Evolution of Heat Shock Protein Expression in a Natural Population of *Daphnia magna*

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ABSTRACT: Populations often face changes in environmental conditions in a relatively short timescale, which may lead to microevolution of traits to cope with these changing selective pressures. Here, we demonstrate microevolution of a physiological trait in a natural population of the water flea *Daphnia magna*. Levels of the stress protein Hsp60 showed genetic variation, indicating in situ evolutionary potential, and the levels increased through time. The observed microevolutionary increase did not fit the historically documented changes in fish predation pressure in this pond, but it paralleled an increase in the load of infective stages of epibionts through time. In line with this, the locally most abundant epibiont caused an induction of Hsp60. Because stress proteins show evolutionary potential and protect organisms against a wide array of environmental factors, microevolution of stress proteins in natural populations is likely to be common.

Keywords: *Daphnia magna*, stress proteins, genetic variation, microevolution, predation risk, parasitism.

Organisms have developed a wide range of behavioral, morphological, physiological, and life-history traits to cope with external environmental factors that would otherwise preclude them from occupying certain habitats. These environmental factors often show pronounced changes in both space and time and thus may act as selective forces driving the microevolution of ecologically relevant traits

(Bijlsma and Loeschcke 1997, 2005). Recently, a number of studies have documented evolutionary changes to environmental factors on an ecological timescale (Hairston et al. 2005). Most of these studies describe experimental evolution to specific selective forces under laboratory conditions. In situ selection studies may, however, give different outcomes. One reason for this difference may be that in nature, environmental factors often act in a group, and trait change is therefore evaluated in a context in which all of the trade-offs associated with a trait are realized (Reznick and Ghalambor 2005). Such in situ studies are scarce, and they potentially suffer from another pitfall, the confounding of in situ evolution driven by selection with other processes such as drift and massive gene flow. Among the notable exceptions that unambiguously show adaptive in situ microevolution in natural populations over time, often in response to predation, are studies on *Anolis* lizards (e.g., Losos et al. 2004), water fleas of the genus *Daphnia* (e.g., Cousyn et al. 2001; Hairston et al. 2001), and guppies (e.g., Reznick et al. 1990, 2004). These studies focused on microevolution of behavioral, morphological, and life-history traits.

Stress proteins (Hsp) function as molecular chaperones and defend organisms at the cellular level against a wide range of environmental factors by enhancing the ability of the cell to cope with increasing concentrations of unfolded or denatured proteins (Sørensen et al. 2003; Kultz 2005). Additionally, molecular chaperones can participate in nonfolding actions outside the cell. Here, they can act as signaling receptors on the cell membrane and as stress cytokines (chaperokine activity) in the extracellular environment, linking the cell response to immunophysiology (Panayi et al. 2004; Asea 2005; Henderson et al. 2006).

Experimental microevolution of stress proteins against thermal stress has been documented, and it can take the form of changes in the constitutive levels or changes in the inducible response (e.g., Bettencourt et al. 1999; Ketola et al. 2004). Further, differences among natural populations in expression of stress proteins consistent with local thermal adaptation have been reported (e.g., Dahlhoff and Rank 2000). Here, we test for in situ microevolution of this physiological trait. Specifically, we evaluate in situ local

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adaptation of the stress protein Hsp60 in the water flea *Daphnia magna* in response to changes in fish predation and epibiont pressure. Predation is an important force in aquatic communities that may drive ecological and evolutionary dynamics of prey populations (Kerfoot and Sih 1987; Tollrian and Harvell 1999; Yoshida et al. 2003). Prey organisms, including the study species, have been shown to express higher levels of Hsp under predation risk, probably to maintain cellular homeostasis under predator stress and to regulate signal transduction through binding at specific receptors (e.g., Kagawa and Mugiya 2002; Pijanowska and Kloc 2004; Pauwels et al. 2005).

Study System and Methods

We used *Daphnia magna* clones hatched from a dormant egg bank of a pond in Oud-Heverlee (Belgium) where strong changes in fish predation pressure have been documented through time (Cousyn et al. 2001). Dormant eggs were isolated for each of the three main periods in the fish-stocking history of this pond: bottom (about 1970–1972), with low predation (newly created pond, before stocking); middle (about 1976–1979), with high predation (stocking of up to 300 kg/ha of planktivorous and benthivorous fish); and top (1988–1990), with reduced predation (reduced levels of stocking; see fig. 1). After hatch-

ing, 12 clonal lineages of each period were kept under standardized conditions in the laboratory for several generations before the experiments, so there is no interference from prior experience or exposure to predators in their natural habitat (Cousyn et al. 2001).

For each clone, five sets of 16 individuals, all born within a 24-h interval, were raised until they were second-instar adults. Then each set was split in two and transferred into 1-L glass jars. One subset was transferred in fish medium, and the other subset was in dechlorinated tap water as a control. Fish medium consists of tap water conditioned by the presence of fish (*Leuciscus idus*) for 24 h, at a density of three fish per 100 L. For each subset, levels of the stress protein Hsp60 were quantified after 6 h, when induction is maximal (Pauwels et al. 2005).

