



## The role of the heat shock proteins (HSP70 and sHSP) in the thermotolerance of freshwater amphipods from contrasting habitats

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### ABSTRACT

The aim of this study was to clarify the significance of HSP70 and sHSP for thermotolerance in freshwater amphipods. We compared four amphipod species from different freshwater habitats and biogeographical regions (Central Europe vs. Lake Baikal). Test individuals were exposed to thermal stress generated by a water temperature of 25 °C. The thermotolerance of the species, determined by median lethal time (LT50), followed in decreasing order by *Gmelinoides fasciatus*, *Echinogammarus berilloni*, *Gammarus pulex*, *Eulimnogammarus verrucosus*. HSP70 and sHSP base level concentrations for the species were determined at control (i.e. non-stress) conditions. For HSP70, the base levels were positively correlated to the species' thermotolerances. For sHSP, however, only thermotolerant *G. fasciatus* showed a high level. Thermal stress at 25 °C water temperature caused a deferred onset of HSP70 and sHSP expression followed by a subsequent offset, delineating a unimodal response curve. The time lag to the expression onset of HSP70 was shorter in the thermosensitive species, compared to thermotolerant ones. Conversely, the time span until the maximum level of HSP70 was variable, not showing a dependence on the thermotolerance properties of the species. The peak concentration in *G. pulex* was distinctly higher than in the other species, whereas *E. verrucosus* did not develop a well-defined response maximum at all. In sHSP, the temporal pattern of expression was even more variable than in HSP70. However, the thermosensitive species *E. verrucosus* showed a time lag of expression onset significantly shorter than the other species and thermotolerant *G. fasciatus* developed the most pronounced response maximum. Basing on these results, the cellular response to thermal stress in amphipods is more consistently reflected by HSP70, compared to sHSP.

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### 1. Introduction

Temperature stress provokes energy-demanding responses on a cellular level, which eventually may reduce the organism's competition and reproduction abilities (Tomanek and Zuzov, 2010; Sorte and Hofmann, 2005). Hence, temperature stress is a significant physiological and ecological factor. In evolution, those species apparently are more successful that better cope with the physiological effects of stress, i.e. respond with less expense of energy (Henkel and Hofmann, 2008). Here, heat shock proteins

(HSPs) are key players, which allow organisms to expand their tolerance to wider range of environmental stress (Kültz, 2003). HSPs perform many tasks in a cell. One of these is to act as molecular chaperones, which bind to denatured and unfolded proteins, prevent aggregation of non-native proteins and repair damaged proteins (Tomanek and Sanford, 2003). On the other hand they also perform folding/refolding and assembly of cell proteins under normal conditions (Becker and Craig, 1994). Stressful conditions, however, such as high temperature, presence of toxic or oxidative substances, and others cause an overexpression of HSPs, by which stress tolerance of cells and organisms, specifically physiological thermotolerance, can be enhanced (Feder and Hofmann, 1999).

On the time scales of evolution, environmental conditions are subject to changes due to natural processes and anthropogenic impacts, and more recently to global warming (Thomas et al., 2004). The ability of organisms to tolerate temperature stress

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depends on the thermal history of their habitat (Feder and Hofmann, 1999). Endemic species were shown to be more susceptible to alterations of the environment than cosmopolitan species, since they mostly occupy specific niches and are adapted to low amplitudes of environmental conditions (Thomas and Wiencke, 1991). Consequently, they may be characterized by different HSP properties than cosmopolitans (Brennecke et al., 1998; Tomanek, 2002).

In comparison to various other aquatic organisms (Sorte and Hofmann, 2005; Fabbri et al., 2008), freshwater amphipods (Crustacea) have been studied sparingly as model organisms of thermotolerance and HSP expression (Timofeyev et al., 2009; Bedulina et al., 2010a). Amphipods occur in almost all types of water bodies under a wide range of environmental conditions, and there they are important elements of the trophic web (Väinölä et al., 2008).

For studying the role of HSPs for thermotolerance we conducted laboratory experiments on amphipod species, each characterized by distinct habitat preference (stream vs. lake), biogeographical subregion (Central Europe vs. Lake Baikal), and dispersal tendency (endemic vs. invasive).

As markers of cellular stress for this study, two different families of heat shock proteins were chosen, namely the 70 kDa HSP (HSP70) and the  $\alpha$ -crystalline-like member of small HSP (sHSP). HSP70 is one of the most conservative and well-studied proteins among the HSPs and is widespread among all types of living organisms (Kültz, 2003). Its main function is that of a molecular chaperon. It performs the task of binding newly synthesized proteins, placing them in the compartments where they are needed, and regulating their folding and refolding (Lindquist and Craig, 1988). Unlike HSP70, the sHSP family is more diverse. All of its members have a conservative  $\alpha$ -crystalline domain, which is structurally similar to the  $\alpha$ -crystallines of vertebrate eye lenses (Sun and MacRae, 2005). They also act as molecular chaperones, by binding to partly denatured proteins and transferring them to other chaperons, such as the HSP70 (Gusev et al., 2002). Both groups of HSPs co-function in the repairing of proteins that have been damaged by heat or by otherwise physiologically adverse conditions. Their synthesis typically is triggered by various forms of cellular stress (Lee and Vierling, 2000), making them viable markers of environmental strain.

