Heat shock response in mulberry silkworm races with different thermotolerances

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Abstract. The thermal sensitivity and heat shock response of the different races of the mulberry silkworm *Bombyx mori* have been analysed. The multivoltine race, strains *C. Nichi* and *Pure Mysore* showed better survival rates than the bivoltine race, strain *NB4D2* exposed to 41°C and above. In general, the fifth instar larvae and the pupae exhibited maximum tolerance compared to the early larval instars, adult moths or the eggs. Exposure up to 39°C for 1 or 2 h was tolerated equally whereas temperatures above 43°C proved to be lethal for all. Treatment of larvae at 41°C for 1 h resulted in a variety of physiological alterations including increased heart beat rates, differential haemocyte counts, enlargement of granulocytes and the presence of additional protein species in the tissues and haemolymph. The appearance of a 93 kDa protein in the haemolymph, fat bodies and cuticle, following the heat shocking of larvae *in vivo* was a characteristic feature in all the three strains examined although the kinetics of their appearance itself was different. In haemolymph, the protein appeared immediately in response to heat shock in *C. Nichi* reaching the maximal levels in 2–4 h whereas its presence was noticeable only after 2–4 h recovery time in *Pure Mysore* and bivoltine races. The fat body from both *C. Nichi* and *NB4D2* showed the presence of 93 kDa, 89 kDa and 70 kDa proteins on heat shock. The haemocytes, on the other hand, expressed only a 70 kDa protein consequent to heat shock. The 93 kDa protein in the haemolymph, therefore could have arisen from some other tissue, possibly the fat body. The 93 kDa protein was detected after heat shock in pupae and adult moths as well, although the presence of an additional (56 kDa) protein was also apparent in the adults. The presence of 46 kDa and 28 kDa bands in addition to the 93 kDa band in the cuticular proteins immediately following heat shock was clearly discernible. The 70 kDa band did not show much changes in the cuticular proteins on heat shock. In contrast to the changes in protein profiles seen in tissues and haemolymph following heat shock *in vivo,* the heat treatment of isolated fat body or haemolymph *in vitro* resulted in protein degradation.

Keywords. *Bombyx mori;* bivoltine silkworms; heat shock proteins; multivoltine silkworms; thermotolerance.

1. Introduction

Heat shock response is a universal physiological phenomenon exhibited by both prokaryotes and eukaryotes (Glover 1982; Ciechanover *et al* 1984; Chirico *et al* 1988; Laminet *et al* 1990; Hightower 1991; Feige and Mollenhauer 1992). It is normally manifested by the appearance of a set of new proteins or an increase in the quantity of certain specific, preexisting proteins in response to exposure of an organism or a cell to thermal- and other forms of stresses. The heat shock proteins

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(HSP) are presumed to ensure survival under stressful conditions by involvement in damage protection or damage repair due to their action as molecular chaperons (Hightower 1991; Morimoto 1993). 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 (Nakayama *et al* 1989; Nath and Lakhotia 1989). Cells which have been preexposed to thermal stress acquire a transient resistance against the killing effect of a subsequent thermal stress and such a thermotolerance is caused by an enhanced resistance of proteins against thermal denaturation and aggregation (Kampinga 1993). The HSPs are also proposed to protect cells depleted in ATP from rapid necrotic death by inhibiting the aggregation of cytoskeletal proteins (Kabakov and Gabai 1994).

The heat shock response of organisms habitating tropical climate is likely to differ from those from temparate climate. This aspect has direct relevance in such commercially important areas as the field rearing of mulberry silkworms for silk production. The native Indian, multivoltine races of *Bombyx mori* (*e.g., Pure Mysore, C. Nicht, Nistari* strains) exhibit much more tolerance to high temperatures, often the field temperatures touching 40°C or more in summer, as against the exotic bivoltine races (e.g., *NB4D2, NB18* strains) of temperate origin (Krishnaswamy *et al* 1977; Pershad *et al* 1986). The latter strains are better yielders of silk fibre in terms of quality and quantity but less popular with the farmers due to their higher susceptibility to environmental fluctuations. Although temperature and humidity have been known to play important role in silk production, not much information is available on the effect of temperature on silkworm physiology. Most of the heat shock studies have been carried out on cell- and tissue cultures or organisms from temperate climate (Ashburner 1970; Lindquist 1980, 1986; Carvalho and Rebello 1987; Sorger 1991). Here we have made a comparative analysis of the effect of heat shock on the tropical and exotic races of silkworm, *B. mori,* at different life stages.

2. Materials and methods

B. mori larvae (bivoltine race, strain *NB4D2* and multivoltine race, strains *C. Nichi* and *Pure Mysore*) were reared in the laboratory on mulberry leaves, at 24–26°C and 60–85% relative humidity. The silkworm eggs, larvae, pupae and the adults were exposed to temperatures ranging from 37°C to 47°C for specified time. The heat treatment was always coupled to a relative humidity of 90%. For every heat shock experiment, at least 20 larvae, 10 pupae or adults, or 100 eggs were used at any time, and each experiment was repeated at least 3 times. After the heat shock, the samples were returned to the rearing temperature and allowed to recover. Survival rate was assessed in the case of eggs by hatchability, larvae by their ability to enter succeeding instar or spin healthy cocoons, pupae by adult emergence, and adult by successful copulation and egg laying.

The effect of exposure to elevated temperature on physiological parameters was analysed. The heart beat rate was counted by examining the dorsal blood vessel of larvae, mounted on plasticin. The protein profiles of the haemolymph, fat body, silk glands and cuticle of the larvae, as well as the total proteins from the pupae and adult moths were analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli 1970).

