Functional Ecology

British Ecological Society

Functional Ecology 2009, **23**, 233–239 doi: 10.1111/j.1365-2435.2009.01541.x

Dynamics of heat-induced thermal stress resistance and Hsp70 expression in the springtail, *Orchesella cincta*

Simon Bahrndorff[1,2,3]*, Janine Mariën[4], Volker Loeschcke[1] and Jacintha Ellers[4]

[1]*Ecology and Genetics, Department of Biological Sciences, University of Aarhus, Ny Munkegade, Building 1540, DK-8000 Aarhus C, Denmark;* [2]*Natural History Museum, Wilhelm Meyers Alle 210, DK-8000 Aarhus C, Denmark;* [3]*Danish Agricultural Advisory Service, Udkærsvej 15, DK-8200 Århus N, Denmark; and* [4]*Department of Animal Ecology, Institute of Ecological Science, Faculty of Earth and Life Sciences, VU University Amsterdam, De Boelelaan 1085, 1081 HV Amsterdam, The Netherlands*

Summary

1. The relationship between thermal resistance and expression of inducible heat shock proteins, especially Hsp70, depends on the species and temperature treatments. The induction of Hsp70 has been shown to be essential for heat stress survival in a number of species, yet the maximum protein expression levels do not coincide with peak survival after heat hardening in *Drosophila*.

2. Here we study the functional relationship between heat-induced expression of the heat shock protein Hsp70, and thermal resistance in adult *Orchesella cincta* by comparing thermal resistance (survival of 37·4 °C for 60 min) with Hsp70 gene and protein expression levels, all three measured at time points 2, 4, 6, 23, 27, 49 h after a heat hardening treatment (35·4 °C for 60 min).

3. Thermotolerance increased over time after heat hardening until 49 h after exposure when the experiment ended. On the other hand the expression of *hsp70* messenger RNA reached a peak within the first 2 h and then sharply decreased after 6 h. Within 23 h *hsp70* expression was back to control levels.

4. Surprisingly, protein levels of Hsp70 followed thermotolerance and reached the highest levels 49 h after heat hardening. A significant positive association was found between thermotolerance and Hsp70 protein levels, but not with *hsp70* mRNA levels.

5. Our results support a strong correlation between Hsp70 expression levels and thermal resistance following a heat hardening treatment. They also show that gene and protein expression follow different dynamics, a difference that may be important for our understanding of the role of candidate genes in functional studies.

Key-words: acclimation, heat shock resistance, collembolan, temperature, plastic responses

Introduction

Thermal acclimation is a plastic response that can occur during development and in different life stages of an organism and can have different durations. Short-term exposure to acclimation is often termed hardening (Loeschcke & Sørensen 2005). Organisms that have been pre-exposed to a non-lethal high temperature treatment exhibit an increased heat shock resistance for a prolonged period of time (Loeschcke *et al.* 1994; Dahlgaard *et al.* 1998). This response is termed as heat hardening and the acquired thermotolerance has been demonstrated in many insects (Chen *et al.* 1990; Carretero *et al.* 1991; Chen *et al.* 1991; Yocum & Denlinger 1992; Dahlgaard *et al.* 1998), yeast (McAlister & Finkelstein 1980), mice (Li & Werb 1982) and mammalian cell cultures (Landry *et al.* 1982). Heat hardening may induce both positive and negative effects with respect to fitness of an organism. The negative fitness effects of heat hardening include a reduced fecundity (Krebs & Loeschcke 1994), and a decrease of growth rate and cell division (Feder *et al.* 1992; Krebs & Feder 1997). Whereas the negative fitness effects often occur instantaneously after exposure and attenuate with time elapsed after treatment (Hercus *et al.* 2003), the positive fitness effects seem to reach their maximum several hours or more after treatment (e.g. Dahlgaard *et al.* 1998). Thus negative and positive fitness effects occur on different time scales. Studies have also shown heat-hardening-induced changes in the metabolic profile (Malmendal *et al.* 2006) and gene expression patterns (Sørensen *et al.* 2005a), where the latter mostly return to pre-hardening values within 4 h after treatment.

*Corresponding author. E-mail: simon.bahrndorff@biology.au.dk

Both the improved heat resistance and the negative fitness effects of heat hardening have often been associated with the induction of heat shock proteins (Dahlgaard *et al.* 1998; Krebs & Feder 1998a; Gregersen *et al.* 2001; Sørensen *et al.* 2003). Heat stress causes thermal denaturation of proteins, which in turn induces heat shock proteins. These proteins help prevent inappropriate protein aggregation in organisms by binding to the denaturated protein; they thus prevent its hydrophobic domains from associating with other damaged proteins (Feder & Hofmann 1999; Sørensen *et al.* 2003). Although under non-stressful conditions accumulated levels of heat shock proteins can interfere with regular protein function and constitute a fitness cost (Georgopoulos & Welch 1993; Sherman & Goldberg 2001), the heat shock response becomes increasingly important during stressed situations.

However, the function of specific heat shock proteins, and Hsp70 in particular, for inducible thermotolerance remains unclear. In certain organisms the levels of Hsp70 gene expression (Bettencourt *et al.* 2008) as well as the synthesis and persistence of higher molecular weight heat shock proteins correlate well with the acquired thermotolerance (Landry *et al.* 1982; Li & Werb 1982). Yet in other organisms this is not the case (Yocum & Denlinger 1992; Dahlgaard *et al.* 1998) and the relative importance of heat shock proteins for the development of thermotolerance in different species has therefore been questioned. Furthermore, the induction of heat shock proteins in response to heat hardening shows dependency on the environment. For example, individuals originating from hot environments express lower levels of heat shock proteins than individuals originating from warmer environments (Sarup *et al.* 2004; Sørensen *et al.* 2005b). Therefore, the heat shock response reflects the severity of the stress experienced by the organism, but also its evolutionary background (Köhler *et al.* 1999; Sørensen *et al.* 2001).

