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Genetic variation in heat resistance and HSP70 expression in inbred isofemale lines of the springtail *Orchesella cincta*

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ABSTRACT: The functional relationship between thermotolerance and heat shock protein (HSP) expression is well described. However, the intraspecific variation in HSP70 expression and heat resistance is not fully understood. Some studies have shown a correlation between thermotolerance and HSP70 expression levels at the intraspecific level, whereas others have not, and results depend on species, developmental stage and environment. The present study extends such correlative studies to species from soil ecosystems, where we have characterized the variation in thermotolerance and HSP70 expression in 18 inbred isofemale lines of the springtail *Orchesella cincta*. The results show ample genetic variation among lines in heat shock resistance as well as in HSP70 protein levels and *hsp70* mRNA expression. However, we did not detect any significant positive correlations between HSP70 expression and thermotolerance. These results indicate that the variation in HSP70 expression and heat resistance at the intraspecific level is dependent on species and/or ecosystem and further studies are needed to clarify this relationship.

KEY WORDS: Heat shock resistance · Collembola · Thermotolerance · Thermal adaptation · Climate change · Evolution

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1. INTRODUCTION

Climate change has the potential to increase the average temperature around the world as well as the likelihood and severity of now rare temperature events. Recently, there has been increased attention on the effects of the abiotic environment on the survival, growth and reproduction of animals; the reason being that climate changes are likely to affect species fitness and distributions (Sinclair et al. 2003, Pertoldi & Bach 2007, Chown et al. 2010 this Special). It is therefore important to gain a better understanding of the responses to environmental stress including both adaptive and plastic responses in a broad range of animals from different ecotypes (see in this Special, Fischer & Karl 2010, de Jong et al. 2010, Scharf et al. 2010, van Doorslaer et al. 2010).

Heat shock proteins (HSPs) play an important role in the cellular heat stress response and are important for protecting the cell against high temperature and other stressors (Lindquist & Craig 1988). During or after heat stress thermal damage occurs due to denaturation of proteins, which induces HSPs that prevent deleterious protein aggregations (Sørensen et al. 2003). HSP70 is one of the main heat-inducible molecular chaperones (Lindquist 1984) and is not expressed before the indi-

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vidual experiences stressors such as heat. However, even though HSP70 is presumably the quantitatively most important HSP, it is not the only one and nor is it the only mechanism that promotes thermotolerance (Krebs & Feder 1997b). The role of HSPs in stress resistance has been documented in a number of studies and is reviewed in Feder & Hofmann (1999).

HSPs have been considered as candidates for a common mechanism underlying the evolution of heat resistance (Sørensen et al. 2003). Krebs & Feder (1997a) showed that extra copy lines of HSP70 in *Drosophila melanogaster* expressing higher levels of HSP70 perform better under hot conditions compared to wild type strains, although too high levels of HSP70 actually decreased thermotolerance (Krebs & Feder 1997a, Bettencourt et al. 2008). However, when applying mild and ecologically relevant heat stress multiple times in selection lines, populations express less HSP70 compared to control populations when subsequently exposed to heat stress (Bettencourt et al. 1999, Sørensen et al. 1999). This suggests that there is a cost associated with HSP70 expression and that populations frequently exposed to stress achieve resistance in other ways (Krebs & Feder 1998). HSP70 expression therefore seems to be important during rare and unexpected stress situations (Sørensen et al. 2003). However, expression is not only restricted to severe stress situations, but also occurs during more regular but less severe stress situations (Sørensen & Loeschcke 2001, 2002, Kristensen et al. 2002). While the functional relationship between thermotolerance and HSP expression is well described, this does not necessarily tell us much about the evolutionary perspectives. Studies have looked at patterns between populations in organisms such as *Drosophila* and collembolans. Köhler et al. (1999) found correlations between stress level and HSP70 expression in *Orchesella bifasciata* and Sørensen et al. (2001a) found genetic differences in HSP70 levels and survival after heat shock in *D. buzzatii*. Jensen et al. (2009) have shown that while there is a genetic basis to variation in HSP70 expression levels, the contribution of variation in HSP70 expression to the genetic variation of heat resistance is unclear at the intraspecific level. Results seem to be dependent on species, developmental stage, earlier stress exposure and time and severity of exposure (Krebs & Feder 1997b, Krebs et al. 1998, Hoffmann et al. 2003).

