



## Impact of temperature on heat shock protein expression of *Bombyx mori* cross-breed and effect on commercial traits

### Author Details

<b>J. Hongray Howrelia</b>	P.G. and Research Department of Advanced Zoology and Biotechnology, Loyola College, Chennai - 600 034, India
<b>Bharat Bhusan Patnaik</b> (Corresponding author)	Central Sericultural Research and Training Institute, Central Silk Board (Govt. of India), Berhampore - 742 101, India e-mail: drbharatbhusan4@gmail.com
<b>M. Selvanayagam</b>	P.G. and Research Department of Advanced Zoology and Biotechnology, Loyola College, Chennai - 600 034, India
<b>S. Rajakumar</b>	Regional Sericultural Research Station, Central Silk Board, Allikuttai (P.O.), Veeranam Road, Salem - 636 003, India

### Abstract

#### Publication Data

Paper received:  
22 October 2009

Revised received:  
16 March 2010

Accepted:  
15 July 2010

The present study investigated the effect of increasing temperature stress on the thermotolerance of *B. mori* cross-breed PM x CSR<sub>2</sub> and tissue specific differential expression of heat shock proteins at IV<sup>th</sup> and V<sup>th</sup> instars. The larvae reared at 25 ± 1°C and 70 ± 5% relative humidity were treated as control. Larvae were subjected to heat shock temperatures of 34, 38 and 42°C for 3 hr followed by 3 hr recovery. Expression of Heat shock protein 72 were analyzed by SDS-PAGE and confirmed by western blotting analysis. The impact of heat shock on commercial traits of cocoons was analyzed by following different strategies in terms of acquired thermotolerance over control. Resistance to heat shock was increased as larval development proceeds and increased thermotolerance is achieved with the induction of Heat shock protein 72 in the V<sup>th</sup> instar larval haemolymph. Relative influence of heat shock temperatures on commercial traits corresponding to the generation of heat shock protein 72 was significantly improved over control. In PM x CSR<sub>2</sub>, cocoon and shell weight significantly increased to 9.90 and 11.90% over control respectively.

### Key words

Commercial traits, *Bombyx mori*, Haemolymph, Heat shock protein, Temperature stress, Thermotolerance

### Introduction

Temperature, a dominant factor in establishing growth, reproduction and distribution of organisms, lacks spatial and temporal constancy in most environments. Consequently, organisms employ diverse adjustments at multiple levels of biological organization to deal with the fluctuating nature of the thermal environment (Hochachka and Somero, 2002; Bhattacharjee, 2008; Kumar and Tripathy, 2009). It has been reported that the ability of organisms to acquire thermotolerance to normally lethal temperature is an ancient and conserved adaptive response (Hong and Vierling, 2000). Silkworm, *Bombyx mori* L. (Lepidoptera: Bombycidae) have been in continuous domestication and therefore have become more susceptible to environmental insults resulting in extensive crop losses particularly in hot and humid climatic conditions. As silk industry has been acknowledged by Indian planners as important sector of the economy, because of its potential for strengthening the rural economy,

providing employment and increasing export earnings (Basavaraju *et al.*, 1995; Bajpai, 2009), a challenging task exists in developing stress and disease-resistant strains.

The success and promise of any new silkworm strain in the field depends on the molecular mechanism of the cell involving the heat shock response. It involves the rapid synthesis of special proteins, the heat shock proteins (Garrido *et al.*, 2001; Kregel, 2002; Park *et al.*, 2008). They act as 'molecular chaperones' to ensure better survival under stressful conditions, including thermotolerance (Santoro, 2000; Parcellier *et al.*, 2003; Mosser and Morimoto, 2004) and have been implicated in immunogenicity to cancers/infectious diseases (Srivastava, 2002).

The heat shock response of organism's habitating tropical climate is likely to differ from those of temperate climate. In tropical and subtropical zones, although there is sufficient supply of mulberry

leaves, most productive strains of silkworm are not adaptable due to extremes of temperature, forcing farmers to rear tolerant strains that are low productive and provide inferior quality silks. In this context, investigations on thermotolerance in silkworms are significant to sericulture industry. Also significantly, most of the heat shock studies have been restricted to cell and tissue cultures or organisms from temperate climate (Sorger, 1991; Lohmann and Riddiford, 1992; Hsieh *et al.*, 1995) and less few works have been related to the commercial traits of *B. mori*. The present study was undertaken in an attempt to study the effect of temperature stress on the late larval stages of *B. mori* cross-breed PM x CSR<sub>2</sub> in relation to the Hsps expressed and its subsequent impact on commercial traits.

