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Application of heat shock protein expression for detecting natural adaptation and exposure to stress in natural populations

Jesper Givskov SØRENSEN*

National Environmental Research Institute, Aarhus University, Department of Terrestrial Ecology, Vejlsøvej 25, P.O. Box 314, DK-8600 Silkeborg, Denmark

Abstract Heat-shock proteins (HSPs) play an undisputed role for maintaining cellular functioning under environmental challenges and protein denaturing conditions. Compelling evidence points to an evolutionary important role of HSPs and a strict evolutionary control of these proteins as a balance between benefits and costs. While there is a great potential for using HSP expression for detecting natural adaptation and exposure to stress in natural populations, some obstacles and key issues await investigation. From an ecological perspective these key issues needs to be resolved in order to fully appreciate the complex responses and adaptations to stress and to increase our understanding of HSPs and other molecular chaperones for stress adaptation and potential use as biomarkers. Here, the current knowledge and understanding of HSPs is reviewed and a number of key issues including the interpretation of elevated HSP levels, the complications of extrapolating between laboratory and field conditions, the effects of choice of traits and methodology and the larger intra-and extracellular networks of interactions that HSPs participate in are discussed [*Current Zoology* 56 (6): 703–713, 2010].

Key words Biomarker, Hsp, Molecular chaperone, Thermal adaptation, Thermal stress

1 Introduction

All organisms are occasionally or regularly exposed to environmental conditions that challenge the physiological functioning of the cells. When this effect becomes severe enough it can be considered as *stressful* and will require counter measures in order to maintain cellular homeostasis/homeodynamics and thus growth, reproduction and survival (see Selye, 1956; Grime, 1979; Sibly and Calow, 1989; Hoffmann and Parsons, 1991; Bijlsma and Loeschcke, 1997 for alternative definitions of stress). On exposure to high temperature cells will mount a strong physiological response, including the heat stress response and the expression of heat shock proteins (HSPs). In addition to heat, the stress response is also activated by a range of other environmental conditions. HSPs and the associated stress response has been shown to be induced by cold and a range of other stresses including insecticides, heavy metals, desiccation, diseases, parasites, inbreeding and habitat condition (see Sørensen et al., 2003, and references therein; Herring et al., 2009). Thus, the heat stress response is considered to be a fundamental component of the physiological response to stress.

The different HSPs and their cellular functions have been reviewed in detail (see e.g. Parsell and Lindquist, 1993; Feder and Hofmann, 1999; Pockley, 2003; Sun and MacRae, 2005). In many organisms Hsp70 is considered to be the major HSP family consisting of solely inducible, constitutive and inducible, and solely constitutive proteins (heat shock cognates). HSPs are molecular chaperones, which are involved in "house-keeping" functions in the cell. These functions include the prevention of aggregation of damaged proteins, folding and unfolding of proteins, transportation and general handling of peptides and proteins and involvement in the degradation of misfolded or aggregated proteins. These functions are performed by constitutively expressed proteins during normal cellular conditions. Under increasingly stressful condition, the need for repair and "house-keeping" is accelerated which is handled by the induction of additional, stress activated HSPs. Mounting the stress response is potentially costly. Costs are thought to arise by the shut down of normal cell functions during the stress response, the extensive use of energy and the toxic effects of high HSP concentrations due to interference with normal cell function (Feder and Hofmann, 1999; Sørensen et al., 2003). However, the extent of the costs are probably minor under mild temperature stress conditions (Sørensen et al., 2008).

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* E-mail: jgs@dmu.dk

The HSPs are characterized by being evolutionary conserved and to occur with high homology within and among all pro- and eukaryote organisms, indicating strong stabilizing evolution (Lindquist and Craig, 1988; Schlesinger, 1990). Due to the generality of the stress genes and responses among organisms and stress types, the heat shock genes have been suggested as obvious candidates for a major role in the protection of cells during or after thermal stress and thus a key component of adaptation to environmental conditions (thermal stress) and as well as biological markers for exposure to stressful conditions. This importance of HSPs for resistance towards heat has been confirmed by several investigations (Lindquist and Craig, 1988; Parsell and Lindquist, 1993; Feder and Hofmann, 1999).

Searching scientific databases for *heat shock protein* yields yearly thousands of new papers. These studies have ensured that we have a thorough understanding of the structure of the *hsp* genes, their regulation, and the *in vivo* and *in vitro* roles for function and survival of organisms exposed to different benign and stressful conditions (see e.g. Parsell and Lindquist, 1993; Feder and Hofmann, 1999; Morimoto et al., 1999; Pockley, 2003; Sørensen et al., 2003). With this in mind it is rather surprising how few of the many papers published each year that are related to evolutionary and ecological perspectives and roles for thermal adaptation. For many years the ecological and evolutionary importance of inducible HSPs for adaptation in natural populations was in most cases assumed and not tested directly. However, during the last ten years focus on ecological and evolutionary studies investigating the conditions for expression, benefits and costs of expression and role for different resistance traits and ultimately the role for natural adaptation has increased. While previous reviews on HSPs often have focused on cellular and molecular aspects of responses and adaptation to stress (Lindquist, 1986; Morimoto et al., 1999; Pockley, 2003; Koorsloot et al., 2004) this paper will focus on the current understanding of HSPs expression levels and the role of the stress response for detecting natural adaptation and exposure to stress in natural populations. Furthermore, the main obstacles for the advancement of this understanding and pinpointing of key areas and directions of future research will also be discussed.

2 The role of HSPs for Adaptation

Data on adaptive variation in thermal resistance in natural populations has accumulated and suggests that stressful conditions are selecting for adaptation in natural populations (Hoffmann et al., 2003). The improved acute heat resistance of organisms across the kingdoms after heat induction of HSP expression is undisputed (Feder and Hofmann, 1999; Basu et al., 2002; Sun et al., 2002), and also include human cells (Fonager et al., 2002). Laboratory studies show that very small amounts of induced HSPs can have effects on life history traits such as development, stress resistance, life span and fecundity (Rutherford and Lindquist, 1998; Sørensen and Loeschcke, 2001; Queitsch et al., 2002; Rutherford, 2003). Adaptive changes in HSP expression over days (Nguyen et al., 1994; Ferguson et al., 1998) and over seasons (Fader et al., 1994; Hofmann and Somero, 1995; Pyza et al., 1997; Minier et al., 2000) also support the ecological significance of HSPs in natural populations. Therefore, HSPs are obvious candidates for playing a large role for adaptation in natural populations. However, while HSPs are very important for survival following heat shock under laboratory conditions, the ecological relevance and adaptive importance under field conditions is less clear and only rarely directly investigated (Dyer et al., 1993; Fader et al., 1994; Gehring and Wehner, 1995; Feder et al., 1997; Kelty and Lee, 1999). Some support for the importance of HSPs for adaptation comes from latitudinal or climatic clines, indicating that natural selection is acting on *hsp* variation. Although variation in the coding regions of the *hsp* genes is low, clinal variation in *hsp* DNA sequences or protein expression is known from e.g. *Drosophila* (Frydenberg et al., 2003; Hoffmann et al., 2003; Sarup et al., 2006), intertidal marine mollusks (Sagarin and Somero, 2006), fish (Hemmer-Hansen et al., 2007) and anurans (Sørensen et al., 2009b).