Haemolymph from a cut proleg of the larvae was collected in a precooled graduated centrifuge tube containing a few crystals of phenyl thiourea (Gupta 1979). The haemolymph was centrifuged at 2000 g for 5 min at 4°C, and the supernatant was analysed for protein contents. For the isolation of haemocytes, the haemolymph from 20 larvae was pooled and centrifuged at 1000 g for 1 min at 4°C to remove the tissue debris, and the supernatant was centrifuged again at 5000 g for 10 min at 4°C. The sedimented haemocytes were examined under the microscope and lysed using the homogenization buffer (200 mM Tris-Cl, pH 7·5; 5 mM $MgCl_2$; and 250 mM NaCl). For analysis of proteins, the fat body, silk gland and the cuticle were dissected from the heat shocked- or control larvae, and washed in Ringer (Irvine 1969). The tissues (1 g/ml of Ringer) were homogenized using a teflon glass homogenizer. NP40 (0·5% v/v) was also included in the buffer while homogenizing the cuticle (Kiely and Riddiford 1985). The homogenates were centrifuged at 5000 g for 10 min at 4°C, and the supernatant after removal of the floating lipid layer was used for protein analysis.

Protein content was quantitated by the dye binding method (Bradford 1976). For *in vivo* labelling, the larvae were heat shocked for 30 min at 41°C and injected with 60 µCi of [^{35}S]Met (specific activity, 8000 Ci/mM) per larvae. The heat shock was continued for an additional 30 min. The larvae were sacrificed and the tissues processed for protein analysis. The [^{35}S]Met labelled proteins after SDS-PAGE were subjected to fluorography (Bonner and Lasky 1974).

In vitro heat shock studies were carried out by exposing the isolated fat body, cuticle (both maintained in insect Ringer) and haemolymph from control larvae to 41°C for lh.

3. Results

3.1 *Effect of high temperature exposure on the survival of the silkworms*

The silkworm *B. mori* when exposed to high temperatures ranging from 37°C to 47°C, showed varying levels of heat tolerance at the different developmental stages viz., the egg, larva, pupa and adult. The later larval instars and the pupal stages tolerated higher temperatures for longer durations as compared to the eggs or adults (figure 1). The data represent the maximum time they could be exposed at any given temperature to give 100% survival. The multivoltine *C. Nichi* strain normally reared at 24–26°C, showed complete tolerance for exposures up to 39°C for 2 h. However, on exposure to temperatures of 41°C or above, the survival rates reduced drastically. Beyond 43°C, even a 10–20 min exposure proved lethal to all the developmental stages of the insect.

The multivoltine *C. Nichi* and *Pure Mysore* larvae were more resistant than the bivoltine *NB4D2,* when they were exposed to different temperatures for varying times (figure 2). The differences begin to appear at elevated temperatures of 41–43°C although they were equally tolerant to exposure at 39°C up to 2h. Between the two multivoltine races examined here, *C. Nichi* appeared to be marginally more tolerant than the *Pure Mysore* strain. In the case of bivoltine even when the larvae survived up to the cocoon spinning stage in some instances, they appeared placid. The fifth instar larvae at 3 days showed more thermotolerance than the younger
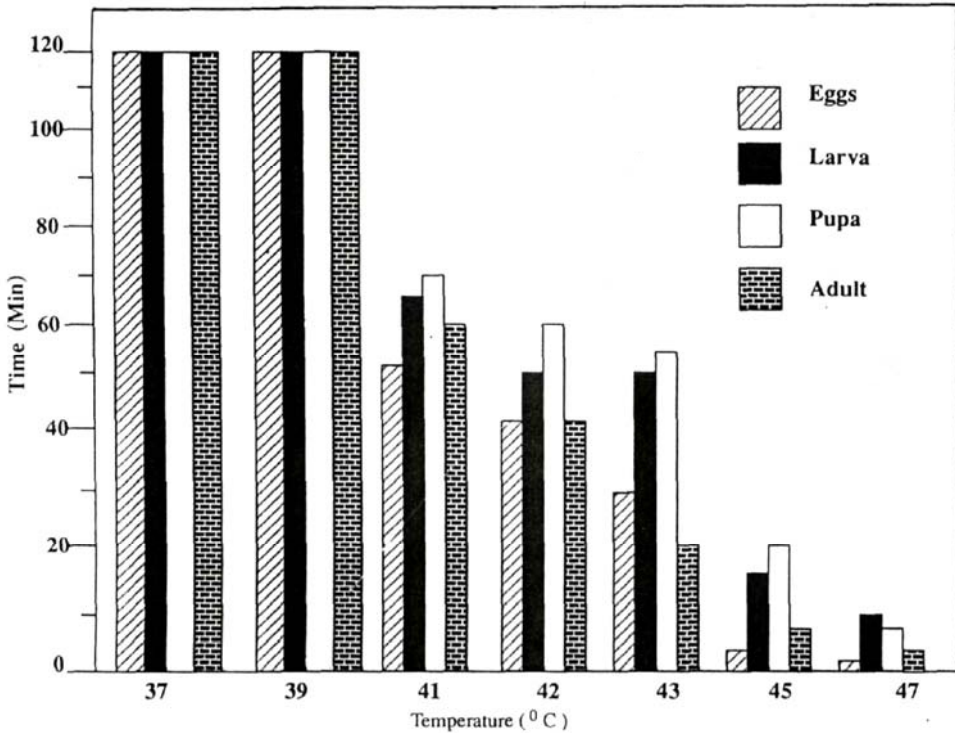
Figure 1   Effect of high temperature exposure on survival of silkworms.