We hypothesized that (1) mortality under temperature stress, being a criterion of thermotolerance of a species, corresponds to the natural temperature regimes of its habitats and to its distribution area. Further, that for the comparison of thermotolerant to thermosensitive species HSP70 and sHSP concentrations exhibit the following patterns: (2) at control conditions base levels are natively higher in thermotolerant species. Under temperature stress (3) the time lag to the onset of expression is shorter in thermosensitive species, and (4) the relative maximum level is higher in thermosensitive species.

## 2. Materials and methods

### 2.1. Test animals

For the experiments four amphipod species from different biogeographic regions were chosen: *Gammarus pulex* (Linnaeus, 1758) and *Echinogammarus berilloni* (Catta, 1878) from the European subregion, *Eulimnogammarus verrucosus* (Gerstfeldt, 1858) and *Gmelinoides fasciatus* (Stebbing, 1899) from the Lake Baikal (Eastern Siberia, Russia) subregion. The species varied with regard to their autecological characteristics: *G. pulex* and *E. berilloni* primarily inhabit running waters (Dedecker et al.,

2006; Meyer et al., 2004). *G. pulex* has been shown to be sensitive to various environmental stressors (Felten et al., 2008) and, therefore, is used as a test-organism for water quality (Kunz et al., 2010) and restoration management (Dedecker et al., 2006). Conversely, *E. berilloni* is characterized by more euryoecious properties. It inhabits water bodies that differ in temperature, organic contents, and pH level on a wide range (Pinkster, 1993). As an invader, it is currently reported to spread in Central European, successfully replacing *G. pulex* (Holdich and Pöckl, 2007). In nature, both species occur under a broad temperature range. *E. berilloni* was even reported to occur at maximum habitat temperatures of up to 31 °C (Holdich and Pöckl, 2007). In laboratory experiments the preferred temperatures were 18–19 °C for *G. pulex* and 19–20 °C for *E. berilloni* (unpublished own data). Unlike the two European gammarid species, the Baikal species *E. verrucosus* and *G. fasciatus* are inhabitants of standing waters mainly. *E. verrucosus* inhabits the littoral and sublittoral zone of Lake Baikal and is sensitive to fluctuations of temperature (Timofeyev et al., 2001), oxygen concentration (Timofeyev, 2002), and other factors (Timofeyev et al., 2008). Under experimental conditions, *E. verrucosus* preferred a temperature of 5–6 °C. In comparison thereto, *G. fasciatus* natively lives in upper littoral zones and is characterized by a greater adaptive capacity (Timofeyev et al., 2001; Timofeyev, 2002). It has been recorded as a invader of the Baltic Sea (Panov and Berezina, 2002). In laboratory experiments, *G. fasciatus* favored a temperature of 17–18 °C (Timofeyev et al., 2001). In natural environment, however, this species can tolerate temperatures up to 29 °C. All test individuals were sampled from their native habitats. Specimens of *G. pulex* were collected from several creeks of Northern Germany in the vicinity of Kiel (Schleswig-Holstein, Germany) in autumn of 2007. *E. berilloni* was sampled at River Alme on the Paderborn Plateau (Westphalia, Germany) in autumn 2008. *G. fasciatus* and *E. verrucosus* were sampled from the littoral of Lake Baikal, close to the settlement of Bolshie Koty (South Baikal, Russia), in summer 2006–2007.

### 2.2. Experimental setup

Prior to the experiments, animals were allowed to acclimate for a minimum of 3 days in aerated keeping tanks, which were filled with water from their natural habitat. To minimize acclimation stress, keeping temperature was adjusted to outside conditions during collection: *G. pulex*: 10 °C, *E. berilloni*: 12 °C, *E. verrucosus* and *G. fasciatus*: 6–7 °C. Near-natural light conditions and an LD cycle of 8:16 was provided by daylight tubes. Animals were fed with commercial food (Tetra-Min, Tetra GmbH, Germany) ad libitum. For thermotolerance and stress response experiments, water temperature was kept constant at 25 °C, at which the level of stress and mortality rate turned out to be balanced. The species were tested separately in the experiments, for which only actively moving animals were selected randomly from the keeping tanks.

#### 2.2.1. Thermotolerance

Each 10 individuals per species were placed into nine aerated test aquaria (18 × 12 cm base area), which were filled with 1 L of filtered water from the sampling site. The aquaria were checked hourly for dead animals during the first 10 h, thereafter in 24 h intervals. When pleopod movement had ceased, individuals were counted as dead and taken out of the tank. Experiments lasted until all animals had died or were terminated after 48 h runtime.

#### 2.2.2. Stress response

Each 30 individuals per species were placed into three 2.5 L aquaria. The individuals for HSP analysis were randomly taken

out after 0.5, 1, 3, 6, 12, and 24 h and immediately fixed in liquid nitrogen for the subsequent analysis. Depending on the body size of the species, 2–6 individuals per time point were selected and blended. Control experiments were carried out in the same type of aquaria, but at keeping temperature. The control animals were fixed at the same time like the test animals. Prior analyses had shown that HSP levels of the control animals did not change significantly over time.

