Heat shock proteins and survival strategies in congeneric land snails (*Sphincterochila*) from different habitats

Tal Mizrahi · Joseph Heller · Shoshana Goldenberg · Zeev Arad

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Abstract Polmunate land snails are subject to stress conditions in their terrestrial habitat, and depend on a range of behavioural, physiological and biochemical adaptations for coping with problems of maintaining water, ionic and thermal balance. The involvement of the heat shock protein (HSP) machinery in land snails was demonstrated following short-term experimental aestivation and heat stress, suggesting that land snails use HSPs as part of their survival strategy. As climatic variation was found to be associated with HSP expression, we tested whether adaptation of land snails to different habitats affects HSP expression in two closely related *Sphincterochila* snail species, a desert species *Sphincterochila zonata* and a Mediterranean-type species *Sphincterochila cariosa*. Our study suggests that *Sphincterochila* species use HSPs as part of their survival strategy following desiccation and heat stress, and as part of the natural annual cycle of activity and aestivation. Our studies also indicate that adaptation to different habitats results in the development of distinct strategies of HSP expression in response to stress, namely the reduced expression of HSPs in the desert-inhabiting species. We suggest that these different strategies reflect the difference in heat and aridity encountered in the natural habitats, and that the desert species *S. zonata* relies on mechanisms and adaptations other than HSP induction thus avoiding the fitness consequences of continuous HSP upregulation.

Keywords HSP · Land snails · Heat stress · Desiccation stress · Environmental stress

Introduction

Land snails are subject to daily and seasonal variations in temperature and in water availability in the terrestrial habitat. Their successful colonization of these habitats depends on a range of behavioural, morphological, physiological and biochemical adaptations for coping with problems of maintaining water, ionic and thermal balance. These problems are especially evident in the severe habitat of the desert where snails are exposed to high temperatures and lack of water during most of the year, and consequently have to face threats of thermal death and/or death from desiccation. As part of their survival strategy, land snails evolved annual cycles of activity and aestivation in relation to seasonal changes in temperature, humidity and water availability. In addition, interspecific differences were found that stem from a variety of adaptations such as snail size, epiphragm thickness, site selection and lifestyle, and osmoregulatory capacities.

Comparative studies in land snails have revealed that, in general, resistance to heat and aridity is correlated with distribution patterns and with abiotic environmental variation (Cameron 1970; Machin 1967). A series of studies on water relations and resistance to experimental desiccation of Israeli land snails have demonstrated that Mediterranean snails are less resistant than desert species and populations and that the distribution pattern of each species and its microhabitat are related to its ability to cope with desiccating conditions (Arad et al. 1992, 1993a, b, 2009). The land snail *Sphincterochila* (Sphincterochilidae) is represented in Israel by five broadly parapatric species that replace one another along a climatic gradient that ranges from the rainy

T. Mizrahi · S. Goldenberg · Z. Arad (✉)
Department of Biology, Technion,
Haifa 32000, Israel
e-mail: zarad@tx.technion.ac.il

J. Heller
Department of Evolution, Systematics and Ecology,
Hebrew University of Jerusalem,
Jerusalem 91904, Israel

Mediterranean environment to the arid desert environment, and were previously found to differ in their susceptibility to desiccation stress (Arad et al. 1989). We designed a series of studies to examine the cellular stress response machinery of *Sphincterochila* snails that allow them to cope with environmental stress. Specifically, we aimed to find whether the heat shock protein (HSP) response is involved in the natural annual cycle of activity and aestivation and following desiccation and heat stress in two closely related *Sphincterochila* species, a desiccation-resistant desert species, *Sphincterochila zonata*, and a Mediterranean-type, desiccation-sensitive species, *Sphincterochila cariosa*. In addition, we tested whether the difference in stress resistance between the two species is correlated with differential upregulation of the HSP response.

