Hydrobiologia (2009) 636:393–400
DOI 10.1007/s10750-009-9968-5

PRIMARY RESEARCH PAPER

Differential expression of heat shock protein 70 in relation to stress type in the flatworm *Schmidtea polychroa*

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Received: 4 August 2009 / Revised: 25 September 2009 / Accepted: 5 October 2009 / Published online: 19 October 2009

Abstract Most heat shock proteins help to cope with stress in organisms ranging from bacteria to vertebrates. Many stress types acting on the intensity of intracellular protein can induce expression of heat shock proteins. Here, we studied changes in expression level of heat shock protein 70 (Hsp70), one of the best investigated stress proteins, in response to five potential stress factors in the planarian flatworm *Schmidtea polychroa*: (1) homogenized planarian tissue, which releases an alarm substance that signals predation injury, (2) physical damage by puncturing, (3) a simulation of ecological competition by adding a mixture of naturally co-occurring species: one *Dendrocoelum* and two *Polycelis* flatworms, one *Asellus* water louse and one leech, and (4) magnesium chloride, which inhibits regeneration ability. We found that alarm substance (1), physical harm (2), and magnesium chloride (4) led to increased expression of Hsp70, while interspecific competition (3) did not result in elevated Hsp70 expression. There was no difference between the experimental negative control and two temporal controls immediately after collection and just before the experiment. Results show that *Schmidtea polychroa* is not sensitive to sampling and lab maintenance. However, planarian homogenate, magnesium chloride and physical harm all caused Hsp70-inducing stress. We conclude that Hsp70 quantification is appropriate to study the current stress level in planaria in response to specific conditions.

Keywords Freshwater ecology · Platyhelminthes · Biomarker · Stress response · Regeneration · Western blotting

Handling editor: K. Martens

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Introduction

Heat shock proteins (Hsps) are a highly conserved family of stress response proteins (Lowe et al., 1983; Lindquist, 1986; Schlesinger, 1990; Boorstein, 1994). They function primarily as molecular chaperones, facilitating the folding of cellular proteins, preventing protein aggregation, or targeting improperly folded proteins to specific degradative pathways (Becker & Craig, 1994; Mayer & Bukau, 2005). Hsps are expressed at low levels under normal physiological conditions, but some isoforms show dramatically increased expression in response to cellular stress (Sanders & Martin, 1993; Feder & Hofmann, 1999). Many stress types have been shown to induce

expression of heat shock proteins. Some relevant inducers of Hsps are high and low temperature, UV radiation, heavy metals, bacterial and viral infection, parasitism, and oxidative stress (Deitch et al., 1995; Jenkins et al., 1997; Köhler & Eckwert, 1997; Gophna & Ron, 2003). Stress factors are usually considered to be extrinsic (environmental), however, there are intrinsic factors known to increase Hsp expression such as inbreeding, deleterious mutations, and aging (Wheeler et al., 1999; Kristensen et al., 2002; Zhao et al., 2002). Moreover, stress proteins have been used to monitor the impact of environmental factors on various animal species, including many invertebrates (e.g., Köhler et al., 1992; Lewis et al., 1999).

The freshwater planarian *Schmidtea polychroa* is a simultaneous hermaphrodite, i.e., one individual possess both fully functional male and female gonads. Planarian flatworms occur in lakes and streams across Europe and have been used for a high number of bioassays due to their importance for aquatic ecology of unpolluted streams, their sensitivity to low concentrations of environmental toxicants, and the presence of a sensitive neurological system (Grebe & Schaeffer, 1991; Schaeffer, 1993; Guecheva et al., 2003; Pagán et al., 2006). Some studies have shown the presence and upregulation of various members of the Hsp family, including the most investigated stress protein Hsp70, in planarians (Wago et al., 1997; Guecheva et al., 2003). However, there is little known about the effect of potential natural stressors on heat shock proteins in planarian species.

Here, we experimentally studied the effect of four potential natural stressors on Hsp70 in the planarian *Schmidtea polychroa* in the laboratory. In order to assess the biological relevance of Hsp70 changes under laboratory conditions, we furthermore compared the expression of the heat shock protein Hsp70 in field samples and in the laboratory, to show that after acclimation these Hsp70 levels are comparable.