### Materials and Methods

**Silkworms and heat shock:** The disease-free layings (DFLs) of silkworm *Bombyx mori* commercial cross-breed PM (Multivoltine) x CSR<sub>2</sub> (Bivoltine) were obtained from silkworm seed development center, Central Silk Board, Morappur, Dharmapuri district, TamilNadu. The hatched larvae were reared in the decontaminated rearing house using disinfected rearing appliances. The feeding, cleaning and sanitation schedule were followed according to Krishnaswami (1988). Rearing of the larvae was conducted under optimum temperatures of 25±1°C and 70±5% relative humidity.

For every heat shock treatment at least 20 larvae were used at one time and each experiment was repeated at least 3 times. The silkworm larvae (IV<sup>th</sup> and V<sup>th</sup> instars) were placed in thin-walled test tubes/beakers and exposed to heat shock temperatures of 34, 38 and 42°C, in water bath for 3 hr. Then the larvae were transferred to room temperature for recovery lasting 3 hr. Thereafter, heat shocked and control larvae were reared until spinning in three replications in controlled environmental conditions.

**Extraction and analysis of heat shock proteins (Hsp):** Haemolymph of silkworm larvae recovering from heat shock were collected by rapid centrifugation method of Nation and Thomas (1965). To inhibit the tyrosinase activity of the haemolymph, phenylthiourea was added. The haemolymph were centrifuged at 750 g to remove haemocytes. Fat body was routinely dissected and were rinsed free of haemolymph using ringer solution.

Proteins were extracted from the tissues after trichloroacetic acid (TCA) precipitation. A 5% homogenate of the tissue samples

was made in cold insect ringer solution. 10% TCA was added to the homogenate and was kept at 0-4°C for 10 min. The sample was then centrifuged at 750 g for 5 min and the sediment thus obtained were washed twice with cold 5% TCA followed by a wash with ethanol-ether (3:1) mixture. The pellet was dissolved in 0.05 N sodium hydroxide (NaOH) and was used for protein estimation (Lowry *et al.*, 1951).

Polyacrylamide gel electrophoresis under denaturing conditions (SDS-PAGE) was performed using 5% stacking and 10% separating gel (Lammelli, 1970). An equal quantity of the samples was treated with SDS-sample buffer for 1 min in boiling water. Electrophoresis was performed for 6 hr at 80 V for stacking gel and 120 V for separating gel. The gel was stained with 0.2% coomassie brilliant blue R250. The quantitative estimation of proteins expressed was performed using a gel documentation unit (Alpha Innotech). Western blotting analysis was performed according to the protocol of Towbin *et al.* (1979) for confirmation of heat shock protein 72 expressions. After electrotransfer of the gel proteins, the nitrocellulose membrane was incubated for 1 hr at room temperature with Rabbit Anti-Hsp70 (Hsp72 polyclonal antibody-Stressgen Biotechnologies, USA) Goat Anti-Rabbit IgG HRP Conjugate at 1:1000 dilution (Bangalore Genei, India) was added to the membrane and incubated for 1 hr at room temperature. For development of immunoblot, the membrane was incubated with Tetramethylbenzidine (TMB) with constant shaking till dark bands appear.

Thermotolerance of heat shock at varied temperatures (34, 38 and 42°C) was assessed based on pre-cocooning parameters such as larval duration, larval weight and silk gland weight. Commercial characteristics like cocoon weight, shell weight and shell ratio were also recorded (Anamed Cocoon Analysis Software) and statistically analyzed using the ANOVA program using SPSS Software.

