Thermotolerance and the heat shock response in *Musca domestica*

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The relative abilities of larvae, pupae and adults of a laboratory population of the tropical housefly, *Musca domestica*, to withstand increasing environmental temperature (37°C to 48°C) were studied in terms of per cent adult survival/emergence. Pupal stage appeared best adapted to higher temperatures (44°C or 46°C), exhibiting significantly higher per cent survival as adult. In contrast, the tolerance of adult flies to higher temperature shock (44°C or 46°C) was poorer. A short period (1 h) of pre-conditioning at 37°C resulted in the development of considerable thermostolerance to higher temperatures (42°C to 46°C). Eight prominent heat shock polypeptides of approximate molecular weights 80, 73, 70, 64, 30, 28, 26 and 20.5 kD were induced upon *in vitro* exposure of the larval or adult tissues at 42°C to 46°C temperature. The 70 kD polypeptide appeared predominant in all the tissues at all temperatures. The pattern of expression (number and relative intensity) of various heat shock proteins at different temperatures of heat shock appeared to be related with the observed per cent values of adult survival at corresponding temperatures. Possible role of the heat-inducible proteins in thermostolerance and adaptation to severe seasonal fluctuations in environmental temperature has been discussed.

Stress response is considered to be the most conserved biological mechanism for maintaining cellular homeostasis during periods of normal cell growth and differentiation and during stresses caused by environmental temperature, heavy metals, amino acid analogues, certain pathophysiological states (e.g. inflammation, infection, ischemia, fever), etc. The heat shock proteins (HSPs), which are specifically synthesized upon exposure of the cells to such environmental insults, are considered to play a key role in keeping the cells composed during transient stresses. A brief exposure to mild heat stress prior to severe heat shock leads to development of thermostolerance, and hence an increased survival at lethal temperatures.

In recent years, considerable interest has emerged on the evolutionary significance of environmental temperature which has an important bearing on ecological adaptations. Temperature is considered as an important environmental factor that influences habitat selection and thus the species distribution, especially in ectotherms. The heat shock response has been assigned significant roles in development of thermostolerance and thermal adaptation in tropical conditions where seasonal fluctuations in ambient temperature are in extreme range as in mid-summer. Our understanding of the regulatory mechanism of thermal adaptation to ambient temperature is still in infancy. The problem of thermoregulation can be more serious in ectotherms whose body temperature is equilibrated with the environmental temperature and might lead to severe consequences if not immediately responded to. The mechanisms of cellular adjustments to sudden and transient thermal shocks involved in natural conditions and that active in *in vitro* cell cultures may not necessarily follow similar kinetics. Detailed probes to find different characteristics of heat shock response at the organisinal level and in natural or as mimicked thermal environment will augment the basics of the underlying mechanisms. Insects, particularly flies, have been regarded as one of the most suitable animal models for such investigations owing to their shorter life span and greater potential to adapt to a wide range of environmental stresses.
This report is based on our observations in respect of (i) the assessment of the relative levels of tolerance of tropical *M. domestica* to wide range of environmental temperatures, as experienced during summer months at different stages of development and (ii) the cellular mechanism to counteract thermal injury caused by extreme ambient temperature.

**Materials and methods**

**Fly culture**

An established colony of *M. domestica* reared in the insectary at 24°C–26°C was used for the experiments.

**Thermotolerance**

Young flies (2 days post eclosion, *n* = 15), pupae (pharate adults, *n* = 25) and larvae (third instar wandering stage, *n* = 25) were separately placed in thin-walled glass tubes and given heat shock in water baths set at 37°C, 42°C, 44°C, 46°C and 48°C for 30 min to 180 min, directly or after pre-treatment for 1 h at 37°C. Each set was taken in triplicate. After heat shock they were allowed to recover at normal temperature and the surviving flies were counted to assess survival percentage (% ± SE). Parallel sets of unstressed individuals were taken as controls.

**Physiological response**

Tissues from third instar wandering stage larvae (salivary glands and Malpighian tubules) and adults (brain, Malpighian tubules, ovaries and testes) were excised in Poels’ salt solution* and heat shocked for 1 h at various temperatures as mentioned above. During the last 30 min of the heat shock, incubation medium was replaced with pre-warmed fresh medium containing 35S-methionine (Act 100 μCi/ml, BRIT, Bombay). Tissues were dissolved in sample buffer (50 mM Tris.HCl, pH 6.8, 2% SDS, 10% glycerol, 100 mM DTT, 2mM PMSF and 0.1% bromophenol blue) by boiling in a water bath for 5 min. For comparison, labelled heat shocked (1 h, 37°C) samples of larval salivary glands or adult ovaries of *Drosophila melanogaster* and control unstressed larvae and adult tissue samples from *M. domestica* were also prepared as above.