Materials and methods

Collection and maintenance

In September 2007, we collected about 300 adults (approx. >1 cm) of the planarian flatworm *S. polychroa* at Wartaweil (WW) on the East shore of lake Ammersee in Bavaria, Germany. Only parthenogenetic biotypes exist at this site (Beukeboom et al., 1996; D'Souza & Michiels, 2006). The location is characterized by high genotypic diversity (Pongratz et al., 2003; D'Souza & Michiels, 2006; D'Souza & Michiels, 2008), caused by occasional sexual exchange between parthenogens (D'Souza et al., 2004; D'Souza et al., 2006). Flatworms were collected from the underside of stones using brushes. Sampling took around 1 h. Subsequently, one group of 20 randomly chosen individuals was fixed in liquid nitrogen (temporal control 1). All others were isolated in 300 ml vials at 16°C and 12:12 h dark:light cycle for 1 week to adapt to laboratory conditions as well as during the subsequent 10 h treatment. Animals were not fed during the study (as they can survive starvation for months, B. Sánchez Navarro, pers. obs.).

Treatments

Individuals were exposed to four different stress factors for a total duration of 10 h. Each treatment group consisted of 20 randomly chosen individuals. Individuals belonging to different treatments were spatially randomized and all tests ran in parallel. Experiments were checked for dead individuals every 2 h. We ran four stress treatments and one control: Each individual in treatment group (1) was kept in water which contained the homogenate of a single *S. polychroa* dissolved in 1 ml culture water. The dissolved planarians were previously selected for homogeneous size. The homogenate comprises an alarm substance that signals predation injury (Wisenden & Millard, 2001), and hence, this substance was expected to cause predator stress in this treatment. The second treatment group (2) was exposed to physical stress by punching a hole onto the planarian's body using a Pasteur pipette. The third treatment group (3) was exposed to a mixture of other freshwater species known to coexist with *S. polychroa* and whose food niches overlap with *S. polychroa* prey preferences (Reynoldson & Davies, 1970; Young, 1981): One leech *(Erpobdella octoculata)*, three planarians (one *Dendrocoelum lacteum* and two *Polycelis nigra/tenuis*), and one isopod (*Asellus aquaticus*). Leeches and other planarians represent food competitors and the isopod a potential prey. All species were collected from the same location and at the same time as

S. polychroa. The fourth and final treatment group (4) was kept in 0.4% $MgCl_2$ culture water. 0.2% $MgCl_2$ solution is known to interfere with wound closure in planarian flatworms (Schürmann & Peter, 1998) and although no detrimental effect on tissue could be observed in previous experiments, we expect at least an osmotic stress response. The negative control group received no treatment except for the change of regular culture water as in the treatments described above. In order to exclude sampling or maintenance bias, we performed two further controls: Temporal control group 1 animals were fixed in the field immediately after sampling (see above). Temporal control group 2 animals were fixed after the acclimation period, directly before the start of the experiment. All individuals survived the 10 h exposure to the treatments and were subsequently fixed in liquid nitrogen. Samples were stored at −80°C for later analysis.

Hsp70 expression assessment

Treatments were randomized and processed as described in Köhler et al. (2005). Whole samples were homogenized on ice with extraction buffer (80 mM potassium acetate, 5 mM magnesium acetate, 20 mM HEPES, pH 7.5). Total protein concentration was determined according to Bradford (1976). Constant protein weights (15 µg) were analyzed by SDS-PAGE (12% acrylamide, 0.12% bisacrylamide (w/v), 15 min at 80 V, 90 min at 120 V) in randomized order and subsequently transferred to nitrocellulose by semi-dry blotting. The filter was blocked for 2 h in 50% horse serum in TBS (50 mM Tris, pH 7.5, 150 mM NaCl) and washed for 5 min in TBS. The filter was then incubated overnight in the first antibody (mouse anti-human Hsp70, Dianova, FRG, dilution 1:5,000 in 10% horse serum in TBS) and again washed for 5 min in TBS. Following the incubation in the second antibody (goat anti-mouse IgG conjugated to peroxidase, Dianova, FRG, dilution 1:1,000 in 10% horse serum/TBS) for 2 h and a washing step in TBS, the antibody complex was visualized with a staining solution (1 mM 4-chloro(1)naphtol and 0.015% H_2O_2 in 30 mM Tris pH 8.5 containing 6% methanol). Evaluation of the Western blot protein bands was performed with a densitometric image analysis system (E.A.S.Y. Win 32, Herolab, FRG). The greyscale value of the Western blot protein bands was related to an Hsp70 standard extracted from the water flea, *Daphnia magna*, run in parallel on each gel. Thus, the Hsp70 levels are expressed as a relative greyscale value (rgv).

