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*Full Length Research Article*

# Comparative Analysis of Protein and Trehalose Contents as Influenced by Thermal Stress at 45°C in the New Bivoltine Lines of *Bombyx mori*

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## ABSTRACT

The yield and quality of the silk cocoons produced by the silkworm have been attributed to several biological, biochemical and environmental parameters. Among biomolecules, protein and trehalose aid in the growth and development of the silkworm and are drastically influenced by the environmental temperature. Considering the significance, we have explored the consecutive impact of multigenerational thermal stress (TS) at 45°C on the protein and trehalose contents of the six new bivoltine lines (NBL) in comparison with their parental breeds. Among six NBL lines, NBL-10B exhibits the highest of 43.07% elevation in the protein content, while it was 23.74% in NBL-2 being lowest on day-4 of the fifth instar larvae. Subsequently, on day-6, it has been increased to 76.30% in NBL-10B being the highest and 21.97% as low in the haemolymph of NBL-4 as a response to TS over their respective control batches. Concomitantly, among NBLs, NBL-5 exhibits an improvement of 24.34% trehalose content and the lowest of 1.99% in TS induced larvae of NBL-10A over their respective control group on day-4. Whereas on day-6, the highest of 37.38% was recorded in NBL-2 and the least of 0.81% was noticed in NBL-4 TS in comparison with their respective control groups. Comparatively, protein and trehalose contents were high and low in NBL-5 and NBL-10B, which have TS induced batches respectively. These salient findings clearly indicate that the NBL possesses high tolerance to withstand the thermal stress without affecting their growth and development compared to their parental breeds.

***Key words***: *Bombyx mori*, Thermal stress, New bivoltine lines, Haemolymph, Protein, Trehalose

In ectothermic organisms - insects, reproductive success and survivability are drastically affected by their environmental temperature. However, change in the environmental temperature beyond the threshold level induce stress affecting their fitness [14] and causing mortality [5]. Thermal stress is responsible for inducing some of the molecular changes activating insects' stress defence mechanism. This ultimately results in the synthesis of a few essential proteins such as heat shock proteins (HSPs), LEA proteins, redox regulating proteins, different compatible solutes, and cytochrome P450s [16].

Proteins are the biological molecules that play an essential role in insect growth, development and metabolism. In addition to their role as enzymes, structural and regulatory proteins are also crucial for the successful completion of an insect's life cycle. During larval development, the fat bodies which are the centre of intermediary metabolism are actively involved in the metabolism of carbohydrates and lipids, and are also responsible for the synthesis and secretion of proteins which are stored in the haemolymph [1], [10]. In addition to proteins, trehalose or mycose is the non-reducing disaccharide which is ubiquitous in the biological systems including bacteria, yeast, fungi, plants, and invertebrates [6]. The involvement of trehalose in the abiotic stress tolerance has been documented in both prokaryotes and eukaryotes. In yeasts, trehalose plays a significant role in osmosis [9], heat and desiccation tolerance [8], and also as a free radical scavenger [3].

Trehalose is the principal sugar present in the haemolymph of insects which has been used as an instant source of energy and is also considered a prominent source to deal with the abiotic stresses. Trehalose also plays a significant role as a compatible solute in preventing protein denaturation and aggregation caused due to low and high-temperature stresses. It can also stabilize the biological membranes by hydrogen bonding with the phosphate groups and hence act as a protectant compound under abiotic stress conditions.

The silkworm, *Bombyx mori* is the only domesticated lepidopteran insect reared under controlled conditions to produce economically important proteinaceous biomaterial - silk. Production of quality silk by the silkworm despite being influenced by several factors, the temperature is the prime

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factor during rearing. Eventually, exposure to extreme temperature induces thermal stress in the silkworm that may further affect the quality and yield of silk. Moreover, induced thermal stress (TS) may result in the production of heat shock proteins (HSPs) and also alter the haemolymph trehalose level in the silkworm, which bring out several physiological changes in them. Consequently, it is essential to understand the manifestation of the protein and trehalose level in the silkworm as a response to TS. Thus, detailed protein and trehalose profiling studies have been made in the New Bivoltine lines (NBLs) of the silkworms which are not been documented so far.