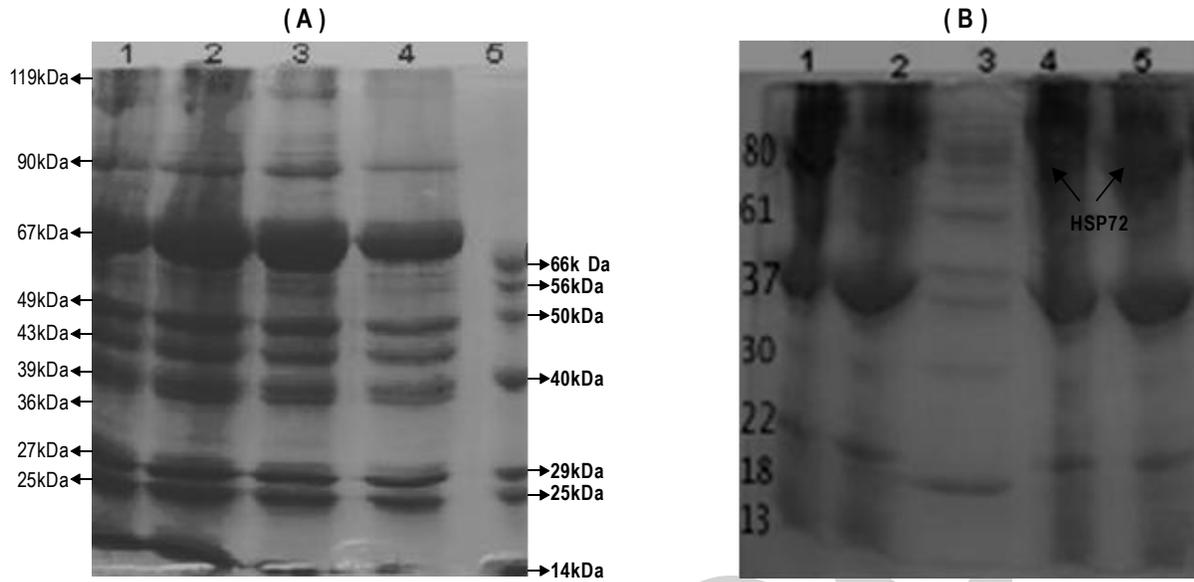
### Results and Discussion

**Differential and tissue specific expression of heat shock proteins:** The prominent presence of eight protein polypeptides (119,90,67,49,43,39,27 and 25 kDa) of relatively higher induction were observed in IV<sup>th</sup> instar haemolymph of silkworm *B. mori* cross breed PM x CSR<sub>2</sub> (Fig. 1A). No significant and abrupt changes were observed in the molecular sizes of bands

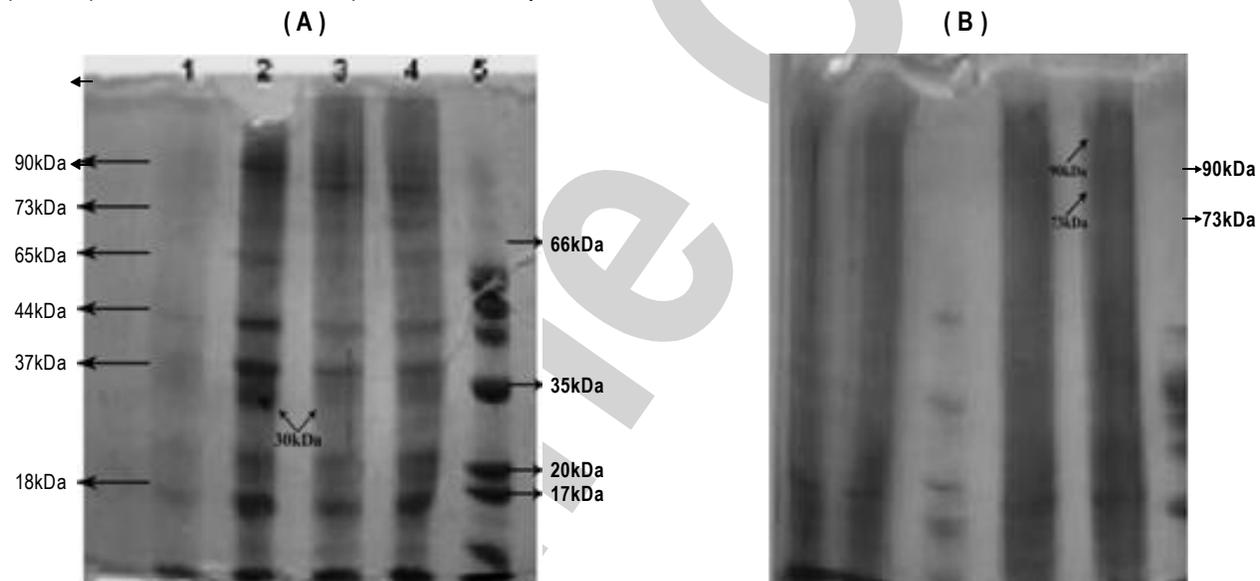
**Table - 1:** Effect of heat shock temperatures on the pre-cocooning and post-cocooning parameters of the silkworm *Bombyx mori* (PM x CSR<sub>2</sub>)