Moving from sequence variation to variation in protein levels the pattern becomes less clear. In some cases clinal studies show seemingly simple and linear relationships between climate or geographical origin and HSP expression (Sørensen et al., 2005b). However, more often non-linear relationships are found or clinal variation is only found for some traits, in some studies, in one gender or life stage or under specific environmental conditions (see e.g. Sørensen et al., 2005b; Sagarin and Somero, 2006; Sarup et al., 2006). Furthermore, conspecifics with overlapping distributions (thus, sharing climatic conditions) have been found to show different levels of thermal resistance and different expression patterns of HSPs (Stratman and Markow, 1998; Sagarin and Somero, 2006). Thus, the relationships between thermal adaptation and *hsp* DNA variation or HSP protein levels are not simple and straightforward.

Some of the factors causing this complexity and making ecological or evolutionary interpretation of HSPs levels difficult will be discussed below.

2.1 Increased HSP levels: Capacity or need?

In an ecological and evolutionary adaptive perspective HSPs are usually measured either under controlled laboratory conditions (common garden experiments) to compare populations or treatments or in individuals collected from the field, where expression levels are measured directly to estimate the amount of stress exposure. In both cases it is important to have clear expectations to be able to interpret the measurements correctly and make sense of them.

It is not always clear when the level of constitutive and inducible HSP expression should be interpreted as reflecting the capacity or ability to mount a strong defense (i.e. as a benefit) or when it should be interpreted as reflecting the need to mount a strong response as the organisms is stressed (i.e. as a cost). In different species of *Drosophila* decreased Hsp70 expression after acute heat stress seems to be the evolutionary consequence if the lines are exposed to chronically stressful high temperature conditions or originate from natural populations that inhabit warm environments (Sørensen et al., 2003). The same pattern was found in invertebrates exposed to continuous heavy metal stress (Köhler et al., 2000). Desert lizards also show a similar pattern of adaptation, with higher threshold for induction of Hsp70 compared to a more northern species (Zatsepina et al., 2000). Interestingly, these desert lizards had increased constitutive levels of Hsp70 suggesting that this stress protein might be important for adaptation to regularly high temperature experiences. The explanation might be that the benefits of mounting the stress response in populations frequently exposed to stress are outweighed by the costs. Thus, natural selection will favor other (and maybe more specific) means of adaptation over expression of HSPs, if these other options are evolutionary available. Inducible HSPs might then only be used when the primary defenses are no longer able to prevent damage and other deleterious effects, and might be most important in connection to rare and unpredictable extreme stress events and not for continuous or frequent exposure. In addition all costs of stress defenses mechanisms and adaptation will have to be traded off against life history traits. Thus, it is not only the use of different stress mechanisms that might evolve differently among species, but the outcome of stress adaptation will affect (and probably depend) on life history evolution as well. This is an important area which is poorly understood and which clearly need much further investigation.

An exception to the hypothesis that HSPs not are used for adaptation to generally extreme conditions might be found among species evolved to tolerate extreme hot or cold environments. In several highly adapted species covering both invertebrates and vertebrates, constitutive levels of HSPs have been found to be increased (Gehring and Wehner, 1995; Rinehart et al., 2006; Evgen'ev et al., 2007). Thus, under extreme circumstances HSPs might be the evolutionary most favorable or even the only possible mean of adaptation, alone or in combination with other behavioral, physiological or molecular mechanisms. High inducible HSP expression levels to stress might thus not reflect a corresponding high level of adaptation in most species, but rather that the organism is severely stressed.

2.2 Extrapolating result from laboratory to field conditions

In comparison to the laboratory situation, the field constitutes a mixture of (known and unknown) environmental factors that are fluctuating unpredictably. Estimates of adaptation, stress factors experienced and responses to these factors (e.g. HSP expression) is usually performed in the laboratory by using correlates for traits assumed to be important in the field: however, the correlates might not always represent traits under selection in the field and are not always tested (Hoffmann et al., 2003). In the field less extreme thermal conditions are likely to occur frequently, and while these mild exposures might not affect survival directly they might have important ecologically and evolutionary effects on mobility (Krebs and Thompson, 2006) and reproduction (Fasolo and Krebs, 2004; Sarup et al., 2004; Shreve et al., 2004; David et al., 2005; Jørgensen et al., 2006). Recent studies have underlined the disparity between results obtained from field and from laboratory investigations of the same populations. Laboratory and field performance was compared in an attempt to link thermal performance and effects of thermal acclimation (Kristensen et al., 2008). Here benefits of cold acclimation were detectable in both field and laboratory, while large costs of acclimation were detected under field conditions but not in the laboratory assays. Another study aimed at investigating the role of inducible HSPs for field performance using a similar set-up. Here, genetically modified lines harboring a mutation that inactivated the heat shock transcription factor (HSF) and thereby the inducible HSPs was compared to the rescued mutant and to wild type controls (Sørensen et al.,

2009a). The main result was that while performing largely as expected in the laboratory standard thermal assays, both genetically modified lines performed extremely poorly under field conditions. Thus, even when care is taken to select and verify the traits investigated in order to reflect natural conditions, results from the laboratory can only in some cases and to some extent be extrapolated to natural field conditions.

