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SHORT COMMUNICATION

Coping with predator stress: interclonal differences in induction of heat-shock proteins in the water flea *Daphnia magna*

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Abstract

Although predation is a strong selection pressure, little is known about the molecular mechanisms to cope with predator stress. This is crucial to understanding of the mechanisms and constraints involved in the evolution of antipredator traits. We quantified the expression of heat-shock protein 60 (Hsp60), a potential marker for predator stress, in four clones of the water flea *Daphnia magna*, when exposed to fish kairomones. Expression of Hsp60 induction increased after 6 h and returned to base levels after 24 h of predator stress. This suggests that it is a costly transient mechanism to temporarily cope with novel predator stress, before other defences are induced. We found genetic variation in the fixed levels and in the fish-induced levels of Hsp60, which seemed to be linked to each clone's history of fish predation. Our data suggest that Hsp60 can be considered part of a multiple-trait antipredator defence strategy of *Daphnia* clones to cope with predator stress.

Introduction

Predation is an important stressor in aquatic communities that may drive not only the ecological but also the evolutionary dynamics of prey populations (Kerfoot & Sih, 1987; Scheffer, 1998; Tollrian & Harvell, 1999). Many studies have demonstrated antipredator traits to be under selection and to possess genetic variation, thereby allowing their adaptive evolution (e.g. De Meester, 1996; Cousyn *et al.*, 2001). A plethora of studies have contributed to the ecological and evolutionary understanding of morphological, life history and behavioural antipredator traits and their impact on predator–prey interactions. In contrast, we have only just started to explore molecular changes associated with defence strategies and little is known about their ecology and evolution. Integration of knowledge on all types of antipredator traits is, however, crucial to understand the mechanisms and constraints involved in their evolution.

Recent studies suggest that heat-shock proteins (Hsp), that make up a ubiquitous and well-studied molecular defence mechanism against stress (Parsell & Lindquist, 1993; Feder & Hofmann, 1999; Pockley, 2003), may also protect prey against cellular damage associated with predator stress (Kagawa & Mugiya, 2002; Pijanowska & Kloc, 2004). Hsps are molecular chaperones that are involved in protein housekeeping: they assist in folding, unfolding, transport and assembling of complex protein units, preventing proteins from getting into 'inappropriate' aggregations, and degradation of misfolded or aggregated proteins (Sørensen *et al.*, 2003). These important functions are fulfilled under normal cellular conditions. In addition, however, their expression can increase in response to all kind of stressors that have the potential to damage cells (Feder & Hofmann, 1999; Sørensen *et al.*, 2003). Hsp protection thus has a constitutive and an inducible component. We consider it an antipredator trait because by maintaining homeostasis, induction of Hsp avoids fitness reduction of the prey under predator stress. Moreover, Hsp may play a role in generating the fight and flight response by not only protecting but also facilitating the binding of messenger molecules and receptors (Kagawa & Mugiya, 2002).

Expression of Hsps has an important cost as it may reduce the amount of resources available for growth and reproduction (Krebs & Loeschcke, 1994; Krebs & Feder,

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1997; Silbermann & Tatar, 2000). It has been shown that even very small amounts of induced Hsps can have effects on life history traits such as development rate, life span and fecundity (Rutherford & Lindquist, 1998; Sørensen & Loeschcke, 2001; Queitsch *et al.*, 2002; Rutherford, 2003). Given that the induction of Hsps may allow organisms to occupy a rich but stressful habitat, and that the induction is costly by itself, it is likely that Hsp induction is a part of ecologically important trade-offs that shape evolutionary strategies. At the moment, the basic information to test this is missing. For example, no studies have so far explored the evolutionary potential of Hsp with regard to predator stress.

In the present study, we focus on Hsps as a constitutive and inducible antipredator mechanism in the water flea *Daphnia magna*. A broad variety of antipredator traits have been shown to be induced by fish kairomones. This plasticity involves life-history traits (size and age at maturity, size and number of eggs, production of sexual eggs), changes in morphology (neck spines) and changes in behaviour (diel vertical and horizontal migration, phototaxis, swarming and escape behaviour). Most of the clones show an induced response to the presence of predators, but there are large differences among clones in the sets of traits used (Boersma *et al.*, 1998). If predation risk is a stressful condition, one might expect the induction of Hsp upon exposure to predators. Pijanowska & Kloc (2004) have indeed reported a change in expression of Hsp60 in *D. magna* upon exposure to fish. We will further document the induction of Hsp by exposure to a fish predator, including the pattern of induction through time. Knowledge of the short-term temporal dynamics is important to better understand the links of Hsp defence with other antipredator traits that vary in their time lag before induction; moreover, any decrease through time may point to costs. By working with clones with known differences in their selection environment with regard to fish predation, and associated differences in fixed and flexible antipredator behaviour (i.e. phototactic behaviour), we can start evaluating the evolutionary potential of Hsp as a mechanism to cope with predator stress as well as the evolutionary links between fish-induced molecular and behavioural changes.