A number of studies have focused specifically on the relevance of Hsp70 in thermotolerance (Krebs & Feder 1998b; Sørensen *et al.* 2003). They have provided important information on the heat shock response and thermotolerance as seen from an ecological and evolutionary perspective. For example, studies using an extra copy strain of *Drosophila melanogaster*, which has an additional 6 *hsp70* transgenes, have shown that these strains perform better under hot conditions than control strains although too high levels of Hsp70 can actually decrease thermotolerance (Krebs & Feder 1998b). This indicates that manipulation of a single trait (Hsp70 expression) is sufficient to alter thermotolerance, yet natural selection may balance benefits and costs of high and low expression levels in natural populations (Sørensen *et al.* 2003). Therefore we expect the specific patterns of the heat shock response to depend on the actual and past thermal conditions of the organism.

Soil organisms are exposed to quite different but well-defined and commonly more stable thermal conditions compared to other model organisms, such as *D. melanogaster*. Because of the winglessness of many soil organisms they usually have a reduced ability to escape unfavourable temperature conditions. Earlier studies have shown that soil organisms show local adaptation to temperature regimes (Bahrndorff *et al.* 2006; Liefting & Ellers 2008), and that there is variation in thermal responses (Bahrndorff *et al.* 2007; Driessen, Ellers & van Straalen 2007; Ellers *et al.* 2008). Unfortunately, hardly any studies address the heat hardening and the associated expression of heat shock proteins in soil ecosystems (but see Selvan *et al.* 1996). Here, we want to describe the heat hardening response in a soil arthropod, *Orchesella cincta* (Collembola) (Fig. 1). We look at the functional relationship between heat resistance and the induction of heat shock protein Hsp70 and *Hsp70* gene expression at various intervals after heat hardening. Our aims are threefold; first, to establish if the heat hardening response in *O. cincta* is similar to above-ground insects such as *D. melanogaster*. We expect that soil-dwelling species such as *O. cincta* respond slower because they live in a thermally more stable environment. Second, we investigate how Hsp70 changes during heat hardening, both at the protein and mRNA level. Based on most other studies we expect that Hsp70, both RNA and protein, will increase shortly after the hardening treatment. The last aim is to relate changes in Hsp70 (both protein and RNA levels) to changes in heat resistance, as this seems to be dependent on the species in question.

Fig. 1. Image of *Orchesella cincta*© Theodoor Heijermans.

Methods

ORIGIN AND MAINTENANCE OF POPULATIONS

A mass population was formed by individuals collected from a site in the Netherlands (Roggebotzand: 52°34′N, 05°47′E). The population was founded by 300–400 individuals to obtain sufficient genetic material. Springtails were held in hard plastic containers containing a substrate of water-saturated plaster of Paris. The culture was kept at 16 ± 1 °C in a 12 : 12 h light : dark regime and fed on small twigs with algae growing on them. Twigs were frozen at –20 °C, 7 days prior being fed to the springtails to avoid biological contamination.

THERMAL EXPOSURE CONDITIONS

Heat hardening treatment

To induce thermotolerance, springtails were transferred to glass vials containing slightly moistened foam at the bottom and moistened

foam stoppers and exposed to a heat hardening treatment (35·4 °C for 60 min in a waterbath). The heat hardening temperature chosen was based on a prior pilot experiment. After heat hardening treatment, springtails were transferred back to control conditions and allowed to recover for different lengths of time (2, 4, 6, 23, 27 and 49 h). Individuals having the longest recovery period were exposed first followed by the other groups so that all groups ended recovery time simultaneously. The control group was not exposed to a heat hardening treatment, but individuals were otherwise kept under the same condition at 16 °C. After the recovery, all experimental individuals were either frozen at –80 °C for future mRNA or protein analyses or assayed for thermotolerance. Ten individuals per vial and 10 vials per treatment were tested for thermotolerance and 10 individuals per vial and five vials per treatment were used for mRNA and protein measurements.

Thermal stress resistance

Springtails assayed for thermal resistance were exposed as above, but to a potentially lethal heat stress (37·4 °C) for 60 min. Ten individuals per vial and 10 vials per treatment were tested. After exposure individuals were allowed to recover for 24 h before mortality was assayed. Individuals that were able to walk in a coordinated fashion after gentle stimuli with a fine brush were considered as survivors.

IMMUNOCHEMICAL ANALYSIS

For determination of Hsp70 protein expression levels each of five vials per treatment was homogenized individually and run on a SDS-page and subsequently analysed by Western-blotting. Springtails were homogenized in ice-cold phosphate-buffered saline (PBS) containing 2 mM PEFA block and 1% (volume) antiprotease cocktail (100 μL mL^{-1} pepstatin A, 50 μL mL^{-1} leupeptin, 10 mM benzamidine, 10 mM sodium metabisulfite). The homogenate was centrifuged for 30 min at 13 000 ***g*** at 4 °C. Subsequently each sample was aliquoted into two replicate samples and frozen at –80 °C. The total protein concentration in the supernatant was determined by means of a BCA assay (Pierce Biochemicals) according to manufacturer's instructions. Constant protein weights were analysed and aliquots of 100 μg for *O. cincta* were loaded in each lane for each treatment. To ensure that equal protein weights were loaded between treatments we incubated samples with monoclonal antibody for actin as well (Affinity BioReagents). These samples did not differ substantially in amount of actin and ensured that comparisons between-treatments and control comparisons could be made. Comparison of Hsp70 protein expression between the different treatments was corrected by incorporating the level of actin.