### 2.3. Determination of HSP levels

For the analysis of HSP70, 35  $\mu\text{g}$  of total protein in *E. berilloni* and *G. pulex*, and 60  $\mu\text{g}$  in *G. fasciatus* and *E. verrucosus* were used. Correspondingly, for the analysis of sHSP, 20  $\mu\text{g}$  of total protein was used in all four species. These amounts were established as optima for the quantification of the resulting HSP levels. For this, each three individuals in *E. berilloni* and *G. pulex*, five individuals in *G. fasciatus*, and two individuals in *E. verrucosus* were melded. The samples were hand-homogenized on ice and centrifuged at 7000g for 15 min at 4 °C. After centrifugation, the supernatant was dissolved in a sample buffer (pH 6.8, containing 0.0625 mol Tris, 1 mmol EDTA, 1% SDS, 20% glycerin, 5%  $\beta$ -mercaptoethanol, and 0.001% bromophenol blue). The total protein concentration was determined for *E. berilloni*, following the assay proposed by Bradford (1976) and for *G. fasciatus*, *E. verrucosus*, and *G. pulex*, according to Lowry et al. (1951). The HSP content of the samples was determined through SDS electrophoresis of equal protein amounts followed by Western blotting using anti-HSP70 primary antibodies (monoclonal anti-heat shock protein 70 antibody produced in mice, Sigma # H9776), which detected both inducible HSP and heat shock cognates (constitutive form of HSP, HSC), and anti- $\alpha$ -crystalline primary antibodies ( $\alpha$  A/B crystalline polyclonal antibody produced in rabbits, Stressgen # SPA-224). SDS electrophoresis was performed in polyacrylamide gel blocks (70  $\times$  80  $\times$  1 mm<sup>3</sup>; Laemmli, 1970) using a Mini-PROTEAN II electrophoretic cell apparatus (BIO-RAD, USA), respectively. Relative molecular weights of the proteins were determined by using a LMW marker kit (Biomol, UK). Western blotting to the PVDF membrane was performed according to Towbin et al. (1979) with the modification as described previously (Timofeyev et al., 2009). Equal loading of protein was verified by staining the membranes with Ponceau Red. After blotting, the membranes were blocked in 2.5% nonfat dry milk solution with addition of sodium azide.

Staining of the membranes was done according to the previously developed protocol for amphipod species as described in Bedulina et al. (2010a). For HSP70 measurement, blots were incubated in primary antibodies, dissolved 1:10000 in blocking solution for 2 h. After two-fold washing, the blots were treated in secondary antibodies (Anti-Mouse IgG:AP Conj., Stressgen # SAB-101), dissolved 1:1000 in blocking solution for 2 h. For sHSP measurement, blots were incubated in primary antibody, dissolved 1:1000 in blocking solution, for 15 min. Thereafter, blots were washed twice in washing solution, containing 0.05% Tween-20, and incubated in secondary antibodies (Anti-Rabbit IgG:AP Conj., Stressgen # SAB-301), dissolved 1:1000 in blocking solution for 15 min.

Base level concentrations of HSP 70 and sHSP at non-stress (control) conditions of temperature were used as a reference for the expression levels determined under subsequent temperature stress. The levels of HSP70 and sHSP were determined relative to the optical density of 100 ng reference protein (WB positive controls). For the visualization of the HSP, the BCIP/nBT system was used. Semi-quantitative analysis of HSP on the Western blot membranes were performed with the "Gel Explorer" software package (DNA-Technology, Russia).

### 2.4. Statistical analysis

All measurements (mortality, HSP concentrations) over time were given as means and standard deviations calculated from independent replicates. Differences between data groups were tested with the asymptotic permutation *t* test (Corcoran and Mehta, 2002). Correlations were calculated as product moments and tested by permutation (*p*-correlation). Variability between measurement points was balanced by fitting non-linear models performing the course over time and 95% confidence intervals. In thermotolerance experiments, the cumulative mortality was reproduced as a log-sigmoid function. Thereupon, thermotolerance was defined as the median lethal time (LT50), i.e. the period at which 50% of the test individuals had died. Expression of HSP70 and sHSP, based on relative concentrations (ng HSP/100  $\mu\text{g}$  total protein) over time, were reproduced for each species by a unimodal (log-normal) model and the 95% confidence intervals. Goodness-of-fit of the models was determined by the Akaike information criterion (AIC), which also includes a penalty for an increasing number of model parameters. Thus, the smaller the AIC the more efficient the fit. In comparison thereto, the common coefficient of determination ( $R^2$ ) is of limited value for most non-linear models, since it lacks responsiveness at higher levels of fit and rewards for inefficient 'overfitting' (Towbin et al., 1979).

Three key descriptors for the species specific HSP expression characteristics were defined as follows: (1) base levels correspond to the mean concentrations measured in control experiments, (2) the time period until onset of HSP expression is defined by the 5th percentile of the modeled response curve, and (3) the absolute and relative (i.e. in relation to the base level) HSP concentrations measured in the experiments. Statistical analysis and graphics were performed with R software (R Development Core Team, 2009).

