Cloning & sequence identification of Hsp27 gene and expression analysis of the protein on thermal stress in *Lucilia cuprina*

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**Abstract**  Hsp27, a highly conserved small molecular weight heat shock protein, is widely known to be developmentally regulated and heat inducible. Its role in thermotolerance is also implicated. This study is a sequel of our earlier studies to understand the molecular organization of heat shock genes/proteins and their role in development and thermal adaptation in a sheep pest, *Lucilia cuprina* (blowfly), which exhibits unusually high adaptability to a variety of environmental stresses, including heat and chemicals. In this report our aim was to understand the evolutionary relationship of *Lchsp27* gene/protein with those of other species and its role in thermal adaptation. We sequence characterized the *Lchsp27* gene (coding region) and analyzed its expression in various larval and adult tissues under normal as well as heat shock conditions. The nucleotide sequence analysis of 678 bps long-coding region of *Lchsp27* exhibited closest evolutionary proximity with *Drosophila* (90.09%), which belongs to the same order, Diptera. Heat shock caused significant enhancement in the expression of *Lchsp27* gene in all the larval and adult tissues examined, however, in a tissue specific manner. Significantly, in Malpighian tubules, while the heat-induced level of *hsp27* transcript (mRNA) appeared increased as compared to control, the protein level remained unaltered and nuclear localized. We infer that *Lchsp27* may have significant role in the maintenance of cellular homeostasis, particularly, during summer months, when the fly remains exposed to high heat in its natural habitat.

**Key words**  heat shock protein; Hsp27; *Lucilia cuprina*; thermal stress

**Introduction**

In an organism, cells and tissues often encounter variety of environmental stresses, including high temperature, which destabilizes the cellular architecture and protein homeostasis. The heat shock response, an evolutionarily highly conserved mechanism, is rapidly activated following exposure to thermal stress (Ritossa, 1962; Satyal & Morimoto, 1998) to cope with its deleterious effects by inducing a set of heat shock genes, encoding heat shock proteins (Hsps) or molecular chaperons (Ellis & van der Vies, 1991; Fulda et al., 2010). Among these Hsps, small or low molecular weight heat shock proteins (approximately 12–43 kDa) constitute a large family of Hsps (Scheibel et al., 1998; Stromer et al., 2003). The Hsp27 gene/protein, is a member of the small Hsp family, consisting of an amino terminal and a variable C-terminal domain of 80–100 amino acids, referred to as α-crystalline domain that participates in the oligomerization of the native form of Hsp27 (Lindner et al., 1998; Quinlan & van den Ijssel, 1999; Denlinger et al., 2001; Heikkila, 2003) The Hsp27 gene/protein sequence (nucleotide/ amino acid)
It was observed that apart from major heat shock proteins (e.g., Hsp70, Hsp60, etc.), several low molecular weight Hsps were also induced when larval and adult tissues were exposed to heat shock temperature, among which 27kDa protein appeared prominent. In this report our aim was to sequence characterize the *Lucilia hsp27* gene to reveal its evolutionary relation with those of other species and to understand its functional significance in germ-line and somatic tissues during normal development and under conditions of heat stress.

### Materials and methods

#### Fly rearing

Flies were reared and maintained in the insectary at 26 ± 2 °C ambient temperature with 50%-60% relative humidity and 12 : 12 h daylight and dark period for normal life cycle. The adult flies were fed on raw goat meat and sugar, and larvae were reared only on raw goat meat until pupation.

#### Genomic DNA isolation

The genomic DNA was isolated from male adult flies. Flies were homogenized in 100 mmol/L Tris-Cl (pH 8.0), 50 mmol/L EDTA, 150 mmol/L NaCl, and 1.0% SDS (Asburner, 1989). The homogenate was treated with 20 mg/mL of proteinase K (Promega, USA), followed by incubation at 37 °C overnight. Subsequently, phenol/chloroform/isoamyl alcohol (25 : 24 : 1) extractions were performed, followed by ethanol precipitation and drying of DNA pellet. The DNA pellets were re-suspended in 100 μL of Tris-EDTA (pH 8.0). RNA was removed by treatment with 20 μg/mL bovine pancreatic ribonuclease A (RNase A) at 37 °C in water bath for 30 min, followed by phenolization to remove RNase. The quality and quantity of total DNA was determined by agarose gel electrophoresis and by UV-visible absorbance.
spectrophotometer (Evolution 220, Thermo Fisher Scientific, USA).