Even though it can be done (McMillan et al., 2005; Sagarin and Somero, 2006), interpreting results from measurements in field collected individuals can be problematic. This is because the natural environment potentially contains a mix of multiple acute and long term stress factors that can affect expression of the stress response. It is therefore important to attempt to verify whether the environmental factors assumed to contribute to the stress induction are actually responsible for the measured levels of HSPs. Another issue is to complement the investigations in order to link the expressed HSPs with the trait in question, i.e. does an increased level of HSPs benefits the resistance towards the stress factor assumed to have induced HSP expression. The establishment of the link between HSP expression and phenotypic trait also needs to consider effects and ecological relevance of the measured age/life stage of the studied organism(s) as these might show specific changes and adaptation. If no such link exists the interpretation of HSP level will be affected. This puts forward demand for detailed knowledge of the ecology and natural conditions experienced by the study organism and can have large implications for the attempted use of HSPs as biomarkers for stress in natural population (Iwama et al., 2004; Herring and Gawlik, 2007), as well as for the understanding the role of HSPs for natural adaptation based on laboratory studies.

2.3 Which traits to measure?

When detecting or describing adaptation by the induction of HSPs the interpretation of the results should be based on whether the treatments or conditions are relevant for the stress experienced in the field by that organism. Therefore the selection of traits to measure as proxies for adaptation to the environment (e.g. tolerance or HSP levels) should be carefully selected, preferable based on in depth knowledge of the species ecology. In *Drosophila* acute heat shock survival is closely related to Hsp70 expression level (but see Jensen et al., 2009), but not closely related to natural adaptation (Hoffmann et al., 2003), while heat knock down resistance is associated with adaptation to high temperature but weakly associated with expression of HSPs (Hoffmann et al., 2003; Nielsen et al., 2005; Johnson et al., 2009). Intra-specific variation of heat tolerance and Hsp70 expression was recently investigated in great detail (Jensen et al., 2009). These authors concluded that Hsp70 expression and heat tolerance was weakly correlated in *D. melanogaster* flies. However, the study used different tolerance assays including acute heat survival, but also heat knock down that previously has been shown to be less dependent of Hsp70 expression, thus affecting the expectation of the strength of the association (Nielsen et al., 2005). Thus, the connection between variation in HSP expression, variation in ecological relevant tolerance assays and life stage specificity require continued study.

Even if acute heat shock survival is closely associated with HSP expression, using this trait (or Hsp70 expression levels) would lead to incorrect conclusions regarding natural adaptation. However the “correct” trait to use is often not known and the precise conditions experienced by the organisms and thereby the selection pressure they are exposed to under natural conditions, are notoriously difficult to assess. Not only the type of trait selected as proxy for natural adaptation but also the exact methodological details might influence results. Critical thermal limits were investigated in two insect species, *D. melanogaster* and the invasive ant species *Linepithema humile* (Chown et al., 2009). Here, species specific effects of acclimation temperature and rates of temperature change (i.e. the exact method used to estimate thermal limits) were found for mean tolerances and their variance estimates. Thus, methodological approach might not only compromise estimates of tolerances, but also estimates of heritable variation due to the effects on variation. Furthermore, different life stages might experience different conditions and selection pressures, and have different sensitivity and different mechanisms to achieve adaptation complicating things further (Hoffmann et al., 2003; Rinehart et al., 2006; Jensen et al., 2009). For smaller organisms measurements are typically performed on whole body homogenates and with increasing body size the separation into different tissues becomes easier. However, regardless of body size different tissues might show different sensitivity and expression of genes and proteins, which can be expected to differ to a large extent (Lakhotia and Prasanth, 2002; Ayroles et al., 2009). When performing whole body measurements mainly signals from the larger tissues can be expected to be detected. Thus, important signals from small and sensitive tissues (e.g. reproductive tissue) might be overlooked.

While survival of acute heat shock is strongly connected to HSPs, the role of the stress response for maintaining fitness under natural conditions is less well understood. Our knowledge about the (tolerance) traits selected for in natural populations and the importance of different environmental factors needs to be improved. Only then can we expect to understand the roles of different stress response mechanisms and the evolutionary trade offs involved in adaptation to stressful environments.

2.4 HSPs in the larger context of the cell

Many studies show large responses of HSPs and the expression of HSPs has in many aspects become synonymous with the heat stress response. However, increased expression of inducible HSPs is one important part of the cellular stress response, which also includes e.g. other molecular chaperones, antioxidases, proteases and DNA repair systems. New 'omics' technology (e.g. transcriptomics, proteomics, metabolomics) already allow for the investigation of many genes, proteins or metabolites compared to what was earlier feasible or even possible. These investigations are becoming every day technologies and are generating vast amounts of data. So far these studies have shown that numerous genes potentially are responding to thermal perturbations (e.g. Qin et al., 2005; Sørensen et al., 2005a, see figure 1). A large number of candidate genes (other than HSPs) and pathways that might be involved in adaptation to environmental conditions have also been identified (Leemans et al., 2000; Morgan and Mackay, 2006; Nielsen et al., 2006; Sørensen et al., 2007; Matzkin and Markow, 2009). Thus, while HSPs might show some of the strongest responses in terms of fold changes (at least to acute heat stress) gene expression array and QTL studies show that many other genes potentially respond, and might respond on different time scales or in different directions than the HSPs. Thus, HSPs are but one part which should be considered when looking at the responses to stress.

2.5 Methodological approaches of measuring HSPs

With increasing development in PCR and sequencing technology, more and more genes can be assayed by high through-put QPCR or array techniques. While lots of data can be produced these methods might overlook or overestimate the effects due to a disparity between gene and protein expression. While gene expression is easy to measure, the functional unit is in most cases an enzyme or protein. Gene and protein expression might differ due to different post transcriptional and