## 3. Results

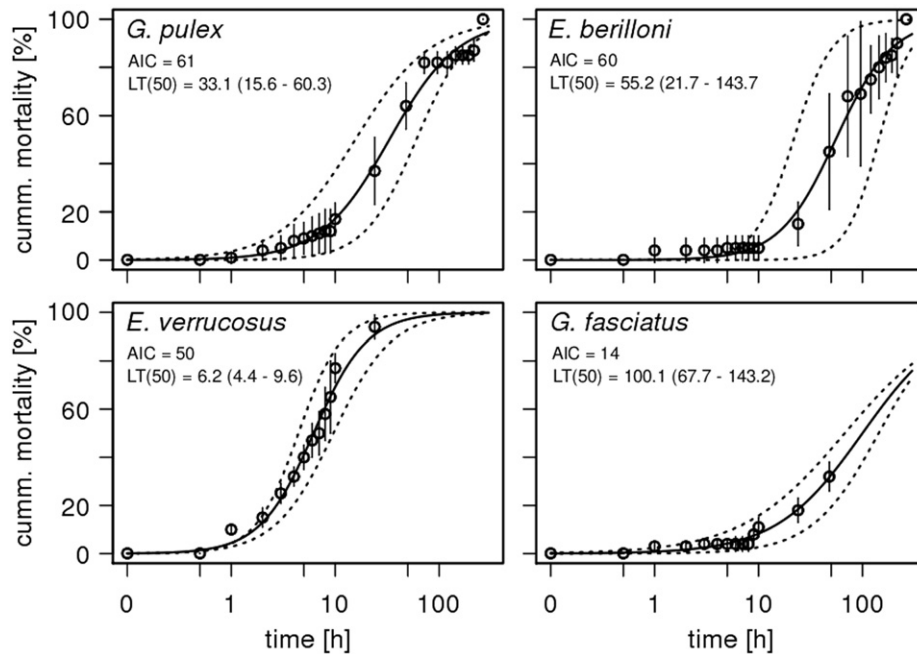
### 3.1. Thermotolerance

The studied species clearly differed from each other with respect of their thermotolerance properties (Fig. 1). The lowest tolerance, i.e. lowest median lethal time, was observed in the Baikal *E. verrucosus*. The median mortality ratio was reached already after 6.2 h. The opposite extreme was represented by *G. fasciatus*, as well a Baikalian amphipod, which proved the greatest thermotolerance of all four test species. The mortality did not exceed 32%, at the end of the experiment after 48 h. However, by the modeled extrapolation, the median mortality was estimated to be reached after 101 h. In the two European species, *G. pulex* and *E. berilloni*, the median lethal time was determined at 33.1 and 55.2 h, respectively. First cases of dead individuals were observed after one or two hours (in *E. berilloni*). According to the modeled interpolation, *E. verrucosus* was the most thermosensitive species with an onset after already 1.1 h of temperature stress.

### 3.2. HSP base levels

#### 3.2.1. HSP70

The base level concentrations of HSP70 split the amphipod species in two groups. The higher levels were found in the more thermotolerant *G. fasciatus* and *E. berilloni*, at concentrations of 34.1 ( $\pm$  15.9) ng and 32.2 ( $\pm$  6.6) ng per 100  $\mu\text{g}$  of total protein, respectively (Table 1). In the more thermosensitive *G. pulex* and *E. verrucosus*, the base level concentrations were as low as only 14.6 ( $\pm$  11.1) and 6.2 ( $\pm$  3.5) ng/100  $\mu\text{g}$  of total protein, being



**Fig. 1.** Mortality data of amphipod species during constant exposure to 25 °C (10 individuals, 9 replicates). Log-sigmoid model (solid line) through mean values and corresponding standard deviations (dotted lines represent the 95% confidence intervals).

**Table 1**

Key parameters of HSP70 expression characteristics (concentrations and standard deviations given in ng per 100 µg total protein) in amphipod species during constant exposure to 25 °C ( $c_{BL}$ : base level at control conditions,  $t_{onset\ mod}$ : modeled time lag (in hours) until onset of expression,  $c_{max}$ : measured peak concentration,  $c_{max}/c_{BL}$ : peak concentration relative to base level,  $t_{max}$ : time point (in hours after start of exposure) of  $c_{max}$ ,  $c_{max\ mod}$ : modeled peak concentration,  $t_{max\ mod}$ : time point (in hours after start of exposure) of  $c_{max\ mod}$ ).

HSP70	<i>G. pulex</i>	<i>E. berilloni</i>	<i>E. verrucosus</i> <sup>a</sup>	<i>G. fasciatus</i>
$c_{BL}$ (ng/100 µg tp)	14.6 ± 11.1	32.2 ± 6.6	6.2 ± 3.5	34.1 ± 15.9
$t_{onset\ mod}$ (h)	4.5	3.1	0.3 (13.2)	7.5
$c_{max}$ (ng/100 µg tp)	378.7 ± 58.3	102.5 ± 62.2	20.7 ± 0.8 (50.1 ± 34.0)	181.7 ± 41.7
$c_{max}/c_{BL}$	25.9	3.2	3.3 (8.1)	5.3
$t_{max}$ (h)	24	6	0.5 (24)	12
$c_{max\ mod}$ (ng/100 µg tp)	406.0	105.4	20.8 (55.1)	200.3
$t_{max\ mod}$ (h)	18.3	5.5	0.5 (29.0)	14.4

<sup>a</sup> Two peaks present in *E. verrucosus*.

