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Acclimatization to Heat Stress in Nistari Race of *Bombyx mori*

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ABSTRACT

An experimental study was conducted to analyze thermal sensitivity in multivoltine mulberry silkworm *Bombyx mori* of Nistari race. Temperature tolerance of the silkworm eggs, larvae, pupae and adults at 17 °C, 33 °C and 43 °C was studied. In general, late stage larvae exhibited maximum tolerance compared to the adult moths and the eggs. Exposure to 17 °C and 33 °C was tolerated equally whereas temperature of 43 °C proved to be lethal. Heat stress resulted in appearance of additional protein species in larval haemolymph. The kinetics of 72 kDa was quite different within and between (IV^{th} and V^{th} instar stage) larval haemolymph after exposure to three different temperatures for 3 consecutive days with 1 hr of duration followed by 24 hr recovery. Increased appearance of 95 kDa protein consequent to heat shock was reported in V^{th} instar larval haemolymph. In Nistari, cocoon and shell weight significantly increased after heat stress over control respectively. This would be due to expression of Hsps at larval stage. These results suggest heat shock protein based breeding strategy for inducing robustness in productive breeds.

Keywords: Heat shock proteins, Multivoltine Nistari race, Robustness, Thermotolerance.

1. Introduction

Silkworm larvae, *Bombyx mori* L. (Lepidoptera: Bombycidae) is an important economic insect which is used as a tool to convert leaf protein into silk[1]. The industrial and commercial use of silk, the historical and economic importance of production and its application in all over the world finely contributed to the silkworm promotion as a powerful laboratory model for the basic research in biology[2]. The development and economic production of sericulture largely and greatly depends on the metabolic modulations and physiological adaptability of silkworm, besides its genetic constitution[3, 4].

The continued domestication for centuries made them susceptible to environment which is always harmful/lethal and produces "stress" or shock to these sensitive insects[5]. Temperature, humidity, air circulation, gases, light, and so forth, show a significant interaction in their effect on the physiology of silkworm depending upon the combination of factors and developmental stages affecting growth, development, productivity, and quality of silk[1]. The biological as well as cocoon-related characters are influenced by ambient temperature, rearing seasons, quality of mulberry leaf, and genetic constitution of silkworm strains[1]. The seasonal differences in the environmental components considerably affect the genotypic expression in the form of phenotypic output of silkworm crop such as cocoon weight, shell weight, and cocoon shell ratio [1].

A number of behavioral and physiological strategies are adapted by insects to avoid or minimize stress [6]. However, to overcome this shock to a considerable level their body accommodates for a protective mechanism by producing kinds of proteins generally known as "Heat shock proteins (Hsps) with the activation of some "genes" located in their chromosomes [7, 8, 9]. Heat shock proteins are present in cells under normal conditions, but they can express at high levels when exposed to a sudden temperature jump or other stress [10]. Heat shock proteins stabilize proteins and are involved in the folding of denatured proteins also [11]. There are reports on the activity of heat shock proteins in silkworm by [12] and [13]. The heat shock response of silkworm habitating tropical climate is likely to differ from those of temperate climate [14]. extremes of temperature of tropics and subtropics, forcing farmers to rear tolerant tropical strains that are low productive and provide inferior quality silks [5].

In this context, investigations on thermotolerance in silkworms are significant to sericulture industry.

The body of literature available on the molecular mechanism of temperature sensitivity and heat shock response in silkworm is rather thin as compared to the enormous work done on other insects. Although temperature and humidity have been known to play important role in silk production [1], not much information is available on the effect of temperature on silkworm physiology. Also significantly, most of the heat shock studies have been restricted to cell and tissue cultures or organisms from temperate climate [15, 16, 17] and less few works have been related to the commercial traits of *Bombyx* mori.

The present study was undertaken in an attempt to study the effect of temperature stress on the different life history stages of *Bombyx* mori of Nistari strain in relation to the Hsps expressed and its subsequent impact on commercial traits.

2. Materials and Methods

2.1. Silkworms and heat shock

Bombyx mori larvae of multivoltine race, Nistari strains were reared in the laboratory on garden fresh mulberry leaves, at 24–26 °C and 60–85% relative humidity [18, 19]. The silkworm eggs, larvae (4th day of 5th instar), pupae and adults were exposed to three different temperatures viz., 17 °C 33 °C and 43 °C for 3 consecutive days with 1 hr of duration. The heat treatment was always coupled to a relative humidity of 90%. For every heat shock experiment, at least 100 eggs, larvae, pupae and adults in triplicates were used. After the heat shock, the samples were returned to the rearing temperature and allowed to recover. Survival of eggs by hatchability, larvae by their ability to spin healthy cocoons, pupae by adult emergence and adult by successful copulation and egg lying was observed. Effects of exposure to elevated temperature on physiological parameters were also analysed.