Labelled polypeptides were separated on discontinuous SDS-PAGE and the vacuum-dried gels were processed for fluorography as described earlier. The approximate molecular weights of the heat shock proteins (HSPs) were calibrated with reference to the co-migrating standard molecular weight markers (Sigma, USA) and *Drosophila* HSPs in the parallel lanes.

**Results and discussion**

**Thermotolerance during development**

The level of tolerance of *M. domestica* larvae, pupae or adults to varying environmental temperature was recorded as per cent adult emergence (for larva or pupa) or adult survival (for adults, regaining of normal activity following recovery). Exposure of the larvae, pupae or adults to 37°C for even a prolonged period (more than 4 h) had no visible effect, however, 42°C (1 h or more) was found critical to the adults but not for the larvae or pupae (Figure 1 a–c). Increase in heat shock temperature to 44°C or above (46°C–48°C) severely affected survival at all stages of development (Figure 1). Even a very short exposure at 48°C (15 min) resulted in a total mortality (data not shown). Pre-treatment of the larvae, pupae or adults to 37°C prior to heat shock at 42°C, 44°C or 46°C, considerably increased the number of surviving individuals as active adults (Figure 1). In case of larvae or adults, however, it was observed that pre-conditioning did not cause any significant change in survival once the extent of thermal stress was significantly above the tolerance level (e.g. 46°C for larva and 44°C for adult; Figures 1 a and c). In contrast, pupae were found much more tolerant to higher temperature shock (44°C or 46°C) and exhibited significant thermotolerance when preconditioned (Figure 1 b). The hard external puparia acting as an effective protective shield and significantly low metabolic status are likely to account for such a high level of thermotolerance in pupae. Earlier observations in *Chironomous* and *Lucilia cuprina* also reported pupae to be much better adapted to higher ambient temperature compared to the larvae or adults. Thus the present findings on the relative levels of adult survival or tolerance during development at varying degrees of thermal stress demonstrate the potential of *M. domestica* to withstand a wide range of thermal fluctuations as experienced in the field during the summer months.

**Heat shock proteins**

Two of the major tissues selected for analysis of the heat shock proteins are the salivary glands (larva) and Malpighian tubules (larva and adult). Both are known to be functionally most active tissues. Adult brain was taken as a differentiated non-mitotic cell type. Testis and ovary are the reproductive organs and disruption in their function might affect fecundity and, to a certain degree, the survival. Mild heat shock partially inhibits general protein synthesis but high temperature shock affects appreciably the ongoing protein synthesis. The selective and increased synthesis of the heat shock proteins during severe temperature shocks therefore,
appear to have a significant role in stabilizing the general translational machinery during the period of heat stress. A total of eight prominent heat inducible polypeptides were observed in various larval and adult tissues examined. The approximate molecular weights of the common polypeptides were 80, 73, 70, 64, 30, 28, 26 and 20.5 kD. Though the presence of a low level of 73, 28, 26 and 20.5 kD HSPs was also noted in unstressed control tissues, their intensities were found to be increased considerably upon heat shock (Figures 2 and 3). Notably,
the major low molecular weight HSPs, such as 20.5, 26, 28 and 30 kD, in most of the larval (Figure 2a) and adult (Figure 3) tissues, appeared as prominent as 70 kD HSP at most of the heat shock temperatures. The optimal level of induction of most of these HSPs was noted at 42°C/44°C; it was much less at 46°C or 48°C except for some of the major HSPs like 80, 73 and 70 kD (Figures 2 and 3). Among these, the 70 kD polypeptide appeared as the most abundant class of HSPs and may possibly correspond to the HSP70 of Drosophila and other organisms. Immuno-characterization of this protein will more conclusively establish homology.

In addition to the common HSPs, larval salivary glands exhibited distinct expression of a 40 kD polypeptide (37°C-44°C) and two low molecular weight HSPs of 19 kD (at 37°C) and 18 kD (at 42°C) whose expression appeared not only specific to salivary glands but also was temperature dependent (Figure 2a). Low-level expression of several other polypeptides of molecular weights 62 kD (37°C-44°C), 59 kD (37°C and 42°C), 30 kD (37°C) and 29 kD (37°C and 42°C) were also noted (Figure 2a). None of these was observed at 46°C or 48°C. In addition, a 24 kD heat-inducible polypeptide was noted only at 37°C and 42°C. Its level, however, appeared relatively higher at 42°C than at 37°C.

Larval Malpighian tubules, however, showed a somewhat different pattern of expression of HSPs. The three major HSPs, 80, 73 and 70 kD were noted maximally induced at 42°C/44°C (Figure 2b). Significant expression of a 52 kD polypeptide was also noted. Interestingly, unlike in other tissues, instead of 73 kD HSP, synthesis of 70 kD HSP, was seen to be completely repressed at 48°C. Whether the 73 kD HSP is an isoform of 70 kD.