**Table 2**

Key parameters of sHSP expression characteristics (concentrations and standard deviations given in ng per 100 µg total protein) in amphipod species during constant exposure to 25 °C ( $c_{BL}$ : base level at control conditions,  $t_{onset\ mod}$ : modeled time lag (in hours) until onset of expression,  $c_{max}$ : measured peak concentration,  $c_{max}/c_{BL}$ : peak concentration relative to base level,  $t_{max}$ : time point (in hours after start of exposure) of  $c_{max}$ ,  $c_{max\ mod}$ : modeled peak concentration,  $t_{max\ mod}$ : time point (in hours after start of exposure) of  $c_{max\ mod}$ ).

sHSP	<i>G. pulex</i>	<i>E. berilloni</i>	<i>E. verrucosus</i> <sup>a</sup>	<i>G. fasciatus</i>
$c_{BL}$ (ng/100 µg tp)	5.2 ± 2.4	5.1 ± 0.6	6.0 ± 3.4	7.7 ± 2.9
$t_{onset\ mod}$ (h)	1.9	19.9	0.0	1.5
$c_{max}$ (ng/100 µg tp)	7.1 ± 0.1	11.7 ± 0.7	15.7 ± 7.4 (18.6 ± 5.4)	24.9 ± 12.7
$c_{max}/c_{BL}$	1.4	2.3	2.6 (3.1)	3.2
$t_{max}$ (h)	6	24	0.5 (6)	6
$c_{max\ mod}$ (ng/100 µg tp)	7.8	15.1	16.0	20.8
$t_{max\ mod}$ (h)	13.4	28.1	4.7	11.0

<sup>a</sup> Two peaks present in *E. verrucosus*.

significantly lower compared to the thermotolerant species (permutation  $t$  test,  $Z = -2.90$ ,  $df = 14$ ,  $p < 0.005$ ).

### 3.2.2. sHSP

Differences in the base level concentrations of sHSP between the species at control conditions, generally were smaller than in HSP70

and did not differ significantly between each other (Table 2). Similar to the above, the highest base level was measured in the thermotolerant Baikal species *G. fasciatus* at a concentration of 7.7 (± 2.9) ng/100 µg of total protein. Conversely, low base levels of sHSP were found in both Central European species *E. berilloni* and *G. pulex* at concentrations of 5.1 (± 0.6) and 5.2 (± 2.4) ng/100 µg of total protein, respectively. The most thermosensitive species

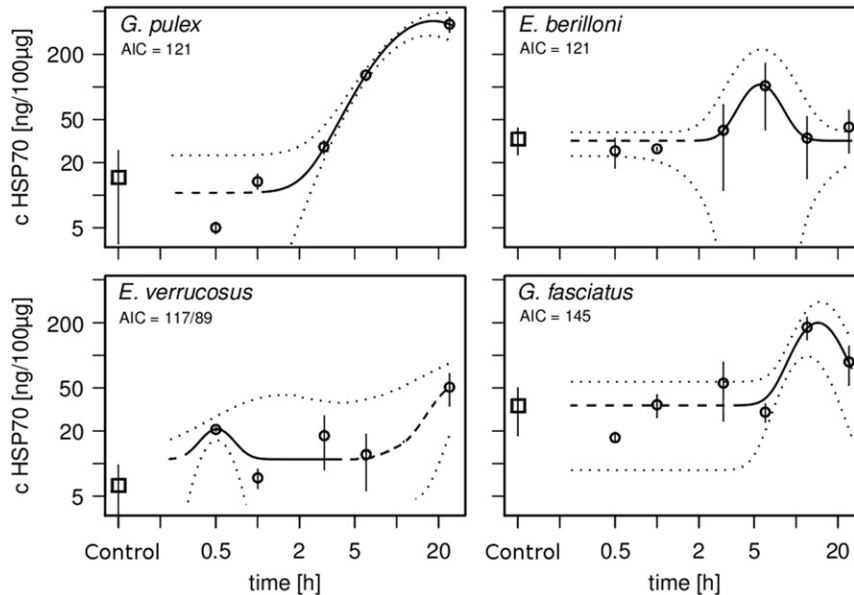
*E. verrucosus*, however, took an intermediate position, with a base level concentration of  $6.2 (\pm 3.4)$  ng/100  $\mu$ g of total protein.