Protein of the total supernatant was separated by mini-gel SDS-page (10% acrylamide, 0·4% bisacrylamide, 1 h at 150 V). Two microlitres of MagiMark™ XP Western Standard (Invitrogen) was used as standard. Protein was transferred to nitrocellulose and the filter blocked in 5% skim-milk in PBS (137 mM NaCl, 2·7 mM KCl, 4·3 mM Na_2HPO_4, 1·47 mM KH_2PO_4, pH 7·1) for 1 h at room temperature. After washing in buffer for 2 × 5 min in 1% non-fat milk PBST, the primary antibody was added and incubated overnight at 4 °C (different incubation times did not affect expression patterns). Immunodetection was performed using two different primary antibodies. Monoclonal antibody (mouse anti-human hsp70; ABR, Affinity BioReagents, dilution 1 : 5000 in PBS) was used for detection of Hsp70 and another monoclonal antibody (mouse anti-*Arabidopsis* actin; ABR, Affinity BioReagents, dilution 1 : 1000 in PBS) was used for detection of β-actin. After repeated washing for 4 × 5 min in 1% non-fat milk PBST the nitrocellulose filter was incubated in secondary antibody goat anti-mouse IgG (H + L) coupled to peroxidase (Pierce, dilution 1 : 1000 in PBS) for 1 h at room temperature. The membrane was subsequently washed 6 × 5 min in 1% non-fat milk PBST as before. Signal was developed with a chemiluminescent system (Super Signal West Dura Extended Duration Substrate, Pierce) according to the manufacturer's instructions. For each treatment, five biological replicates were analysed for generalities in expression patterns. To enable us to compare controls and treated replicates these samples were always run on the same gel and with equal amounts of protein loaded onto each lane. Quantification of protein bands (Hsp70 and actin) on the blots was done using Versa Doc Imaging System (Biorad) and levels of Hsp70 were calculated relative to the actin level.

PARTIAL GENE SEQUENCE OF *ORCHESELLA CINCTA* HSP70 AND DESIGN OF QPCR PRIMERS

To obtain the (partial) sequence of the inducible *Hsp70* gene of *O. cincta* we designed degenerate primers (Table 1) on the Hsp70 homologues of *D. melanogaster* (GenBank: AE014297) and *Folsomia candida* (Collembase.org: Fc_nor_51C12 and Fc_phe_02A11 (Timmermans *et al.* 2007)). PCR was performed using the GoTaq® Flexi DNA polymerase system of Promega. Cycling parameters were: 5 min 95 °C, 30 × (15 s 95 °C, 45 s 54 °C, 1 min 72 °C), 5 min 72 °C. The PCR product was cloned using the pGEM®-T Vector System of Promega in XL-1 Blue subcloning-grade competent cells of Stratagene. Plasmids were isolated from four positive clones and the inserts were sequenced with BigDye® Terminator v1·1 chemistry (Applied Biosystems) on an ABI PRISM 3100 Genetic Analyzer. The sequences were deposited in GenBank under the Accession numbers FJ009069–FJ009072 and the consensus sequence was used for further analysis. To confirm the identity of the consensus Hsp70 sequence, it was subjected to blastn and blastx (Altschul *et al.* 1990) comparison with the GenBank non-redundant nucleotide (nr/nt) and protein (nr) data base, respectively.

Quantitative Real Time PCR primers were developed on the consensus Hsp70 sequence using software package Primer Express version 1·5 (Applied Biosystems) to have an annealing temperature of 60 °C, and to amplify an amplicon of 80–120 bp with 45–55% GC content (Table 1).

In order to determine PCR efficiency, standard curves were obtained in triplicate for the RT-PCR primer set with fourfold dilutions of a standard batch cDNA (Pfaffl 2001). Quantitative RT-PCR was performed on a DNA engine Opticon (MJ Research), using universal cycling conditions (15 min at 95 °C; 15 s at 95 °C, 1 min 60 °C,

Table 1. Degenerate primers of the *Hsp70* homologues of *D. melanogaster* and *F. candida* and RT-PCR primers developed on the consensus *Hsp70* sequence

Primer	Sequence
Degenerate	
Hsp70-1580F	5′-TCC ATC AAC CCR GAY GAR GCW GT-3′
Hsp70-2023R	5′-CGW CCC TTG TCG TTY TTG ATS G-3′
RT-PCR	
Hsp70-RT-F	5′-ATC AAA GTT TAT GAG GGA GAG CGA-3′
Hsp70-RT-R	5′-AAG GTG ACT TCA ATT TGT GGC AC-3′

40 cycles) with a single fluorescence measurement and a melting curve program (60–90 °C with a heating rate at 0·1 °C s^{-1} and fluorescence measurement every second).