Collembolans, more specifically the species *Orchesella cincta*, show substantial variation in heat resistance among populations (Bahrndorff et al. 2006). Some work has also been conducted on the functional relationship between induced heat resistance and expression of HSP70 in *O. cincta* (Bahrndorff et al. 2009b). However, as with other organisms, studies looking at associations between HSP70 expression and heat shock resistance within natural populations are lacking.

The aim of the present study was to investigate the link between the heat shock response at the molecular and phenotypic levels by using a set of inbred isofemale lines of the collembolan *O. cincta*. This species is a soil-dwelling arthropod that occurs in a wide variety of seasonal habitats (Timmermans et al. 2005, Bahrndorff et al. 2006). It is exposed to quite different thermal conditions, as compared to model organisms such as *Drosophila melanogaster* (Liefting & Ellers 2008, Bahrndorff et al. 2009a), and can therefore provide valuable information on heat shock resistance and expression of HSP70 in species living in the soil. We wanted to:

(1) establish the variation in heat resistance, induction of HSP70 and *hsp70* gene expression between isofemale lines;

(2) correlate protein and gene expression with heat shock resistance between isofemale lines;

(3) look at the effect of inbreeding on both HSP70 expression and heat shock resistance.

2. MATERIALS AND METHODS

2.1. Animals and inbreeding of populations

A total of 30 inbred isofemale lines were set up to obtain numerous individuals of nearly identical genotype required for the experiment. Each line was initiated by one parental pair from a mass-bred population that had been cultured in the laboratory for 5 generations (Driessen et al. 2007). The mass-bred population initially consisted of a mix of 4 populations: Poland (Pilica: 50° 29.1′ N, 19° 39.6′ E), Sweden (Ringarum: 58° 21.5′ N, 16° 13.7′ E) and The Netherlands (Roggebotzand: 52° 34.4′ N, 05° 47.9′ E; Amsterdamse Waterleidingduinen: 52° 1.5′ N, 04° 33.1′ E). Subsequently, the isofemale lines were inbred for 15 generations with 5 selected individuals each generation, which should reduce strong deleterious inbreeding effects. Because not all 5 individuals necessarily contributed to the next generation, the coefficient of inbreeding F was at least 0.83 after 15 generations of inbreeding (Falconer 1989). Inbreeding was relaxed for 5 to 6 generations before individuals were used in the present study. In addition to the inbred isofemale lines, 3 outbred lines were used to test for possible effects of inbreeding.

Collembolans were held in hard plastic containers containing a substrate of water-saturated plaster of Paris. The culture was kept at 16 ± 1°C under a 12 h light:12 h dark regime and fed on small twigs with algae growing on them. Twigs were frozen at –20°C 7 d prior to being fed to the collembolans, to avoid biological contamination.

2.2. Exposure conditions

2.2.1. HSP70 induction

To induce HSP70, collembolans were transferred to glass vials containing slightly moistened foam at the bottom and moistened foam stoppers and exposed to the following heat-shock profile: 35.2 ± 0.5°C for 1 h in a waterbath succeeded by 1 h of recovery at 16 ± 0.1°C before being snap frozen at −80°C; 10 individuals per vial and 5 vials per treatment were used for mRNA and protein measurements, respectively.

2.2.2. Thermal stress resistance

Collembolans assayed for thermal resistance were exposed as above, but to a potentially lethal heat stress (37.4°C) for 1 h. Again, 10 individuals per vial and 5 vials per isofemale line were tested. After exposure, individuals were allowed to recover at 16 ± 0.1°C before mortality was assayed after 22 and 42 h of recovery. Individuals that were able to walk in a coordinated fashion after gentle stimulation with a fine brush were considered as survivors. A total of 18 inbred isofemale lines and 3 control lines were tested for thermal stress resistance.