### 3.3. Temperature stress and HSP expression

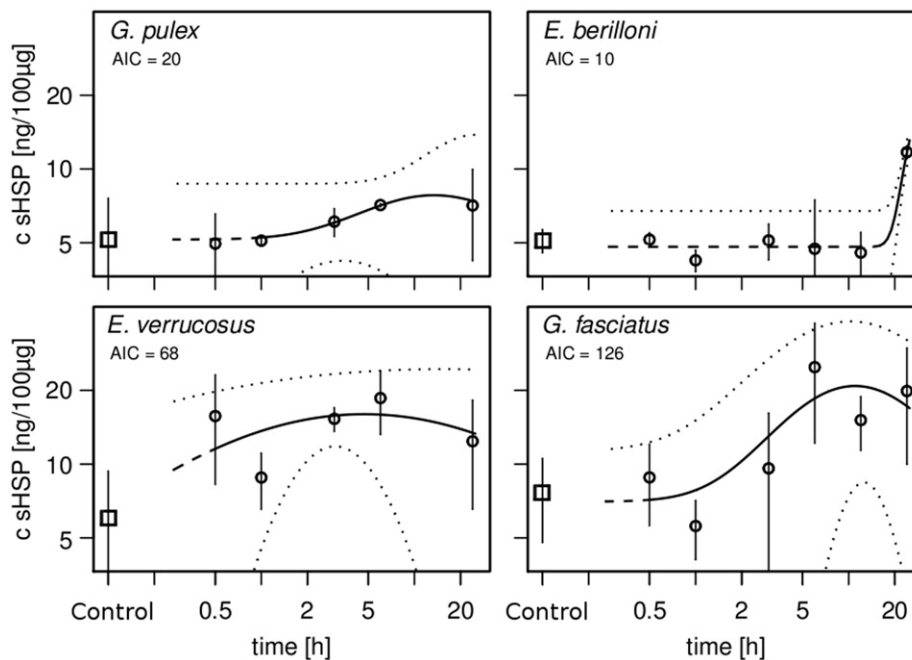
In all species, the exposure to an elevated water temperature of 25 °C caused an increase in both HSP70 and sHSP concentrations. Onset times and relative concentration maxima as key descriptors for the expression of the HSP variants, however, rendered a more heterogeneous picture than the base levels.

#### 3.3.1. HSP70

The expression of the HSP70 type was characterized by a time lag to the onset and an increase of concentration. This was followed by a peak, the maximum expression level, and a subsequent offset, i.e. a decline of the concentration (Table 1, Fig. 2). In all species, the shortest time lag of HSP70 expression onset was noted in the thermosensitive Baikalian *E. verrucosus*. The following expression pattern of the species, however, was heterogeneous. The first measurement peak occurred after 0.5 h and corresponded to a relative increase of 330% ( $20.7 \pm 0.8$  ng/100  $\mu$ g of total protein), being significantly higher than the base



**Fig. 2.** HSP70 expression characteristics of the amphipod species during constant exposure to 25 °C. Log-normal distribution model (solid line) through mean values and corresponding standard deviations (the square shows the base level concentration at control conditions, dotted lines represent the 95% confidence intervals).



**Fig. 3.** sHSP expression characteristics of the amphipod species during constant exposure to 25 °C. Log-normal distribution model (solid line) through mean values and corresponding standard deviations (the square shows the base level concentration at control condition, dotted lines represent the 95% confidence intervals).

concentration (permutation  $t$  test,  $Z = -2.33$ ,  $df = 5$ ,  $p < 0.02$ ). Onset time for this expression peak was 0.3 h only. A second peak at 810% of the base level concentration ( $50.8 \pm 16.6$  ng/100  $\mu$ g of total protein) was determined at 24 h after the start of the experiment. Expression onset of this peak was not until 13.2 h. The HSP70 expression characteristics, in comparison to *E. verrucosus*, showed more consistence in the other species. *G. fasciatus*, the most thermotolerant of the four species, showed a clear onset of HSP70 expression after 7.5 h, ending in a peak concentration of 530% ( $181.7 \pm 41.7$  ng/100  $\mu$ g of total protein) at 12 h after beginning of the stress exposure. Compared by the 95%-confidence intervals, expression onset in *G. fasciatus* appeared significantly later compared to the European species *G. pulex* and *E. berilloni*. In the latter two, the time lags of onset were determined at 4.5 and 3.1 h, respectively. The peak concentration of HSP70 reached 310% ( $102.5 \pm 62.2$  ng/100  $\mu$ g of total protein) after 6 h in *E. berilloni*, being not significantly different from that in *G. pulex*. Remarkably, however, *G. pulex* developed an extremely high concentration maximum ( $102.5 \pm 62.2$  ng/100  $\mu$ g total protein) after 24 h, corresponding to 2590% of the base level, after all significantly exceeding the peak concentrations of the other species (permutation  $t$  test,  $Z = 2.81$ ,  $df = 9$ ,  $p < 0.005$ ).

### 3.3.2. sHSP

In contrary to the pattern of HSP70 expression, sHSP showed a more arbitrary characteristics (Table 2, Fig. 3). As for HSP70, the most distinct, though also heterogeneous expression was observed in the thermosensitive Baikalian species *E. verrucosus*. A first concentration peak, relative to the control level, reached 260% ( $15.7 \pm 7.4$  ng/100  $\mu$ g total protein) already after 0.5 h, and a second peak of 310% ( $18.6 \pm 5.4$ ) appeared after 6 h. The longest onset period of 19.9 h was found in *E. berilloni*, increasing to a relative peak concentration of 230% ( $11.7 \pm 0.7$  ng/100  $\mu$ g total protein) reached after 24 h, after the ending of the experiment. In the two species *G. pulex* and *G. fasciatus*, expression set on after time lags of 1.9 and 1.5 h, respectively. The relative expression maximum of *G. pulex* peaked at only 140% ( $7.1 \pm 0.1$  ng/100  $\mu$ g total protein) after 6 h. In the more thermotolerant species *G. fasciatus* the peak concentration after onset of sHSP expression reached a level of 320% ( $24.9 \pm 12.7$  ng/100  $\mu$ g total protein), after 6 h, too, and thus was significantly higher than in the other species (permutation  $t$  test,  $Z = 2.63$ ,  $df = 10$ ,  $p < 0.01$ ).