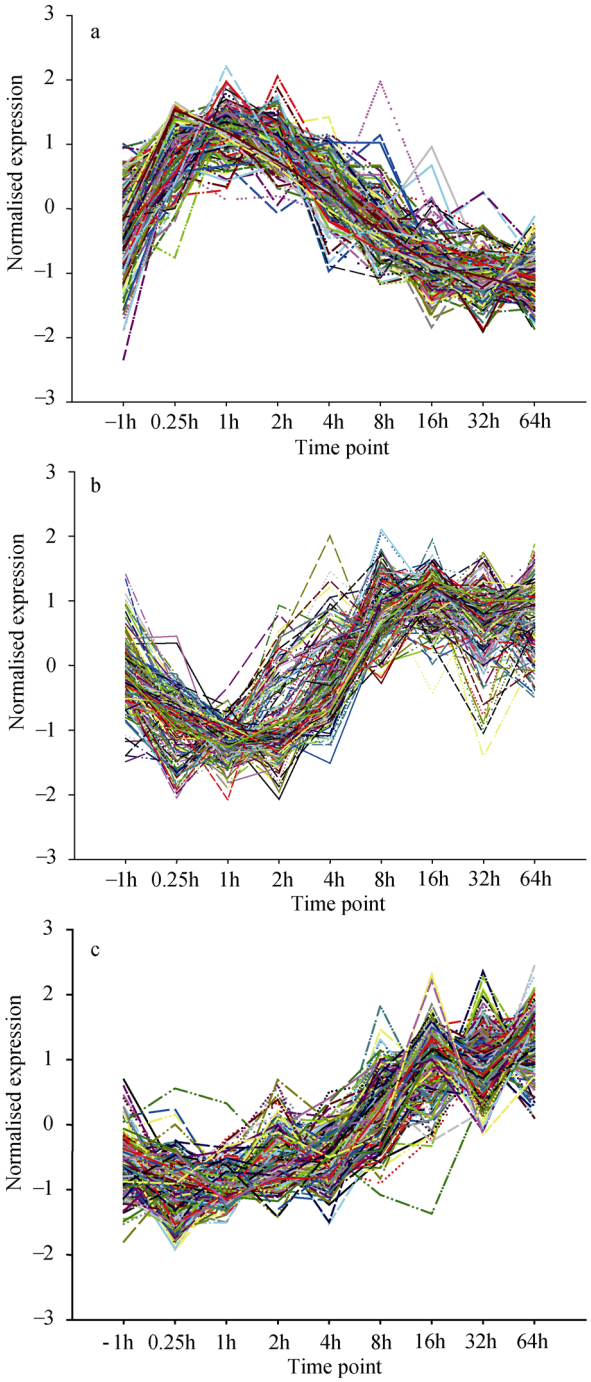

Fig. 1 Gene expression profile in *Drosophila melanogaster*, before and in a temporal series after a heat stress (Sørensen et al., 2005a)

Three patterns of stress responsive genes were found. They were a) Early up-regulated genes. Here, the HSPs representing the classical heat shock response were found in addition to around 250 other genes. b) Early down-regulated genes. Around 500 genes, primarily genes involved in metabolism, responded to heat stress by immediately down-regulation. c) Late up-regulated genes. Around 225 genes started to become up-regulated four hours after the heat shock. At this time point most early responding genes had returned to near control levels. In total nearly 1000 genes were found to respond to the heat shock, with approximately equal number of down- and up-regulated genes (Figure from Sørensen et al., 2005a).

translational regulation, or due to different dynamics, e.g. on a temporal scale (Feder and Walser, 2005). An example of such a disparity was observed as a delayed response in protein compared to gene expression levels of Hsp70 in heat stressed springtails (Bahrndorff et al., 2009) and in cold stressed insects (Koštál and Tollarová-Borovanská, 2009). In both cases the mRNA levels peaked immediately after the treatment, while protein level peaked after the mRNA level had returned to near normal levels. The level of Hsp70 protein was closely related to heat tolerance while mRNA level were not (Bahrndorff et al., 2009). Caution is therefore needed when making inferences about mechanisms in transcriptional studies as key genes may be overlooked, e.g. due to lack of temporal sampling (Heckmann et al., 2010) or due to post transcriptional and translational regulation (Feder and Walser, 2005).

Given the disparity between measuring mRNA and protein levels it could be argued that the protein levels should always be the target of investigation. However, this is not always practically feasible or even possible for technical reasons. Protein measurements techniques like Western blotting and Enzyme-linked immunosorbent assays (ELISA) rely on the availability of antibodies, which, for many species and classes of HSPs have not been commercially developed. mRNA techniques, like e.g. QPCR or microarrays, rely on sequence information, which, with the technological development of sequence techniques (e.g. 4-5-4 sequencing) is more likely to be present or developed for the species in question with relatively limited investment of time and money. Thus, mRNA can be representative of changes in expression and also relevant at the protein level in some circumstances. Especially acute exposure leading to upregulation of mRNA is likely to lead to similar (but probably temporally delayed) expression of proteins. The dynamics of long-term or chronic exposures, on the contrary, is less predictable and under these conditions the interpretation of mRNA levels should be made cautiously.

Even, if antibodies are available, the technique should be validated before generating results. ELISA might be preferred over Western blotting as ELISA immediate generates quantitative results and often allows for a higher through-put set-up. However, it is important to go through validation steps for both techniques. Western blotting has the advantage over ELISA that unspecific binding can be detected as additional bands and can be used to verify the expected size of detected bands. Thus, Western blotting should precede the use of a new antibody or a new species tested on ELISA. Furthermore, as un-expected interactions might occur between antibody, target protein and the remaining cellular/extracellular matrix, optimally a linear relationship between signal and content of target protein should be established. Direct dilution of samples is not preferred as both target protein and all other proteins are diluted to the same extent. Samples spiked with additional levels of purified target protein and/or mixtures of samples with high and low levels of target protein (e.g. stress induced and control samples) provide the best validation of both Western blotting and ELISA: showing that the signal of the assay represents the amount of target protein in the sample. Note that the linear relationship should not be assumed to be valid outside the concentrations tested.

The next generation of protein quantification will allow large scale identification and quantification of proteins and their post-translational modifications. However, the proteomics technology still needs some development in order to achieve coverage of large parts or the full proteome. Still, multiple protein measurements are possible and can solve some of the problems associated with single protein measurements. When developed further proteomics has the potential to be a strong tool for identifying candidate proteins for ecologically relevant traits, and increase our understanding of the role of these proteins for adaptation to environmental conditions.

Exposure to environmental stress not only affects genes (and thereby proteins) but also energy reserves, metabolites and membrane compositions (Ohtsu et al., 1998; Koštál et al., 2003; Malmendal et al., 2006; Overgaard et al., 2006; Holmstrup et al., 2007). These changes are all likely to contribute to the responses and adaptations to environmental and especially thermal stress. It might be so that some mechanisms play a relatively larger role for some organisms, for certain stress factors or under some conditions; however, this needs much more investigation before a clear pattern is likely to emerge.