2.2.3. Immunochemical analysis

For determination of HSP70 protein expression levels, each of 5 vials per isofemale line was homogenized individually and run on a sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and subsequently analysed by western blotting. Collembolans were homogenised in ice-cold phosphate-buffered saline (PBS) containing 2 mM 4-(2-aminoethyl)-benzenesulfonylfluoride (PEFA) block and 1% (volume) antiprotease cocktail (100 µl/ml pepstatin A, 50 µl/ml 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 2 replicate samples and frozen at −80°C. The total protein concentration in the supernatant was determined by means of a bicinchoninic acid (BCA) assay (Pierce Biochemicals) according to the manufacturer's instructions. Aliquots of 100 µg of protein were loaded in each lane for each treatment. To ensure that equal protein weights were loaded between isofemale lines, we incubated samples with monoclonal antibody for β-actin as well (Affinity BioReagents). These samples did not differ substantially in the amount of β-actin and ensured that comparisons between isofemale lines could be made. Comparison of HSP70 protein expression between the different treatments was corrected by incorporating the level of β-actin. HSP70 protein levels were established in 17 inbred isofemale lines and 3 control lines.

Protein of the total supernatant was separated by mini-gel SDS-PAGE (10% acrylamide, 0.4% bisacrylamide, 1 h at 150 V); 2 µl 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 phosphate buffered saline with Tween 20 (PBST), the primary antibody was added and incubated overnight at 4°C. Immunodetection was performed using 2 different primary antibodies. Monoclonal antibody (mouse anti-human HSP70; Affinity BioReagents, dilution 1:5000 in PBS) was used for detection of HSP70 and another monoclonal antibody (mouse anti-*Arabidopsis* actin; 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 isofemale line, 5 biological replicates were analysed for expression patterns. To enable us to compare isofemale lines, samples were always run on the same gel and with equal amounts of protein loaded onto each lane. Quantification of protein bands (HSP70 and β-actin) on the blots was done using Versa Doc Imaging System (BioRad) and levels of HSP70 were calculated relative to the actin level.

2.3. RNA extraction and quantitative RT-PCR analysis

Animals were crushed and RNA was isolated using the 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. We used quantitative real time PCR (RT-PCR) primers for HSP70 RNA expression developed by Bahrndorff et al. (2009b). 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 (MNE) value was calculated from the C_t values with the Q-Gene module using β-actin as a reference gene for normalization of input cDNA. β-actin was recently validated as the most stable reference gene in *Orchesella cincta* for RT-PCR gene expression measurements under temperature stress (de Boer et al. 2009). A total of 17 inbred isofemale lines and 3 control lines were tested for *hsp70* expression.

2.4. Statistical analysis

Survival fractions were arcsin-square root transformed to improve normality. Levene's test showed equal variance and subsequently we used 1-way ANOVA to test for treatment effects on survival and mRNA data. Scheffe's *post hoc* test was used to test for differences between treatments. Due to no detectable levels of HSP70 protein in some isofemale lines, a non-parametric Kruskal-Wallis test was used to test for treatment effects. Correlation analysis (Spearman's rank correlation) was used to test for associations between thermotolerance and HSP70 protein levels and *hsp70* mRNA expression. Heat shock resistance, *hsp70* mRNA and HSP70 protein levels were available for 16 inbred isofemale lines and these were used for the correlation analysis. Significant results were corrected for multiple comparisons following the standard Bonferroni correction (Rice 1989). Analyses were performed using SPSS 11.0 (SPSS 2001).

3. RESULTS

3.1. Thermal heat resistance

There was a large difference in heat resistance among lines (Table 1): heat resistance ranged from 5 to 60% in the inbred lines and from 42 to 51% in the outbred lines at 22 h past exposure. After 42 h of recovery, heat resistance ranged from 15 to 74% in the inbred lines and from 45 to 57% in the outbred lines (Table 1). Mean heat resistance was lower in the inbred lines compared to the outbred lines; 1-way ANOVA showed a significant effect of line after 22 h of recovery ($F_{20,189}$ = 8.89, $p < 0.001$) and also after 42 h of recovery ($F_{20,189}$ = 7.12, $p < 0.001$).