2.2. Extraction and analysis of heat shock proteins (Hsp)

Haemolymph from punctured proleg of the heat exposed larvae (3rd day of 4th instar and 4th day of 5th instar) after 24 hr of recovery was collected in a pre-cooled graduated centrifuge tube containing a few crystals of phenylthiourea [20]. The haemolymph was centrifuged at 3000 *g* for 10 min at 4°C. The protein profiles of the haemolymph were analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) [21].

2.3. Statistical analysis

Each assay was replicated 3 times. Values were expressed as mean ±SE (Standard error) of replication and Student's *t*-test was applied to locate differences between treated and control groups. To determine the differences in thermotolerance at varied temperatures, mean values of various life history stages obtained from different heat treatments were subjected to a two way analysis of variance test (two way ANOVA). In each test difference at $P<0.05$ was considered significant.

3. Results

3.1. Effects of temperature exposure on the survival of the silkworms

Temperature tolerance of the eggs, larvae, pupae and adults of the Nistai strain at different temperatures (17 °C, 33 °C and 43 °C) for 3 consecutive days with 1 hr of duration was shown as survival percentage (Table 1). The silkworm *Bombyx mori* showed varying levels of heat tolerance at the different developmental stages viz., the egg, larva, pupa and adult. The rates of survival of the insect at various heat treatments were low in comparison with those incubated at room temperature (Table 1, Pair data *t*-tests, $P<0.05$). Except for the pupae, 17 °C was found to be suitable for all the life cycle stages of the respective strain (Table 1). The multivoltine Nistari strain normally reared at 24–26 °C, showed good tolerance for exposures to 33 °C for 3 hr (Table 1). However, on exposure to temperatures of 43 °C or above, the survival rates reduced drastically (Table 1, two way ANOVA, $P<0.05$). The later larval instars tolerated higher temperatures for longer durations as compared to the eggs or adults (Table 1).

Table 1: Effect of heat stress on survival of silkworms (Nistari strain, n= 9)

Temperature	Survival (%) of Nistari race of *Bombyx mori*				Duration of temperature exposure (In 3 consecutive days)
	Egg	Larvae(4th)	Pupa	Adult	
Control[a] (24-26 °C)	100±0.32	100±0.32	100±0.32	100±0.32	
17 °C[a]	89.75±0.25**	95±0.26**	50±0.26**	94±0.26**	(1+1+1) hours
33 °C[a]	88±0.32**	95±0.26**	70.12±0.22**	75±0.26**	(1+1+1) hours
43 °C[b]	2±0.26**	40±0.26**	15±0.26**	15±0.26**	(1+1+1) hours

Values are mean ± SE of three replications. Significant *p<0.05, **p<0.01 compared to control in the same column. Comparison is also between mean values among treatments in different rows. Heat treatments marked with same letter are statistically not significantly different (Two factor ANOVA, P<0.05). n= Number of samples

Table 2: Effect of heat stress on post- cocooning parameters of silkworms (Nistari strain, n=9)

Temperature Exposure	Nistari race of mulberry silkworm		
	Wt. of Single cocoon (g)	Wt. of Single cocoon shell (g)	Shell%
Control (24-26 °C)	.533±0.0004	.08±0.003	14.95±0.003
17 °C	.682±0.0003** (27.476)	.106±0.0003** (32.5)	15.54±0.003 (3.946)
33 °C	.594±0.0003** (11.028)	.095 ±0.0003 (18.75)	15.99±0.003* (6.956)
43 °C	.633±0.0003** (18.317)	.098±0.0003 (22.5)	15.48±0.003 (3.545)

Values are mean ± SE of three replications. Significant *p<0.05, **p<0.01 compared to control in the same column, Percentage increase/decrease over control are indicated in parenthesis, n= Number of samples

3.2. Effects of heat stress on commercial traits

Weight of the cocoon spun by larvae after heat shock was found to increase over their respective control population (Table 2). The highest cocoon weight of 0.682 g with 27% improvement over its control was noticed at 17 ^{0}C (Table 2, Pair data *t*-tests, P<0.05). The shell weight also increased up to 32.5% (Pair data *t*-tests, P<0.05) and 22.5% at 17 ^{o}C and 42 ^{0}C compared to control (Table 2). Markedly, the increased shell ratio was recorded to the level of 6.95% at 33 ^{0}C (Table 2, Pair data *t*-tests, P<0.05).

3.3. Physiological changes

The heart beat (per min) of larvae ranged from 54±1. On exposure to high temperature, the rate of heart beat increased (Figure 1). The haemolymph pH 6·3 and did not show any variations.