## MATERIALS AND METHODS

*Maintenance of silkworms*

Six new bivoltine silkworm lines – NBL-2, NBL-4, NBL-5, NBL-9, NBL-10A, NBL-10B and their parental breeds $CSR_2$ and $CSR_{27}$ were used in the present investigation. Disease-free layings of NBLs were incubated under optimum environmental conditions (25 ± 1ºC temperature and 75 ± 5% relative humidity) followed by black-boxing until hatching. The larvae were fed with mulberry leaves and reared following a standard rearing procedure [4].

*Induction of thermal stress*

Day-4 and day-6 fifth instar larvae were placed in plastic petri-dishes and exposed to 45°C at 75 ± 5% of relative humidity in a water bath for 2 hours. After two hours of recovery at room temperature, the haemolymph was collected from the silkworm larvae for the estimation of protein and trehalose contents.

*Estimation of total proteins*

The total protein contents in the haemolymph of silkworm larvae were estimated by the method prescribed by [11] using bovine serum albumin (BSA) as a standard. 0.1 ml of haemolymph was diluted with 0.9 ml of distilled water to derive an optimum solution. To this 5 ml of protein-reagent was added and incubated at room temperature for 15 min. 0.5 ml of Folin's reagent was added and the mixture was allowed to stand for about 30 min. The optical density (OD) of the sample along with the standard was recorded at 660 nm using a spectrophotometer. The concentration of the protein was estimated by plotting a standard curve and the results were expressed in µg of protein per µl of haemolymph.

*Estimation of trehalose*

The trehalose estimation was carried out following the method described by [15]. Briefly, 25 µl of haemolymph was first mixed with 0.5 ml of 20% NaOH and kept in boiling water for about 10 min followed by cooling at room temperature. To this 5 ml of anthrone reagent (0.05% anthrone in 70% sulphuric acid) was added and mixed thoroughly. The sample was then kept in boiling water for about 15 min for colour development and then cooled at room temperature. The intensity of colour was measured at 620 nm in a spectrophotometer considering trehalose as a reference standard.

*Statistical analysis*

The data collected from the above experiments were analyzed employing one-way ANOVA ($p \leq 0.05$) by using SPSS statistical package Version: 28.0.0.0 (190).

## RESULTS AND DISCUSSION

*Protein profiling as a response to thermal stress*

A gradual increase in the protein content was recorded from day-4 to day-6 of the fifth instar NBL larvae exposed to the thermal stress of 45°C (Fig 1). The highest 52.80 µg/µl protein content was recorded in the haemolymph of NBL-5 after HS compared to that of control which was accounting for 40.53 µg/µl on day-4 of fifth instar larvae. Similarly, a significant increase in the protein content, as a response to TS, recorded was 51.93 µg/µl in NBL-9, 49.67 µg/µl in NBL-10A, 45.67 µg/µl in NBL-4, and 41.00 µg/µl in NBL-2 respectively. However, the low protein content of 38.53 µg/µl was recorded in the NBL-10B subjected to TS while it was 26.93 µg/µl in the respective control (Fig 1).