Table 1. *Orchesella cincta*. Heat shock resistance after 22 and 42 h of recovery, levels of HSP70 protein (ratio to actin) and mean normalized expression levels of *hsp70* mRNA (mean ± SE) in inbred and control lines. (–) Analyses not possible due to insufficient sample size

Regime / Line	Heat shock resistance 22 h	Heat shock resistance 42 h	HSP70 protein	*hsp70* mRNA
Control (outbred)				
E1a	0.51 ± 0.03	0.57 ± 0.05	0.372 ± 0.006	0.048 ± 0.006
E1	0.45 ± 0.05	0.48 ± 0.05	0.285 ± 0.006	0.036 ± 0.007
E2	0.42 ± 0.07	0.45 ± 0.07	0.346 ± 0.008	0.066 ± 0.008
Overall mean	0.46 ± 0.03	0.50 ± 0.04	0.335 ± 0.026	0.050 ± 0.009
Inbred				
15b	0.14 ± 0.05	0.23 ± 0.06	0.374 ± 0.006	0.035 ± 0.006
16b	0.35 ± 0.08	0.39 ± 0.08	0.393 ± 0.008	0.093 ± 0.008
17b	0.07 ± 0.04	0.22 ± 0.05	0.560 ± 0.012	0.079 ± 0.012
19b	0.05 ± 0.02	0.15 ± 0.03	0.982 ± 0.06	0.060 ± 0.006
21b	0.17 ± 0.07	0.48 ± 0.06	0.815 ± 0.003	0.015 ± 0.003
25b	0.35 ± 0.07	0.38 ± 0.08	0.722 ± 0.008	0.052 ± 0.008
26a	0.48 ± 0.08	0.55 ± 0.06	0.393 ± 0.006	0.032 ± 0.006
27a	0.13 ± 0.07	0.31 ± 0.07	0.929 ± 0.007	0.031 ± 0.007
32b	0.45 ± 0.10	0.55 ± 0.06	0.000 ± 0.000	0.004 ± 0.001
39b	0.27 ± 0.04	0.31 ± 0.10	0.592 ± 0.199	0.047 ± 0.001
40b	0.61 ± 0.04	0.64 ± 0.06	0.764 ± 0.192	0.091 ± 0.005
41b	0.31 ± 0.07	0.46 ± 0.06	1.008 ± 0.057	0.051 ± 0.007
45b	0.55 ± 0.06	0.69 ± 0.04	0.309 ± 0.191	0.047 ± 0.004
50b	0.24 ± 0.05	0.40 ± 0.06	1.202 ± 0.089	0.057 ± 0.005
53a	0.58 ± 0.04	0.74 ± 0.03	0.571 ± 0.236	0.041 ± 0.009
21a	0.33 ± 0.07	0.60 ± 0.08	0.683 ± 0.231	0.024 ± 0.007
22a	0.28 ± 0.05	0.29 ± 0.06	0.244 ± 0.244	–
37a	0.57 ± 0.06	0.72 ± 0.06	–	0.031 ± 0.003
Overall mean	0.31 ± 0.04	0.45 ± 0.04	0.619 ± 0.071	0.046 ± 0.006

3.2. *hsp70* mRNA expression after heat shock

MNE of *hsp70* varied substantially among lines and ranged from 0.004 to 0.09 (Table 1). The mean expression of *hsp70* was slightly lower in the inbred lines compared to the outbred lines; 1-way ANOVA showed a significant effect of line on *hsp70* expression ($F_{19,80}$ = 13.17, $p < 0.001$).

3.3. HSP70 protein levels after heat shock

The expression of protein HSP70 varied among lines, and in one line (32b) there was no detectable level of HSP70, whereas Line 50b showed the maximum level of HSP70 of 1.2 (Table 1). The mean relative HSP70 expression was lower in the outbred lines compared to the inbred lines. A Kruskal-Wallis test showed a significant effect of line on HSP70 expression (df = 19, χ^2 = 35.52, $p < 0.05$).