**RNA isolation and cDNA preparation**

RNA was isolated from control and heat shocked larval salivary gland, Malpighian tubules and adult Malpighian tubules using Tri-reagent (Sigma, St Louis, MO, USA) according to manufacturer’s instructions. DNA was removed from RNA by RNase free DNaseI (1U/μL) (Fermentas, USA), followed by phenolization. The quality and quantity of RNA was determined by Nanophotometer™ P-Class (Implen, GmbH, Germany) and stored at -80°C.

cDNA was prepared from 1 μg of the isolated RNA by reverse transcriptase PCR. The RNA was reverse transcribed in 20 μL volume using superscript II reverse transcriptase (Invitrogen, USA) and 3 μg/μL random primer mix (Invitrogen, USA) as per manufacturer’s instructions.

**Isolation and amplification of Lchsp27**

Fly genomic DNA and cDNA was used for hsp27 gene amplification. The primers for amplifications were designed from hsp27 gene sequence of Drosophila (GenBank accession no. NM_079276.2) using an offline program Genetool. The primers were synthesized commercially (Bioserve, Hyderabad, India) and used for amplification. Two sets of primer sequences spanning the complete coding region are (i) forward (F) 5'-GTCAATTATACCCTCTGTCG-3' and reverse (R) 5'-AGTCCCATTTCTCTGCC-3'; (ii) forward 5'-CGTCGCCGCAGCAGCAGTCTA-3'; and reverse 5'-CTTCCTCTCCATGCACAGTCTT-3'.

The PCR amplifications were performed in 25 μL volume reaction mix using 100 ng of genomic DNA, PCR buffer with 1.5 mmol/L MgCl₂, 200 μmol/L dNTPs, 10 pmol/L of each primer, and 1.0 U Taq polymerase (Fermentas, USA). The cycling conditions were: initial denaturation for 2 min at 94 °C, denaturation at 95 °C for 30 sec, followed by annealing for 30 sec at 54.6 °C and extension for 1 min at 72 °C for 30 cycles. The amplified amplicons were run on 1% agarose gel to verify the presence of specific bands of predicted size. The intensities (quantity) of the bands (amplicons) were analyzed using image lab software (Bio-Rad, USA).

**Cloning, sequencing and alignment of Lchsp27**

The Lchsp27 PCR amplicons were cloned into a vector containing T overhang (pTZ57R/T) or using InstAclone PCR product Cloning kit (Fermentas, USA), following manufacturer’s instructions. The ligated product was transformed in E. coli (DH5α) by calcium–chloride mediated transformation (Sambrook & Russell, 2001) and positive colonies were identified using blue/white selection.

The recombinant colonies were used for plasmid isolation using mini plasmid kit (K0502, Fermentas, USA), following manufacturer’s instructions. Plasmid containing Lchsp27 insert was sequenced commercially (M/s Bioserve India Pvt. Ltd., India) using both forward and reverse primers of hsp27 (mentioned above). The obtained sequences were aligned with those of other hsp27 species to verify Lchsp27 sequence, and then submitted to GenBank for public access (Gene accession no. KJ840796).