RNA EXTRACTION AND QUANTITATIVE RT-PCR ANALYSIS

After the treatments animals were frozen in liquid nitrogen and stored at –80 °C. Animals (10 individuals per vial) were crushed and RNA was isolated using SV Total RNA isolation system (Promega). For cDNA synthesis 5 µL of total RNA (approximately 100 ng RNA μL^{-1}) was reverse transcribed using 200 U MML-V reverse transcriptase (Promega) and 0·5 µg oligo(d)T, according to the manufacturer's instructions. The cDNA samples were 1 : 3 diluted and 2 µl was used in 20 µL PCR reaction volumes containing forward and reverse Hsp70-RT primers and *Power* SYBR Green PCR Master Mix (Applied Biosystems). Quantitative RT-PCR was performed in triplicate for each sample as described above. A mean normalized expression value (MNE) was calculated from the obtained Ct values with the Q-Gene module[29] using β-actin as a reference gene for normalization of input cDNA.

STATISTICAL ANALYSIS

Survival fractions were acrsin-square root transformed to prevent the variance to be a function of the mean and improve normality. Levene's test was used to test for equality of variances. Subsequently we used one-way ANOVA to test for treatment effects on survival and Scheffe's *post hoc* test to test for differences between treatments. Welch test was used when assumptions of equality of variance was not met (expression of *Hsp70*) and Games–Howell *post hoc* test to test for differences between treatments. Due to no detectable levels of Hsp70 protein in some groups of treatments, a nonparametric Kruskal–Wallis test was used to test for treatment effects. Correlation analysis (Spearman correlation coefficient) was used to test for associations between thermotolerance and Hsp70 protein levels and mRNA expression. Analyses were performed using the statistical package SPSS 14·0 (SPSS Inc., Chicago, IL).

Results

INDUCTION OF THERMAL HEAT RESISTANCE AFTER HEAT SHOCK

In the control group (Fig. 2), which did not experience prior heat hardening, survival after heat shock was 31%. Survival gradually increased with time after hardening and reached a maximum in the 27 and 49 h group. One-way ANOVA shows that time after hardening explains a significant proportion of survival ($F_{6,69} = 10{\cdot}65$, $P < 0{\cdot}0001$). *Post hoc* test revealed that survival of the control group without hardening was significantly lower compared to 23, 27 and 49 h after hardening ($P < 0{\cdot}05$) and survival 2 and 4 h after hardening were significantly lower compared to 27 and 49 h past hardening ($P < 0{\cdot}05$) (Fig. 2).

INDUCTION OF HSP70 mRNA EXPRESSION AFTER HEAT SHOCK

The partial *Hsp70* gene sequence obtained for *O. cincta* was 443 bp long and showed highly significant similarity to homologous Hsp70 nucleotide (*Crassostrea gigas*; AF144646; 7e-56; 76% nucleotide identity) and protein (*D. melanogaster*; AAW34354; 1e-59; 84% amino acid identity).

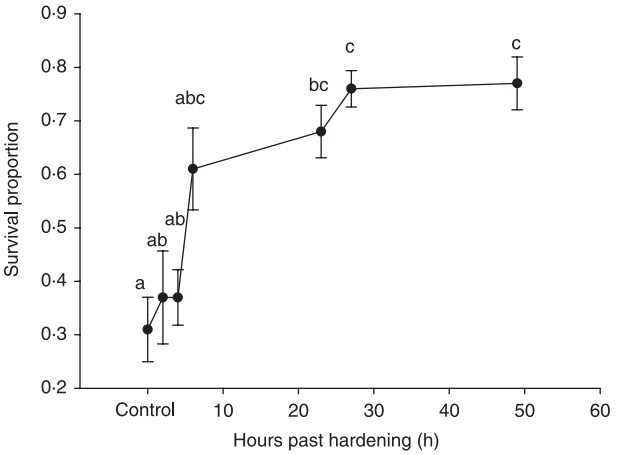

Fig. 2. Survival proportion after 60 min exposure at 37·4 °C (24 h of recovery; means ± SE). Survival was measured at different times (h) elapsed between heat hardening and exposure to heat shock. The control hour treatment did not receive any prior heat hardening treatment.

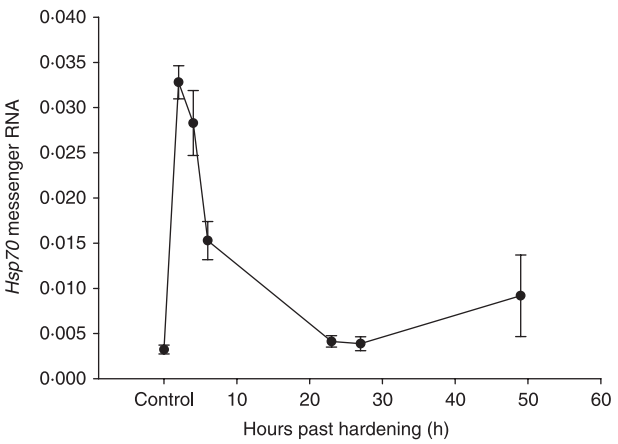

Fig. 3. Levels of *Hsp70* (mRNA) (in percentage of standard) in *Orchesella cincta* at different times elapsed after heat hardening. Means ± SE. *Hsp70* was measured at different times elapsed after heat hardening (35·4 °C for 60 min in a waterbath). After heat hardening treatment, springtails were allowed to recover for different lengths of time (2, 4, 6, 23, 27 and 49 h) at control conditions (16 °C). The control hour treatment did not receive any prior heat hardening treatment.