Data analysis

Differences in the replicate numbers for the different treatments resulted from insufficient protein concentration in some samples due to the protein extraction method. Since samples were also randomized for Hsp70 expression assessment drop-outs occurred in all treatments. Nested ANOVAS with "treatment" as a fixed factor and "gel run" as a nested factor were applied to correct for the methodological variability. Since the different gel runs did not affect the band intensities, we performed one-way analyses of variance (ANOVA). We first compared the three controls to test for sampling and handling effects. We then compared the treatments with the negative control. To this end, we performed multiple post hoc pairwise comparisons between all treatment groups and the negative control using Dunnett's test (Dunnett, 1955). The results are displayed as a Least Significant Difference Threshold Matrix (LSD) (Fisher & Bennett, 1990) which is a statistical procedure that determines whether the difference between two groups is due to the treatment or simply due to chance. Statistical analysis was performed using JMP IN version 5.1.2 (SAS Institute Inc., Cary, NC, USA).

Results

Temporal controls

Hsp70 levels in the controls after field sampling, before the treatment and after the treatment (negative control) did not differ significantly (ANOVA, $F_{2,46} = 1.2211$, $P = 0.30$) (Fig. 1), suggesting that collecting and handling did not significantly stress the individuals.

Treatments

The overall ANOVA showed a significant difference among the treatment groups (ANOVA, $F_{4,84} = 3.3043$, $P = 0.0145$). The post hoc analysis revealed that homogenized planarian tissue, physical stress

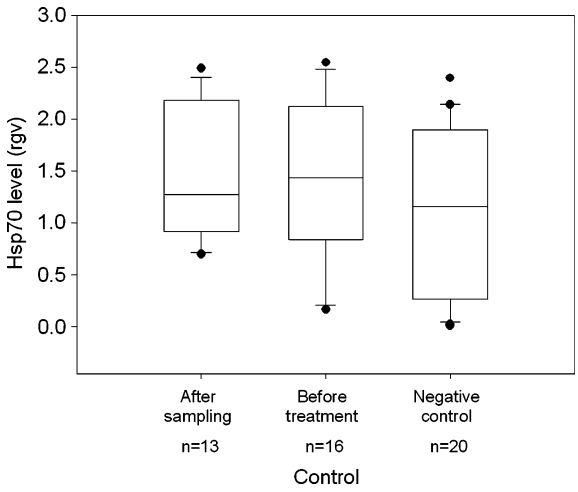

Fig. 1 Box-plot showing Hsp70 levels (relative grey values [rgv] of the Western blot protein bands) in the two temporal controls after field sampling and before the treatment, and the negative control after the treatment. *Boxes* represent the median and the interpercentile range; the *bars* represent the 10th and 90th percentile. *Closed circles* represent outliers. There is no significant difference among the three controls (ANOVA, $F_{2,46} = 1.2211$, $P = 0.30$)

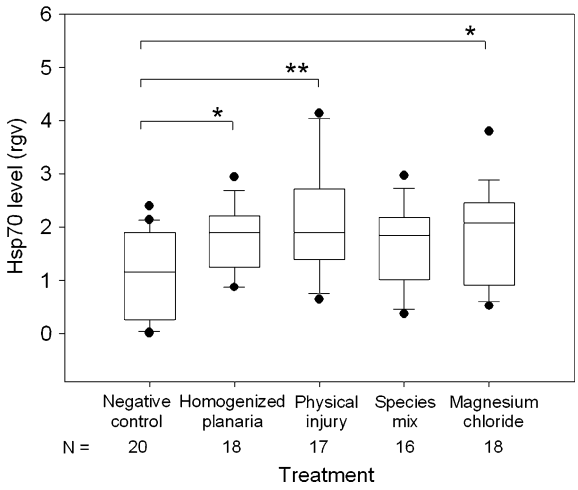

Fig. 2 Box-plot showing Hsp70 expression (relative grey values [rgv] of the Western blot protein bands) for the negative treatment control and four treatments. Pairwise comparisons were done for each treatment with the negative control using Dunnett's test. *Boxes* represent the median and the interpercentile range; the *bars* represent the 10th and 90th percentile. *Closed circles* represent outliers. *Single asterisks* show significant differences between the treatment and the negative control after correction for multiple testing ($P < 0.05$), *double asterisks* represent $P \leq 0.01$