Since predation could not explain the observed Hsp pattern (see next section), we also specifically tested for another biotic interaction changing through time, parasitism. In this additional experiment, two clones of the bottom period and two clones of the top period were exposed to *Amoebidium parasiticum*, the most abundant ectoparasite in the the Oud-Heverlee pond (Decaestecker et al. 2004). Sets of five newborn *Daphnia* were placed in 60-mL jars and assigned to one of three treatments. Besides a parasite treatment where *Daphnia* were exposed to freshly sampled sediment containing sporangiospores of

Figure 1: Changes in dormant egg density, density of stocked fish, infection rates by epibionts, and Hsp60 levels of *Daphnia magna* in the Oud-Heverlee pond during a period of 26 years after its creation (1970–1996). Fish stock densities are aligned with sediment record of dormant egg abundance after Cousyn et al. (2001). Infection rates (%) by *Amoebidium parasiticum* are taken from Decaestecker et al. (2004). Hsp60 levels of subpopulations are given as least squares means (with 95% confidence intervals) corrected for HeLa cell concentration.

Figure 2: Levels of Hsp60 (average optical density, with 95% confidence interval) for 12 *Daphnia magna* clones from each of three periods differing in fish-stocking history (bottom [1970–1972], low predation; middle [1976–1979], high predation; top [1988–1990], reduced predation). For each clone, Hsp60 levels are given after 6 h exposure to fish kairomones (open circles) or control water (solid circles). Within each subpopulation, the clones are ordered according to increasing values of Hsp60 expression in the absence of fish, and bars indicate subpopulation means (based on clonal means, with 95% confidence interval). Given are least squares means corrected for HeLa cell concentration.

A. parasiticum, we used a control with autoclaved sediment, where all infective agents were killed, and a control without sediment. Half of the medium was refreshed every 2 days, and in order to avoid crowding effects, newly born brood was removed. After 15 days, Hsp60 levels were quantified for 10 sets of five animals per treatment. For the parasite treatment, only sets with all five animals infected were used.

To quantify Hsp60, groups of eight (first experiment; historical reconstruction) or five (second experiment; induction by parasites) *Daphnia* were blotted dry, and buffer was added. Total homogenates were stored at -80°C , and then $40\text{ }\mu\text{g}$ of the protein sample was used for analysis. After SDS PAGE (Criterion Precast Gel, 10% Tris-HCl; Bio-Rad, Hercules, CA) and Western blotting, the optical densities of the Hsp60 bands, visualized after immunodetection with antibodies, were quantified using image analyzer software (Image-Pro Plus). All samples were randomized over blots. The antibody used is a broad-spectrum anti-Hsp60 that quantified all members of the Hsp60 family and their posttranslational modifications. For details on quantification of Hsp60, we refer to Pauwels et al. (2005).

Optical densities for the different treatments were analyzed using ANCOVAs, with period and clone nested in period as independent variables. To correct for any variation among blots we also ran a control HeLa cell sample on each gel and included its optical density as a covariate in the analysis. Clone nested in period and its interaction with fish kairomones/parasite treatment were considered random variables. Parasite treatment was included as an independent variable. For the fish kairomones treatments,

the coupled observations of subsets of eight individuals in the absence and presence of fish kairomones were treated as repeated measures. A significant effect of the within-subject factor would then indicate an effect of fish kairomones. Models were run in PROC MIXED in SAS, version 9.1. To test the significance of a random effect, we performed a one-sided likelihood ratio test that compared the model where the variance of this variable was unconstrained with the model where the variance was constrained to zero. The difference of -2 res loglikelihood between these models is χ^2 distributed, with 1 degree of freedom (Littell et al. 1996). Corrected degrees of freedom were obtained with the Satterthwaite option. Nonsignificant interactions were removed from the final model. As we had three levels for the parasite treatment, we tested two a priori orthogonal contrasts: one comparing the parasite exposure treatment with both controls and one comparing both controls.

Results and Discussion

Heat shock protein 60 levels steadily increased through time (period: $F = 9.08$, $df = 2, 327$, $P < .001$; fig. 2). Duncan post hoc tests showed significant differences in Hsp60 levels between all periods (all $P < .05$). Because previous work showed no genetic subdivision based on microsatellite markers among periods, clones isolated from different sediment depth belong to subpopulations of one single population (Cousyn et al. 2001). Genetic differentiation in Hsp60 levels therefore reflects in situ microevolution and not genetic drift or massive gene flow. We observed genetic variation for Hsp60 levels within the subpopula-

tions of each period (clone [period]: $\chi^2 = 7.8$, $df = 1$, $P < .01$; fig. 2), which may have fueled the evolutionary response. The associated rate of evolutionary change in Hsp60 levels between the different subpopulations was 0.12 Haldane units between bottom and middle and 0.08 Haldane units between middle and top subpopulation, indicating rapid microevolution (Kinnison and Hendry 2001; these estimates are based on the assumption that one calendar year is equal to one generation, which is a reasonable assumption given the parthenogenetic reproduction cycle of *Daphnia magna* involving a yearly bout of sexual reproduction).