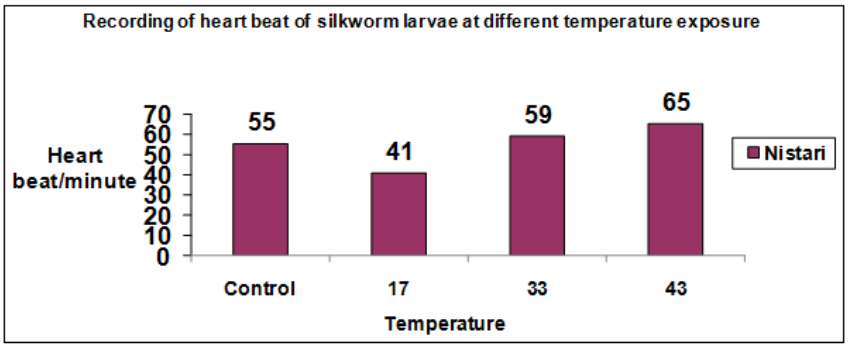

Fig 1: Recording of heart beat of silkworm larvae at different temperature exposure

3.4. Ethological changes

5th instar larvae of Nistari race maintained solitary existence with the exposure of temperature stress at 17 °C for 1 hour during 3 consecutive days. Exposure to 33 °C produced no significant ethological changes whereas to 43 °C larvae produced upright posture and circumvent behavior.

3.5. Protein profiles from heat shocked larvae

Most significant was the continued expression of heat inducible polypeptide of 72 kDa in IVth instar larval haemolymph during the 24 hr recovery time at 43 °C following the heat shock. (Figure 2A). Expression of 67 kDa band was higher in heat shocked samples than control (Figure 2A). Differential expression of 49 and 43 kDa protein consequent to heat shock (Figure 2A) was evident at heat shock temperatures of 43 ^{o}C. The profile of non-heat shock proteins was not changed in heat shocked larvae (Figure 2A). Comparatively the protein profiles in Vth instar larvae were critically down regulated (Figure 2B). The densities of 95 kDa band appeared light in control, but became dense in heat stressed Vth instar larvae (Figure 2B). Same findings were reported for 72, 35, 29 and 14 kDa (Figure 2B) proteins.

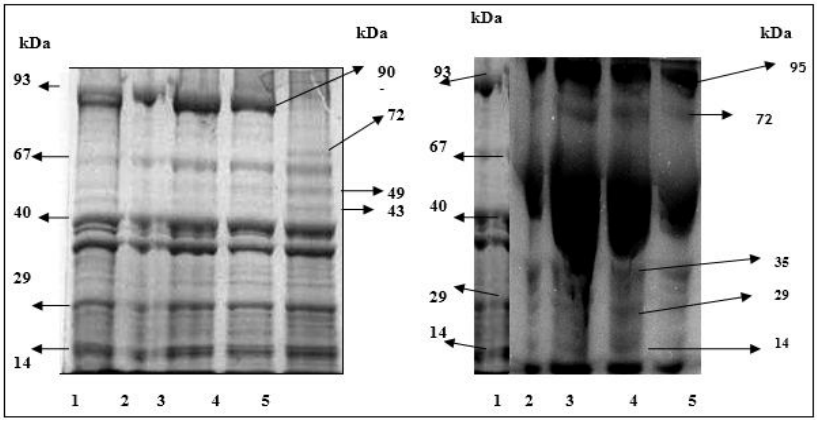

Fig 2: Silkworm larvae (4th instar and 5th instar) were exposed to 17 °C, 33 °C and 43 °C for 1 h in 3 consecutive days. After recovery period of 24 hours, the hemolymph was collected and the proteins were separated on SDS-PAGE (10%) and stained with Coomassie brilliant blue (lanes 1–5).

A--- (4th instar)Lanes: 1, control (no heat shock); Lane2, marker; Lane3, 17 °C ; Lane 4, 33°C ; Lane 5, 43 °C

B-- (5th instar) Lanes: 1, marker ; Lane2, control (no heat shock); Lane3, 17°C ; Lane 4, 33°C; Lane 5, 43°C

4. Discussion

It is quite evident that Silkworm in all stages of its growth, excluding egg stage, is characterized as a stenothermal animal, according to its demands for environmental temperatures (22-28 ^{0}C)[22]. Nistari is an indigenous multivoltine strain selected for the study has history of exposure to high temperatures in their natural environment. Differential levels of tolerance to thermal exposure (other than their normal growth temperature) were reported to be dependent on the life stages of the silkworm[23]. The fifth instar larvae were proved to be more tolerant compared to the pupae and the adult moths. The eggs were also very sensitive to temperature of 43 °C, when the hatchability was reduced almost to zero. Similar findings were also reported by[23] and [12].