**Bioinformatics and sequence homology analysis**

The NCBI, BLAST Alignment Tools (www.ncbi.nlm.nih.gov/blast.cgi) (Altschul et al., 1990) were used for alignment of Lchsp27 sequence with those of Drosophila, mice, rat, human and other dipteran species, using ClustalW2 (www.ebi.ac.uk/services/clustalw2) (Thompson et al., 1994). The accession numbers of the hsp27 gene from various species used for phylogeny study and blast analysis are: D. melanogaster (J01101.1), L. cuprina (KJ840796), C. riparius (KC495957), C. capitata (ACD76913.1), B. mori (KF547930.1), S. scrofa (AY789513.1), H. sapiens (AB020027.1), M. musculus (NM_0011647081), R. norvegicus (M86389.1), D. rerio (EU000061.1), C. lupus (KJ947842.1), X. laevis (DQ473544.1), T. tropicalis (NM_001079349), G. gorilla (XM_004045619), G. gallus (XM_001231557), P. tetrodogydes (XM_001167201.2), C. jacchus (XM_000761310), M. mulatta (XM_001257754.2). The phylogenetic tree and molecular evolutionary analysis was performed by using the programs Bio-Edit (Hall, 1999) and MEGA4 (Kumar et al., 2004, Tamura et al., 2011). The inferred amino acid sequence was analyzed with expert protein analysis system (http://www.ebi.ac.uk/clustalW2).

**Quantitative real time PCR**

The mRNA expression of Lchsp27 gene was analyzed by quantitative PCR (Q-RT PCR or real time PCR). A housekeeping gene, Q28S rRNA was used to normalize the values of mRNA with respect to hsp27 gene for quantification of the hsp27 transcript (Bagnall & Kotze, 2010). The primers used for quantitative expression analysis (Q-RT PCR) were, Q27F 5'-GTCCATGCCCCAGCAGTCTGTT-3' and Q27R 5'-CG-ACACATCCATGCACAGTCTT-3'. 28S rRNA (Q28S
primers Q28S forward 5'-CCAAAGAGTCGTGTTGC-TTG-3', and Q28S reverse 5'-ATTCAAGGTTCATCGG-GCTTTA-3') were used as an endogenous control. The larval and adult tissues were given heat shock at 42 °C for 1 h and the control tissues were incubated at room temperature (25 ± 1°C) and used for cDNA preparation.

The real-time quantitative PCR was performed in a final volume of 20 μL with the template diluted 10 folds (10 ng template [cDNA], 2.5 pmol/L each of forward and reverse primers, and 5 μL 2× Fast SYBR Green master Mix) (Applied Biosystem, Life technologies, UK). The PCR conditions were: denaturation for 30 sec at 94 °C, annealing for 30 sec at 52 °C and extension for 30 sec at 72 °C for a reaction of 30 cycles. A melting curve program was run immediately after the PCR program. For both control and heat shock samples, 3 separate reactions were set, each set was in triplicates to rule out any statistical bias. The amplification reactions were run in ABI 7500 Real-Time PCR System (Applied Biosystem, USA).

The obtained data was analyzed by using SDS 2.1 software, considering the parameters that include absolute quantification (standard curve, melting curve), relative quantification and background thresholds. The mean of triplicate samples were used to determine the threshold cycle (C_T) values, and relative expression of hsp27 in each sample was determined by ΔC_T. The fold change in expression level was estimated by 2^{(-ΔΔC_T)} method (Livak and Schmittgen, 2001), where the lowest C_T value for each gene was set to be 1 for each sample. For control (nonheat shock), ΔΔC_T was taken zero and thus, 2^{(-ΔΔC_T)} was one.

**Western blotting**

Western blotting was performed to detect the expression level of Hsp27 in different tissues of *Lucilia*. The larva/adult tissues were heat shocked at 42 °C for 1 h and lysed in the tissue lysis buffer (Tris lysis buffer; 1 mol/L Tris-Cl pH 6.8, 10% SDS, 20% glycerol and 5% β-mercaptoethanol). Simultaneously, the control sets of larval tissues were incubated at normal (room) temperature (25 ± 1°C) and then lysed in the lysis buffer. The concentration of protein in each sample was determined by Bradford’s method (Bradford, 1976) using BSA as standard. The polyepitides in the lysed tissue samples were separated on 12% denaturing gel and transferred to polyvinylidene fluoride membrane (PVDF, GE Healthcare, UK) by electroblotting. The membrane was incubated in blocking solution containing BSA, followed by incubation with Hsp27 (primary) monoclonal antibody (IAP-28, ab49919, Abcam, UK) in 1 : 800 dilution and anti β-actin antibody in 1 : 1000 dilution (C4, sc-7778, Santa Cruz Biotechnology, Inc, USA) for 1 h. After removing the primary antibody, followed by brief washing, the membrane was incubated with horseradish peroxidase (HRP) labeled antimouse IgG in 1 : 2000 dilution (SAB-100; Assaydesigns StressGen Biotechnologies, USA). The Hsp27 specific signals were visualized using enhanced ECL plus reagent (Amersham Bioscience, USA). Quantification and data analyses were carried out by using Image J software provided with the BioRad XR+ gel doc system that measures integrated density of bands nullifying the background intensity.