Although we find evidence for significant microevolution of Hsp60 levels, our results do not point to fish predation as the key selective factor driving this microevolution. The steady increase of Hsp60 is not consistent with the fish-stocking history: although Hsp60 increased from the bottom to the middle period, it had an even greater increase when going to the top period, when fish predation pressure was reduced. Because stress proteins are costly (Sørensen et al. 2003), one would expect a quick decrease in Hsp60 levels when the stressor was relieved. That the link between fish predation pressure and Hsp60 expression may have been weak in this population is also suggested by the fact that exposure to fish kairomones did not result in an increase of Hsp60 levels (fish kairomones: $F = 0.26$, $df = 1, 322$, NS). There were also no significant interactions of fish kairomones and period ($F = 0.59$, $df = 2, 320$, NS) or of fish kairomones and clone nested within period ($\chi^2 = 0.0$, $df = 1$, NS; fig. 2). Previous studies on *D. magna* did show induction of Hsp60 in response to fish kairomones (Pijanowska and Kloc 2004; Pauwels et al. 2005). However, these studies also showed clonal differences in expression levels, with some clones not responding with increased Hsp60 levels to predator stress. Predator induction of Hsp60 can be seen as part of the multitrait antipredator defense strategies of particular clones (Boersma et al. 1998; Pauwels et al. 2005), where different sets of antipredator mechanisms can develop in response to predation.

If it is not predation, then which selective factor or combination thereof is driving the evolution of Hsp60 in this population? Given the steady increase of Hsp60 through time, candidate selective forces should show the same pattern. A strong candidate selective factor may be parasite load. Decaestecker et al. (2004) reconstructed the changes in abundance of infective stages of microparasites and epibionts by exposing *Daphnia* to sediments of different age (depth in a sediment core) retrieved from the study pond. They observed a steady increase in the abundance of *Daphnia* infected by the most common (more than 95% of observations) epibiont, *Amoebidium parasiticum*, with time (see fig. 1). Their study could not dif-

ferentiate between differences in abundance and effects of declining infectivity with aging. Yet in other ponds that were studied in the same way, not all epibiont infections showed a consistent decline with age, as was observed in the Oud-Heverlee pond, which suggests that this pond indeed experienced an increasing abundance of epibionts with time (Decaestecker et al. 2004). Epibionts, when abundant, have negative effects on zooplankton by impeding movement and reducing buoyancy (Green 1974; Chiavelli et al. 1993) and reproduction (Threlkeld and Willey 1993).

In line with a role of epibionts in underlying the microevolutionary increase in Hsp60, we find a significant increase in Hsp60 levels in the *Daphnia* exposed to *A. parasiticum* (mean $\pm$ SE: optical density = $19,900 \pm 1,800$) compared with the controls with autoclaved sediment ($10,900 \pm 2,000$) and without sediment ($13,300 \pm 1,700$; contrast: $F = 13.56$, $df = 1, 90$, $P < .001$). We cannot fully exclude the possibility that other parasites, not yet detectable at 15 days of infection, coinfect the *Daphnia* in our experiment. Therefore, we suggest that epibionts or parasites can induce an increase in Hsp60. This induction by parasite infection is consistent with previous studies documenting a role for stress proteins in defense against parasites (Merino et al. 2002; Rinehart et al. 2002; Tomás et al. 2005). Heat shock protein 60 and its homologue in prokaryotes, GroEL in particular, have been shown to be involved in regulatory systems that integrate cell stress with key cellular systems designed to cope with major stresses, such as infectious agents (Wallin et al. 2002; Panayi et al. 2004; Henderson et al. 2006).

Although suggestive, our observation of congruence in the patterns of epibiont abundance and Hsp60 levels is no proof of a causal relationship. Temporal changes in other factors known to affect levels of stress proteins (Sørensen et al. 2003), such as a decrease in water quality (increase of nitrogen and phosphorus or associated hypoxia), an increase in environmental pollution (e.g., increase in pesticide load, associated with the accumulating sediments), or climate change (increased in situ temperature extremes in summer), may also have acted as selective forces. We cannot rule out an impact from these factors. However, an impact from increased pollution seems unlikely. First, we have no indications of pollution in the study pond, which is located in a protected area. Moreover, we see no differences in Hsp60 levels between *Daphnia* exposed to autoclaved sediment of the pond and *Daphnia* not exposed to sediment (contrast: $F = 0.96$, $df = 1, 90$, NS).

To conclude, we provide evidence of rapid in situ microevolution of stress protein levels in a natural population. Given that several studies report evolutionary potential of stress proteins (e.g., Bettencourt et al. 1999; Pauwels et al. 2005; this study) and their role in maintaining cellular ho-

meostasis against a wide range of stressors (Sørensen et al. 2003), we hypothesize that microevolution of stress proteins may be a widespread phenomenon. Physiological traits in general have been studied less than behavioral, morphological, and life-historical traits when documenting microevolution. The ability of organisms to cope with certain environmental factors requires the integration of all these different types of traits, which may be traded off against each other. To better understand how organisms adapt to changing environmental factors, an important challenge is therefore to include physiological traits in studies focusing on joint evolution of integrated sets of phenotypes.

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