Plasticity in depth selection behavior and heat shock proteins in *Daphnia*

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**Abstract** Habitat selection behavior by aquatic and terrestrial animals is influenced by both abiotic (e.g., temperature) and biotic (e.g., threat from predators) environmental factors. In this study, the mechanisms underlying the variability in behavior of habitat selection of *Daphnia* under environmental stress were examined. Experiments were conducted using five *Daphnia* clones with different environmental preferences and, consequently, with a different width of the reaction norm. These clones also showed variation in their constitutive levels of stress-related heat shock proteins (HSP60, HSP70 and HSP90), but none of the tested stress factors had any direct effect on their expression. However, behavioral plasticity was significantly positively correlated with the constitutive level of HSP70. It is likely that animals with a high constitutive HSP70 level can cope better with sudden changes in environment conditions that they experience, e.g., during vertical migrations. In contrast, non-migrating animals with low HSP levels do not allocate energy to the synthesis of stress proteins and have a narrow range of behavioral plasticity.

**Keywords** *Daphnia* · HSP · Phenotypic plasticity · Temperature

**Introduction**

In general, animals differ in their norms of reaction to environmental changes, which differentiate their ability to exploit habitat heterogeneity, patterns of habitat choice and relative fitness in the current environmental context. The freshwater crustacean *Daphnia* is an excellent model organism to study phenotypic plasticity displayed under the impact of various stress factors (Colbourne et al. 2011; Ebert 2011), including the plasticity of habitat selection behavior (Pijanowska 1993; Lampert 2011). In response to a range of environmental changes, *Daphnia* shows a wide norm of reactions (Weider and Pijanowska 1993). Separate clones display different reactivity to environmental stress and, consequently, a different range of phenotypic plasticity. *Daphnia* populations are typically under strong pressure from predators. To avoid visual predation by fish, *Daphnia* have evolved a variety of anti-predator mechanisms, of which seeking refuge in deep water is one of the most common. In
response to the presence of fish, some *Daphnia* migrate vertically during the day into deeper layers of the lake (Zaret and Suffern 1976). Due to the low temperature and scarcity of food in such hypolimnetic refuges, there are costs associated with this migration: the time of development increases and the number of offspring decreases (Loose and Dawidowicz 1994). At night, *Daphnia* return to the warm and food-rich surface layers of the lake. These daily changes in the selected habitat are known as diel vertical migrations (Lampert 1989; Pijanowska 1993; Dawidowicz and Pijanowska 2018). *Daphnia* depth selection behavior may also be influenced by a number of other factors, such as presence of cyanobacteria or toxic compounds. Filamentous cyanobacteria, which disrupt the process of *Daphnia* food collection by filtration (Gliwicz and Siedlar 1980), are most common in the warm upper layers of the lake. In response to the presence of these cyanobacteria, *Daphnia* descend to deeper and cooler layers to avoid contact with the filaments (Gliwicz and Siedlar 1980) and/or to take advantage of the increased water viscosity which, as suggested by Abrusán (2004) and Bednarska and Dawidowicz (2007), facilitates the filtration process when the food suspension is contaminated with cyanobacterial filaments.

In an earlier paper, we showed that the presence of toxic polychlorinated biphenyls (PCBs) also causes *Daphnia* to alter its depth selection behavior and may disrupt natural prey-predator communication (Bernatowicz and Pijanowska 2011). When migrating in summer, between warm upper lake layers and deeper and colder waters, *Daphnia* must overcome the thermocline with noticeable thermal gradient, often of over 10 °C, twice a day. There are some mechanisms that may protect the physiology of migrating zooplankton individuals from heat or cold shock. First is a transcription and translation of proteins, especially enzymes, of their specific activity adjusted to adequate temperature. These mechanisms are known in marine copepods (Freese et al. 2012). They are, however, costly (old proteins must be decomposed and new ones must be synthesized) and slow (exchange of proteins set may take a long time). Another mechanism is protection of already existing proteins during thermal shock. This implies that migratory behavior in response to environmental factors may be linked to the expression and protective function of heat shock proteins (HSPs).

HSPs perform a number of vital functions in all living cells: (i) they play a role in the correct folding of newly synthesized proteins, (ii) they facilitate the renaturation of proteins improperly folded as a result of thermal shock, toxins or other stress factors, (iii) they participate in the induction of proteins required for responses to environmental stress, and (iv) they form complexes with other proteins and assist in their transport to the appropriate cell compartments (Schlesinger 1990). Since HSP expression changes under adverse environmental conditions, they are reliable molecular indicators of an environmental stress response (Cruz-Rodríguez and Chu 2002; Pijanowska and Kloc 2004) and it is believed that these proteins are involved in protection against temperature changes in Crustacea (Lejeusne et al. 2006). Most recently, the unique roles that different proteins of HSP70 group play throughout *Daphnia* development (both continuous and interrupted by diapause) was demonstrated by Chen et al. (2021).