through puncturing, and $MgCl_2$-enriched water resulted in an upregulation of Hsp70. The presence of other species at high density did not significantly affect Hsp70 levels compared to the negative control (Fig. 2, Table 1).

Table 1 Multiple pairwise comparisons with the negative control using Dunnett's method

Level	LSD	*P*
Negative control	−0.67	1
Homogenized planarian tissue	0.004	0.0482
Physical injury	0.244	0.0042
Species mix	−0.11	0.1253
Magnesium chloride	0.018	0.0423

Discussion

We demonstrated that the sampling procedure and maintenance in the laboratory did not affect Hsp70 levels in *Schmidtea polychroa*, suggesting that laboratory assays yield results comparable to those obtained directly from the field. This implicitly assumes the freshly collected planarians to show a low baseline Hsp70 expression, which is supported by the fact that Hsp70 levels increased in three of the experimental stress treatments. We demonstrate this for the first time in freshwater planarians. Homogenized planarian tissue, physical damage by puncturing, and $MgCl_2$-enriched water caused a significant increase in Hsp70 expression. Independent of the different characters of the stressors, we propose that all this represents a response to stress involving proteotoxicity and that Hsp70 levels likely are a suitable stress proxy for individuals in natural populations of this species.

Sampling and handling stress

Experimental procedures such as handling, sampling, and other physical stressors might be stressful. Hence, when studying stress in aquatic organisms it is important to establish whether the procedures themselves are affecting the Hsp response (Iwama et al., 2004). In this study, we show for the first time that Hsp70 expression in planarian flatworms was not affected by sampling and handling as has been occasionally reported for fish (Vijayan et al., 1997; Washburn et al., 2002; Iwama et al., 2004). Therefore, we can safely assume that the Hsp70 level measured in the field and after the acclimation period represents the natural baseline Hsp70 level of the population. Since

in this study we focus on Hsp70 exclusively, we can not exclude that handling influenced expression levels of other Hsps.

Hsp70 as an alarm response

In aquatic organisms, many interactions, e.g., predator–prey interaction, also involve chemical communication (Brönmark & Hansson, 2000). The ability to assess predator risk is an essential process since inefficient predator avoidance increases mortality risk. Planarians are known to show an escape response to conspecific injury-released cues (Wisenden & Millard, 2001). We also demonstrated a reduced locomotory activity of the planarians when exposed to the homogenized conspecific tissue (S. Blötscher and T. G. D'Souza, unpublished results). Our finding of elevated Hsp70 levels in the presence of homogenized conspecific tissue substantiates this observation. Further research is needed to elucidate the exact signal transduction pathway, but it is likely that Hsp70 plays a role in the cellular alarm response triggered by alarm substances.

The stress of regeneration

Freshwater planarians possess remarkable regenerative abilities (Newmark, 1998; Reddien & Sánchez Alvarado, 2004). Regeneration after an injury is enabled by a group of undifferentiated cells called neoblasts (Saló et al., 2008). Diverse intracellular proteins including Hsps have been shown to be expressed after injury (Patruno et al., 2001, in echinoderms) and, more specifically, Hsp60 is involved in wound healing in planarians (Wago et al., 1997). The injury treatment in this study caused the highest Hsp70 response compared to the control and to the other treatments, indicating that injury activates a potent stress response in *S. polychroa*. However, it was impossible to differentiate whether the injury per se caused the high Hsp70 level or whether the secondary effect of wound healing through regeneration and consequently, the formation of freshly synthesized protein contributed to its expression.