HSPs are a selected group of proteins that are induced in diverse organisms by different environmental stressors (Feder and Hofmann 1999; Lindquist and Craig 1988; Sørensen et al. 2003). HSPs range in molecular mass from 15 to 110 kDa and are divided into families based on both size and degrees of homology. The 70-kDa family is considered the most prominent eukaryotic family of stress proteins, and several isoforms exist including the constitutively expressed heat shock cognate protein 70 and the heat-inducible Hsp70, whereas Hsp90 is one of the most abundant cytosolic proteins in eukaryotes. Under non-stress conditions, Hsp70 and Hsp90 display essential roles in the cell, chaperoning proteins during folding, assembly, intracellular trafficking and degradation, and are involved in cell regulatory pathways (Csermely et al. 1998; Kiang and Tsokos 1998; Nollen and Morimoto 2002). It is generally accepted that HSPs protect organisms from the detrimental effects of heat and possibly other stressors including various chemicals, heavy metals, oxidative stress and desiccation, and that stress tolerance depends on the synthesis of HSPs (Kregel 2002; Somero 1995; Lindquist 1986; Feder and Hofmann 1999).

Our studies in *Sphincterochila* species indicate the involvement of the HSP machinery following stress conditions and as part of the natural annual cycle of activity and aestivation (Mizrahi et al. 2010, 2011, 2012; Arad et al. 2010). The expression of most tested HSPs (members of the 70-kDa, 90-kDa and small HSPs family) was induced in response to experimental desiccation, heat stress and arousal from long-term aestivation, suggesting that land snails use HSPs as part of their survival strategy. However, our studies demonstrated delayed and limited expression of HSPs in the desert-inhabiting *S. zonata*, which is naturally exposed to extreme environmental conditions of heat and aridity. We suggest that in *Sphincterochila* species, evolution in harsh environment may have resulted in selection for reduced HSPs expression, probably due to fitness costs associated with the activation of the HSP machinery in snails that regularly experience environmental stress (Sørensen et al. 2003).

Natural annual cycle of heat shock proteins expression

During aestivation there is a marked decrease in metabolic activity that allows land snails to survive even in extreme arid conditions (Pakay et al. 2002; Storey 2002; Schmidt-Nielsen et al. 1971). The onset of activity occurs with the beginning of the rainy season, when snails replenish their water reserves. The mechanisms of metabolic depression include coordinated suppression of protein synthesis and degradation and enhancement of defence mechanisms that stabilize macromolecules (e.g. antioxidants, chaperone proteins, protease inhibitors). In a study related to stress proteins and short-term experimental aestivation and arousal in land snails, Brooks and Storey (1995) suggested that in *Otala lactea*, aestivation-specific proteins (members of the 30-, 50-, 70- and 90-kDa families) may be involved in the transition to a depressed metabolic state. Ramnanan et al. (2009) showed that the expression of some HSPs increased following 2 weeks of experimental aestivation in the land snail *O. lactea*, and suggested that this upregulation is part of the mechanism for stabilizing proteins that contribute to long-term metabolic stability during aestivation. However, Reuner et al. (2008) found that in the Mediterranean grunt snail (*Cantareus apertus*), long-term laboratory aestivation had no effect on the level of Hsp70 in the foot tissue. The authors concluded that Hsp70 does not have an essential function during aestivation in this species except for possible basal metabolic activities, and that the upregulation of HSPs in aestivating *O. lactea* as demonstrated by Brooks and Storey (1995) might be limited to the transition from an active to a depressed state and might be not apparent in animals in the dormant state.

Our study in naturally active and aestivating snails of *S. cariosa* and *S. zonata* supports this suggestion (Arad et al. 2010). We found a general downregulation of the HSP levels in the foot, kidney and hepatopancreas tissues in aestivating compared to active snails, with the exception of Hsp90 in the foot tissue whose expression was higher during aestivation. We suggested that the stress protein machinery is upregulated during arousal in anticipation for possible oxidative stress ensuing from the accelerating metabolic rate and the exit from the deep hypometabolic state characterizing aestivation. Our results suggest that land snails use HSPs as important components of the aestivation mechanism, and as part of their survival strategy after arousal. Our study also indicates that adaptation to different habitats results in the development of distinct strategies of HSP expression. The Mediterranean-type species *S. cariosa* had higher standing stocks of Hsp70 and of small HSPs (sHSPs) in the foot, kidney and hepatopancreas after arousal, and in the foot and hepatopancreas also during aestivation, whereas the desert species *S. zonata* had higher stocks of Hsp70 and Hsp90 in the kidney during aestivation. This differential HSP

response may reflect the relative vulnerability and the physiological function of different tissues. It seems that in this respect, *S. cariosa* is more sensitive than *S. zonata* and therefore maintains higher standing stocks of Hsp70 and of sHSPs. We suggest that the desert-adapted *S. zonata* developed a distinct strategy of HSP expression in the kidney to improve its water economy, while maintaining lower standing stocks of sHSPs and Hsp70 in other tissues.