**In situ localization of Hsp27 protein**

The immune-localization of Hsp27 protein was done by immunofluorescence staining of control (25 ± 1°C) and heat shocked (42°C/1 h) larval/adult tissues following the method given in Ashburner (1989). The tissues were fixed in 4% paraformaldehyde, followed by washing in PBT (phosphate buffer with 0.1% tween-20) 3 times for 5 min each. After washing, the tissues were incubated in blocking buffer to reduce the nonspecific binding and incubated with primary antibody, anti-Hsp27 monoclonal (ab49919, abcam, UK), in 1 : 100 dilution at 4 °C overnight, followed by washing in PBT 3 times for 10 min each. Tissues were then incubated in secondary antibody, antimouse IgG TRITC conjugate (RTC-3, Bangalore Genei, Bangalore, India) in 1 : 200 dilution at room temperature for 1 h in dark, then washed thrice in PBT for 10 min each and mounted with Vectashield mounting medium that also contain DAPI (Diamidino-2-phenylindole) to counter stain nuclei. The slides were observed under Leica fluorescence microscope (Germany) at 554 nm/576 nm and 359 nm/461 nm excitation/emission range for TRITC and DAPI, respectively. All the images were assembled using Adobe Photoshop 7.0.

**Statistical tests**

The hsp27 gene expression and the level of protein were quantified by densitometry using Image J. Graphs were plotted using Sigma plot ver. 10. All quantitative data were expressed as mean ± SEM and comparisons between control and heat shocked samples were done using standard Student’s t-test. The data was subjected to homogeneity test before applying Student’s t-test. The statistical significance was defined as P value, where P ≤ 0.05. Three separate sets of experiments or reactions were carried out, each set was in triplicates for both control and heat shock conditions.
Molecular characterization of hsp27 in L. cuprina

Fig. 1 (A) Nucleotide sequence of hsp27 gene of L. cuprina. (B) Phylogenetic tree constructed from nucleotide sequences of hsp27 gene from different species (D. melanogaster [J01101.1], L. cuprina [KJ840796], C. riparius [KC495957], C. capitata [ACD76913.1], B. mori [KF547930.1], S. scrofa [AY789513.1], H. sapiens [AB020027.1], M. musculus [NM_0011674081], R. norvegicus [M85639.1], D. rerio [EU000061.1], C. lupus [KJ497482.1], X. laevis [DQ473544.1], X. tropicalis [NM_001079349], G. gorilla [XM_004045619], G. gallus [XM_001231557], P. troglodytes [XM_001167201.2], C. jaccus [XM_002761310], M. mulatta [NM_001257754.2]). The phylogeny was generated by using Phylip program of ClustalW2.

Results

Lucilia cuprina hsp27 gene/amino acid sequence identification, sequencing and alignment

The L. cuprina hsp27 gene sequence was isolated from Lucilia genomic DNA/cDNA using specific PCR primers designed from Drosophila hsp27 cDNA (Gene accession no: NM_079276.2). The obtained amplicon size was of 678 base pairs. The nucleotide sequence of the cloned Lchsp27 gene was confirmed by sequencing. The obtained sequence (Gene accession no: KJ840796) (Fig. 1A) showed initiation (16th bp) and termination codon (664th bp) and the conserved nucleotides. The Lchsp27 sequence was aligned with those of Drosophila, lower vertebrates and other eukaryotic species to determine its phylogenetic relationship with them.