The multivoltine race of *B. mori*, strain *C. Nichi* was reared in the laboratory between 24–26°C. At different life stages of the organism, viz., eggs, larvae (fifth instar, day 3), pupae (2 days old and removed from the cocoon) and the adult moths were exposed to different temperatures from 37°C to 47°C for varying times (within 2 to 5 min increments at any given temperature to record 100% survival) as shown. The survival in the case of larva was judged by its capacity to enter the succeeding moult or to spin cocoon depending on the age of larvae. Hatchability was taken as the criterion for eggs. The survival of pupae was assessed by adult emergence and that of adult by successful copulation and egg laying.

larvae in the 41–43°C range in both multivoltine and bivoltine races when exposed for different times. Therefore, this age of larvae was chosen for all further heat shock studies.

3.2 *Physiological changes*

The heart beat (per min) of larvae ranged from 54 ± 3 in multivoltine to 58 ± 3 in bivoltine races. On exposure to high temperature, the heart beat rates increased in both races, the most pronounced one being the multivoltine *Pure Mysore* strain (table 1). Temperatures of 43°C or more, however, reduced the heart beat rate and the larva become placid. The haemolymph pH (6·4, in all three strains examined) and volume (1·0 ±0·1 ml in *C. Nichi* and *Pure Mysore,* and 1·6 ±0·1 ml in *NB4D2*)
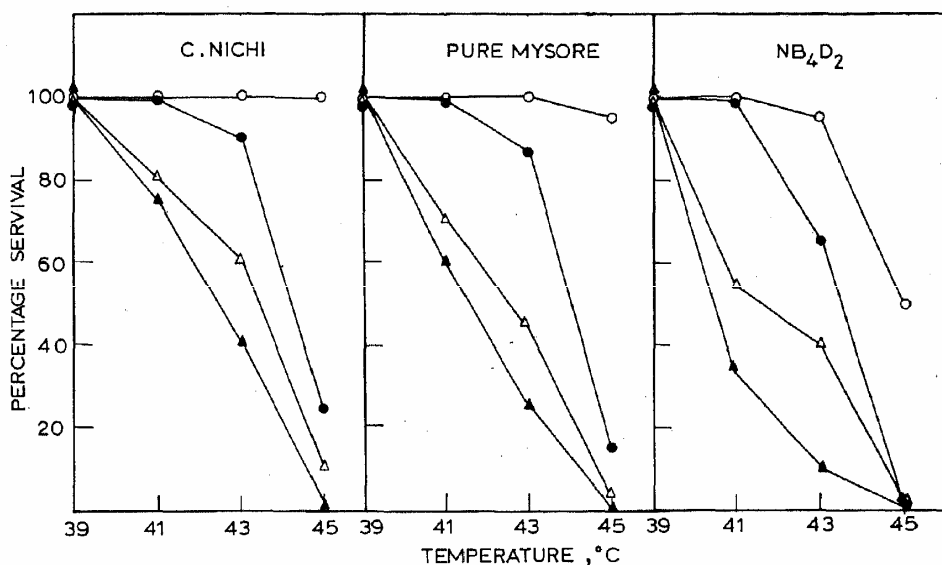
Figure 2. Thermal tolerance for varying exposure times.

Silkworm larvae (multivoltine, strains *C. Nichi* and *Pure Mysore,* and bivoltine, strain *NB4D2*) normally reared at 24–26°C up to the fifth larval instar (3 days) when exposed to 39°, 41°, 43° and 45°C for varying times (30, 60, 90 or 120 min) as indicated. After heat shocking the larvae were brought back to normal rearing temperature. Survival of the larvae was assessed by their capacity to spin cocoons. Duration of exposure at different temperatures is indicated by: (O) 30 min; (●) 60 min; (Δ) 90 min and (▲) 120 min

Table 1. Effect of temperature shock on physiological parameters.

| Silk worm strain	Population of haemocytes							
	Granulocyte (%)		Plasmatocyte (%)		Oenocytoid + sperulocyte (%)		Heart beat (per min)	
	26°C	41°C	26°C	41°C	26°C	41°C	26°C	41°C
C. Nichi	46 ± 4	40 ± 6	47 ± 6	53 ± 11	7 ± 1	7 ± 2	$54 \cdot 1 \pm 3 \cdot 6$	$79 \cdot 6 \pm 7 \cdot 3$
Pure Mysore	44 ± 2	30 ± 9	48 ± 8	53 ± 11	8 ± 2	8 ± 1	$54 \cdot 8 \pm 3 \cdot 3$	$87 \cdot 7 \pm 5 \cdot 1$
NB4D2	47 ± 5	43 ± 11	44 ± 10	48 ± 14	9 ± 1	9 ± 1	$58 \cdot 5 \pm 3 \cdot 4$	$82 \cdot 6 \pm 4 \cdot 4$

The different strains of silkworm *B. mori,* reared at 24–26°C were exposed to 41 °C for 1 h and the physiological parameters were enumerated. The number of haemocytes per mm^2 *NB4D2, Pure Mysore* and *C. Nichi* on an average was 1642, 1730 and 1875 respectively. The percentage of different populations of haemocytes was calculated on this basis.

did not show variations consequent to heat shock. The total protein contents in different tissues or haemolymph were also not affected by heat shock (data not shown).

The total haemocyte population did not show much variation in the heat shocked larvae. However, the differential count of plasmatocyte and granulocyte showed variations, the former decreasing by 8–12% and the latter showing a corresponding

increase, in all three strains (table 1). The counts of spherulocyte + oenocytoids did not change. The haemocytes from control larvae always showed a characteristic feature of crowding when a droplet of the haemolymph was placed on a glass slide but this character was significantly reduced in haemolymph from heat shocked larvae. The haemocytes of bivoltine *NB4D2* were generally larger than their counterparts from the multivoltine C. *Nichi* (figure 3). The size of granulocyte increased on heat shock in both races, but the enlargement was more prominent in the latter. In *C. Nichi,* on an average the granulocyte diameter increased from $3\cdot5 \pm 0\cdot8\mu m$ to $5\cdot1\pm 0\cdot4\mu m$ whereas in *NB4D2,* the increase was from $5\cdot1 \pm 0\cdot6$ μm to $5\cdot5\pm0\cdot9\mu M$.