The aim of this study was to test the hypothesis about the association between HSP expression and plasticity of depth selection behavior in *Daphnia*. We assume that clones with high phenotypic plasticity of habitat choice experience relatively large depth-related temperature variability and may, therefore, need the protective buffer of higher HSP expression. We further assume that *Daphnia* individuals with broad behavioral plasticity might also exhibit variability in HSPs levels under different environmental conditions.

**Material and methods**

Five clones from the *Daphnia longispina* group were selected for the experiments, among them clones of *D. longispina*, *D. galeata* and their hybrids (as determined by allozyme analysis). The founder individuals were either caught in the epilimnion or hatched from ephippia originating from the lowland European lakes Konstanz (South Germany), Świecąt and Roś (North Poland) (Table 1). These clones are known to exhibit different phenotypic plasticity in their depth selection behavior (Bernatowicz and Pijanowska 2011). Two experiments were conducted in parallel: one to measure HSPs expression in *Daphnia* exposed to adverse factors and the second to monitor *Daphnia*...
depth selection behavior in the presence of these factors.

(a) Analysis of HSP expression
Newborn *Daphnia* (aged 8 h) were kept in glass jars containing 0.75L of a medium (25 individuals per jar) until they reached sexual maturity. Five experimental treatments were set up. The control medium consisted of water from eutrophic Lake Szczęśliwice located near the University campus in Warsaw. Before use, water was stored for 6 months in a large (10 m³) aerated underground tank, then filtered through a Whatman GFC membrane filter and enriched with *Acutodesmus obliquus* at a concentration of 1 mg Corg L⁻¹, which served as food. The algae were cultured in Z4 mineral medium (Zehnder and Gorham 1960). In other treatments, the medium was supplemented with 0.5 mg CorgL⁻¹ filaments of non-toxic clone of cyanobacterium *Cylindrospermopsis raciborskii* (Abrusán 2004; Bednarska and Dawidowicz 2007), PCB150 (10 lgL⁻¹), PCB52 (10 lgL⁻¹) or planktivorous fish kairomone, respectively. Water with fish kairomone was produced by keeping one individual of *1+ rudd* (*Scardinius erythropthalmus*, in the second year of life) per five liters for 24 h. Five replicates of each treatment were performed. The determination of the expression level of HSP proteins was carried out in five stages, according to the procedure developed for *Daphnia* by Pijanowska and Kloc (2004) and Mikulski et al. (2009):

- **Preparation of the sample Daphnia** were homogenized in Eppendorf tubes in RIPA buffer (with the addition of COMPLETE (Roche) buffer (which is a mixture of proteases inhibitors). Then, after centrifugation (10,000 rpm for 30 s), the supernatant (60 µl) was transferred to another tube, 60 µl Sampling Buffer (Sigma) was added and the sample was boiled for 5 min. A small amount of the supernatant (5 µl) was placed in titration plate for further analysis of the protein content.
- **Total protein content analysis** The protein content of the sample was determined using the DC Protein Assay kit (Bio Rad) according to the method recommended by the manufacturer. The obtained results were used to calculate the volume of the sample to be applied on the polyacrylamide gel before electrophoresis.
- **Electrophoresis** The electrophoresis was run on a polyacrylamide gel (4% and 10%) for 60 min at a voltage of 180 V and a current of 100 mA in RUNNING buffer. The samples (containing 0.012 mg Corg proteins) were transferred to wells. In addition, the colored molecular marker and the lysate from human HeLa cells previously exposed to heat shock were separated in parallel. The marker allowed the observation of electrophoresis progress, and the lysate was so-called positive control allowing to determine whether the antibodies used in immunodetection worked properly.
- **Western Blotting** After electrophoresis, proteins were transferred to a PVDF membrane (Amersham). This process was carried out for 75 min at a voltage of 100 V and a current of 300 mA in the BLOTTING buffer. After transfer, the membrane was stained with Ponceau dye to reveal the associated protein (correctness verification of the transfer).
- **Immunodetection** After the Ponceau dye was rinsed with distilled water, the membrane was blocked with cow’s milk proteins. Then, after washing with TBST buffer, the membrane was immersed in a solution of primary antibodies (polyclonal rabbit IgG against