Treatment at V <sup>th</sup> instar	Larval duration (hr)	Larval body weight (g)	Silk gland weight (g)	Cocoon weight (g)	Shell weight (g)	Shell ratio (%)
Control (25 ± 1°C)	216 ± 0.008	4.46 ± 0.028	0.85 ± 0.005	1.92 ± 0.09	0.42 ± 0.12	21.76 ± 0.04
Heat shock (34°C)	218 ± 0.019 (0.92)	4.48 ± 0.016 (0.48)	0.87 ± 0.019 (2.35)	1.97 ± 0.15 (2.60)	0.44 ± 0.003 (4.76)	22.1 ± 0.67 (1.56)
Heat shock (38°C)	224 ± 0.017** (3.70)	4.86 ± 0.014** (8.97)	0.95 ± 0.12** (11.76)	1.96 ± 0.24 (2.08)	0.43 ± 0.012 (2.38)	21.96 ± 0.63 (0.91)
Heat shock (42°C)	228 ± 0.057** (5.55)	4.91 ± 0.14** (10.09)	0.95 ± 0.23** (11.76)	2.11 ± 0.14** (9.90)	0.47 ± 0.09** (11.90)	22.01 ± 0.97 (1.14)

Values are mean ± SE of five replications. Significant \*p < 0.05, \*\*p < 0.01 Percentage increase/decrease over control are indicated in parenthesis



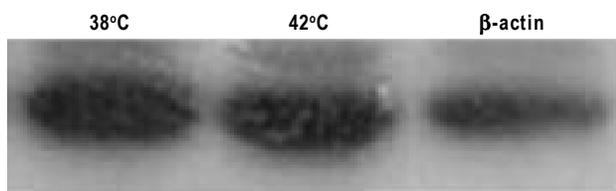
**Fig. 1:** Heat shock protein profile derived from IV<sup>th</sup> and V<sup>th</sup> instar larval haemolymph of *Bombyx mori* (PM x CSR<sub>2</sub>) (A) IV<sup>th</sup> instar haemolymph (lane1: control, lane2: heat shocked at 34°C, lane3: heat shocked at 38°C, lane4: heat shocked at 42°C, lane5: marker (B) V<sup>th</sup> instar haemolymph (lane1: marker, lane2: control, lane3: heat shocked at 34°C, lane4: heat shocked at 38°C, lane5: heat shocked at 42°C. Tissue specific expression of 72 kDa heat shock protein is indicated by arrow



**Fig. 2:** Heat shock protein profile derived from IV<sup>th</sup> and V<sup>th</sup> instar larval fat body of *Bombyx mori* (PM x CSR<sub>2</sub>), (A) IV<sup>th</sup> instar fat body (lane1: control, lane2: heat shocked at 34°C, lane3: heat shocked at 38°C, lane4: heat shocked at 42°C, lane 5: marker. Expression of 30 kDa protein is indicated by arrow, (B) V<sup>th</sup> instar fat body (lane1: control, lane2: heat shocked at 34°C, lane 3: marker, lane4: heat shocked at 38°C, lane5: heat shocked at 42°C. Expression of 90 and 73 kDa protein is indicated by arrows

in between control and heat shocked samples. Expression of 67 kDa band was significantly higher in control and heat shock samples. Comparatively the protein profiles in V<sup>th</sup> instar larvae were critically down regulated in response to increased heat shock conditions. Exposure of the larvae to 34°C brought about sustained proteolysis and reduction in number of protein bands. Most significant were the expression of heat-inducible polypeptide of 72 kDa in 38 and 42°C heat shock samples (Fig. 1B). In V<sup>th</sup> instar larval haemolymph, differential expression of