The fitness character of a race is determined among other things by its ability to preserve the protein structure against heat shock[6]. In the present study expression of 72 kDa was persisted in the haemolymph of IVth instar larvae after they were returned to normal temperature following exposure to 43 ^{0}C (Figure 2A). The same protein (72 kDa Hsp) was not observed in haemolymph after recovery from 17 and 33 ^{0}C (Figure 2A). This clearly showed that

the induction of Hsp72 is facilitating the silkworm larvae to acquire thermo tolerance against heat shock. This lays credibility to the fact that survivability of the silkworm *Bombyx mori* at extremes of heat relates to counter effects of heat shock proteins, serving as molecular chaperones assisting in refolding of denatured proteins [24]. On the other hand in Vth instar larval haemolymph the 72 kDa protein was present constitutively and on heat shock increased substantially (Figure 2B). It proves that expression of heat shock proteins varies depending on the stage of development and also explains the reasons behind the higher levels of temperature tolerance by the late age silkworms. The level of 49 and 43kDa remained high during the 24 hr recovery time of 43 °C heat shocked samples compared to control and other stressed samples (Figure 2A). Whether the continued presence of theses Hsps in Nistari strain is responsible for its better resistance to high temperature needs investigation. Surprisingly 90 kDa Hsps were not detected from the haemolymph of 43 ^{0}C heat shocked samples after 24 hr recovery (Figure 2A.). The same protein was found in control and other stressed samples (17 ^{0}C and 33 ^{0}C, Figure 2A). 90 kDa Hsps are moderately conserved in eukaryotes and are usually expressed following exposure to temperature extremes but appear nonessential for thermotolerence[25]. Synthesis of 95 kDa polypeptide was quite pronounced in Vth instar larvae (Figure 2B). Increased appearance of this high molecular weight protein consequent to heat shock was reported in the haemolymph, fat bodies and cuticle of multivoltine and bivoltine races of silkworms by [7], [8], [9] and [23]. Another significant feature in the haemolymph protein profiles was persistence of non–heat shock haemolymph proteins at all the temperature treatments (Figure 2A). Lack of repression of non-heat shock protein synthesis is an adaptation response observed in the insects inhabiting hot climates[26].

There is ample literature stating that good quality cocoons are produced within a temperature range of 22-27 °C and above these levels makes the cocoon quality poorer[18, 27]. On the contrary, in temperatures below 20 ^{0}C, silkworms are consuming small quantity of food and their growth prolonged, since their normal growth rate is decreased, resulting in abnormal growth and sensitivity against several diseases[28]. Consequently, in the present experiment as a novel strategy, heat shocked larvae (whole organisms) when allowed to grow under natural environmental conditions they spun better quality cocoons than the non-heat shocked larvae reared in natural environmental conditions[12]. Nistari strain was proved as highly tolerant as judged by their survival and capacity to spin cocoons after 3 consecutive days of heat shock. The increased cocoon weight with 18% improvement over control and shell weight with 22.5% increase over its respective control (Table 2) is attributed to the expression of Hsp72 at late larval stage. Further due to induced tolerance, heat shock larvae spun better cocoons than their respective controls. This study therefore proves that after heat shock, if these larvae are reared under natural environmental conditions where frequent fluctuations occur, their performance will be better in relation to quality of cocoons than the non-heat shocked individuals. These investigations highlighted the fact that knowledge obtained from model organisms under normal laboratory conditions does not always reflect what happens out in the field, where conditions are continuously changing and unpredictably hostile. Interestingly, the increased cocoon weight and shell weight over control, reflects the positive correlation between heat shock responses and silk protein content in the cocoon. Abramova IYU *et al*. [29] reported suppression of fibroin synthesis in the silk gland following heat shock, but recently[30] identified HSP90, HSP70, and HSP60 in the silk glands of *Bombyx mori*, offering the opportunity for further systematic investigation in different breeds of silkworm. None of the larvae recovered from heat shock at 45 °C [12] and 46 °C [16], were able to spin cocoons. However, in these aspect further investigations to determine species-specific responses to heat shock is required. The practical application of this phenomenon will need to be explored positively and systematically (using multivoltine and bivoltine silkworm strains) in laboratory and field conditions in order to achieve stabilized sericulture farming in tropics.

5. Conclusion

The experiment again proved that Nistari the native multivoltine strain of West Bengal can withstand adverse environmental condition and give a handful return to the rearers. Several researchers observed that resistance to high temperature is a heritable character[31]. On the other end temperature sensitive bivoltine silkworms produced superior qualities of silk which is too a heritable racial character[14]. So, effort can be made to introduce crossbreeds with Nistari (Nistari x Bi) throughout the year in the tropical countries like India. This study was an attempt to bridge gap in the relationships between Heat shock proteins and commercial traits in the silkworm *Bombyx mori* opening up a new avenue to study the correlations between heat shock, heat shock proteins, stress tolerance and commercial traits.

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