### Table 1 Description of five *Daphnia* clones used in the experiment

| Clone | Origin        | Allozymes | Taxon           |
|-------|---------------|-----------|-----------------|
| A     | Roś (Poland)  | ff sf     | *D. galeata*    |
| B     | Święcajty (Poland) | sf ff | *D. galeata*    |
| C     | Konstanz (Germany) | sf sf | hybrid          |
| D     | Roś (Poland)  | sf ss     | *D. longispina* |
| E     | Konstanz (Germany) | ss ss | *D. longospina* |
proteins HSP 60 (StressGen, # SPA-805), HSP 70 (StressGen, # SPA-812) and HSP 90 (StressGen, # SPA-846) and GPDH (Santa Cruz Biotechnology, # B0106)). The amount of the GPDH proteins (the so-called loading marker) was a control of the analysis and its level was detected each time. After incubation for 12 h at 4 °C, the membranes were washed with TBST buffer. Then, they were immersed in the secondary antibody solution (alkaline phosphatase conjugated goat anti-rabbit IgG (Rockland)). After 4 h incubation at room temperature and washing the membrane with TBST buffer, test protein bands were visualized by adding SIGMA FAST, a substrate of the reaction leading to a colored and water-insoluble compound performed by an enzyme conjugated to secondary antibodies. After drying, the membranes were stored in the dark. The intensity of the immunoreactive bands on the blots was quantified using the Bio-Rad imaging system with Quantity One (Bio-Rad) software.

(b) Depth selection

*Daphnia* neonates (aged from 0 to 8 h) were transferred to a system of glass flow-through tubes (60 cm long and 1.5 cm diameter): so-called plankton organ, a device that simulates the thermal conditions in a lake, which has been widely used to study *Daphnia* migratory behavior (Dawidowicz and Loose 1992). The 16L:8D photoperiod and thermal stratification set up in the tubes (8 °C at the bottom and 24 °C close to the surface) mimicked conditions in a dimictic lake pelagial in the temperate climate zone during the summer.

Eight newborn *Daphnia* individuals were placed in each tube of the plankton organ. In the experiment, we used the same treatments as in the case of HSP analysis. Each treatment had two replications in the plankton organ (space replications) and was repeated twice (time replications). Following the standard procedure proposed by Loose et al. (1993), after 6 days, when the *Daphnia* reached maturity, the depth of each individual in the water column was recorded, 3 h after switching on the light. The mean values were calculated by pooling all observations for each tube of the plankton organ (for 8 individuals). The phenotypic plasticity index of each clone was calculated using the modified algorithm of Valladares et al. (2000): \[ PP = \frac{(\text{max} - \text{min})}{d} \] (PP–phenotypic plasticity, max–maximum mean depth (out of the 5 treatments), min–minimum mean depth (out of the 5 treatments), d–height of the water column available for migrating *Daphnia*).

(c) Statistical analysis

The results were analyzed using the GLM (general linear model; models used: \[ \text{HSP}(i) = \text{clone} + \text{treatment} + \text{clone} \times \text{treatment}, \] where \( i \) is an HSP proteins (HSP60, HSP70, HSP90) and \( \text{Depth} = \text{clone} + \text{treatment} + \text{clone} \times \text{treatment} + \text{space_replication} + \text{time_replication} \)) and Bonferroni post-hoc test. A Pearson correlation between the level of HSPs in the tested clones and their phenotypic plasticity was calculated. All analyses were done using IBM SPSS Statistics 21 software.

Results

We found that *Daphnia* clones differed significantly \((p < 0.001)\) in their levels of all proteins studied: HSP60, HSP70 and HSP90 (Figs. 1 and 2). Post-hoc test showed that the concentration of HSP60 was significantly higher in A and E clones than in B, C and D. In case of HSP70 the concentration was higher in A and B than C, D and E. There were no statistically significant differences between clones in HSP90 concentration. The applied factors (presence of fish, cyanobacteria, PCB 153 or PCB 52) did not exert a significant effect on these stress proteins \((p_{\text{HSP60}} = 0.96, \ p_{\text{HSP70}} = 0.93 \text{ and } p_{\text{HSP90}} = 0.17, \) respectively). There was also no statistically significant interaction between clone affiliation and its treatment. This indicates that there was no direct effect of any of the stress factors on any HSP expression in the *Daphnia longispina* group.

The tested *Daphnia* clones showed significant \((p < 0.001)\) difference in their depth selection (Fig. 3). The post-hoc test showed that there was a statistically significant difference in selected depth
only between clones D and E. There was also a statistically significant effect of treatment \((p < 0.001)\) and interaction between treatment and clone affiliation \((p < 0.001)\). We did not find any differences resulting from space or time replications. Basing on post-hoc test results, we found that only presence of fish and cyanobacteria affected depth selection behavior by forcing *Daphnia* to migrate deeper in the water column. None of PCB congeners caused such effect.