Heat shock proteins and resistance to desiccation

Stress conditions associated with lack of water were previously found to induce the expression of HSPs in different organisms. For example, induction of various HSPs was found during short-term experimental aestivation in the land snail *O. lactea* (Brooks and Storey 1995; Ramnanan et al. 2009). The genes of sHSPs, Hsp70 and Hsp90 were upregulated in response to dehydration in the Antarctic midge *Belgica antarctica* (Lopez-Martinez et al. 2009), and induction of Hsp70 mRNA was also found in the eutardigrade *Milnesium terdigradum* in the transitional stage between activity and desiccation (cryptobiosis, Schill et al. 2004).

Similarly, our study in *Sphincterochila* snails revealed a general induction of various HSPs (members of the 70-kDa, 90-kDa and sHSPs families) in response to experimental desiccation in a species-dependent and tissue-dependent manner (Mizrahi et al. 2010). As long-term aestivation revealed general downregulation of the HSP levels compared to active snails, these findings suggested that the observed upregulation of HSPs during experimental desiccation was transient and might be limited to the early stages following desiccation stress as part of the survival strategy and as an important component of the aestivation mechanism in the transition from activity to dormancy.

In addition, we found variations in the HSP response in both timing and magnitude between the desiccation-resistant *S. zonata* and the desiccation-sensitive *S. cariosa*. The desert-inhabiting *S. zonata* exhibited a delayed response of Hsp72, Hsp74 and Hsp90 to desiccation stress, together with an enhanced response of sHSPs compared to *S. cariosa*. These findings suggest that *Sphincterochila* species that differ in their resistance to experimental desiccation developed distinct strategies of HSP expression that reflect the difference in aridity encountered in their natural habitats.

Heat shock proteins and resistance to heat

Previous studies demonstrated heat induction of HSPs in various populations of Mediterranean land snails. Thus, upregulation of both Hsp70 protein and mRNA was detected in the foot tissue of grunt snail (*C. apertus*) after 1 h of

recovery following heat stress at 37°C (Reuner et al. 2008), and the levels of Hsp70 and Hsp90 were upregulated in populations of *Xeropicta derbentina* and *Theba pisana* in response to 8 h at 38 to 43°C (Köhler et al. 2009). Likewise, our recent work in *Sphincterochila* snails shows induction of HSPs in the Mediterranean-type species *S. cariosa* in response to heat stress (Mizrahi et al. 2012). However, while in *S. cariosa* heat exposure at 42°C caused rapid induction of Hsp70 proteins and Hsp90 in the foot and kidney tissues, the desert-inhabiting species *S. zonata* displayed limited and delayed upregulation of Hsp70 proteins in the foot and upregulation of hsp90 alone in the kidney.

The stress needed to induce HSPs is strongly related to the niche of the organism in question (Feder and Hofmann 1999). Interspecific comparisons of ectothermic species from different habitats have shown that typically, species adapted to a higher temperature niche were more heat tolerant, induced synthesis of HSPs at higher temperatures and had higher upper thermal limits of protein synthesis (Tomanek and Somero 1999; Nakano and Iwama 2002; Evgen'ev et al. 2007; Dong et al. 2008). For example, the temperatures at which enhanced synthesis of Hsp70 first occurred were higher in the more heat-tolerant Australian embiid species than in the less heat-tolerant rainforest species (Edgerly et al. 2005), and in species of marine snails of the genus *Tegula* from warmer habitats (Tomanek and Somero 1999). Our studies in *Sphincterochila* species exposed to either desiccation or heat stress follow the same pattern. These studies suggest the development of distinct strategies of HSP expression in response to environmental stress, namely the delayed induction of Hsp70 and Hsp90 in the desert-inhabiting species.