The Blast analysis for sequence similarity showed that Lucilia hsp27 has maximum identity, score, and coverage (86.11) with Drosophila hsp27 as compared to those of other species (Fig. 1B). A search based on Blast analysis showed that LcHsp27 also belongs to the α-crystallin domain/sHsp superfamily. Sequence comparison between D. melanogaster, C. riparius, C. capitata, and H. sapiens revealed the base sequence from 271 to 504 to be relatively more conserved among these species that corresponds to α-crystallin domain of the Hsp27. The putative dimer interface on the conserved α-crystalline domains, as present in metazoans, showed 8 of 12 residues at position 217–273, 292–294, 298–300, 304–309, 418–420, and
481–486 to be conserved in Lucilia. The matched score between Lucilia and other species showed significantly decreased, particularly, with those of higher species, like human (47.27%), mice (45.54%), rat (43.96%), and zebrafish (42.43%) (Fig. 1B). Multiple sequence alignment was performed to determine sequence similarity between Lucilia and Drosophila hsp27 genes, which showed, apart from coding region, several nucleotide mismatches in the 5'–3' ends of the Lchsp27 transcript. However at 3' end of Lchsp27, fewer sequence substitution were observed, such as T to A change at 573th base position, insertion of T at 576th base, insertion of C at 588th base, G to C change at 593rd base, insertion of GCA at 595–597, C to A change at 599th positions, etc.

The deduced polypeptide sequence consists of a single open reading frame (ORF) of 226 amino acids with an approximate molecular weight of 24.31 kDa. Like nucleotide sequence, the amino acid sequence of LcHsp27 (Figs. 2A and B) was also analyzed using ClustalW2 multiple sequence alignment and amino acid blast between L. cuprina, D. melanogaster, and several other eukaryotic species, which showed maximum (86.25%) homology with Drosophila. Based on homology analysis, the LcHsp27 amino acid sequence from 86 to 163 was correspond to α-crystallin domain and at 86, 93, 95, 97–98, 135, 156, and 157 positions, with the putative dimer–dimer interface sequences. The amino acid sequence homology was found to be significantly reduced when compared with those of higher eukaryotic species, like human (29.27%), rat (28.78%), zebrafish (28.78%), and mice (20.45%) (Fig. 2B). The C-terminal region of LcHsp27 also showed variations in amino acid sequences with those of Drosophila and other vertebrate species.

Elevated temperature increases the induction of hsp27 and modulates its expression pattern in a tissue specific manner

In order to determine the pattern of expression of LcHsp27, both at mRNA as well as protein level, quantitative real-time PCR and immunofluorescence, respectively, were performed in larval and adult tissues exposed to heat stress at 42 °C for 1 h or incubated at 25 ± 1 °C (control). Quantitation of hsp27 transcripts in larval salivary glands (SGs) and Malpighian tubules (MTs) from 3rd instar larvae showed that the expression level of hsp27 was increased slightly upon heat shock as compared to that at control (RT) temperature (Fig. 3). 28S rRNA was taken as an internal loading control in each case. The relative fold change in the mRNA expression was determined by change in \( C_T \) that showed a significant increase in the hsp27 transcripts, almost 2 fold in larval Malpighian tubules (\( P > 0.02 \)) in larval salivary glands (\( P > 0.10 \)) and in adult Malpighian tubules (\( P > 0.10 \)) upon heat shock (Fig. 3). Fold change in expression level of Lchsp27 was calculated by \( 2^{(-\Delta\Delta C_{T})} \). The efficiencies of the reactions were 100%. The \( C_T \) standard deviation was determined by SDS v.2.1
Molecular characterization of hsp27 in L. cuprina

Fig. 4 Pattern and level of expression of Hsp27 in larval salivary gland (SGs) of L. cuprina under control and heat shock conditions. (A) and (D) show anti-Hsp27 antibody staining (red). Images in inset in (A)–(D) are at higher magnification (40×). Nuclei were counterstained with DAPI (blue) as shown in (B) and (E). (C) and (F) represent the merged images of the cells expressing Hsp27 (red) with DAPI (blue). Scale bar in (A) represents 50 μm (inset) and 10 μm is applied to images in (B)–(F). (G) is the western blot of protein from salivary gland of L. cuprina under control and heat shock conditions showing relative levels of Hsp27 (upper row). β-actin (lower row) was used as an internal loading control. Histogram in H indicates (mean ± SE, n = 3) level of Hsp27 relative to that in control sample. Paired Student’s t-test was performed in order to estimate significance level (*) by keeping P ≤ 0.05.