Figure 2. Synthesis of heat shock proteins in M. domestica larval salivary gland (a) and Malpighian tubule (b), heat shocked (1 h) at various temperatures (37, 42, 44, 46 and 48°C). For molecular weight reference, heat shocked (37°C/1 h) sample of Drosophila larval salivary gland is shown in lane DR (Drosophila, a). Approximate molecular weights are indicated in kD.
HSP and is relatively more thermostable than HSP70 in larval Malpighian tubules at higher temperatures, needs further investigation. Notably, a major (constitutive) protein of approximately 62 kD was synthesized at all temperatures at elevated levels in larval Malpighian tubules and even appeared more prominent than the 73 kD HSP at 48°C (Figure 2 b). Significantly, the level of low-molecular weight (LMW) HSPs in larval MTs was lower than that in the larval salivary glands or in either of the adult tissues. The general pattern of the HSP induction at 43°C in the adult Malpighian tubules was observed similar to that in the other tissues except that the synthesis of the 20.5 kD was reduced compared to other LMW HSPs. In this tissue two polypeptides of 62 kD (constitutive) and 64 kD (heat inducible) appeared to be very significantly labelled (Figure 3).

The general pattern of induction of HSPs at 43°C in the adult brain tissue was similar to that in the other tissues (Figure 3). The adult ovary or testis, heat shocked at 43°C, exhibited significant expression of all the HSPs as observed in larval salivary glands. Interestingly, the 64 kD protein showed an increased level of expression, particularly in the testis. However, the 62 kD polypeptide could not be detected either in the testis or in the ovary. No sex-specific variation in the HSPs could be found except that the 30 kD HSP appeared absent in the ovary while being a prominent LMW HSP in the testis (Figure 3). Selective and significant expression of the HSP64 in the adult tissues thus appears to be developmental stage (adult) specific.

The pattern of induction of different HSPs (number) and their relative levels at various temperatures (42°C to 48°C) was generally found to be related to the percent adult survivals at the corresponding temperatures. Positive correlation between the cellular concentration of the HSP70 and the degree of induced thermotolerance has also been reported for the cells in culture.\textsuperscript{19-22} In a recent observation on an intertidal mussel \textit{Mytilus trossulus},\textsuperscript{23} seasonal increase in the level of the endogenous HSPs has been indicated to confer thermotolerance. It should be noted that apart from the HSP70 and some other high molecular weight HSPs (e.g. HSPs 60 kD and 104 kD), role of certain low molecular weight HSPs (e.g. 28 kD and 30 kD) in the development of heat tolerance has also been implicated\textsuperscript{24-26}.

Pre-exposure to mild heat shock temperature is shown to induce a low level of some of the major heat shock proteins (as also noted in this study, see HSPs at 37°C), which are suggested to generate thermotolerance at higher temperature\textsuperscript{13,17}. The natural populations of housefly that

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\caption{Synthesis of heat shock proteins in \textit{M. domestica} adult brain, Malpighian tubule, testis and ovary heat shocked (1 h) at 37°C and 43°C. Approximate molecular weights are indicated in kD.}
\end{figure}
remain exposed to a wide range of thermal fluctuations during summer months, ranging from mild to severe heat shock conditions, may therefore, be capable of developing better resistance against severe ambient temperature.

The physiological response to heat shock in *M. domestica*, appears to be generally comparable to that observed in *Drosophila* or *L. cuprina*¹, except for some differences which may be attributed to species-specific differences in their genetic, behavioural and morphological characteristics.

Though the general pattern of HSPs in *L. cuprina* and *M. domestica* appeared similar, certain interesting differences were quite evident. Unlike in *L. cuprina*, where the Malpighian tubule-specific 62 kDa (a homologue of HSP60 family) and 64 kDa species of polypeptides were constitutive and not much affected by temperature shock¹ in *M. domestica* these polypeptides were heat inducible. Further, while the 62 kDa polypeptide was Malpighian tubule-specific, the 64 kDa HSP was seen, in addition to the Malpighian tubules, in adult reproductive tissues as well, although this protein was absent in the adult brain. In *Drosophila* also, a 64 kDa polypeptide was reported to be induced specifically by heat shock in Malpighian tubules only²². This polypeptide was found homologous to the HSP60 family proteins and although induced by heat shock only in the Malpighian tubules, it was constitutively present in all the tissue types of *Drosophila*²⁹. Though immunological identities of the 62 and 64 kDa HSPs in *M. domestica* are currently not known, we expect at least one of them to be a member of the HSP60 family. Significance of their tissue specificity remains to be examined.


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