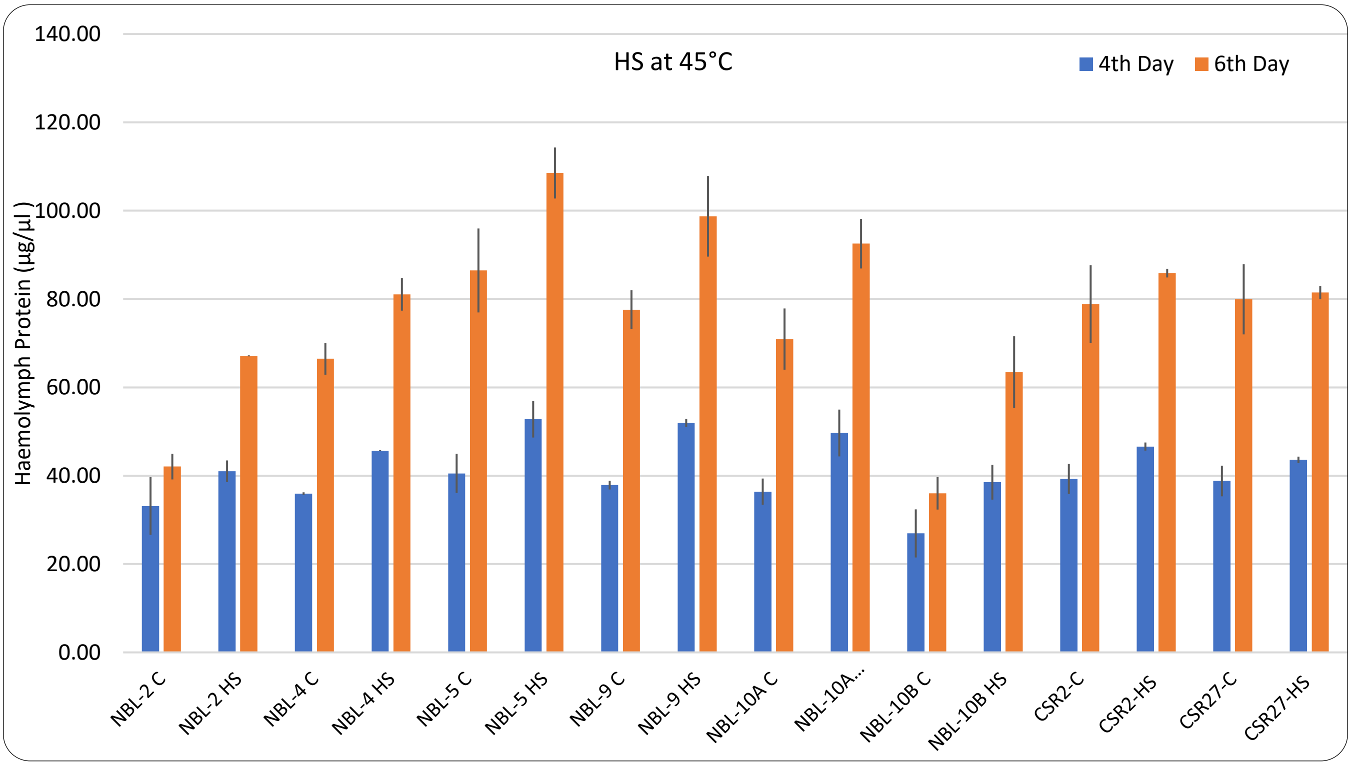


Fig 1 Haemolymph protein content in the new bivoltine lines and its parental breeds of *Bombyx mori* as a response to thermal stress at 45°C

The highest content of protein accounting for 108.53 µg/µl was recorded in TS induced NBL-5 larvae compared to 86.47µg/µl of its respective control. On day-6 protein content was 98.73µg/µl while recorded from TS induced larvae of NBL-9 it was 92.53µg/µl in NBL-10A, 81.07µg/µl in NBL-4, and 67.13 µg/µl in NBL-2 compared to its respective controls (77.60, 70.93, 66.47 and 42.07 µg/µl). The low protein content of 63.47 µg/µl was reported in NBL-10B subjected to TS compared and 36.00 µg/µl in non-TS induced larvae. In addition to this, the parent breeds $CSR_2$ and $CSR_{27}$ exhibited 46.60 and 43.60 µg/µl of haemolymph protein against their control batches on day-4, while it was 85.87 and 81.47 µg/µl on day-6 respectively.

*Total trehalose profiling in response to heat shock*

A gradual increase in the trehalose content was recorded from day-4 to day-6 of the fifth instar larvae of NB lines exposed to the heat shock of 45°C and its respective control. The highest of 12.60 µg/µl trehalose was observed in TS induced NBL-5 larvae, while it was 10.13 µg/µl in control. 9.95 µg/µl trehalose being low was noticed in TS induced NBL-10B but slightly higher than its control (9.20 µg/µl). NBL-9, NBL-10A, NBL-4 and NBL-2 subjected to TS have shown 10.67, 10.27, 10.20 and 10.00 µg/µl trehalose content against their control batches measuring 10.13, 10.07, 9.53 and 9.47 µg/µl respectively (Fig 2).