72 kDa protein consequent to heat shock (Fig. 3) was evident at heat shock temperatures of 38 and 42°C. These observations are in conformity with the studies of Evgenev *et al.* (1987), which attributed the synthesis of Hsp after exceptionally "heavy" heat shock in *B. mori* cells *in vitro* and *in vivo*. Such an induction was not evident in the larval fat body of V<sup>th</sup> instar larvae of *B. mori*. This clearly showed that the induction of Hsp72 is tissue specific and differentially regulated, facilitating the silkworm larvae to acquire thermotolerance against heat shock. This lays credibility to the fact that



**Fig. 3:** Western blot of tissue-specific Hsp72 profile in the V<sup>th</sup> instar haemolymph of *Bombyx mori* (PM x CSR<sub>2</sub>) exposed to heat shock temperatures of 38, 42°C and positive control  $\beta$ -actin)

survivability of the silkworm *B. mori* at extremes of heat relates to counter effects of heat shock proteins, serving as molecular chaperones assisting in refolding of denatured proteins (Samad *et al.*, 2005). Also the haemolymph being an open circulatory system, most of the tissues including the fat bodies and silk gland laid bathed in haemolymph within the *B. mori* larvae. Consequently, several proteins synthesized in the fat body find their way into the haemolymph (Dean *et al.*, 1985). In this respect, the presence of Hsp72 in the haemolymph of *B. mori* can be considered as a desirable feature in conferring thermotolerance to the larvae.

Expression of heat shock proteins in different tissues also varies depending on the stage of development or even the temperature at which the exposure was given (Joy and Gopinathan, 1995). The total concentration of Hsp and their redistribution to specific intracellular sites are considered as most important factors in the acquisition of thermotolerance (Kampinga, 1993).

The synthesis of proteins in fat body induced by heat shock at different temperatures was analyzed. Seven polypeptides with apparent molecular weights of 90, 73, 65, 44, 37, 22 and 18 kDa were observed in the IV<sup>th</sup> instar larval fat body of *B. mori* cross-breed PM x CSR<sub>2</sub> (Fig. 2A). Heat shock led to the synthesis of 30 kDa polypeptide which was restrictively-regulated. The study of V<sup>th</sup> instar permitted the identification of developmentally regulated proteins of 90 kDa. Synthesis of 90 kDa polypeptide, which was quite pronounced in IV<sup>th</sup> instar larvae declined in the V<sup>th</sup> instar. Joy and Gopinathan (1995) reported the presence of 93,89 and 70 kDa polypeptides in the fat body of multivoltine and bivoltine silkworm and 84,62,60,47 and 33 kDa polypeptides was reported in the bivoltine silkworms (Chavadi *et al.*, 2006) on heat shock. Good expression of 70 and 64 kDa polypeptide without heat shock was observed in different tissues of cockroach (Singh and Lakhotia, 1999) and may therefore have significant implications in silkworm.

The susceptibility of economically important organisms such as silkworms to environmental fluctuations of temperature, humidity *etc.*, attain significance in their field rearing for the commercial production of silk. Silkworm *B. mori* cross-breed PM x CSR<sub>2</sub> (Multi x Bivoltine) show different levels of tolerance for exposure to temperatures higher than normal growth temperatures. Generally, the heat shock response depends on the magnitude of temperature elevation and duration of exposure and is relative to the environmental temperature at which the organism normally survives

(Nath and Lakhotia, 1989; Bijlsma and Loeschecke, 1997). Therefore differential expression may exist in the synthesis of Hsp among various cell types in the same organism.