## 4. Discussion

The four amphipod species were selected for this comparative study, since their biogeographic origins and actual distributions gave support to the assumption that they differ in terms of temperature preferences and tolerances. Three of the species, *G. pulex*, *E. berilloni*, and *G. fasciatus*, are known as invaders (Meyer et al., 2004; Panov and Berezina, 2002; Piscart et al., 2009), and one species, *E. verrucosus*, as endemic (Timoshkin, 2004).

The temperature of 25 °C for the stress exposure experiments was chosen as it marks an extreme situation that occasionally arises in the native environments of the test species, and thus can be expected to produce significant physiological results and ensure the survival of the test individuals. Mortality data of the tested species, which were obtained from longterm exposure to temperature stress, turned out to be highly consistent due to a low inter-individual variability. Against this background, we decided to define the median lethal time (LT50) as an adequate and robust criterion of species specific thermotolerance.

As hypothesized, the thermotolerance properties of the species conform rather well to their spatial distribution and dissemination dynamics. However, to a lesser extent to their temperature

preferences. According to that, *E. verrucosus*, the representative of a distinctly endemic distribution pattern, was determined as the least thermotolerant species of the tested gammarids. In its primary habitat, the littoral and sublittoral of Lake Baikal, temperature conditions are characterized by a high constancy (Timofeyev et al., 2008). Newer findings of the species were reported also from the rivers Angara and Yenisey, as well as from various close by water bodies (Timoshkin, 2004), but can be supposed not to represent stable populations. On the other hand, *G. fasciatus*, the most thermotolerant species, nowadays covers a huge area of distribution and is distinguished by a great invasive potential. Currently it is successfully spreading into the estuaries of the Baltic Sea from the Volga basin and the adjacent great lakes, where it had been introduced about 50 years ago (Panov and Berezina, 2002). In the two European species, *G. pulex* and *E. berilloni*, the situation is less obvious. Their thermotolerance properties correspond better to the degree of invasiveness, as in the Baikal species, but not to the covered area of geographic distribution. And also neither a correspondence exists to the temperature characteristics of their habitats nor to the experimentally determined temperature preferences. Both species are known as invaders, however *G. pulex*, a natively Central European gammarid, only recently has been found as an invader in North-West Europe (Piscart et al., 2009; Kelly et al., 2006). *E. berilloni*, in comparison thereto, has been spreading from South-West Europe over vast parts of Western and Central Europe, already since the 1920s (Boeker, 1926), outcompeting *G. pulex* in some catchment areas (Holdich and Pöckl, 2007).

Thus, the Baikal *G. fasciatus* and European *E. berilloni*, the coevally more potent invaders and partly wider distributed species in fact were distinguished by a greater thermotolerance.

How far these clear findings, as well as all characteristics of stress response, were subject to the specific conditions the test individuals had lived in and were taken from prior to the experiments, cannot be determined a posteriori, but shall be a matter of discussion. The temperatures that characterize the habitats of the species *G. pulex* and *E. berilloni*, can vary substantially (0–25 °C) between winter and summer. Thus, the thermotolerance properties of the species also might differ between the seasons and generations, respectively. On this account, the two European species were sampled strictly in autumn. In case of the Baikal species, the temperatures that characterize their habitats, the deeper littoral, fluctuate within a much smaller range (5–7 °C) between the seasons. Given this fact, we assumed that the thermotolerance properties of the Baikal species remain widely constant throughout the year.

The increasing mortality rates, as here observed under enduring temperature stress, is a result of gradually advancing cell damage within the organism. At the initial sublethal stages of stress the chaperones HSP70 and sHSP are already activated and fulfill the task of preventing cell damage (Becker and Craig, 1994; Feder and Hofmann, 1999). Thus, thermotolerant, compared to thermosensitive species, were expected to sustain higher levels of HSPs, even under control conditions, i.e. during the absence of temperature stress. This phenomenon was observed, however, as a clear linkage found only in HSP70 and particularly in the thermotolerant species *G. fasciatus*. This correspondence between thermotolerance and HSP70 base levels has been described previously for two other amphipod species from the Baltic Sea (Bedulina et al., 2010a), as well as for several other taxa of different geographical origin (Ulmasov et al., 1992; Dutton and Hofmann, 2009). These findings support the conclusion that the HSP70 base level provides a pre-adaption to fluctuations of the environmental conditions. Based upon that, we considered HSP70 a physiological key player that defines the potential of a species to successfully seize new habitats as an invader.