Cost, benefits and survival strategy

HSPs display essential roles under non-stress conditions, and their upregulation may enhance survival under stress exposure by rescuing critical proteins and reducing the energetic cost associated with protein damage. It is generally accepted that thermotolerance depends on the synthesis of HSPs (Moseley 1997; Kregel 2002; Krebs and Bettencourt 1999; Nollen et al. 1999; Bahndorff et al. 2009). Interspecific studies revealed correlations between a greater heat tolerance and higher constitutive levels of HSPs (Evgen'ev et al. 2007; Nakano and Iwama 2002). Zatssepina et al. (2000), for example, found higher levels of both Hsp70 mRNA and protein in desert-dwelling lizard species than in non-desert species. Similarly, in the study of four limpets of the genus *Lottia*, Dong et al. (2008) found higher constitutive levels of Hsp70 in the two high-intertidal species compared to the low and mid-intertidal species. These studies suggest that organisms occupying extreme environments will employ a “preparative defence”

strategy involving maintenance of high constitutive levels of HSPs in their cells as a mechanism for protection against periods of extreme and unpredictable stress. The existence of large pools of HSPs may explain the rather limited HSP response to heat observed in these organisms.

However, the reduced Hsp70 response to desiccation and heat in *S. zonata* foot cannot be attributed to the existence of large pools of Hsp70, since we found smaller pools of Hsp70 in the foot of *S. zonata* compared to *S. cariosa* during aestivation and after arousal. Sørensen et al. (2003) proposed that the expression level of HSPs in each species and population is a balance between benefits and costs, and overexpression of HSPs may exert a negative impact on growth, development rate and fertility (Krebs and Bettencourt 1999; Krebs and Feder 1997; Sørensen et al. 1999). For example, Krebs and Loeschke (1994) found reduced fecundity in *Drosophila melanogaster* females conditioned with a mild thermal stress, and heat-induced Hsp70 expression in *D. melanogaster* was associated with a reduction in egg hatching among progenies of exposed mothers (Silbermann and Tatar 2000). These studies suggest that a trade-off between heat resistance in the form of Hsp70 expression and fertility is responsible for the level of Hsp70 expression. Thus, since HSP expression may incur fitness costs on individuals that regularly experience environmental stress, evolution in harsh environments may result in selection for reduced HSP expression. This concept is supported by our studies in *Sphincterochila* snails and by studies in other organisms. For example, lower levels of Hsp70 were found in lines of *Drosophila* continuously exposed to high temperatures (Bettencourt et al. 1999; Sørensen et al. 1999), in natural populations of *Drosophila* from warmer climates (Zatsepina et al. 2001; Sørensen et al. 2001) and in aquatic and soil invertebrates exposed to heavy metals (Köhler et al. 2000; Haap and Köhler 2009).

The desert-inhabiting species *S. zonata* developed a variety of effective morphological adaptations to withstand the severe habitat of the desert (Machin 1967; Schmidt-Nielsen et al. 1972). In an interspecific study of *Sphincterochila*, Arad et al. (1989) showed that *S. zonata* was the most resistant species to desiccation, characterized by the lowest rates of water loss, thickest epiphragm, lowest epiphragm area-specific water vapour conductance and the most favourable surface-to-volume ratio, compared to all other congeners, including the Mediterranean-type *S. cariosa*. We suggest that *S. zonata* relies on mechanisms of adaptation other than HSP induction in order to avoid the fitness costs associated with continuous HSP upregulation. Similarly, in a study of another non-model organism, the copper butterfly (*Lycaena tityrus*), Karl et al. (2009) observed reduced plasticity with respect to Hsp70 expression in response to long-term heat exposure in the high-altitude butterflies that are exposed to harsher environmental conditions in their natural environment, and suggested

that due to energy costs associated with the heat shock response, the high-altitude butterflies seem to rely more on genetically fixed thermal stress resistance.

Summary

While studying the contribution of HSP expression to resistance of whole organisms to environmental stressors, it is important to take into account the fitness cost associated with the activation of the HSP machinery. Our studies in the land snail *Sphincterochila* demonstrate upregulation of HSPs in response to stress conditions in both the desert-adapted species *S. zonata* and the Mediterranean-type species *S. cariosa*. However, *S. zonata* maintained lower standing stocks of Hsp70, and displayed reduced induction of Hsp70 proteins and Hsp90 in response to desiccation and heat stress compared to *S. cariosa*. These findings suggest that adaptation to different habitats results in the development of distinct strategies of HSP expression in response to stress, and support the concept that evolution in harsh environments will result in selection for reduced HSP expression. Thus, while it seems that both species use HSPs as part of their survival strategy, we suggest that the energetically driven trade-offs between HSP induction and other mechanisms of adaptation enable *S. zonata* snails to survive in the harsh environment of the desert.

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