In the control group without heat hardening, expression of *Hsp70* mRNA was low. However, expression of *Hsp70* sharply increased and reached its peak after only 2 h of recovery (Fig. 3). At 4 and 6 h after hardening treatment expression was elevated but decreasing. After 23 and 27 h from hardening treatment expression levels were back to the control level. The Welch test shows that time after hardening explains a significant proportion of *Hsp70* mRNA ($F_{6,12{\cdot}015} = 40{\cdot}547$, $P < 0{\cdot}001$). *Post hoc* tests revealed that *Hsp70* mRNA 2, 4 and 6 h after hardening treatment was significantly higher than in the control 23 and 27 h past hardening ($P < 0{\cdot}05$). Furthermore,

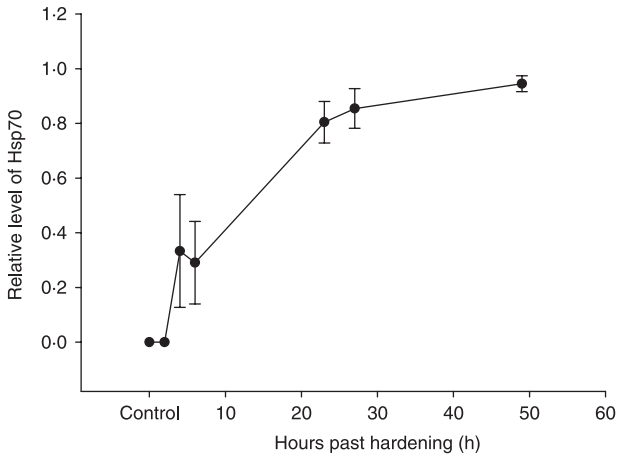

Fig. 4. Levels of Hsp70 (protein) (protein: to actin ratio) in *Orchesella cincta* at different times elapsed after heat hardening. Means ± SE. Hsp70 was measured at different times (h) elapsed after heat hardening (35·4 °C for 60 min in a waterbath). After heat hardening treatment, springtails were allowed to recover for different lengths of time (2, 4, 6, 23, 27 and 49 h) at control conditions (16 °C). The control hour treatment did not receive any prior heat hardening treatment.

Hsp70 mRNA 2 h after hardening was significantly higher ($P < 0{\cdot}05$) compared to all treatments except at 4 h.

INDUCTION OF HSP70 PROTEIN LEVELS AFTER HEAT SHOCK

Levels of protein Hsp70 were not detectable in the control treatment and 2 h after hardening treatment. However, levels of Hsp70 gradually increased thereafter (Fig. 4) and at 4 and 6 h after the hardening treatment expression was elevated but with large variation. The protein level continued to increase at 23 and 27 h after the hardening treatment and reached its peak at 49 h after hardening. A Kruskal–Wallis test revealed a significant effect of treatment ($\chi^2 = 25{\cdot}191$, df = 6, $P < 0{\cdot}001$).

THE ASSOCIATION BETWEEN HSP70 AND THERMAL TOLERANCE

We performed correlation analyses to test if higher levels of Hsp70 (mRNA and protein) were associated with increased thermotolerance. The correlation coefficients revealed that there was a highly significant correlation between survival and Hsp70 protein levels ($r = 0{\cdot}918$, $n = 7$, $P < 0{\cdot}01$), but not for *Hsp70* mRNA ($r = -0{\cdot}126$, $n = 7$, $P = 0{\cdot}788$).

Discussion

Heat hardening treatment induces thermotolerance in a wide range of organisms. The induced heat tolerance persists for different lengths of time depending on the species being examined (Landry *et al.* 1982; Li & Werb 1982; Carretero *et al.* 1991; Dahlgaard *et al.* 1998). The present results show that a heat hardening treatment induces thermal resistance of *O. cincta* to temperatures which are otherwise lethal (Fig. 2). Protein levels of Hsp70 followed thermotolerance and reached the highest levels 49 h after heat hardening, whereas this was not the case for *Hsp70* mRNA, highlighting that protein levels of Hsp70 are temporally associated with thermotolerance in *O. cincta* (Figs 3 and 4).

The response to the hardening treatment conformed to the general heat hardening pattern found in other organisms, although in *O. cincta* heat resistance was maintained for a relatively long period of time. Thermal resistance reached its peak after 27 h and remained elevated for at least 49 h, when the experiment ended (Fig. 2). This pattern was closely matched by the protein levels of Hsp70. Such a prolonged and stable response is not exceptional: in some fish species high levels of Hsp70 were maintained for at least 5 days after a 2-h heat-shock (Bierkens 2000). Also, in chironomids thermotolerance remained elevated until 72 h past hardening and Hsp70 protein expression remained elevated 12–34 h after the hardening treatment (Carretero *et al.* 1991). In contrast, in *D. melanogaster* and in mammalian cell cultures Hsp expression and heat resistance generally return to almost normal after 24–32 h (Dahlgaard *et al.* 1998; Feige *et al.* 1996). It should however be pointed out that the results are based on individuals from one population of *O. cincta* and recent results have shown genetic variation for plastic traits in this species (Liefting & Ellers 2008; Bahrndorff *et al.* 2008).