We expected that the amphipods studied here, owing to their distinct autecological properties, not only to differ in their HSP base levels, but also to reveal specific patterns of expression of HSP. Since higher base levels of HSP70 in thermotolerant species allow for a more effective buffering of stress effects, less tolerant species should react more immediately to stress, consequently by a faster onset of HSP70 expression. However, the species specific time lags of expression revealed only a tendency to be shorter in thermosensitive species. This effect was discernible, though weakly, only in the two Baikal species. Unfortunately, the HSP70 expression pattern of *E. verrucosus* did not allow for a sound analysis and interpretation. The two concentration peaks, which this species developed during thermal stress, were most probably due to the high variability of the measurement results close to the lower detection limit of the method. The other two parameters of HSP expression, the maximum level and the corresponding time location of the peak, as well did not show a clear link to the thermotolerance properties in all four species. Similar inconsistency was described in other studies, too, though only between higher taxonomic levels. In two amphipod species from contrasting environments of the Baltic Sea, the intensity of HSP70 expression was found to be positively correlated to the HSP70 base level (Bedulina et al., 2010b). Conversely, in sculpins (Cottoidea), the more thermotolerant species maintained higher HSP70 base levels, but produced weaker increases of HSP70 under heat stress (Nakano and Iwama, 2002). And comparably, southern populations of the killifish (*Fundulus heteroclitus*) showed higher base levels of HSP70 and less inducibility by heat shock compared to more thermosensitive northern ones (Fangue et al., 2006).

The expression maximum normally is followed by an exhaustion of the HSP system, leading to a decrease and low continuance of the HSP70 level. Whether or not a temporal proximity existed between mortality probability and the course of HSP expression over time, was tested for each species separately. The best correspondence was found for *G. pulex* ( $p$ -correlation,  $r=0.96$ ,  $p < 0.02$ ) and *G. fasciatus* ( $p$ -correlation,  $r=0.99$ ,  $p < 0.002$ ) owing to a late onset of HSP expression, compared to that of the other species ( $p$ -correlation,  $r < 0.60$ , n.s.). The deviant HSP70 expression pattern in *G. pulex*, characterized by a deferred and extreme maximum might have been a result of overexpression, caused by an accumulation of HSP70. As observed in various other mostly thermotolerant species, this phenomenon may appear in conjunction with a high constancy of stress tolerance (Bedulina et al., 2010a). In the case of *G. pulex*, however, the dynamics of HSP70 expression cannot be explained straightforwardly on the basis of the available background data. Possibly, more and/or different environmental factors involved than temperature triggered the HSP70 expression in this species. The high thermotolerance of *G. fasciatus* may be discussed against its history as tertiary relict of Lake Baikal (Takhteev, 2000). The climatic condition of East Siberia during the Tertiary were close to subtropical. Early inhabitants of the ancient Lake Baikal may have developed or maintained wider adaptive abilities, characterizing them until now.

For sHSP, expression onset upon thermal stress was also observed in all tested species. However, the patterns of expression were rather arbitrary, probably due to a high variation over time. As hypothesized, the thermotolerant species *G. fasciatus* had both the highest base level, but conversely also the highest peak level of sHSP. Data points did not differ significantly between each other, showing coefficients of variation (CV) between replicate measurements of  $1.1 < CV < 3.5$ . Also the differences between the base levels of the species were relatively small compared to the high variabilities of the replicate measurements. Similarly in *E. verrucosus*, the short onset time, as hypothesized for thermosensitive species, could be reproduced only with low accuracy of determination for the model ( $R^2=0.34$ ). With respect to the

temporal proximity between the probability of mortality and the course of sHSP expression over time, only *G. pulex* showed a good correlation ( $p$ -correlation,  $r=0.93$ ,  $p < 0.05$ ), compared to the other species ( $p$ -correlation,  $r < 0.50$ , n.s.). Hence, in *G. pulex* the expression of both HSP types showed a close temporal coupling to the increasing probability of mortality, whereas in the other species this relation was less distinct. Whether or to what extent the greater inter-individual variations of the sHSP levels and/or technical shortcomings of the blotting method were responsible for the heterogeneity of the results, could not be decided with certainty. We assume that both factors may have played a role and seem to mark the limitations of the method or the experimental design. A statistical corroboration of the established relationships could be carried out in parts only. A better generalization of the observed relationships only would become feasible, if the number of test species is doubled, at least. A meta-analysis, based on the joined results from other studies on stress response of gammarid species, still suffers from incompatibilities in data quality and experimental design.

In conclusion, the results obtained in this study at least partly support the hypothesis of a consistent functional properties in HSP as a mechanism of thermotolerance in amphipod species. As previously mentioned, HSP proteins to a certain degree protect the functioning of cells against fluctuations of the environmental conditions. In this context, HSP70 proved a greater efficiency to operate as molecular chaperones than sHSP. Apparently, this polyfunctionality makes the HSP70 protein a more efficient protector against temperature stress. The stronger relationship between HSP expression and thermotolerance found in the Baikal species, compared to the European species, obviously is due to the greater difference with respect to their ecological ranges. Unexplainable deviations from the expected patterns of HSP expression probably were consequences of principal shortcomings, e.g. the relatively great variation of the data, especially at the lower limit of detection. In addition, thermal stress, even in standardized experiments, may not be considered as the only environmental trigger of cellular stress response. A better resolution of the results, however, can be attained by additional control of ecological and biological factors, such as age, sex, and a largely standardized conditioning of the test animals for the experiments. In this respect, thermotolerance and HSP expression characteristics of amphipod species do reveal a complex dependency on various physiological as well as environmental factors, which still requires a more detailed study on this phenomenon.

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