A significant increase in the content of trehalose was recorded on day-6 larvae as a response to the TS. The highest of 9.57 µg/µl was recorded in TS induced NBL-5 but it was measuring 8.83 µg/µl in its control batches. A low trehalose level of 5.61 µg/µl was observed in NBL-10B which was lesser than that of its respective control (5.23 µg/µl). Further, NBL-9, NBL-10A, NBL-4, and NBL-2 exhibit increased trends accounting for 8.32, 7.53, 6.09 and 7.77 µg/µl against their respective control group measuring 7.63, 6.09, 6.04 and 5.65 µg/µl (Fig 2).

Further, the trehalose content in the parents $CSR_2$ and $CSR_{27}$ were reported to be 10.93 and 10.00 µg/µl against their control batches on day-4, while it was 7.19 and 6.59 µg/µl respectively on day-6.

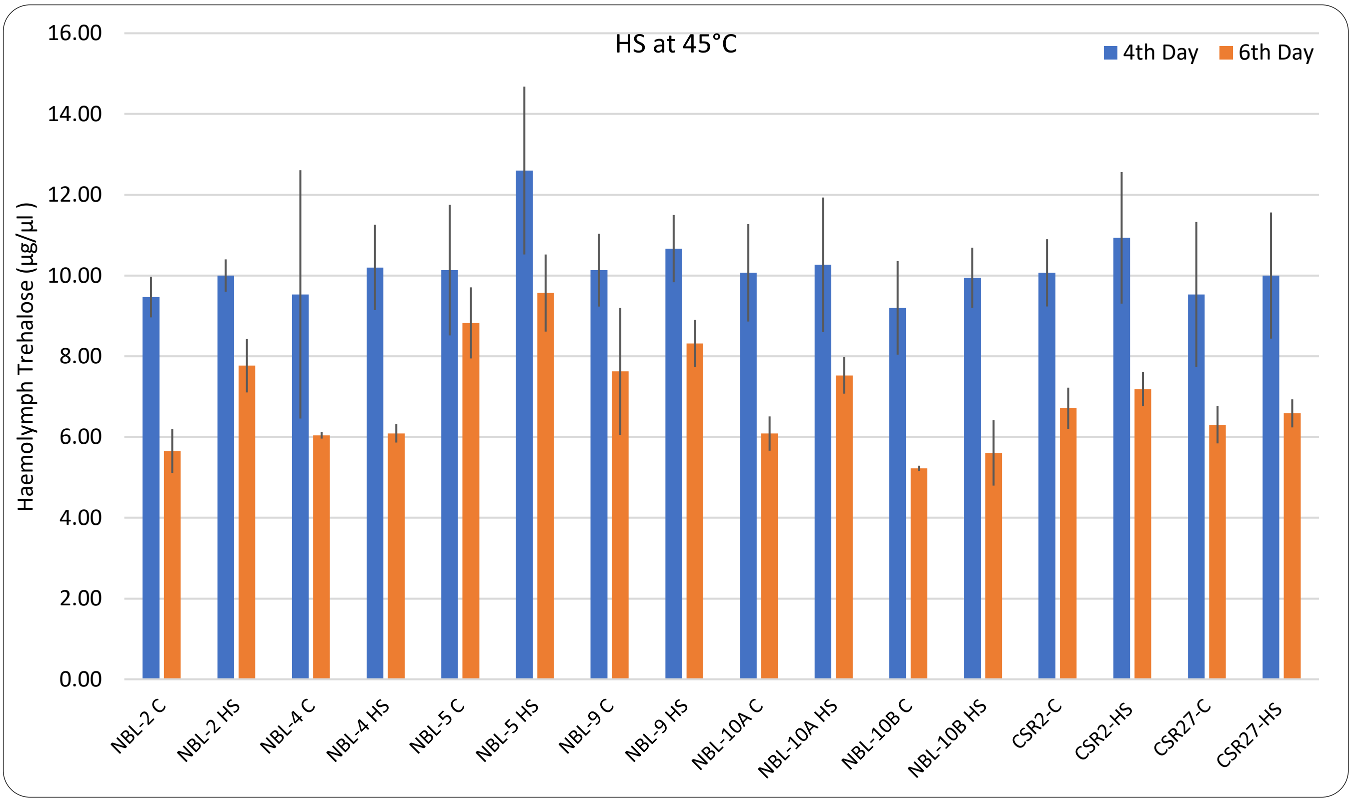


Fig 2 Haemolymph trehalose content in the new bivoltine lines and its parental breeds of *Bombyx mori* as a response to thermal stress at 45°C

Insect cellular activities are greatly vulnerable to the fluctuating environmental temperature and became susceptible to the diseases upon continuous domestication, for example, the silkworm, *Bombyx mori*. Optimal thermal stress often triggers the synthesis of a certain set of cellular proteins to overcome critical temperature such that there will be an elevation in the total protein contents of the cell. Basically, the ontogenic pattern of haemolymph proteins noticed during larval development is probably to balance the synthesis, storage, transport and degradation of structural proteins [7]. An increase in the protein level during the fifth instar facilitates the ontogenic development of larva which is characterised by the production and secretion of new sets of proteins in the haemolymph [12]. However, a higher amount of protein/proteins accumulation in the body and/or haemolymph leads to the death of an organism, which might have exceeded its limits to operate autophagy [13].

In the present study, thermal stress induced at 45°C explicit varied levels of protein contents in the NBLs indicating all the NBLs and their parents have different levels of response to it that acquired through multigenerational thermal stress. Interestingly, protein content increased due to the overexpression of certain heat shock protein genes that are triggered by thermal stress. Of all the NBLs, NBL-10B exhibited higher levels of 43.07 and 76.30% in its protein content on day-4 and day-6 respectively, while NBL-2 exhibited a low rate of 23.74% on day-4 and NBL-4 record 21.97% on day-6 despite higher than that of its non-thermal stress induced fifth instar larvae (Fig 1). This varied level of protein contents suggests that response to thermal stress varies among different new bivoltine breeds, while NBL-10B response is higher than the rest of the NBLs.