2.6 HSPs as components of an intracellular network

Traditionally, expression of HSPs has primarily been considered to cover a range of essential housekeeping and cytoprotective functions (Feder and Hofmann, 1999; Pockley, 2003). However, recent studies indicate that the functions of HSPs are much more diverse and that HSPs might function as immunoregulatory agents (Pockley, 2003). Thus, HSPs seem to be part of a larger network of stress responsive and protective network that also in-

clude hormonal and immune responses (Pockley, 2003; Collier et al., 2008). The question remains what the extracellular HSPs measured in the blood represents and what the relation between intra- and extracellular levels of HSPs are. For instance how are intra- and extracellular HSP concentrations associated with stress exposure, disease resistance or the general health or state of individual organisms? Regardless of these open questions, the discovery that HSPs can be found in e.g. the blood gives an optimistic perspective for, especially, vertebrate biologists who wish to use measurements of HSPs in larger animals and in the field. These samples can be collected, repeatedly through time, and are relatively un-invasive.

We can imagine two mechanisms by which HSPs can end up in the extracellular matrix of an organism. Either it can be present due to passive release from the cells, where proteins e.g. are leaked from necrotic cells. In this case extracellular HSP levels can be thought to represent the intracellular levels. Alternatively, HSPs are released into the circulation system by an active release mechanisms (Asea, 2007; Collier et al., 2008). In this case extracellular HSP levels might not represent intracellular levels, and might have functions that are different from the intracellular functions.

Some insight into the significance of HSPs in the blood of vertebrates has been reached. A number of HSPs in the circulatory system seem to have stimulatory effects on immune and inflammatory responses and may also have some regulatory effects on co-activated molecules (Pockley, 2003; Asea, 2007). The prevailing theory, supported by increased HSP levels in organisms affected by various diseases, suggests that HSPs originating from stressed or damaged cells serve as a danger signal supposed to activate defenses, inclusive immune responses (see Asea, 2007 for review). Intriguingly, even potential danger or fear induced by presence of predators or predator cues can induce the production and release of HSPs suggesting that this response is not only a passive result of leaking from damaged cells (Fleshner et al., 2004; Pauwels et al., 2005).

Thus, although much is still to be investigated and understood in greater detail, blood levels generally seem to be relevant and the most easily and least invasive way to measure HSPs in larger animals and under field conditions (Kristensen et al., 2004). However, the measurements of blood levels do not necessarily give the same results as assaying tissue samples. Kristensen and Løvendahl (2006) measured Hsp72 in high temperature exposed blood and muscle samples of Jersey *Bos taurus* calves. They found large differences in the expression profile among the two types of measurement and only found a weak association between plasma and muscle samples. In the study of Kristensen and Løvendahl (2006), cellular Hsp72 levels were the appropriate indicator of the heat stress and disruption of homeostasis. In fish, hypoxic conditions have been shown to induce Hsp70 production in some tissues and in the blood of juvenile Nile tilapia *Oreochromis niloticus*, while no change was detected in other types of tissues (Delaney and Klesius, 2004). The Hsp70 results reported in the mentioned examples above suggest that the cellular content often can be different from the blood levels. In insects whole animals are often investigated due to smaller size; however, this is mainly for practical reasons as the tissue specificity can be expected to be equally relevant for all organisms regardless of body size. Another perspective of HSPs measured in blood that might cause problems of interpretation is the fact that measurements of HSPs from blood might include the expression of both extra-cellular network (measured in plasma) and intra-cellular production (measured in erythrocytes). The latter case is much more complex as the clot might contain HSPs from leukocytes as well and the status of immune system activation (Martinez-Padilla et al., 2004; Blanco et al., 2006).

More studies and further development of these issues are clearly needed for future use of HSPs in vertebrates in order to clarify interpretation of HSP levels. These questions include if and when high or low cellular and blood levels of HSPs are associated with each other and with increased performance or adaption under field conditions.

HSPs are also likely to play a role in connection to anoxia and hereby following oxidative stress, as oxidative stress is a cellular consequence of stressful conditions and anoxia is known to induce Hsp70 (Jedlicka et al., 1997). It can be hypothesized that a link exists between heat shock proteins and oxidative stress. It is unknown to what degree the beneficial effects of HSPs are specific in connection to oxidative stress damages and to what degree HSPs maintain a general line of defense that is secondary to the more specific oxidative stress scavengers. However, HSPs and oxidative stress damage does have specific interactions and does, under some circumstances, play a role in the protection against oxidative damages under some circumstances (Koorsloot et al., 2004; Kalmar and Greensmith, 2009; Rodgers et al., 2009). It has been suggested that oxidative stress or oxidative limitations could be a principal

determinant of both high and low temperature thermal tolerance (Pörtner, 2001). However, while oxidative limitation might affect the maintenance of homeostasis or homeodynamics during long term, mildly stressful conditions, HSPs and other stress responses will probably be of major importance under more acute exposures to severe conditions. Thus, both factors might play roles for adaptation and tolerance in natural populations under field conditions.

2.7 HSPs as proxy for individual quality

The role of HSPs and other cellular maintenance systems has been studied with respect to humans. This is because many human diseases are caused by protein folding problems potentially relating to molecular chaperones (reviewed in Gregersen et al., 2003). Individual variation in the strength or efficiency of response to protein folding challenges (either due to genetic disease or environmentally induced) has been shown to correlate with specific variation in the protein quality control system (Gregersen et al., 2003). Thus, individual quality is affected by a complex association and interaction between HSP expression, genotype and phenotype.

In birds, some studies have established associations between the physiological condition, stress response and immuno- or infection status and resistance. Increased blood levels of Hsp60 were significantly associated with parasite infections in blue tit *Parus caeruleus* nestlings (Arriero et al., 2008). In pied flycatcher *Ficedula hypoleuca* females increased levels of Hsp60, but not Hsp70, were associated with lower humoral and cell-mediated immune responses. The authors suggest that the strength of the immune defense was traded-off against investments in protection from physiological stress through the induction of the stress response due to the costs of activating these systems. However, it is also possible that both mechanisms contribute to defense in a complementary fashion so that less of one mechanism is needed when the other mechanism is more active (Morales et al., 2006).

3 Conclusions and Future Perspectives

The HSPs are involved in intra- and extracellular responses to stress and have the potential to be developed into a key biomarker in ecological and evolutionary research for detecting natural adaptation and exposure to stress in natural populations. However, in order to achieve this some obstacles and key issues await investigation. These revolve around the interpretation of elevated HSP levels, the complications of extrapolating between laboratory and field conditions, the choice of traits and methodology and the larger intra- and extracellular networks of interactions that HSPs participate in. Thus, measuring an array of responses, genes or proteins are highly preferred to measurements and interpretations based on single genes and proteins. The technological advancements that are, and will become, available gives us a good starting point for the investigations that are needed to increase our knowledge and push forward our understanding of responses and adaptation to natural stressful conditions.

