gland. The immediate and proximate source of water for silk biosynthesis is haemolymph and the consequent volume reduction of haemolymph requires elimination of cations to maintain the osmotic pressure [2]. The haemolymph zinc concentration is held relatively high in the non-feeding pupal stage due to non functional excretory system in pupal stage. Akao [35] found that the concentration of zinc in the blood of *Bombyx mori* triplet at the start of pupation. He related this change to the special function of zinc in the blood in the reproductive organs and showed that the concentration of zinc in the blood at the end of pupal life was four times as great in ovariectomized females as in normals, where it was incorporated into the sex organs.

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