The results of immunofluorescence also supported the above observation. Hsp27 protein was seen to be expressed in cytoplasm as well as in the nucleus under normal conditions in 3rd instar larval salivary glands (SGs) (Fig. 4A). The protein was also found accumulated at the nuclear periphery. Heat shock remarkably elevated the level of Hsp27 protein in the cytoplasm with abundance in the salivary gland polytene nuclei (Fig. 4D). Western blots also showed elevation in the relative expression of Hsp27 protein upon heat shock. The difference in the expression level was also apparent from the relative intensities of immunofluorescence observed in heat shocked and normal control tissues (Figs. 4G and H).

Interestingly, the western blots of both control and heat shocked larval (Figs. 5M and O) or adult (Figs. 5N and O) Malpighian tubules (MTs) did not show any significant difference in the level of expression of Hsp27 protein. However, the immunofluorescence observations revealed an interesting pattern of intracellular localization of the protein. There was an increased presence (fluorescence intensity) of Hsp27 in the polytene nuclei, more so in the perinuclear space, upon heat shock, in both larval (Fig. 5D) and adult Malpighian tubules (Fig. 5J), as compared to their respective controls (Figs. 5A and G). Surprisingly, while the hsp27 transcript level was seen to be elevated upon heat shock (Fig. 4), no significant increase in the level of Hsp27 protein could be observed.

In larval/adult fat bodies, the expression level of Hsp27 protein was found significantly high, both in cytoplasm and in the nucleus, appearing as small granular structures when tissues were incubated at normal temperature (Figs. 6A and G). An increased amount of protein was observed near nuclear periphery in both larval and adult fat bodies, similar to that noted in larval salivary gland. Upon heat shock (42 °C for 1 h), fat bodies from larva showed elevated expression of Hsp27 in the cytoplasm and nucleus both (Figs. 6D and J). Interestingly, while the protein appeared diffused throughout (larval) fat body nuclei following heat shock, it appeared as granular structures inside the nuclei of nonheat shocked tissue (Fig. 6A). Adult fat bodies showed relatively higher constitutive expression of Hsp27 than larval fat bodies. In heat shocked

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adult fat bodies, Hsp27 was seen present in the nucleus and in cytoplasm both at a relatively higher concentration (Fig. 6J). The increase in the level of Hsp27 protein was further corroborated by western blots of both larval (Figs. 6M and O) and adult (Figs. 6N and O) tissues.

Immunostaining of developing oocytes (stages 4th–5th, 9th–10th, 11th–12th, and 13th–14th) from 10-d old flies also showed enhanced expression of Hsp27 protein upon heat shock in both oocyte as well as nurse cells (Figs. 7A, M, G, and S), when compared to their corresponding controls (Figs. 7D, P, J, and V). The enhancement in the level of Hsp27 protein in oocytes was further confirmed by western blots of proteins extracted from oocyte stages 0–4, 5–8, and 9–14 (Fig. 7Y), followed by densitometry to estimate the relative proportion of Hsp27 protein expressed in various stages of oogenesis (Figs. 7Y and Z). Further, the distribution pattern of Hsp27 protein in nurse cells and oocytes region following heat shock was also seen to be differential at different oocyte stages. For example, in stage 4–5, presence of Hsp27 was observed in both nurse cells and oocyte region at control temperature (Fig. 7A) (nonheat shocked), but its level was elevated upon heat shock in both the compartments (Fig. 7D). In stages 9–12, nurse cells showed predominance of Hsp27 staining upon heat shock (Fig. 7P). In stages 13–14, the expression of Hsp27 was modest in control conditions (Fig. 7S), but was found enhanced after heat shock (Fig. 7V).