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References

Arriero E, Moreno J, Merino S, Martinez J, 2008. Habitat effects on physiological stress response in nestling blue tits are mediated through parasitism. Physiol. Biochem. Zool. 81: 195–203.

Asea A, 2007. Mechanisms of HSP72 release. J. Biosciences 32: 579–584.

Ayroles JF, Carbone MA, Stone EA, Jordan KW, Lyman RF et al., 2009. Systems genetics of complex traits in *Drosophila melanogaster*. Nat. Genet. 41: 299–307.

Bahrndorff S, Mariën J, Loeschcke V, Ellers J, 2009. Dynamics of heat-induced thermal stress resistance and hsp70 expression in the springtail *Orchesella cincta*. Funct. Ecol. 23: 233–239.

Basu N, Todgham AE, Ackerman PA, Bibeau MR, Nakano K et al., 2002. Heat shock protein genes and their functional significance in fish. Gene 295: 173–183.

Bijlsma R, Loeschcke V, 1997. Environmental Stress, Adaptation and Evolution. Basel: Birkhäuser Verlag.

Blanco G, Frias O, Martinez J, Lemus JA, Merino R et al., 2006. Sex and rank in competitive brood hierarchies influence stress levels in nestlings of a sexually dimorphic bird. Biol. J. Linn. Soc. 88: 383–390.

Chown SL, Jumbam KR, Sørensen JG, Terblanche JS, 2009. Phenotypic variance, plasticity and heritability estimates of critical thermal limits depend on methodological context. Funct. Ecol. 23: 133–140.

Collier RJ, Collier JL, Rhoads RP, Baumgard LH, 2008. Genes involved in the bovine heat stress response. J. Dairy Sci. 91: 445–454.

David JR, Araripe LO, Chakir M, Legout H, Lemos B et al., 2005. Male sterility at extreme temperatures: A significant but neglected phenomenon for understanding *Drosophila* climatic adaptations. J. Evolution. Biol. 18: 838–846.

Delaney MA, Klesius PH, 2004. Hypoxic conditions induce Hsp70 production in blood, brain and head kidney of juvenile Nile tilapia *Oreochromis niloticus* (L.). Aquaculture 236: 633–644.

Dyer SD, Brooks GL, Dickson KL, Sanders BM, Zimmerman EG, 1993. Synthesis and accumulation of stress proteins in tissues of arsenite-exposed fathead minnows *Pimephales promelas*. Environ. Toxicol. Chem. 12: 913–924.

Evgen'ev MB, Garbuz DG, Shilova VY, Zatsepina OG, 2007. Molecular mechanisms underlying thermal adaptation of xeric animals. J. Biosciences 32: 489–499.

Fader SC, Yu Z, Spotila JR, 1994. Seasonal variation in heat shock proteins (hsp70) in stream fish under natural conditions. J. Therm. Biol. 19: 335–341.

Fasolo AG, Krebs RA, 2004. A comparison of behavioural change in *Drosophila* during exposure to thermal stress. Biol. J. Linn. Soc. 83: 197–205.

Feder ME, Blair N, Figueras H, 1997. Natural thermal stress and heat-shock protein expression in *Drosophila* larvae and pupae. Funct. Ecol. 11: 90–100.

Feder ME, Hofmann GE, 1999. Heat-shock proteins, molecular chaperones, and the stress response: Evolutionary and ecological physiology. Annu. Rev. Physiol. 61: 243–282.

Feder ME, Walser J-C, 2005. The biological limitations of transcriptomics in elucidating stress and stress responses. J. Evolution. Biol. 18: 901–910.

Ferguson IB, Snelgar W, Lay Yee M, Watkins CB, Bowen JH, 1998. Expression of heat shock protein genes in apple fruit in the field. Aust. J. Plant Physiol. 25: 155–163.

Fleshner M, Campisi J, Amiri L, Diamond DM, 2004. Cat exposure induces both intra- and extracellular Hsp72: The role of adrenal hormones. Psychoneuroendocrino. 29: 1142–1152.

Fonager J, Beedholm R, Clark BFC, Rattan SIS, 2002. Mild stress-induced stimulation of heat-shock protein synthesis and improved functional ability of human fibroblasts undergoing aging in vitro. Exp. Gerontol. 37: 1223–1228.

Frydenberg J, Hoffmann AA, Loeschcke V, 2003. DNA sequence variation and latitudinal associations in *hsp23*, *hsp26* and *hsp27* from natural populations of *Drosophila melanogaster*. Mol. Ecol. 12: 2025–2032.

Gehring WJ, Wehner R, 1995. Heat shock protein synthesis and thermotolerance in *Cataglyphis*, an ant from the Sahara desert. P. Natl. Acad. Sci. USA 92: 2994–2998.

Gregersen N, Bolund L, Bross P, 2003. Protein misfolding, aggregation and degradation in disease. 232: 3–16.

Grime JP, 1979. Plant Strategies and Vegetation Processes. Chichester: John Wiley.

Heckmann LH, Baas J, Jager T, 2010. Time is of the essence. Environ. Toxicol. Chem. 29: 1396–1398.

Hemmer-Hansen J, Nielsen EE, Frydenberg J, Loeschcke V, 2007. Adaptive divergence in a high gene flow environment: Hsc70 variation in the European flounder (*Platichthys flesus* L.). Heredity 99: 592–600.

Herring G, Gawlik DE, 2007. The role of stress proteins in the study of allostatic overload in birds: Use and applicability to current studies in avian ecology. TheScientificWorldJo. 7: 1596–1602.

Herring G, Gawlik DE, Rumbold DG, 2009. Feather mercury concentrations and physiological condition of great egret and white ibis nestlings in the Florida Everglades. Sci. Total Environ. 407: 2641–2649.

Hoffmann AA, Parsons PA, 1991. Evolutionary Genetics and Environmental Stress. New York: Oxford University Press.

Hoffmann AA, Sørensen JG, Loeschcke V, 2003. Adaptation of *Drosophila* to temperature extremes: Bringing together quantitative and molecular approaches. J. Therm. Biol. 28: 175–216.

Hofmann GE, Somero GN, 1995. Evidence for protein damage at environmental temperatures: Seasonal changes in levels of ubiquitin conjugates and Hsp70 in the intertidal mussel *Mytilus trossulus*. J. Exp. Biol. 198: 1509–1518.