Concomitantly, the total trehalose content was also found to elevate differently as a response to thermal stress on day-4 and day-6 of fifth instar larvae of NBLs. High trehalose levels protect the organism against dehydration stress at high ambient temperatures with increased haemolymph osmolarity [2]. Upon exposure to higher temperatures, monovalent cations like Na+ and K+ were hyper-regulated and divalent cations like Ca2+ and Mg2+ were hypo-regulated in order to prevent the abnormal rise in the levels of haemolymph osmotic pressure [12]. Among all the NB lines, NBL-5 exhibit a higher rate of 24.34%, and the lowest of 1.99% in NBL-10A day-4 TS induced larvae. Whereas on day-6, the highest rate of 37.38% trehalose content was noticed in NBL-2 HS, while it was low (0.81%) in NBL-4 compared to control batches (Fig. 2). This change is due to the fact that trehalose is a major haemolymph sugar component that may accumulate in high quantities in both freeze-tolerant and freeze-averse insects, which act as a cryoprotectant and supercooling agent [17] and also plays a significant role in preventing protein degradation and aggregation as a protectant compound under abiotic stress conditions [9].

A steady rise in trehalose level along with glucose level of haemolymph suggests increased demand for the fuel reserves for larval-pupal and pupa-imaginal transformation occurring during pupal development. Thus, the enhanced trehalose content in the NB lines in response to the thermal stress might be beneficial in preventing the degradation and aggregation of normal cellular proteins [9], which are essential in the larval growth and development as well as spinning of good quality cocoons.

## CONCLUSION

Taken together, the present study confirms the presence of certain biochemical modifications with respect to proteins and trehalose levels in the NB lines developed in presence of multigenerational thermal stress. These further aid in the NB lines to endure the fluctuating high temperature without declining their cocoon economic traits compared to their parental breeds. Thus, we suggest using all the NBLs as potential breeds for large scale commercial exploitation in the field.

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