Each of the tested clones had a different range of phenotypic plasticity (Fig. 3). We also found a clear statistically significant correlation (with Bonferroni correction, \(p = 0.0054\)) between the constitutive (measured under control conditions) level of HSP70 and phenotypic plasticity in depth selection. The higher expression level of HSP70, the greater behavioral plasticity. However, no such correlation was found for either HSP60 or HSP90 (Fig. 4).

**Discussion**

The lack of any effect of the tested environmental conditions on the level of HSP expression in clones from the *Daphnia longispina* group was not expected. In a closely related species (*D. magna*), the expression of HSPs was found to change in the presence of a predator (Pijanowska and Kloc 2004; Pauwels et al. 2005), high temperatures (Mikulski et al. 2009) and toxic substances (Haap et al. 2008). It is possible that smaller size members of the *D. longispina* group respond to stressors using a different mechanism. *D. magna* live in temporary fishless habitats without strong predatory pressure, in contrast to *D. longispina* from relatively stable lake environments but with high pressure of planktivorous fish.

It is known that the constitutive level of HSPs may vary among closely related species, e.g., in morphologically similar *Hydra* species (Bosch et al. 1988) and among separate clones of the same species, e.g. *Daphnia magna* (Pauwels et al. 2005). Now we confirmed this phenomenon using *Daphnia* clones from other species. These differences may either be the cause or the consequence of different reactions of the animals to environmental conditions.

The behavioral reactions of examined *Daphnia* clones were very similar to those observed by Bernatowicz and Pijanowska (2011). The strongest reactions were performed in the presence of planktivorous fish and cyanobacteria. In the current study, we also observed significant diversity of clones reactions to both, abiotic and biotic treatments. This implies interclonal differences in the observed range of phenotypic plasticity. These results confirm other data demonstrating high diversity of phenotypic plasticity in the *Daphnia longispina* group (Wolińska et al. 2007).

Though the physiological basis of phenotypic plasticity is not yet fully characterized (Fusco and Minelli 2010; Hales et al. 2017), we identified a significant correlation between the constitutive level of HSP70 and behavioral plasticity in *Daphnia*. The constitutively high level of this heat shock protein might allow *Daphnia* to respond to a stress related to the temperature gradient they experience during diel vertical migration and to induce tolerance to such temperature changes. HSP70 participates in the protection of other proteins against temperature stress.
Therefore, individuals with high level of HSP70 may be able to select a preferable depth regardless of the thermal conditions, and safely migrate across the thermal gradient. Starrett and Waters (2007) showed that the function of HSP70 is related to heat avoidance behavior in spiders. Moreover, Hamdoun et al. (2003) demonstrated that the thermal limits of Pacific oysters are relatively plastic, and that these limits are correlated with changes in the expression of HSP70s. These findings on diverse organisms are analogous to those of the present study, which describe the correlation between Daphnia behavior and the constitutive level of HSP70 responsible for protecting these animals against sudden and abrupt changes in temperature.

Transcription, translation and other molecular processes require time and Daphnia that perform relatively rapid diel vertical migrations have to be prepared for sudden thermal changes at the molecular level. In the Daphnia longispina group, the constitutive level of HSP70 is apparently more important than induced expression of this protein. The results of an earlier study on the transcriptional regulation of a stress-inducible heat shock protein (HSP70) in the scleractinian coral Pocillopora verrucosa (Poli et al. 2017) indicated that local habitat conditions may influence transcription of stress-related genes. Corals exhibiting higher basal HSP70 levels displayed enhanced tolerance toward environmental stress factors. Also, basing on response to heat stress of Atlantic ribbed mussel, Geukensia demissa inhabiting contaminated urban waterways, Ravaschiere et al. (2017) suggested that the HSP70 are involved in the unique adaptive mechanisms enhancing tolerance to
variability of conditions in impacted habitats. Similarly to corals and mussels, migrating *Daphnia* have to cope with highly unpredictable changes in their environment with changes of broad amplitude in the experienced conditions. Broad behavioral plasticity requires maintenance of HSP machinery on standby, in case of abrupt and sudden environmental changes often experienced on their migration route. In non-migrating *Daphnia*, the continuous synthesis of HSPs at a high level would be an unnecessary waste of resources and energy. This type of selection of clones may fix the positive correlation between behavioral activity and HSP level.

Results from this study contribute to the better understanding of physiological basis of behavioral plasticity and provide fresh insight into the mechanisms of adaptation to the challenging environments.

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Declarations

Conflict of interest The authors declare that they have no conflict of interest and all institutional and national guidelines for the care and use of laboratory animals were followed. The data that support the findings of this study are not openly available and are available from the corresponding author upon reasonable request.

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