3.4. Correlation among traits

Heat shock resistance at 22 and 42 h past hardening were positively correlated (r_S = 0.86, $p < 0.01$). How-

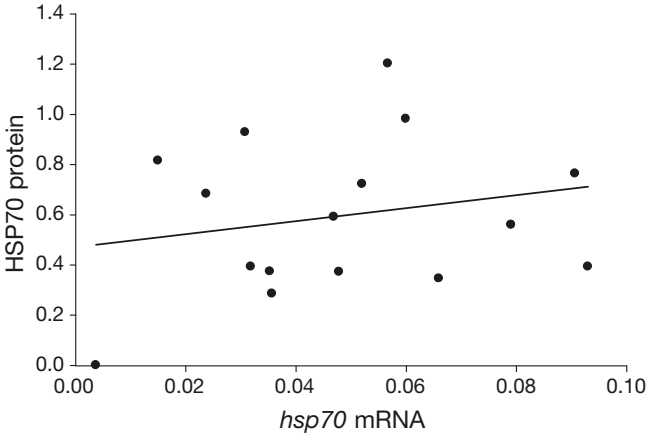

Fig. 1. *Orchesella cincta*. Correlation of HSP70 protein and *hsp70* mRNA levels in inbred isofemale lines. Each data point is the mean value of each isofemale line. $r^2 = 0.16$, $p = 0.5$

ever, there was no significant correlation between HSP70 protein levels and *hsp70* mRNA ($r_S = 0.20$, $p = 0.46$) (Fig. 1), heat shock resistance (after 22 h of recovery) and HSP70 protein levels ($r_S = -0.39$, $p = 0.14$) or heat shock resistance (after 22 h of recovery) and *hsp70* mRNA ($r_S = -0.01$, $p = 0.98$) (Figs. 2 & 3). At 42 h of recovery neither HSP70 protein ($r_S = -0.22$, $p = 0.42$) nor *hsp70* mRNA ($r_S = -0.28$, $p = 0.30$) were correlated with heat shock resistance (Figs. 2 & 3).

4. DISCUSSION

Soil invertebrates live in a variable environment (Joosse & Verhoef 1987) where the intensity and frequency of extreme temperature events experienced by the individual depend on the microhabitat and geo-

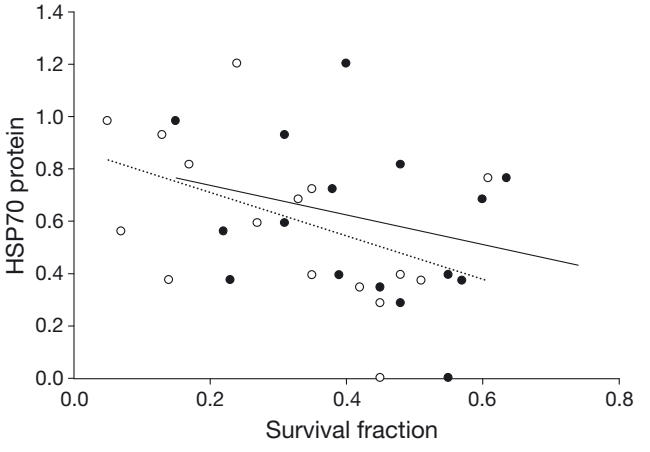

Fig. 2. *Orchesella cincta*. Correlation of HSP70 protein and heat shock resistance measured at 22 h (●) and 42 h past exposure (○) in inbred isofemale lines. Each data point is the mean value of each isofemale line. $r^2 = -0.47$, $p = 0.03$ (22 h, dotted line); $r^2 = -0.29$, $p = 0.23$ (42 h, solid line)

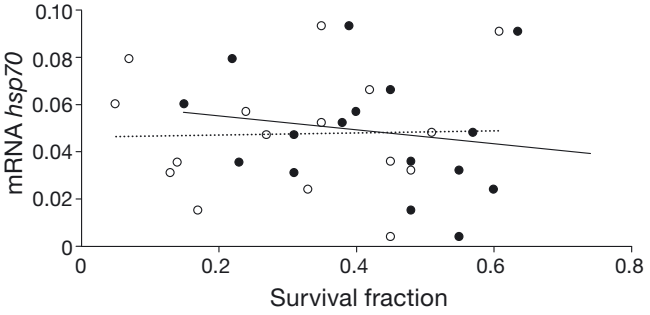

Fig. 3. *Orchesella cincta*. Correlation of *hsp70* mRNA and heat shock resistance measured at 22 h (●) and 42 h past exposure (○) in inbred isofemale lines. Each data point is the mean value of each isofemale line. $r^2 = -0.03$, $p = 0.89$ (22 h, dotted line); $r^2 = -0.27$, $p = 0.26$ (42 h, solid line)