The rate of induced protein production after heat hardening in *O. cincta* is particularly slow: although elevated protein levels could be detected 4–6 h after heat hardening, protein expression levels still increased until the end of the experimental period (49 h) (Fig. 4). Hsp70 protein levels peak much faster in *D. melanogaster*, that is 2 h past hardening, which is even before any increases in thermotolerance are apparent (Dahlgaard *et al.* 1998). It is unlikely that the different rates of protein production are due to changes in the speed of mRNA *Hsp70* expression. The temporal dynamics of mRNA *Hsp70* levels found in the present study (Fig. 3) are in agreement with transcriptional profiling of heat-induced thermotolerance in *D. melanogaster* (Sørensen *et al.* 2005a), where mRNA levels of most stress proteins are almost back to control levels within 6–8 h after the heat hardening treatment. Rather, protein production seems exceptionally fast in *D. melanogaster*, as other taxa also show a delayed onset of protein production starting after 6 to12 h of recovery. For example, chironomids (*C. thummi*) reach maximum Hsp70 elevation 12 h after the hardening treatment (Carretero *et al.* 1991), bay scallops reach maximum Hsp levels after 6 h (Brun *et al.* 2008), and trout Hsp72 peak between 6 and 24 h after heat hardening (Werner *et al.* 1996).

A possible explanation for the striking discrepancy in temporal dynamics of protein levels and heat resistance between *D. melanogaster* and *O. cincta* can be found in the natural thermal regime experienced by the different species used. Under natural conditions, *O. cincta* lives on the soil surface, but it can retreat to deeper soil layers where the temperature is less variable and more predictable (Bahrndorff *et al.* 2008). The thermal regime of soil habitats is more buffered than the ambient temperatures experienced by most insects (Liefting

& Ellers 2008). Based on the predictions of Krebs & Feder (1998b) individuals of *O. cincta* should therefore produce Hsp70 slower than *D. melanogaster*. Also, the temperature conditions during the recovery period can affect the speed of protein production. In Dahlgaard *et al.* (1998) *Drosophila* individuals were kept at 25 °C after the hardening treatment and in the present study individuals were kept at 16 °C, slowing the turnover rate of Hsp70 protein.

Another cause of variation in Hsp70 protein levels is the severity of the hardening treatment. In *D. melanogaster* cell lines recovery and synthesis of Hsp70 is proportional to the severity of the stress experienced (DiDomenico *et al.*1982). A similar relation between stress exposure and Hsp70 levels is found in the Collembolan *O. bifasciata*, when exposed to different concentrations of heavy metals. These soil invertebrates show low concentrations of Hsp70 levels after moderate exposure, while induced levels of Hsp70 increase with heavy metal exposure until reaching a non-compensation zone where Hsp70 levels decrease (Eckwert *et al.* 1997; Köhler *et al.* 2000). Hence, at high levels of metal exposure Hsp70 actually decreases which is likely to be due to pathological cell damage. Whether such an effect could explain the present results is uncertain.

The association between inducible thermotolerance and Hsp70 protein levels has been described in a number of studies using different organisms, exposures and time scales. Some studies have found a strong relation between thermal tolerance and Hsp70 protein expression (Carretero *et al.* 1991; Eckwert *et al.* 1997; Landry *et al.* 1982; Li & Werb 1982), whereas other studies have not (Dahlgaard *et al.* 1998; Krebs *et al.* 1998b; Yocum *et al.* 1992). The complexity is exemplified in a study by Krebs & Feder (1998b) comparing a control and extra-copy lines of *D. melanogaster*, where a tight association was found between inducible thermotolerance and Hsp70 levels until critically high levels of Hsp70 were reached. The correlation not only illustrates the role of Hsp's in thermotolerance at moderate stress levels, but also highlights that expression of Hsp70 comes at a cost (Sørensen *et al.* 2003). Furthermore, Hsp70 expression is eliminated when stress is absent (Solomon *et al.* 1991) and also after the recovery phase has ended (DiDomenico *et al.* 1982). As pointed out by Krebs & Feder (1998b) variation in Hsp70 has positive and negative effects and the trade-offs between them can affect regulatory control. The benefits and costs of different regulatory patterns may vary with different thermal environments. The temporal dynamics of Hsp70 expression levels following heat hardening in the present study also raises a number of questions concerning costs and benefits of heat hardening in soil organisms. Contrary to results obtained on *Drosophila* Hsp70 levels do not seem to disappear fast in *O. cincta*. This obviously indicates a different temporal dynamics of cost and benefit functions between these organisms.

The present results highlight that we need to increase our understanding of the heat hardening response. Transcriptomic and metabolomic tools have yielded valuable information on the heat hardening response in fruit flies (Sørensen *et al.* 2005a; Malmendal *et al.* 2006). However, we need to broaden the use of model systems to include species from different thermal niches and with different patterns of hardening responses and the underlying cost : benefit ratios. At present we do not have a full understanding of why some species show a similar hardening response whereas others do not.

Acknowledgements

SB was supported by a grant from the graduate school ISOBIS and Natural History Museum of Aarhus and an exchange grant from the European Science Foundation (ESF). JE was supported by the Netherlands Organization for Scientific Research, VIDI-grant 864·03 003. This study was further supported by grants from the Danish Natural Science Research Council (VL).

References

Altschul, S.F., Gish, W., Miller, W., Myers, E.W. & Lipman, D.J. (1990) Basic local alignment search tool. *Journal of Molecular Biology*, **3**, 403–410.

Bahrndorff, S., Holmstrup, M., Petersen, H. & Loeschcke, V. (2006) Geographic variation for climatic stress resistance traits in the springtail *Orchesella cincta*. *Journal of Insect Physiology*, **52**, 951–959.