Food competition

During this treatment, we expected a stress response due to food competition in the experimental vial. Previous studies showed the diet of leeches and some triclad species to overlap considerably (Reynoldson & Davies, 1970). In contrast to our expectations, the presence of other freshwater planarians and a leech as food competitors and an isopod as a potential prey resulted in a moderate, non-significant increase in Hsp70 levels only. One possibility that could explain this result is that although individuals were not fed for 1 week, this time may not be sufficient to cause a situation of competition for food. However, Collins & Gerald (2009) showed that hunger levels do not influence planarian activity toward different prey cues. Furthermore, leeches are more successful at capturing live prey (Seaby et al., 1995) and this could again cause stress for *S. polychroa* when competing for food. On the other hand, a live *Asellus* may not be a suitable prey for this experiment. *S. polychroa* and *Polycelis spec*. are more active as predators than *D. lacteum* (Reynoldson & Young, 1963). Nevertheless, they react more actively when they perceive prey cues (Seaby et al., 1995). The lack of predator behavior of the triclad species could be explained by the fact that the prey was not injured and no body fluids were leaking as the experiment started. Moreover, the different predation modes of the triclad species and the leech might solve the stress of food competition between these species under these conditions. In order to elucidate the stress effect of food competitors on *S. polychroa* further studies choosing a more suitable prey should be done.

Salinity stress

Magnesium chloride is commonly used as an anaesthetic for aquatic organisms and can be lethal when applied in high dosage. Being a freshwater organism, *S. polychroa* is sensitive to salinity fluctuations and its abundance depends on osmoregulatory capacities (Velde et al., 1986). Schürmann & Peter (1998) showed that 0.2% $MgCl_2$ solution already has severe effects on planarian regeneration. When confronted with 0.4% $MgCl_2$ flatworms showed an increased stress response. A high dosage of $MgCl_2$ may also activate an alarm response to desiccation stress even though the actual mechanism behind the response shown here is unknown.

Hsp70 as a biomarker for health in nature

Biomarkers are used to monitor the relationship between exposure to a stressor and impaired health.

The expression of the heat shock protein Hsp70 has been used as a biomarker of effect in several studies (Clayton et al., 2000; Nadeau et al., 2001; Köhler et al., 2005). In this context, the characterization of the Hsp70 expression in aquatic organisms is a reliable method to monitor the proteotoxic action of toxic substances which may occur even at very low concentrations. Although Hsps are quite frequently used in ecotoxicological assays, little is known about the effects of natural stressors on the Hsp70 response in some taxa such as Turbellaria. In general, it is commonly accepted that the unstressed organism produces these proteins, which are required in various aspects of protein metabolism in a constitutive manner (Sørensen et al., 2003). The stress needed to induce Hsps is strongly related to the ecological niche of the organism (Feder & Hofmann, 1999). Under natural conditions, individual Hsp response differs according to different factors, e.g., the stressor, the developmental stage and the season. This variation in Hsp expression levels may not only reflect the general stress status integrating overall proteotoxicity, it could also represent the health status of the population.

When an organism undergoes a stressful situation, many other mechanisms will be activated in addition to the Hsp response (Sørensen & Loeschcke, 2007). Thus, the Hsp70 level might decline while the stressor is still present. This should be considered when characterizing the stress response in an organism under natural conditions. To our knowledge, there are no studies for *S. polychroa* which elucidate how long an elevated Hsp70 level is maintained after exposure to a stressor. However, such an approach was also beyond the scope of this study.

Conclusion

The comparison of Hsp70 levels in nature and under laboratory conditions and the characterization of the Hsp70 expression in the freshwater planarian *Schmidtea polychroa* demonstrate the reliability of using this stress protein as an indicator for current proteotoxic stress levels in a natural population. Since Hsp70 expression depends on the stressor and its mode of action, we should be careful when choosing stress factors for further studies. Although Hsp70 may not represent the best stress indicator in every situation, it offers a new approach to study stress in situ.

Acknowledgments We thank Milosz Olczak and Gregor Schulte for collecting flatworms and Nadine Timmermeyer for the assistance during the experiment. We are grateful to Rebecca Schulte and Nadine Timmermeyer for fruitful discussions and valuable comments to improve previous versions of this manuscript. This study was supported by a grant from the German Science Foundation (DFG) no. MI 482/11 and the Volkswagen Foundation (Initiative Curriculum Evolutionary Biology: Tübingen in Evolution, Evolution in Tübingen).

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