Materials and methods

Study organism

The large cladoceran *D. magna* is a key model organism for evolutionary-ecological studies and functional genomics (Feder & Mitchell-Olds, 2003). We will compare the induction of Hsp60 in four *D. magna* clones that differ strongly in their phototactic behaviour in the absence of fish kairomones, as well as in their induced change in phototactic behaviour in response to fish kairomones (Table 1). Fish are visual predators. As a result, *Daphnia* that stay deeper in the water column are safer with regard to fish predation. A negative phototactic behaviour is thus a habitat selection behaviour effective against fish predation (De Meester *et al.*, 1999). In the absence and presence of fish, clone $P_1$32,96 (hereafter called A^-: see Table 1) shows a very safe habitat selection behaviour, while clone $C_1$242 (C^+) remains positively phototactic, which is a risky habitat behaviour. $P_1$32,85 (A^+) shows a very risky habitat selection behaviour in the absence of fish, but responds drastically in response to fish to become almost as safe as clone A^-. Finally, clone Bl_{20} (B^0) is intermediate in the absence of fish and slightly negatively phototactic in the presence of fish.

These four genotypes were kept as clonal lines in the laboratory for many generations prior to the experiments. During this period, they were cultured in the absence of fish. By using lines that were deprived of predator stress since their isolation from the field, we could look directly for genetic differences and exclude any maternal effects. Clones were kept in the laboratory under standardized conditions (30 individuals per litre; 20 ± 2 °C; 14 h light/10 h dark photoperiod; food concentration: 1.5×10^5 *Scenedesmus acutus* cells mL^{-1}) (De Meester, 1991). Fish-conditioned medium was made as described in Kieu *et al.* (2001).

Temporal pattern of induction

Groups of 16 first-instar adult *Daphnia* of clone A^+ were placed in 0.5-L jars and continuously exposed for 0, 1, 2, 3, 4, 5, 6 and 24 h to fish kairomones. For each

Table 1 Characteristics of the four *Daphnia magna* clones used in this study. Phototactic behaviour is coded on a scale from very positively phototactic (+++) towards very negatively phototactic (−−−), the intermediate neutral level is indicated by '0'.

Clone	Complete code of clone	Phototactic behaviour		Origin/fish predation pressure	Reference
		No fish	With fish		
A^+	$P_1$32,85	+++	−−	Fishpond/low and variable	De Meester, 1993; De Meester & Cousyn, 1997; Kieu *et al.*, 2001
A^-	$P_1$32,96	−−−	−−−	Fishpond/low and variable	De Meester, 1993; Decaestecker *et al.*, 2002
B^0	Bl_{20}	0	−	Fish lake/high	De Meester, 1996; Boersma *et al.*, 1998
C^+	$C_1$242	+++	++	Fishless pond/absent	De Meester, 1991; Kieu *et al.*, 2001

exposure time, there were four replicate observations (except for 0 h, where two replicas were lost). To make sure this temporal pattern was not due to other factors like handling stress, we added a control. The control involved animals that were similarly handled and kept for 6 h (i.e. at the peak of the induction) except for the fact that no fish kairomones were present during that period.