Bahrndorff, S., Loeschcke, V., Pertoldi, C., Beier, C. & Holmstrup, M. (2008) The rapid cold hardening response of Collembola is influenced by thermal variability of the habitat. *Functional Ecology*, **2008**, in press. DOI: 10.1111/j.1365-2435.2008.01503.x.

Bahrndorff, S., Petersen, S.O., Loeschcke, V., Overgaard, J. & Holmstrup, M. (2007) Differences in cold and drought tolerance of high arctic and subarctic populations of *Megaphorura arctica* Tullberg 1876 (Onychiuridae: Collembola). *Cryobiology*, **55**, 315–323.

Bettencourt, B.R., Hogan, C.C., Nimali, M. & Drohan, B.W. (2008) Inducible and constitutive heat shock gene expression responds to modification of *Hsp70* copy number in *Drosophila melanogaster* but does not compensate for loss of thermotolerance in *Hsp70* null flies. *BMC Biology*, **6**, 5.

Bierkens, J.G.E.A. (2000) Applications and pitfalls of stress-proteins in biomonitoring. *Toxicology*, **153**, 61–72.

Brun, N.T., Bricely, V.M., MacRae, T.H. & Ross, N.W. (2008) Heat shock protein responses in thermally stressed bay scallops, *Argopecten irradians*, and sea scallops, *Placopecten magellanicus*. *Journal of Experimental Marine Biology and Ecology*, **358**, 151–162.

Carretero, M.T., Carmona, M.J. & Diez, J.L. (1991) Thermotolerance and heat-shock proteins in Chironomus. *Journal of Insect Physiology*, **37**, 239–246.

Chen, C.P., Lee, R.E. & Denlinger, D.L. (1990) A comparison of the responses of tropical and temperate flies (Diptera, Sarcophagidae) to Cold and Heat-Stress. *Journal of Comparative Physiology B-Biochemical Systemic and Environmental Physiology*, **160**, 543–547.

Chen, C.P., Lee, R.E. & Denlinger, D.L. (1991) Cold shock and heat-shock – A comparison of the protection generated by brief pretreatment at less severe temperatures. *Physiological Entomology*, **16**, 19–26.

Dahlgaard, J., Loeschcke, V., Michalak, P. & Justesen, J. (1998) Induced thermotolerance and associated expression of the heat-shock protein Hsp70 in adult *Drosophila melanogaster*. *Functional Ecology*, **12**, 786–793.

DiDomenico, B.J., Bugaisky, G.E. & Lindquist, S. (1982) The heat-shock response is self-regulated at both the transcriptional and posttranscriptional levels. *Cell*, **31**, 593–603.

Driessen, G., Ellers, J. & van Straalen, N.M. (2007) Variation, selection and heritability of thermal reaction norms for juvenile growth in *Orchesella cincta* (Collembola : Entomobryidae). *European Journal of Entomology*, **104**, 39–46.

Eckwert, H., Alberti, G. & Köhler, H.R. (1997) The induction of stress proteins (hsp) in *Oniscus asellus* (Isopoda) as a molecular marker of multiple heavy metal exposure 1. Principles and toxicological assessment. *Ecotoxicology*, **6**, 249–262.

Ellers, J., Marien, J., Driessen, G. & van Straalen, N.M. (2008) Temperature-induced gene expression associated with different thermal reaction norms. *Journal of Experimental Zoology*, **310B**, 137–147.

Feder, J.H., Rossi, J.M., Solomon, J., Solomon, N. & Lindquist, S. (1992) The consequences of expressing Hsp70 in *Drosophila* cells at normal temperatures. *Genes and Development*, **6**, 1402–1413.

Feder, M.E. & Hofmann, G.E. (1999) Heat-shock proteins, molecular chaperones, and the stress response: Evolutionary and ecological physiology. *Annual Review of Physiology*, **61**, 243–282.

Feige, U., Morimato, R.I., Yahara, I. & Polla, B.S. (1996) *Stress Inducible Cellular Responses*. Birkhauser Verlag, Basel.

Georgopoulos, C. & Welch, W.J. (1993) Role of the major heat-shock proteins as molecular chaperones. *Annual Review of Cell Biology*, **9**, 601–634.

Gregersen, N., Bross, P., Andresen, B.S., Pedersen, C.B., Corydon, T.J. & Bolund, L. (2001) The role of chaperone-assisted folding and quality control in inborn errors of metabolism: Protein folding disorders. *Journal of Inherited Metabolic Disease*, **24**, 189–212.

Hercus, M.J., Loeschcke, V. & Rattan, S.I.S. (2003) Lifespan extension of *Drosophila melanogaster* through hormesis by repeated mild heat stress. *Biogerontology*, **4**, 149–156.

Köhler, H.R., Eckwert, H., Triebskorn, R. & Bengtsson, G. (1999) Interaction between tolerance and 70 kDa stress protein (hsp70) induction in collembolan populations exposed to long-term metal pollution. *Applied Soil Ecology*, **11**, 43–52.

Köhler, H.R., Zanger, M., Eckwert, H. & Einfeldt, I. (2000) Selection favours low hsp70 levels in chronically metal-stressed soil arthropods. *Journal of Evolutionary Biology*, **13**, 569–582.

Krebs, R.A. & Feder, M.E. (1997) Deleterious consequences of Hsp70 over-expression in *Drosophila melanogaster* larvae. *Cell Stress and Chaperones*, **2**, 60–71.

Krebs, R.A. & Feder, M.E. (1998a) Experimental manipulation of the cost of thermal acclimation in *Drosophila melanogaster*. *Biological Journal of the Linnean Society*, **63**, 593–601.