In pupae (removed from cocoon), exposure to high temperature increased the movement of the posterior part of the body. Beyond 43°C, the adult emergence was reduced to 5%. The adult moths showed enhanced wing fluttation on increase in environmental temperature and fecundity was reduced to 30% by 41°C. The eggs kept above 41°C changed to a brick red colour and the hatchability was reduced to zero.
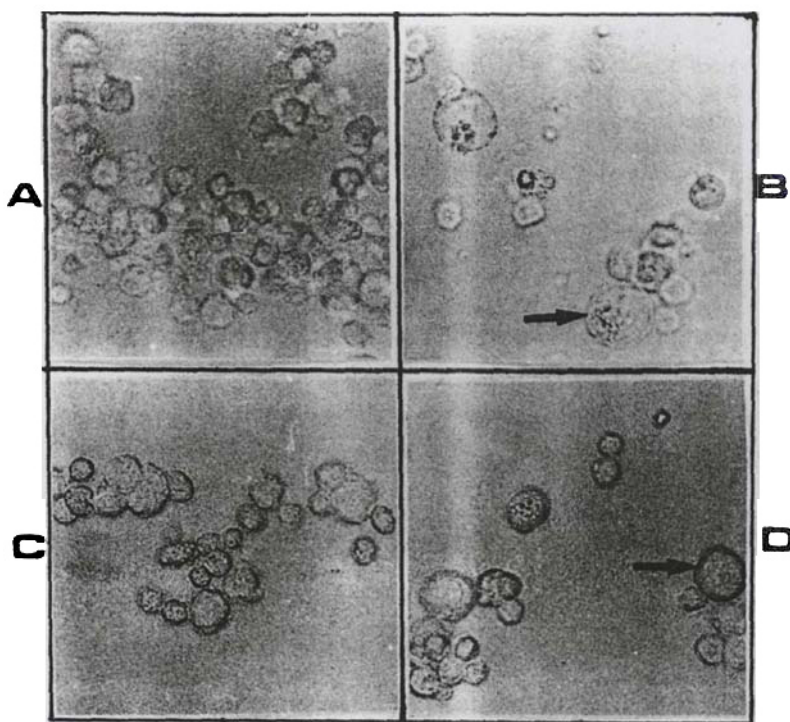
Figure 3. Haemocytes from control and heat shocked larvae.

The larvae (fifth instar, day 3) were subjected to a thermal shock of 41°C, 1 h and the haemolymph was collected immediately. The haemocytes were isolated by centrifugation at 5000 *g* and examined under phase contrast microscope (X 1600). The enlargement in granulocytes is clearly seen (indicated by arrow).

Haemocytes, from *C. Nichi.* (**A, B**) and *NB4D2* (**C, D**), control and heat shocked.

3.3 *Protein profiles from heat shocked larvae*

3.3a *Haemolymph:* The protein profiles of haemolymph from heat shocked larvae in comparison to the control (nonheat shocked) or those allowed to recover from the heat shock are presented in figure 4 (A–C). A total of 12–15 protein bands were present in *C. Nichi, Pure Mysore* as well as *NB4D2* strains. The appearance of new 93 kDa protein band consequent to exposure to high temperature was clearly discernible in all of them but their kinetics differed. In *C. Nichi* this protein band appeared immediately on exposure to 41°C and its level remained high during the 2–4 h recovery time at 26°C following the heat shock. In *Pure Mysore* and bivoltine *NB4D2* strains, the 93 kDa protein band could be seen only after 2 h recovery from thermal shock. There were no noticeable changes in any of the other protein bands. Exposure of larvae to temperatures up to 39°C for 1 h did not result in any change in the haemolymph protein profiles compared to the controls.

The appearance of the 93 kDa protein band in the haemolymph of *C. Nichi* was confirmed also by *in vivo* [^{35}S]Met labelling studies (figure 4A, lanes 5 and 6). In contrast to the *in vivo* result, when the haemolymph isolated from control larvae (reared at 26°C) was exposed to 41°C for 1 h (*in vitro* heat shocking) the heat shock resulted in protein degradation.

Since the HSPs are known to be nonsecretory, the appearance of a new protein band (93 kDa) in haemolymph was unexpected. In order to see whether this protein band had arisen from the haemocytes which populate the haemolymph, the protein pattern of the isolated haemocytes from such heat shocked larvae was examined. Only a few protein bands were seen in haemocytes but the appearance of a new 70 kDa protein band was conspicous in *C. Nichi* haemocytes, 2 h post recovery from thermal shock (figure 4D, lanes 1–3). This protein band was clearly absent in *NB4D2* haemocytes (figure 4D, lanes 4–6). However, the 93 kDa protein band was not seen in either race.

3.3b *Heat shock proteins from fat bodies:* The protein profiles of fat bodies derived from control larvae and those exposed to 41°C for 1 h, of multivoltine *C. Nichi* and bivoltine *NB4D2* races are presented in figure 5. As seen in the haemolymph, the most characteristic change was in the appearance of the 93 kDa band in both *C. Nichi* (figure 5A), and *NB4D2* (figure 5B) following heat shock. In addition, a 89 kDa band was also observed. In *C. Nichi,* the 70 kDa protein was detectable in the control larvae, which increased on heat shock and stayed at high levels during the entire recovery time (figure 5A). Although the 70 kDa band was apparent also in the bivoltine *NB4D2,* the changes in its level on heat treatment did not vary as conspicously as in *C. Nichi.* The fat body proteins from larvae heat shocked at 39°C also did not show any change in their profiles. Subjecting the isolated fat bodies from control larvae to heat shock for 1 h at 41°C, again resulted in protein degradation as was observed in the case of haemolymph.