To harvest the *Daphnia*, they were retained on plankton gauze (250 μm) and blotted dry with paper to remove as much water as possible. Whole cell extracts were prepared by homogenizing the 16 *Daphnia* with a plastic pestle in 80 μl RIPA buffer [0.15 M NaCl, 1% deoxycholic sodium salt, 1% Triton X-100, 0.1% sodium dodecyl sulphate (SDS), 0.01 M Tris, pH 7.2] to which a general protease inhibitor cocktail was added (Sigma-Aldrich, Steinheim, Germany). The homogenate was flash-frozen in liquid nitrogen. The whole procedure takes <1 min, thereby avoiding interference by handling stress. The protein concentration of the samples was determined by the Bio-Rad protein assay (Bio-Rad, München, Germany). We loaded 40 μg of the protein sample on SDS polyacrylamide gels (30% acrylamide/bis solution 37.5 : 1), and transferred the samples from gels to nitro-cellulose membranes by Western blotting (as described in Lundebye *et al.*, 1995; Pijanowska & Kloc, 2004). The membranes were blocked for 1 h in 3% skimmed milk in TBS (25 mM Tris–HCl; 500 mM NaCl). Hsp expression was detected indirectly using a monoclonal antibody recognizing Hsp60 (dilution 1 : 1000, SPA 805, StressGen®, Victoria, BC, Canada). The membranes were incubated with the primary antibody for 1.5 h, and washed in 3% skimmed milk in TBS; two times for 5 min and one time in TBS with 0.5% Tween-20. Thereafter, the membranes were incubated for 1 h with an anti-rabbit, AP-conjugated secondary antibody (polyclonal Goat anti-Rabbit Ig, D0487, DakoCytomation®, Glostrup, Denmark). Washing was repeated as described above. Colouring of alkaline phosphatase substrate was carried out by enzymatic reaction using NBT/BCIP solution. The blots were scanned and analysed with Image Pro plus® v. 5.0 (MediaCybernetics, Silverspring, MD, USA). Optical densities were calculated by using the density sum (sum of each density measurement in the selected range). To correct for variation between blots, we ran a control sample of 10 μL Hela Cell Lysate (Heat shocked; Stressgen®) on every blot.

We verified the linearity of the response curve for optical density against concentration of Hsp60 by establishing a concentration gradient of Hsp60. This was done by scoring Hsp60 in mixtures containing different proportions of stressed and unstressed animals of a single clone (A^+). More specifically, we made the following gradient (number of stressed animals : number of unstressed): 1 : 15, 5 : 11, 8 : 8, 11 : 5 and 15 : 1. This method enabled us to load the same amount of total proteins in each run. The readings showed a strong linear relationship ($R^2 = 0.91$, $n = 5$, $P = 0.012$).

Interclonal variation

For this experiment, laboratory cultures of each clone were split into two groups: one was exposed to fish kairomones, the other one not. Hsp60 was quantified after 6 h in each of these two groups. We used an exposure time of 6 h because the former experiment suggested that induction was maximal then (see Results). We ran 10 replicates (16 animals pooled in one replica)

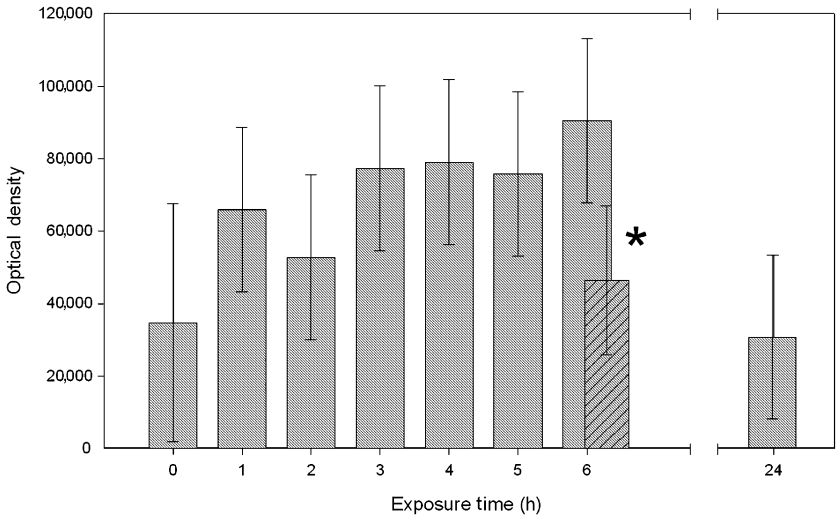

Fig. 1 Hsp60 levels (average optical density corrected for Hela Cell concentration, with 95% confidence interval) in *Daphnia magna* clone A^+ as a function of exposure time to fish kairomones. The shaded bar (*) shows Hsp60 level in the control condition after 6 h exposure.

per combination of clone and kairomone treatment. We quantified Hsp as described above.

Data analysis

Optical densities for the different treatments were analysed using ANCOVAS, with the optical densities of the control Hela Cell sample as a covariable. This way, we corrected for variation among blots. For the temporal pattern of induction, we carried out a one-way ANCOVA with exposure time as independent variable. In the experiment on interclonal differences, we first tested separately for differences in Hsp60 concentration among clones in the absence and presence of fish kairomones using one-way ANCOVAS with clone as an independent variable. Next, we tested for differential induction patterns among clones with a two-way ANCOVA with clone and exposure treatment as the two independent variables. In all ANCOVAS there was parallelism of slopes (all interactions with the covariable, n.s.).