Holmstrup M, Overgaard J, Bindesbøl AM, Pertoldi C, Bayley M, 2007. Adaptations to overwintering in the earthworm *Dendrobaena octaedra*: Genetic differences in glucose mobilisation and freeze tolerance. Soil Biol. Biochem. 39: 2640–2650.

Iwama GK, Afonso LOB, Todgham A, Ackerman P, Nakano K, 2004. Are hsps suitable for indicating stressed states in fish? J. Exp. Biol. 207: 15–19.

Jedlicka P, Mortin MA, Wu C, 1997. Multiple functions of *Drosophila* heat shock transcription factor in vivo. Embo J. 16: 2452–2462.

Jensen LT, Cockerell FE, Kristensen TN, Rako L, Loeschcke V et al., 2009. Adult heat tolerance variation in *Drosophila melanogaster* is not related to Hsp70 expression. J. Exp. Zool. Part A 313: 35–44.

Johnson TK, Cockerell FE, Carrington LB, Rako L, Hoffmann AA et al., 2009. The capacity of *Drosophila* to heat harden associates with low rates of heat-shocked protein synthesis. J. Therm. Biol. 34: 327–331.

Jørgensen KT, Sørensen JG, Bundgaard J, 2006. Heat tolerance and the effect of mild heat stress on reproductive characters in *Drosophila buzzatii* males. J. Therm. Biol. 31: 280–286.

Kalmar B, Greensmith L, 2009. Induction of heat shock proteins for protection against oxidative stress. Adv. Drug Deliver. Rev. 61: 310–318.

Kelty JD, Lee RE, 1999. Induction of rapid cold hardening by cooling at ecologically relevant rates in *Drosophila melanogaster*. J. Insect Physiol. 45: 719–726.

Köhler HR, Zanger M, Eckwert H, Einfeldt I, 2000. Selection favours low Hsp70 levels in chronically metal-stressed soil arthropods. J. Evolution. Biol. 13: 569–582.

Koorsloot A, van Gestel CAM, van Straalen NM, 2004. Environmental Stress and Cellular Response in Artropods. Boca Ratton, Florida: CRC Press.

Koštál V, Berková P, Šimek P, 2003. Remodelling of membrane phospholipids during transition to diapause and cold-acclimation in the larvae of *Chymomyza costata* (Drosophilidae). Comp. Biochem. Phys. B 135: 407–419.

Koštál V, Tollarová-Borovanská M, 2009. The 70 kDa heat shock protein assists during the repair of chilling injury in the insect *Pyrrhocoris apterus*. PLoS ONE 4: e4546. doi:4510.1371/ journal.pone.0004546.

Krebs RA, Thompson KA, 2006. Direct and correlated effects of selection on flight after exposure to thermal stress in *Drosophila melanogaster*. Genetica 128: 217–225.

Kristensen TN, Hoffmann AA, Overgaard J, Sørensen JG, Hallas R et al., 2008. Costs and benefits of cold acclimation in field-released *Drosophila*. P. Natl. Acad. Sci. USA 105: 216–221.

Kristensen TN, Løvendahl P, 2006. Physiological responses to heat stress and their potential use as indicators of reduced welfare in Jersey calves. Acta Zool. Sinica 52: 681–689.

Kristensen TN, Løvendahl P, Berg P, Loeschcke V, 2004. Hsp72 is present in plasma from Holstein-Friesian dairy cattle, and the concentration level is repeatable across days and age classes. Cell Stress Chaperon. 9: 143–149.

Lakhotia SC, Prasanth KV, 2002. Tissue- and development-specific induction and turnover of hsp70 transcripts from loci 87A and 87C after heat shock and during recovery in *Drosophila melanogaster*. J. Exp. Biol. 205: 345–358.

Leemans R, Egger B, Loop T, Kammermeier L, Haiqiong H et al.,

2000. Quantitative transcript imaging in normal and heat-shocked *Drosophila* embryos by using high-density oligonucleotide arrays. P. Natl. Acad. Sci. USA 97: 12138–12143.

Lindquist S, Craig EA, 1988. The heat-shock proteins. Annu. Rev. Genet. 22: 631–677.

Lindquist SL, 1986. The heat-shock response. Annu. Rev. Biochem. 55: 1151–1191.

Malmendal A, Overgaard J, Bundy JG, Sørensen JG, Nielsen NC et al., 2006. Metabolomic profiling of heat stress: Hardening and recovery of homeostasis in *Drosophila*. Am. J. Physiol.–Reg I 291: R205–R212.

Martinez-Padilla J, Martinez J, Davila JA, Merino S, Moreno J et al., 2004. Within-brood size differences, sex and parasites determine blood stress protein levels in Eurasian Kestrel nestlings. Funct. Ecol. 18: 426–434.

Matzkin LM, Markow TA, 2009. Transcriptional regulation of metabolism associated with the increased desiccation resistance of the cactophilic *Drosophila mojavensis*. Genetics 182: 1279–1288.

McMillan DM, Fearnley SL, Rank NE, Dahlhoff EP, 2005. Natural temperature variation affects larval survival, development and Hsp70 expression in a leaf beetle. Funct. Ecol. 19: 844–852.

Minier C, Borghi V, Moore MN, Porte C, 2000. Seasonal variation of MXR and stress proteins in the common mussel *Mytilus galloprovincialis*. Aquat. Toxicol. 50: 167–176.

Morales J, Moreno J, Lobato E, Merino S, Tomas G et al., 2006. Higher stress protein levels are associated with lower humoral and cell-mediated immune responses in pied flycatcher females. Funct. Ecol. 20: 647–655.

Morgan TJ, Mackay TFC, 2006. Quantitative trait loci for thermotolerance phenotypes in *Drosophila melanogaster*. Heredity 96: 232–242.

Morimoto RI, Jolly C, Satyal S, Mathew A, Shi Y et al., 1999. Molecular chaperones and the heat shock response. Brit. J. Cancer 80: S18.

Nguyen HT, Joshi CP, Klueva N, Weng J, Hendershot KL et al., 1994. The heat-shock response and expression of heat-shock proteins in wheat under diurnal heat stress and field conditions. Aust. J. Plant Physiol. 21: 857–867.

Nielsen MM, Overgaard J, Sørensen JG, Holmstrup M, Justesen J et al., 2005. Role of HSF activation for resistance to heat, cold and high-temperature knock-down. J. Insect Physiol. 51: 1320–1329.