Relationships between heat shock, protein polymorphism and fitness characters have been recorded in number of organisms (Watt, 1992; Dahlhoff and Rank, 2000). The effect of heat shock temperatures on the V<sup>th</sup> instar larval duration, larval body weight and silk gland weight of *B. mori* cross-breed PM x CSR<sub>2</sub> is depicted in Table 1. With the rise in heat shock temperatures, the larval duration increased significantly. At 42°C, maximum larval duration of 228 hr was reported and it prolonged the larval period by 10 hours when compared with control ( $p < 0.01$ ). Significant increases in the larval body weight over control were also noticed with the increase in heat shock temperatures with maximum weight of the larvae noted to be of 4.91 g at 42°C. It was 10.09% improvement over the control body weight of 4.46 g ( $p < 0.01$ ). The effect of heat shock temperatures led to significant change in the silk gland weight. It was 0.95 g at 42°C, an increase of 11.76% over control.

Weight of the cocoon spun by larvae after heat shock was found to increase over their respective control population (Table 1). The highest cocoon weight of 2.11 g with 9.90% improvement over its control was noticed in PM x CSR<sub>2</sub>. The shell weight also increased significantly in response to heat shock up to 11.90% at 42°C ( $p < 0.01$ ). Markedly, the increased shell ratio was recorded to the level of 1.56 and 1.14% at 34 and 42°C over control. This study was an attempt to bridge gap in the relationships between Heat shock proteins and commercial traits in the silkworm *B. mori* opening up a new avenue to study the correlations between heat shock, heat shock proteins, stress tolerance and commercial traits. The increased cocoon weight with 9.90% improvement over control and shell weight with 11.90% increase over its respective control is attributed to the expression of Hsp72 at 5<sup>th</sup> instar 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.

### Acknowledgments

J.H. Howrelia is thankful to Jawaharlal Nehru Memorial Fund for awarding Jawaharlal Nehru Scholarships (Ref. No. SU/1/6/2004-2005/823; dated 23/12/2004) for this work.

### References

- Basavaraju, H.L., S. Nirmal Kumar, M.N. Reddy, K.M.M. Giridhar and R.K. Datta: New productive bivoltine hybrids. *Ind. Silk*, **34**, 5-9 (1995).
- Bajpai, A.K.: Mulberry sericulture in eastern and north-eastern India: Prospects, problems and development strategies. Proceedings of National Conference on Vanya Silk, Jorhat, Assam, India (2009).
- Bhattacharjee, S.: Triadimefon pretreatment protects newly assembled membrane system and causes up-regulation of stress proteins in salinity stressed *Amaranthus lividus* L. during early germination. *J. Environ. Biol.*, **29** (2008).