In vivo labelling with [^{35}S]Met during heat shock confirmed the 89 and 93 kDa proteins in fat bodies of *C. Nichi* although no prominent change was apparent in the level of 70 kDa protein (figure 5A, lanes 5 and 6).

3.3c *Cuticles:* The cuticle protein pattern from the *C. Nichi* strain is presented in figure 6. The protein profiles were more complex due to the presence of larger

number of protein species. Nevertheless, in C. *Nichi,* the presence of additional protein bands at 93 kDa, 46 kDa and 28kDa immediately on exposure to 41°C was distinct. The 70 kDa protein band was clearly seen but did not show much change in the level on heat shock. The appearance of these protein bands in *C. Nichi* following heat shock was also confirmed from the *in vivo* [35S]Met labelling studies (lanes 5 and 6). These changes were similar in cuticle proteins from the *Pure Mysore* and *NB4D2* strains (data not shown).

3.3d *Silk gland proteins:* The silk gland proteins from the heat shocked larvae did not show any clear changes in the protein profiles. Fifth instar larvae start synthesizing large quantities of proteins from third day onwards and these proteins
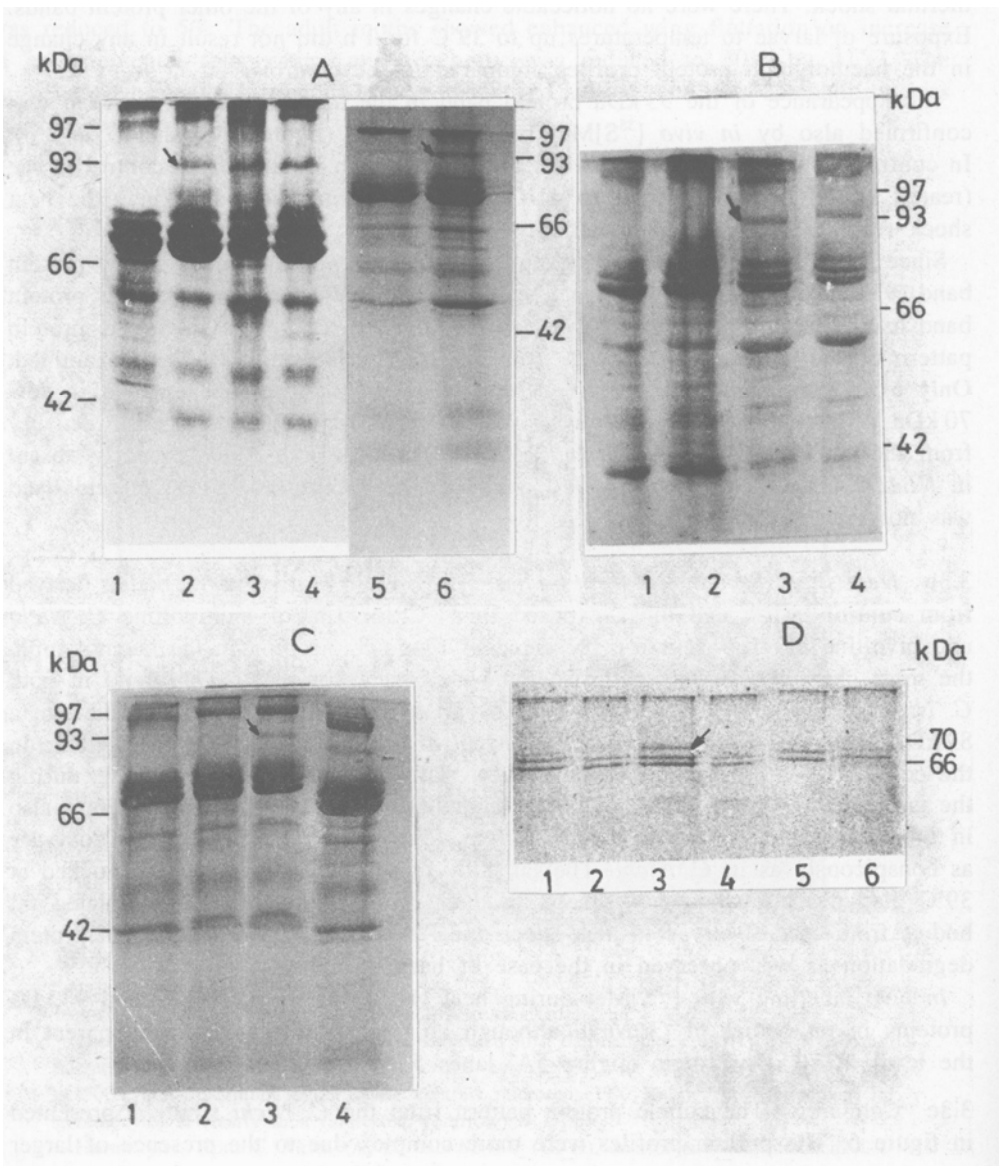
could have masked the HSPs. Alternatively, the absence of HSPs could be attributed to the silk glands not sensing the shock due to their location within the larvae, covered by haemolymph, fat body and cuticle. Due to such insulation, the heat stress may not even reach the silk glands, when the whole larvae is subjected to heat shock. The absence of HSPs in the silk gland could be a consequence of this and may not reflect the inability of the tissue to synthesize them. However, it should be noted that the haemocytes from *C. Nichi* though not *NB4D2* showed the presence of HSP.