Nielsen MM, Sørensen JG, Kruhøffer M, Justesen J, Loeschcke V, 2006. Phototransduction genes are upregulated in a global gene expression study of *Drosophila melanogaster* selected for heat resistance. Cell Stress Chaperon. 11: 325–333.

Ohtsu T, Kimura MT, Katagiri C, 1998. How *Drosophila* species acquire cold tolerance-qualitative changes of phospholipids. Eur. J. Biochem. 252: 608–611.

Overgaard J, Sørensen JG, Petersen SO, Loeschcke V, Holmstrup M, 2006. Reorganization of membrane lipids during fast and slow cold hardening in *Drosophila melanogaster*. Physiol. Entomol. 31: 328–335.

Parsell DA, Lindquist S, 1993. The function of heat-shock proteins in stress tolerance: Degradation and reactivation of damaged proteins. Annu. Rev. Genet. 27: 437–496.

Pauwels K, Stoks R, De Meester L, 2005. Coping with predator stress: interclonal differences in induction of heat-shock proteins in the water flea *Daphnia magna*. J. Evol. Biol. 18: 867–872.

Pockley AG, 2003. Heat shock proteins as regulators of the immune response. Lancet 362: 469–476.

Pörtner HO, 2001. Climate change and temperature-dependent biogeography: Oxygen limitation of thermal tolerance in animals. Naturwissenschaften 88: 137–146.

Pyza E, Mak P, Kramarz P, Laskowski R, 1997. Heat shock proteins (HSP70) as biomarkers in ecotoxicological studies. Ecotox. Environ. Safe. 38: 244–251.

Qin W, Neal SJ, Robertson RM, Westwood JT, Walker VK, 2005. Cold hardening and transcriptional change in *Drosophila melanogaster*. Insect Mol. Biol. 14: 607–613.

Queitsch C, Sangster TA, Lindquist S, 2002. Hsp90 as a capacitor of phenotypic variation. Nature 417: 618–624.

Rinehart JP, Hayward SAL, Elnitsky MA, Sandro LH, Lee RE et al., 2006. Continuous up-regulation of heat shock proteins in larvae, but not adults, of a polar insect. P. Natl. Acad. Sci. USA 103: 14223–14227.

Rodgers KJ, Ford JL, Brunk UT, 2009. Heat shock proteins: Keys to healthy ageing? Redox Rep. 14: 147–153.

Rutherford SL, 2003. Between genotype and phenotype: Protein chaperones and evolvability. Nat. Rev. Genet. 4: 263–274.

Rutherford SL, Lindquist S, 1998. Hsp90 as a capacitor for morphological evolution. Nature 396: 336–342.

Sagarin RD, Somero GN, 2006. Complex patterns of expression of heat-shock protein 70 across the southern biogeographical ranges of the intertidal mussel *Mytilus californianus* and snail *Nucella ostrina*. J. Biogeogr. 33: 622–630.

Sarup P, Dahlgaard J, Norup AM, Jørgensen KT, Hebsgaard MB et al., 2004. Down regulation of Hsp70 expression level prolongs the duration of heat-induced male sterility in *Drosophila buzzatii*. Funct. Ecol. 18: 365–370.

Sarup P, Sørensen JG, Dimitrov K, Barker JSF, Loeschcke V, 2006. Climatic adaptation of *Drosophila buzzatii* populations in southeast Australia. Heredity 96: 479–486.

Schlesinger MJ, 1990. Heat shock proteins. J. Biol. Chem. 265: 12111–12114.

Selye H, 1956. The Stress of Life. New York: McGraw-Hill.

Shreve SM, Kelty JD, Lee RE, 2004. Preservation of reproductive behaviors during modest cooling: Rapid cold-hardening fine-tunes organismal response. J. Exp. Biol. 207: 1797–1802.

Sibly RM, Calow P, 1989. A life-cycle theory of responses to stress. Biol. J. Linn. Soc. 37: 101–116.

Sørensen JG, Kristensen TN, Loeschcke V, 2003. The evolutionary and ecological role of heat shock proteins. Ecol. Lett. 6: 1025–1037.

Sørensen JG, Loeschcke V, 2001. Larval crowding in *Drosophila melanogaster* induces Hsp70 expression, and leads to increased adult longevity and adult thermal stress resistance. J. Insect Physiol. 47: 1301–1307.

Sørensen JG, Loeschcke V, Kristensen TN, 2009a. Lessons from the use of genetically modified *Drosophila melanogaster* in ecological studies: Hsf mutant lines show highly trait–specific performance in field and laboratory thermal assays. Funct. Ecol. 23: 240–247.

Sørensen JG, Nielsen MM, Kruhoffer M, Justesen J, Loeschcke V, 2005a. Full genome gene expression analysis of the heat stress response, in *Drosophila melanogaster*. Cell Stress Chaperon. 10: 312–328.

Sørensen JG, Nielsen MM, Loeschcke V, 2007. Gene expression profile analysis of *Drosophila melanogaster* selected for resistance to

environmental stressors. J. Evolution. Biol. 20: 1624–1636.

Sørensen JG, Norry FM, Scannapieco AC, Loeschcke V, 2005b. Altitudinal variation for stress resistance traits and thermal adaptation in adult *Drosophila buzzatii* from the New World. J. Evolution. Biol. 18: 829–837.

Sørensen JG, Pekkonen M, Lindgren B, Loeschcke V, Laurila A et al., 2009b. Complex patterns of geographic variation in heat tolerance and Hsp70 expression levels in the common frog *Rana temporaria*. J. Therm. Biol. 34: 49–54.

Sørensen JG, Sarup P, Kristensen TN, Loeschcke V, 2008. Temperature induced hormesis in *Drosophila*. In: Le Bourg E, Rattan SIS ed. Mild Stress and Healthy Aging. Netherlands: Springer Verlag, 65–79.

Stratman R, Markow TA, 1998. Resistance to thermal stress in desert *Drosophila*. Funct. Ecol. 12: 965–970.

Sun W, van Montagu M, Verbruggen N, 2002. Small heat shock proteins and stress tolerance in plants. Biochim. Biophys. Acta 1577: 1–9.

Sun Y, MacRae TH, 2005. Small heat shock proteins: Molecular structure and chaperone function. Cell. Mol. Life Sci. 62: 2460–2476.

Zatsepina OG, Ulmasov KA, Beresten SF, Molodtsov VB, Rybtsov SA et al., 2000. Thermotolerant desert lizards characteristically differ in terms of heat-shock system regulation. J. Exp. Biol. 203: 1017–1025.