- Bijlsma, R. and V. Loeschecke: Environmental stress, Adaptation and Evolution. Birkauser, Verlag, Basel (1997).
- Chavadi, V.B, A.H. Sosalegowda and M.H. Boregowda: Impact of heat shock on heat shock proteins expressions, biological and commercial traits of *Bombyx Mori*. *Ins. Sci.*, **13**, 243-250 (2006).
- Dahlhoff, E.P. and N.E. Rank: Functional and physiological consequences of genetic variation at phosphoglucose isomerase: heat shock protein expression is related to enzyme phenotype in a montane leaf beetle. *Proc. Natl. Acad. Sci. USA*, **97**, 10056-10061 (2000).
- Dean, R.L, M. Locke and J.V. Collins: Structure of the fat body: *In: Comprehensive insect physiology, biochemistry and pharmacology* (Eds. G.A. Kerkut and L.I. Gilbert). Pergamon Press, New York. pp. 55-210 (1985).
- Evgenev, M.B, V.S. Sheinker and A.V. Levin: Molecular mechanisms of adaptation to hyperthermia in higher organisms. Synthesis of heat shock proteins in cell cultures and larvae of certain silkworm species. *Mol. Biol.*, **21**, 484-494 (1987).
- Garrido, C, S. Gurbuxani, L. Ravagnan and G. Kroemer: Heat shock proteins: Endogenous modulators of apoptotic cell death. *Biochem. Biophys. Res. Comm.*, **286**, 433-442 (2001).
- Hochachka, P.W. and G.N. Somero: *In: Biochemical adaptation*, Oxford University Press, New York (2002).
- Hong, S.W. and E. Vierling: Mutants of *Arabidopsis thaliana* defective in acquisition of tolerance to high temperature stress. *Proc. Nat. Acad. Sci. USA*, **97**, 4392-4397 (2000).
- Hsieh, F.K, S.J. Yu, S.Y. Su and S.J. Peng: Studies on the thermotolerance of the silkworm. *Bombyx Mori. Chin. J. Entomol.*, **15**, 91-101 (1995).
- Joy, O. and K.P. Gopinathan: Heat shock response in mulberry silkworm races with different thermotolerances. *J. Biosci.*, **20**, 499-513 (1995).
- Kampinga, H.H.: Thermotolerance in mammalian cells: Protein denaturation and aggregation and stress proteins. *J. Cell Sci.*, **104**, 11-17 (1993).
- Kregel, K.C.: Heat shock proteins: Modifying factors in physiological stress responses and acquired thermotolerance. *J. Appl. Physiol.*, **92**, 2177-2186 (2002).
- Krishnaswami, S.: Development of an appropriate technology in India for tropical sericulture. International Congress on Tropical Sericulture Practices. Bangalore, India (1988).
- Kumar, G. and R. Tripathy: Influence of heat stress on genome of grass pea (*Lathyrus sativus* L.). *J. Environ. Biol.*, **30**, 403-408 (2009).
- Lammelli, U.K.: Cleavage of structural proteins of the head of bacteriophage T4. *Nature*, **227**, 680-685 (1970).
- Lohmann, C.M.F. and L.M. Riddiford: Synthesis and secretion of low molecular weight cuticular proteins during heat shock in the tobacco horn worm *Manduca sexta*. *J. Experiment. Zool.*, **262**, 374-382 (1992).
- Lowry, O.H, N.J. Rosenbergh, A.L. Farr and R.J. Randall: Protein measurement with the folin phenol reagent. *J. Biol. Chem.*, **193**, 265-275 (1951).
- Mosser, D.D. and R.I. Morimoto: Molecular chaperones and the stress of oncogenesis. *Oncogene*, **23**, 1105-1116 (2004).
- Nath, B.B. and S.C. Lakhota: Heat shock response in tropical chironomous: Seasonal variation in response and the effect of developmental stage and tissue type on heat shock protein synthesis. *Genome*, **32**, 676-686 (1989).
- Nation, J.L. and K.K. Thomas: Quantitative studies on purine extraction in the greater wax moth, *Galleria mellonella*. *Annu. Rev. of Entomol. Soc. America*, **58**, 833-885 (1965).
- Parcellier, A., S. Gurbuxani, E. Schmitt and C. Garrido: Heat shock proteins, cellular chaperones that modulate mitochondrial cell death pathways. *Biochem. Biophys. Res. Commun.*, **304**, 505-512 (2003).
- Park, E.M, Y.O. Kim, B.H. Nam, H.J. Kong, W.J. Kim, S.J. Lee, Y.J. Jee, J.S. Kong and T.J. Choi: Cloning and expression analysis of a small Hsp26 gene of Pacific abalone (*Haliotis discus Hannai*). *J. Environ. Biol.*, **29**, (2008).
- Samad, H. El, H. Kurata, J.C. Doyle, C.A. Gross and M. Khammash: Surviving heat shock: Control strategies for robustness and performance. *Proc. Natl. Acad. Sci. USA*, **102**, 2736-2741 (2005).
- Santoro, M.G.: Heat shock factor and the control of stress response. *Biochem. Pharmacol.*, **59**, 55-63 (2000).
- Singh, A.K. and S.C. Lakhota: Tissue specific variations in the induction of Hsp70 and Hsp64 by heat shock in insects. *Cell Stress and Chaperones*, **56**, 90-97 (1999).
- Sorger, P.K.: Heat shock factor and heat shock response. *Cell*, **65**, 363-366 (1991).
- Srivastava, P.: Interaction of heat shock proteins with peptides and antigen presenting cells: Chaperoning of the innate and adaptive immune responses. *Annu. Rev. Immunol.*, **20**, 395-425 (2002).
- Towbin, H., T. Staehelin and J. Gordon: Electrophoretic transfer of proteins from Polyacrylamide gels to nitrocellulose sheets: Procedure and some application. *Proc. Nat. Acad. Sci. USA*, **76**, 4350-4354 (1979).
- Watt, W.B.: Eggs, enzymes and evolution: Natural genetic variants change insect fecundity. *Proc. Natl. Acad. Sci. USA*, **89**, 10608-10612 (1992).