3.4 *Heat shock response in pupae and adult moths*

The protein profiles of pupae and the adult moths of *C. Nichi* when exposed to 41°C for 1 h are presented in figure 7(A,B). At both stages, the 93 kDa proteins appeared immediately on exposure to the high temperature. In adult moth, additionally the appearance of a 56 kDa band was clear (figure 7B, lane 2 and 3). Further, some of the low molecular weight protein bands nearly disappeared after heat shock of the moth.

4. Discussion

Exposure of an organism or tissue to temperatures higher than the optimal growth temperature disturbs their normal function, ultimately resulting in death. An early consequence of heat shocking is the appearance of a set of highly conserved proteins, the HSPs, ubiquitous to all organisms examined so far. The HSPs are considered to confer protection against the adverse effect of heat shock (Hightower 1991). The susceptibility of economically important organisms such as the silkworms to environmental fluctuations of temperature, humidity etc., attain significance in their field-rearing for the commercial production of silk. The three strains of *B. mori* examined here, viz., *C. Nichi* and *Pure Mysore* of the multivoltine race, and *NB4D2* of the bivoltine race show different levels of tolerance for exposure to

Figure 4. Haemolymph proteins from heat shocked larvae.

Silkworm larvae (fifth instar, day 3) were exposed to 41°C for 1 h. The haemolymph was collected and the proteins were separated on SDS-PAGE (12·5%). The proteins were stained with Coomassie brilliant blue (lanes 1–4). For *in vivo* labelling of haemolymph proteins [^{35}S]Met (60 µCi/animal) was injected to *C. Nichi* larvae (fifth instar, day 3) half way through the heat shock at 41°C for 1 h. The haemolymph was collected immediately after heat shock and subjected to SDS-PAGE (12·5%). For controls the silkworms maintained at 26°C were injected .with [^{35}S]Met (60 µCi/animal) and the haemolymph was collected after 30 min. The labelled proteins were detected by fluorography (lanes 5 and 6 in figure 5A). (**A**) *C. Nichi*; (**B**) *Pure Mysore*; (**C**) *NB4D2*. Lanes: 1, control (no heat shock); 2, immediately after heat shock (no recovery time); 3 and 4, recovery at 26°C after heat shock for 2 and 4h respectively. Lanes 5 and 6 (figure 5A), labelled haemolymph proteins from control and *in vivo* heat shocked larvae. The 93 kDa protein is indicated by arrow. (**D**) Haemocyte proteins. The haemocytes were isolated from control or heat shocked (41°C, 1 h) larvae (fifth instar, day 3), lysed and the proteins were analysed on SDS-PAGE (7·5%). Lanes 1–3, haemocyte proteins from C. *Nichi,* control (no heat shock), immediately after heat shock and 2h recovery after heat shock, respectively; Lanes 4–6, haemocyte proteins from *NB4D2,* control (no heat shock), immediately after heat shock and 2h recovery after heat shock, respectively. The 70 kDa protein band is indicated by arrow.

temperatures higher than their normal growth temperature. *C. Nichi* was the most tolerant of the three while the exotic strain *NB4D2* was most labile, as judged by their survival and capacity to spin cocoons after a 1 h heat shock. Temperatures above 43°C proved lethal for all of them. These observations are in general agreement with the commercial field rearing experience on the silkworms. The sensitivity to thermal exposure was also dependent on the life stages of the silkworm.
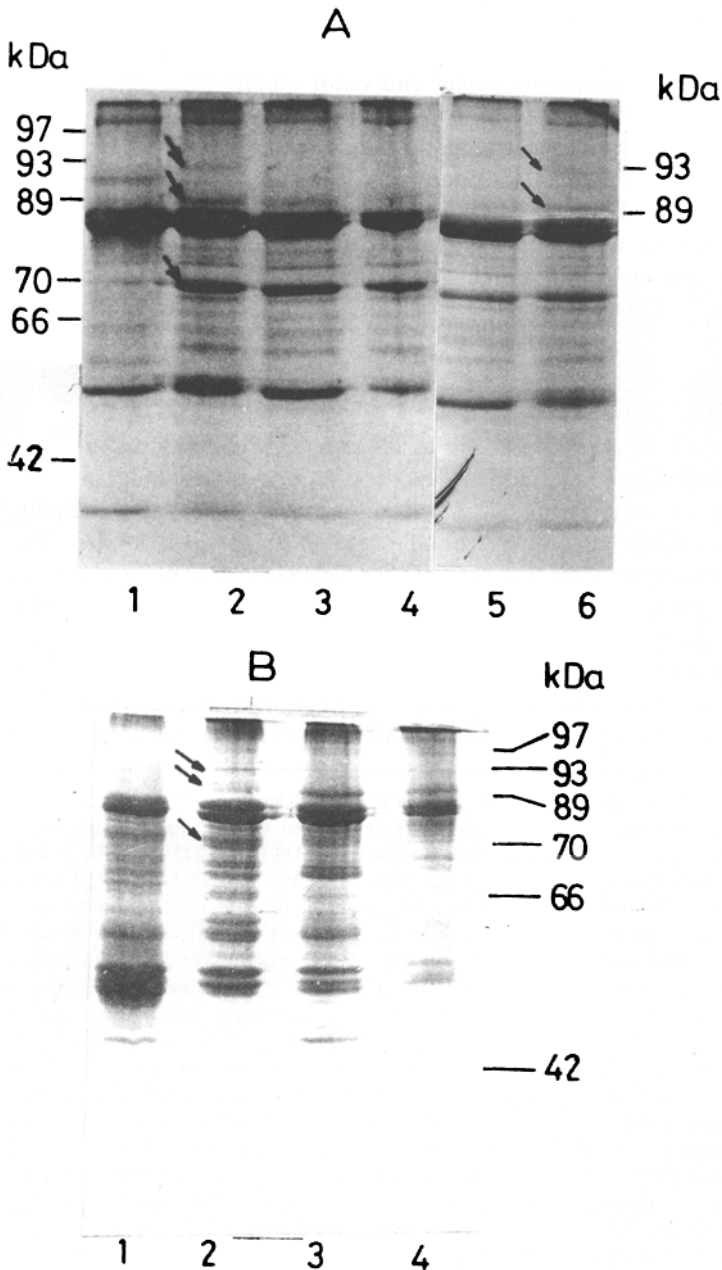
The fifth instar larvae and the pupae were more tolerant compared to the young larvae or the adult moths. The eggs were also very sensitive to temperature of 39°C and above, when the hatchability was reduced to zero.