Results

Temporal pattern of induction

Figure 1 shows that Hsp60 is induced in clone A^+ when continuously exposed to fish kairomones (ANCOVA, time effect: $F_{7,21} = 3.54$; $P < 0.05$). The induction gradually builds up during the first 6 h. The control condition, where animals were kept for 6 h in the absence of fish kairomones, does not differ from the initial condition after 0 h (ANCOVA, $F_{1,3} = 1.40$, n.s.), and has a lower level of Hsp60 compared with the condition where animals were exposed for 6 h to fish kairomones (ANCOVA, $F_{1,5} = 14.25$; $P < 0.05$; Fig. 1). This indicates the temporal pattern is truly reflecting an induction pattern under predator stress. After 24 h, Hsp60 concentration has decreased to levels before induction.

Interclonal variation

Figure 2 shows the Hsp60 levels in control and 6 h fish-exposed animals of the four different clones. In the control animals that were not exposed to fish kairomones, Hsp60 levels differed among clones (ANCOVA, clone: $F_{3,35} = 3.17$, $P < 0.05$). Duncan *post-hoc* tests showed that clone B^0 had higher Hsp60 levels than clones A^- ($P < 0.01$) and C^+ ($P < 0.05$), and clone A^+ had a higher level than clone A^- ($P < 0.05$). Clones reacted differently to fish kairomones with regard to expression of Hsp60 (clone × exposure: $F_{3,71} = 3.022$, $P < 0.05$; Fig. 2). Clones A^+ and A^- showed a significant increase in Hsp60 after 6 h exposure to fish kairomones (ANCOVAS, A^+: $F_{1,17} = 9.29$, $P < 0.01$; A^-: $F_{1,17} = 8.87$, $P < 0.01$). This increase was not significant for clone C^+. Clone B^0 showed no increase in Hsp60 level after exposure. After 6 h of exposure to fish kairomones, clones had similar Hsp60 levels (ANCOVA, clone: $F_{3,35} = 1.33$, n.s.).

Discussion

There is increasing evidence that Hsps, in addition to their importance in keeping cellular homeostasis, also play an important role in the every-day ecology of

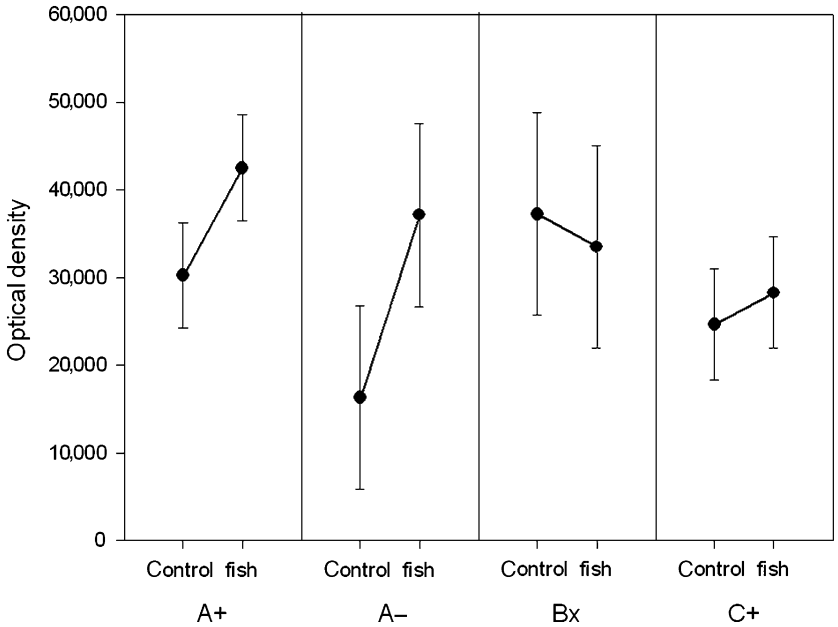

Fig. 2 Hsp60 levels (average optical density corrected for Hela Cell concentration, with 95% confidence interval) in the absence and presence (6 h exposure) of fish kairomones in the four studied *Daphnia magna* clones (see Table 1).

organisms (Huey & Bennett, 1990; Feder, 1996; Sørensen *et al.*, 2003). Fish are very efficient predators on large-bodied zooplankton, and the presence of fish kairomones induces many antipredator traits in *Daphnia* (Tollrian & Harvell, 1999). Exposure to fish no doubt induces stress, and our study confirms the recent observation by Pijanowska & Kloc (2004) that exposure to fish kairomones induces Hsps. Our study builds further on these initial observations by documenting the pattern of induction during the first hours after exposure as well as showing differences among genotypes in the constitutive and induced levels of Hsp60.