In addition to salivary gland, Malpighian tubules and fat body, the expression level of Hsp27 was also examined in adult brain under normal as well as heat shock conditions (Fig. S1), which also showed elevated level of protein in brain cells upon heat shock as compared to controls (Figs. S1A and B).

Discussion

Our study has revealed several interesting features of hsp27 gene of L. cuprina (Lchsp27), including its evolutionary relationship with those of other species and
the likely role in thermal protection. The *Lchsp27* gene consists of a coding region of 678 bps. The *Lchsp27* nucleotide sequence alignment with other species showed maximum similarity with *Drosophila hsp27* (85.11), and relatively lesser with those of other species, like Mediterranean fruit fly (59.53%), chironomus (53.38%), human (47.27%), mice (45.54%), rat (43.96%), and zebrafish (42.43%). ClustalW2 multiple sequence alignment also showed increased sequence similarity between *L. cuprina* and *D. melanogaster*, both being members of the same insect order, that is, diptera. Similar to nucleotide sequence, the amino acid sequence encoding the polypeptide, also showed substantial homology with that of *D. melanogaster* (86.85%), as compared to those of other species, such as Mediterranean fruit fly (56.07%), chironomus (44.62%), human (29.27%), rat (28.78%), zebrafish (28.78%), and mice (20.45%). Significantly, the N-terminal segment of *Hsp27* showed maximal sequence conservation as compared to C-terminal domain, which seems to indicate that *hsp27* gene might have diverged through gene duplication, early during evolution (Kokolakis *et al*., 2008). The N-terminal segment of Hsp27 is reported to be involved in both, oligomerization and chaperone functions (Wegele *et al*., 2004). Among sHsps, the N-terminal region is most variable in different species studied. It also varies in protein quaternary conformation, while its functional form is further configured by C-terminal region (Salerno *et al*., 2003, Eifert *et al*., 2005).

Although small Hsps are widely reported to be induced upon heat stress, their functional significance in stressed cells has still remained a question to be investigated further. Several studies in *Drosophila* have shown expression of Hsp27 to be induced by heat shock over a broad temperature range (30–37 °C), reaching its maximum level at 35 °C (Lindquist, 1980; Vazquez *et al*., 1993). Recently, in *Chironomous*, it was observed that both heat shock (35 °C/2 h) and cold shock (4 °C) cause significant upregulation of *hsp27* mRNA transcript (Martinez-Paz *et al*., 2014). The above observation is significant as no
specific report is available as yet on the cold shock induce expression of hsp27 mRNA, indicating the functional importance of the protein in thermal regulation. In our earlier study in L. cuprina, the most optimal induction of Hsps (Hsp60, Hsp70, Hsp90 and small Hsps, including Hsp27) was observed at 42 °C, which is 5 °C higher than that of Drosophila (Sharma et al., 2006), indicating its higher tolerance potential against thermal stress, which is again critical for the survival of Lucilia during extreme environmental conditions encountered in its natural habitat.