Having assessed the differences in thermal tolerance of the various races, the effect of heat shocking the whole larvae on various physiological parameters, and the protein profiles of individual tissues or haemolymph of *B. mori* were analysed on a comparative basis. The approach used here is different from those attempted previously which were confined mostly to cells maintained in cultures. In haemolymph, fat bodies and cuticle, the appearance of a 93 kDa protein consequent to heat shock was evident in the multivoltine and bivoltine races of silkworms analysed. However, there were differences in the time of their appearance, whether immediately on heat shock or after permitting them to go through a recovery period. The protein band made the appearance instantaneously after the heat shock in haemolymph of the more thermotolerant *C. Nichi* compared to the bivoltine *NB4D2* where the protein appeared only after 2 h, during recovery. The 70 kDa protein was present constitutively in fat body and cuticle of both races. On heat shock, the levels of 70 kDa protein showed substantial increase in the fat bodies, from *C. Nichi.*

The fat body is the reservoir and active synthetic tissue for most of the haemolymph and cuticular proteins (Shigematsu 1968; Wyatt and Pan 1978; Tojo *et al* 1981; Plantevin *et al* 1987). Only a small number of haemolymph proteins are synthesized by the haemocytes (Palli and Locke 1987; Shigematsu 1968; Dean *et al* 1985; Wyatt and Pan 1978). In the haemocytes from heat shocked *C. Nichi,* only the 70 kDa. was heat induced but the 93 kDa protein was absent. There were no signs of haemocyte lysis or damage consequent to heat shocking. Since the 93 kDa protein is seen after heat shock in the haemolymph, fat bodies and the cuticle, it is likely that the protein is synthesized in the latter tissues and found its way into the haemolymph. But so far, none of the HSPs have been shown to be secretory in nature. The haemolymph being an open circulation system most of the tissues including the fat bodies and silk glands lie bathed in haemolymph within the *B. mori* larvae. Consequently several proteins synthesized in the fat body find their way into the haemolymph (Shigematsu 1968; Dean *et al* 1985). Even nonsecretory proteins from the fat bodies may thus enter the haemolymph due to the immersion of the former in the haemolymph. This is a most likely reason for the presence of 93 kDa protein in haemolymph.

The differences in the pattern of expression of HSPs in different tissues of silkworm, seen here are quite unusual. A complex regulatory pattern is seen in the

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Figure 5. Effect of heat shock on fat body proteins.

Silkworm larvae (fifth instar, day 3) were subjected to heat shock at 41°C for 1 h and the fat bodies were dissected out. The total proteins were extracted in homogenization buffer and subjected to SDS–PAGE (12·5%). For *in vivo* labelled proteins from fat bodies, *C. Nichi* larvae were injected with [^{35}S]Met (60 µCi/animal) midway during heat shock at 41°C for 1 h, and the fat bodies were isolated immediately afterwards. For controls, the animals were injected with [^{35}S] Met and maintained at normal rearing temperature (26°C) for 30 min before dissection. The positions of 93 kDa, 89 kDa and 70 kDa proteins are indicated by arrows.

(**A**) *C. Nichi;* (**B**) *NB4D2.* Lanes: 1, control larvae; 2, immediately after heat shock; 3 and 4, recovery at 26°C for 2 and 4 h respectively after heat shock. Lanes 5 and 6 (in **A**), labelled proteins from fat bodies of control and *in vivo* heat shocked larvae.
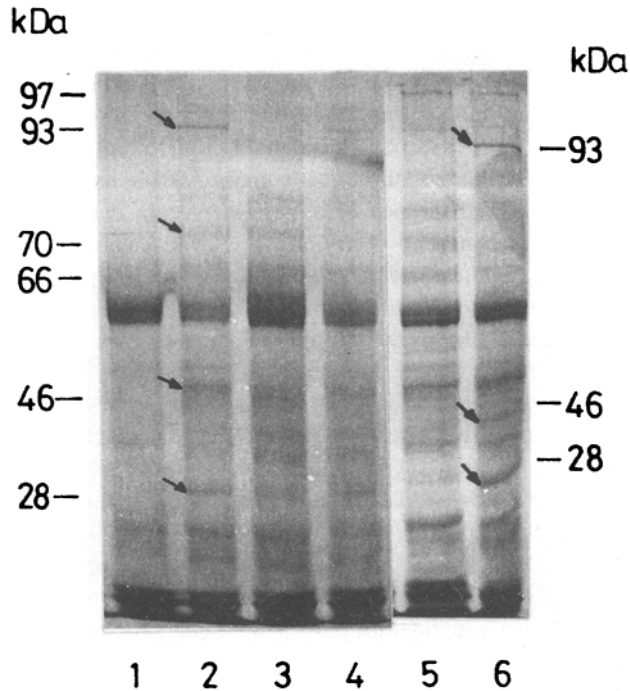
Figure 6. Cuticular proteins.