Krebs, R.A. & Feder, M.E. (1998b) Hsp70 and larval thermotolerance in *Drosophila melanogaster*: how much is enough and when is more too much? *Journal of Insect Physiology*, **44**, 1091–1101.

Krebs, R.A. & Loeschcke, V. (1994) Costs and benefits of activation of the heat-shock response in *Drosophila melanogaster*. *Functional Ecology*, **8**, 730–737.

Landry, J., Bernier, D., Chretien, P., Nicole, L.M., Tanguay, R.M. & Marceau, N. (1982) Synthesis and degradation of heat-shock proteins during development and decay of thermotolerance. *Cancer Research*, **42**, 2457–2461.

Li, G.C. & Werb, Z. (1982) Correlation between synthesis of heat-shock proteins and development of thermotolerance in chinese-hamster fibroblasts. *Proceedings of the National Academy of Sciences USA-Biological Sciences*, **79**, 3218–3222.

Liefting, M. & Ellers, J. (2008) Habitat-specific differences in thermal plasticity in natural populations of a soil arthropod. *Biological Journal of the Linnean Society*, **94**, 265–271.

Loeschcke, V. & Sørensen, J.G. (2005) Acclimation, heat shock and hardening – a response from evolutionary biology. *Journal of Thermal Biology*, **30**, 255–257.

Loeschcke, V., Krebs, R.A. & Barker, J.S.F. (1994) Genetic-variation for resistance and acclimation to high-temperature stress in *Drosophila buzzatii*. *Biological Journal of the Linnean Society*, **52**, 83–92.

Malmendal, A., Overgaard, J., Bundy, J.G., Sorensen, J.G., Nielsen, N.C., Loeschcke, V. & Holmstrup, M. (2006) Metabolomic profiling of heat stress: hardening and recovery of homeostasis in *Drosophila*. *American Journal of Physiology-Regulatory Integrative and Comparative Physiology*, **291**, R205–R212.

McAlister, L. & Finkelstein, D.B. (1980) Heat-shock proteins and thermal-resistance in yeast. *Biochemical and Biophysical Research Communications*, **93**, 819–824.

Pfaffl, M.W. (2001) A new mathematical model for relative quantification in real-time RT-PCT. *Nucleic Acids Research*, **29**, 2003–2007.

Sarup, P., Dahlgaard, J., Norup, A.M., Jorgensen, K.T., Hebsgaard, M.B. & Loeschcke, V. (2004) Down regulation of Hsp70 expression level prolongs the duration of heat-induced male sterility in *Drosophila buzzatii*. *Functional Ecology*, **18**, 365–370.

Selvan, S., Grewal, P.S., Leustek, T. & Gaugler, R. (1996) Heat shock enhances thermotolerance of infective juvenile insect-parasitic nematodes *Hererorhabditis bacteriophora* (Rhabditida: Heterorhabditidae). *Experientia*, **52**, 727–730.

Sherman, M.Y. & Goldberg, A.L. (2001) Cellular defenses against unfolded proteins: A cell biologist thinks about neurodegenerative diseases. *Neuron*, **29**, 15–32.

Solomon, J.M., Rossi, J.M., Golic, K., Mcgarry, T. & Lindquist, S. (1991) Changes in Hsp70 alter thermotolerance and heat-shock regulation in *Drosophila*. *New Biologist*, **3**, 1106–1120.

Sørensen, J.G., Dahlgaard, J. & Loeschcke, V. (2001) Genetic variation in thermal tolerance among natural populations of *Drosophila buzzatii*: down regulation of Hsp70 expression and variation in heat stress resistance traits. *Functional Ecology*, **15**, 289–296.

Sørensen, J.G., Kristensen, T.N. & Loeschcke, V. (2003) The evolutionary and ecological role of heat shock proteins. *Ecology Letters*, **6**, 1025–1037.

Sørensen, J.G., Nielsen, M.M., Kruhøffer, M., Justesen, J. & Loeschcke, V. (2005a) Full genome expression analysis of the heat stress response in *Drosophila melanogaster*. *Cell Stress and Chaperones*, **10**, 312–328.

Sørensen, J.G., Norry, F.M., Scannapieco, A.C. & Loeschcke, V. (2005b) Altitudinal variation for stress resistance traits and thermal adaptation in adult *Drosophila buzzatii* from the New World. *Journal of Evolutionary Biology*, **18**, 829–837.

Timmermans, M.J.T.N., de Boer, M.E., Nuckols, J., de Boer, T.E., Mariën, J., Klein-Lankhorst, R.M., van Straalen, N.M. & Roelofs, D. (2007) Collembase: a repository for springtail genomics and soil quality assessment. *BMC Genomics*, **8**, 341.

Werner, I., Viant, M.R., Rosenblum, E.S., Gantner, A.S., Tjeerdema, R.S. & Johnson, M.L. (1996) Cellular responses to temperature stress in steelhead trout (*Onchorynchus mykiss*) parr with different rearing histories. *Fish Physiology and Biochemistry*, **32**, 261–273.

Yocum, G.D. & Denlinger, D.L. (1992) Prolonged thermotolerance in the flesh fly, *Sarcophaga crassipalpis*, does not require continuous expression or persistence of the 72 Kda Heat-Shock Protein. *Journal of Insect Physiology*, **38**, 603–609.

Received 6 November 2008; accepted 9 January 2009
Handling Editor: Frank Messina