graphical location (Liefting & Ellers 2008, Bahrndorff et al. 2009a). It is therefore not surprising that species and populations exhibit adaptation to their thermal habitats (Fischer & Karl 2010). Several studies have shown variation in thermotolerance among collembolan species (van der Woude & Verhoef 1986, Joosse & Verhoef 1987) and populations (Bahrndorff et al. 2006, 2007, Liefting & Ellers 2008). In an earlier study it was shown that *Orchesella cincta* differed by 45% in heat shock resistance between 2 populations collected along a latitudinal gradient in Europe (Italy and Denmark; Bahrndorff et al. 2006). This is in accordance with results from the present study which show genetic variation in heat shock resistance.

The question now is whether or not expression of HSPs can explain inter- and intrapopulation differences in heat shock resistance. In other taxa such as *Drosophila*, several studies have investigated the role of HSPs in adaptation to heat stress (Feder & Hofmann 1999, Sørensen et al. 2003). HSP70 levels are higher during heat shock exposure and also show improved thermotolerance when receiving a thermal pretreatment in genetically engineered *Drosophila* lines with extra copies of *hsp70* (Feder et al. 1996). However, looking at intrapopulation variation in heat shock resistance and HSP70, correlations depend on species and developmental stage. Krebs & Feder (1997b) found a correlation between HSP70 and thermotolerance in larvae, but not in adults (Krebs et al. 1998b, Jensen et al. 2009). The results from the present study support the latter contention that neither HSP70 protein nor mRNA are correlated with adult thermotolerance in the inbred isofemale lines and therefore at the intrapopulation level.

There could be several explanations for the lack of correlation between thermotolerance and HSP70.

(1) Higher expression of HSP70 might not be associated with higher thermotolerance, seen over many generations of exposure. Köhler et al. (1999) looked at

HSP70 expression in natural populations of *Orchesella bifasciata* originating from more or less contaminated sites (heavy metals). They found that when these populations were subsequently exposed to stressors the population from the most contaminated site did not express the highest levels of HSP70 compared to the population from the least contaminated site. Instead, the population originating from the most contaminated site had achieved increased resistance in other ways. It is therefore likely that thermal resistance seen on an evolutionary scale is achieved in other ways due to the costs of producing elevated levels of HSP70 (Feder & Krebs 1998, Köhler et al. 1999, 2000). Other ways of achieving resistance could be through behavioural, morphological and physiological mechanisms (Hoffmann & Parsons 1997). In *Drosophila*, a recent study by Jensen et al. (2009) showed a lack of correlation between thermotolerance and HSP70 protein levels in 3 independent experiments and using populations originating from 3 different sites. The authors suggested that the importance of HSP70 to intraspecific heat tolerance variation might be life-stage specific.

(2) The experimental design is important in establishing correlations between HSP70 and thermotolerance. Timing of measurements and exposure can affect the results. Bahrndorff et al. (2009b) showed that HSP70 protein expression and induced thermotolerance are coupled in *Orchesella cinta*, but HSP70 peaks rather late during the experiment, highlighting that the expression level of HSP70 is dependent on when it is measured. Since we only measured HSP70 protein and mRNA levels at a single time point in the present experiment, the lack of correlation between HSP70 protein levels and thermotolerance may well be due to our experimental setup. However, as shown by Bahrndorff et al. (2009b), *hsp70* mRNA expression and heat shock resistance do not coincide and therefore one might not necessarily expect a strong correlation in the present study.

These results therefore show the need for follow-up studies to gain a better understanding of HSP70 expression and thermotolerance in soil organisms, specifically for HSP70 protein levels and heat shock resistance.

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