B. mori larvae (fifth instar, day 3), *C. Nichi* strain, were subjected to heat shock at 41°C for 1 h and the cuticles were separated out after removal of the internal tissues and organs. The cuticular proteins were extracted by homogenization and separated by SDS-PAGE (12·5%). Coomassie blue staining (lanes 1–4). For *in vivo* labelling of cuticular proteins, the experimental details were as under figure 5. The *in vivo* labelled cuticular proteins from *C. Nichi* separated on SDS-PAGE (12·5%) were subjected to fluorography.

Lanes: 1, control (no heat shock); 2, immediately after heat shock; 3 and 4, recovery for 2 and 4 h at 26°C following heat shock; 5 and 6, [^{35}S]Met labelled cuticle proteins from control (no heat shock) and immediately after heat shock. The arrows indicate the 93 kDa, 70 kDa, 46 kDa and 28 kDa bands.

case of *hsp* gene expression in the flesh fly, *Sarcophaga crassipalpis* (Joplin and Denlinger 1990). Here the expression of the different HSPs in different tissues vary depending on the stage of development or even the temperature at which the exposure was given. Coulon-Bublex and Mathelin (1991) had reported the appearance of a 70 kDa stress response protein after heat shock or acid treatment of the diapausing embryos of *B. mori,* which was also present during deep diapause.

HSPs provide protection to cells against the heat induced damage (Pelham 1986) and the synthesis of HSPs is related to heat resistance (Bosch *et al* 1988; Lohmann and Riddiford 1990). Thermotolerance is expressed as an attenuation of damage formation or as a better repair of protein damage, termed facilitated recovery (Kampinga 1993). The total concentration of HSPs (both constitutive and inducible) and their redistribution to specific subcellular sites are considered as more important factors in the acquisition of thermotolerance (Kampinga 1993). In this respect, the
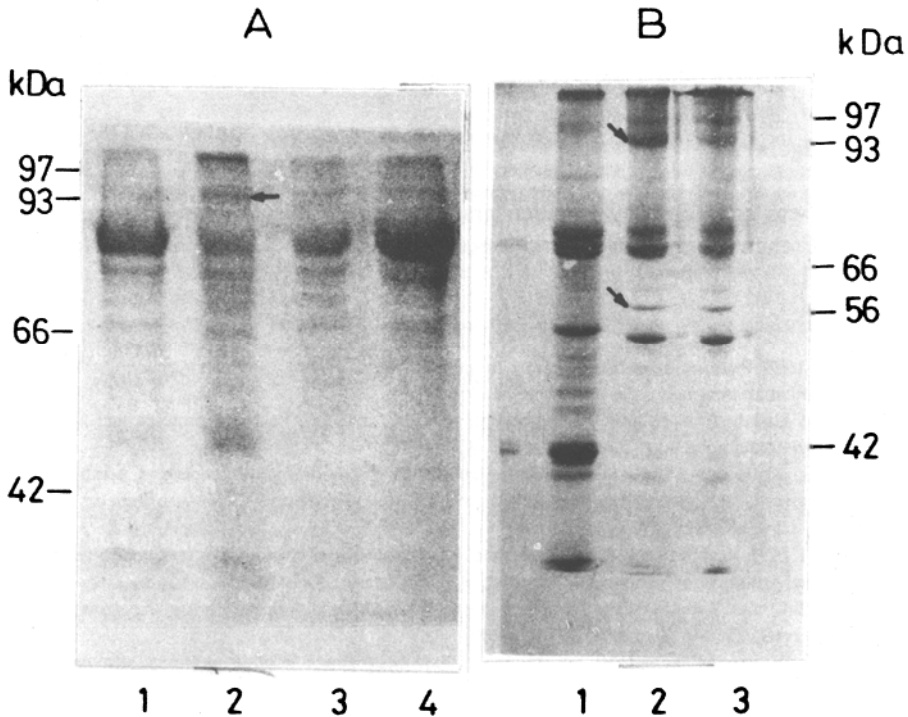
Figure 7. Heat shock response in *B. mori* pupae or adult moths.

C. Nichi pupae (2 day old) removed from the cocoon (**A**), or the adult moth [on the day of emergence (**B**)] were subjected to heat shock at 41°C for 1 h. The wings, legs and head were clipped off from the adults before protein extraction. The pupae and adults (an equal number of males and females mixed) were frozen in liquid nitrogen and the proteins were extracted by grinding thoroughly with homogenization buffer. The debris was removed by centrifugation at 8000 g and the supernatant was subjected to SDS-PAGE.

Lanes: 1, control; 2, immediately after heat shock; 3, recovery at 26°C for 2 h following heat shock; 4 (in A), recovery for 4 h at 26°C. The additional protein bands appearing after heat shock are marked by arrows.

presence of HSP in the haemolymph of *B. mori* can be considered as a desirable feature in conferring thermotolerance to the larvae.

Integuments consisting of the cuticle and the underlying epidermis are the external tissues which act as initial defense organ in most insects. Response of the integument to heat shock will therefore be an integral part of the survival of the silkworm. The appearance of 93 kDa, 70 kDa, 46 kDa and 28 kDa proteins was conspicous in all strains examined consequent to exposure to high temperature. The small molecular weight HSPs in cuticle of *Manduca sexta* have been speculated to provide an alteration in the relative water loss allowing evaporative cooling during heat shock (Lohmann and Riddiford 1992).

Our results here show that *in vitro* heat shocking of the isolated tissue or haemolymph at 41°C from control animals reared at 24–26°C, generally resulted in protein degradation whereas subjecting the whole larvae to thermal shock for

an identical time and temperature, resulted in the appearance of the additional protein bands. The synthesis of these proteins *in vivo,* in response to heat shock may therefore play important role in the thermal tolerance of the silkworms.

Acknowledgements

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Corresponding editor: M S SHAILA