In D. melanogaster, the downregulation of hsp27 is not reported to have any significant effect on development, but loss of hsp27 function was found to be associated with increased sensitivity toward starvation stress (Wang et al., 2004; Hao et al., 2007). Further, Hao et al. (2007) reported that overexpression of Hsp27 could provide cells significant resistance against thermal as well as oxidative stress. Though we have not probed it further, as reported above, we presume that the significantly increased expression of Hsp27 protein in salivary gland, fat body and oocytes upon heat shock might have similar implications in L. cuprina as well. Our results show a tissue specific pattern of expression of Hsp27. In larval salivary gland, fat body and oocytes, its presence was both cytoplasmic and nuclear,
with significant enhancement in the staining after heat shock. However, the pattern of Hsp27 expression appeared surprisingly different in Malpighian tubules. Although the level of hsp27 transcript was found elevated in both larval and adult MTs upon heat shock, the protein level was found unaltered. This might either be due to the instability of the transcripts or some posttranscriptional regulatory mechanism unique to Malpighian tubules. An observation was also made in Drosophila Malpighian tubules on heat shock proteins (Hsp70 and Hsp64) on heat shock (Singh & Lakhotia, 2000). In D. melanogaster, the protein is found mainly concentrated in the nuclei of heat shocked adult MTs (Arrigo et al., 1988; Loktionova et al., 1996, Bryantsev et al., 2002; Wong 2000; Bryantsev et al., 2007). Significantly, it is reported that Hsp27 overexpression facilitates disaggregation of nuclear proteins rendered insoluble by heat shock in D. melanogaster (Borrelli et al., 1996b; Kampinga et al., 1994). Though we have not made a detailed investigation, a similar function may also be possible for LcHsp27 protein, which was observed, sequestered in the Malpighian tubule polytene nuclei of both larva and adult following heat shock. The nuclear translocation of Hsp27 protein may likely be required for rapid disaggregation of nuclear proteins that were denatured and aggregated by thermal stress, as also indicated in some of the earlier studies (Bryantsev et al., 2007; Michaud et al., 2008). The Hsp27 protein expression appeared dynamic and varying with oocyte stages, suggesting its significant role in oogenesis that need further probing. Our observations in oocytes finds support from some of the earlier studies, which showed that the level of Hsp27 protein in Drosophila somatic tissues as well as in ovarian nurse cells (from where the protein is transported to mature oocytes) remains same during first hour of embryonic development (Zimmerman et al., 1983), however, decreases during mid-embryonic stages (Thomas & Lengyel, 1986). Interestingly, in later stages of embryogenesis, the level of Hsp27 protein expression increases considerably (Arrigo & Pauli, 1988; Arrigo & Tanguay, 1991). A similar pattern of Hsp27 expression was also found in different embryonic stages of C. capitata at high temperature (Mintzas et al., 2008; Theodoraki et al., 2008). The pattern is found to be similar in vertebrates too. It is reported that in early stage embryos of Xenopus, heat shock did not cause any appreciable change in the expression of hsp27 mRNA, but during later embryonic stages, heat shock results in the increased accumulation of hsp27 mRNA (Lang et al., 1999). Thus, apart from other constitutive cellular functions in which hsp27 has essential contribution, it plays significant role in maintaining the cellular homeostasis under conditions of thermal stress in L. cuprina.

The present results indicate the Lchsp27 gene/protein sequence structure and cellular functions to be evolutionarily highly conserved. The phylogenetic analysis showed Lchsp27 to be closer to Drosophila as compared to other insect species. A recent study on small Hsps in lower vertebrates revealed recognizable orthologs of certain small Hsps, suggesting that small Hsps may have evolved much before the species divergence came into account (Franck et al., 2004). Our ongoing studies are likely to elucidate the structure of UTRs and upstream regulatory sequences of Lchsp27 gene, which may help us to understand the mechanism of its transcriptional regulation, in general, and in L. cuprina, in particular. The increase in the level of Hsp27 protein in developing oocytes as well as in other somatic tissue types clearly indicates that it might have some protective function during heat-induced cellular injury and, hence, may be considered as an adaptive cellular response that helps organisms to survive (Bijlsma & Loeschecke, 1997; Singh & Lakhotia, 2000). Further investigations are required to get more insight into the role of accumulation of Hsp27 in the nuclei of heat shocked cells (e.g., MTs) in L. cuprina. Our ongoing studies on Lchsp27 are expected to shed more light on the various unanswered questions.

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Conflict of interest

Authors declare no interest of conflict.

References


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**Supporting Information**

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site:

**Fig. S1.** Expression analysis of *hsp27* in adult brain of *L. cuprina*. (A) represents the western blot of total protein from adult brain under control and heat shock conditions to show the relative level of Hsp27 (upper row). β-actin (lower row) was used as an internal loading control. Histogram in (B) indicates the mean (± SE, n = 3) level of Hsp27 relative to that in control sample. Paired Student’s *t*-test was performed in order to estimate significance level (*) by keeping *P* ≤ 0.05.

**Table S1.** Table showing fold change in mRNA expression level of *Lucilia hsp27* in different tissues.