We observed an increase in Hsp60 concentration during the first hours after the start of the exposure to fish kairomones. Apparently, gene expression is activated immediately following exposure. This is a similar dynamic of induction of Hsp as found in other species towards other stressors (e.g. heat-shocked *Drosophila*; Feder & Hofmann, 1999). After 24 h, we observed that the amount of Hsp60 was again similar to that prior to induction. It is known that Hsp induction is energetically very costly (Sørensen *et al.*, 2003; Korsloot *et al.*, 2004). This may explain why Hsp levels decrease after induction: *Daphnia* apparently cannot afford to maintain a constantly high level. Our results suggest that the induction of Hsp can be seen as a transient mechanism to cope with novel predator stress, enabling the animal to bridge the time lag before other defences are induced. Once a safer phenotype has been induced, the cost of maintaining a high level of Hsp may be too high compared with its benefits. For instance, animals of clone A^+ have been shown to gradually change their habitat selection behaviour through time, as the animals become completely negatively phototactic, hiding in the deep and darker water layers, within a period of approximately 12 h (De Meester & Cousyn, 1997). Our results thus support the idea that the adaptive role of Hsp is related to coping with stress during periods of sudden increases in stress exposure, while other mechanisms take over under chronic stress (Sørensen & Loeschcke, 2002; Sørensen *et al.*, 2003).

We observed differences in the strength of induction of Hsp60 when exposed to fish kairomones. These differences among clones imply genetic variation in the possibility to induce Hsp60 upon exposure to fish. The degree of Hsp60 induction may be linked with the specific antipredator strategies of particular clones (Boersma *et al.*, 1998). It is not unlikely that costly changes in habitat selection or other predator-induced traits are traded off against the costly increase in Hsp levels, and that this trade-off contributes to the shaping of the speed with which antipredator defences are induced. Although the number of clones studied is obviously low for making any strong inferences, our data may point to a relationship in the degree of Hsp induction and predation risk in the habitats the clones were derived from. Clones A^+ and A^- are derived from a habitat with low and variable fish predation (De Meester, 1996), and show a strong induction pattern. Clone C^+ was isolated from a fishless pond and showed a weaker response. Clone B^0, isolated from a lake in which fish predation pressure is high, showed no induction of Hsp. This latter clone does show, however, a high constitutive Hsp level (Fig. 2). *Daphnia magna* occurs only at very low, marginal densities in this habitat (Declerck *et al.*, 1997), and other antipredator defence mechanisms are strongly developed in this population too (De Meester, 1996; Boersma *et al.*, 1998). Our results may thus suggest direct selection on the level of Hsp60 in connection to fish presence. Alternatively, selection may have been indirect, with clones that are frequently exposed in their natural habitat being little stressed by fish (and thus express little Hsp60), while clones not used to fish exposure being much more affected by the presence of fish and thus express more Hsp60 due to the higher stress. However, indirect selection, for example, cannot explain the lower induction of Hsp60 in clone C^+ that originated from the fishless pond.

As we also observed significant differences in the amount of Hsp60 in the control condition, our data indicate that there are genetic differences in the constitutive amount of Hsp60 and that selection may operate both on this constitutive amount and on the increase in Hsp60 upon exposure to fish. The fact that Hsp60 levels are quite similar for all four clones in the presence of fish kairomones may be due to the fact that the costs of maintaining high levels of Hsp are too high, and all clones have reached the physiological maximum (Krebs & Loeschcke, 1994; Krebs & Feder, 1997). Costs of Hsp production are due to the shutdown of normal cell functions during stress response, the extensive use of energy, and the toxic effects of high concentrations due to interference with normal cell function (Feder & Hofmann, 1999; Sørensen *et al.*, 2003).

In summary, our results show a dynamic induction of Hsp upon exposure to cues indicating predation risk, and show genetic differences in the constitutive level of Hsp60 as well as in the increase in Hsp60 upon exposure to fish. We suggest that Hsp60 induction is part of the multi-trait antipredator defence strategies developed by *Daphnia* clones to cope with one of the major stresses in their life: predation by fish. Studies involving larger number of clones are needed to reveal whether there are patterns of genetic covariation between Hsp induction and other predator-induced defences.

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