The heat shock response in congeneric land snails (*Sphincterochila*) from different habitats

Tal Mizrahi · Joseph Heller · Shoshana Goldenberg · Zeev Arad

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Abstract Land snails are subject to daily and seasonal variations in temperature and in water availability, and use heat shock proteins (HSPs) as part of their survival strategy. We used experimental heat stress to test whether adaptation 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 findings show that in *S. cariosa*, heat stress caused rapid induction of Hsp70 proteins and Hsp90 in the foot and kidney tissues, whereas the desert-inhabiting species *S. zonata* displayed delayed induction of Hsp70 proteins in the foot and upregulation of Hsp90 alone in the kidney. Our study suggests that *Sphincterochila* species use HSPs as part of their survival strategy following heat stress and that adaptation to different habitats results in the development of distinct strategies of HSP expression in response to heat, namely the reduced induction of HSPs in the desert-dwelling species. We suggest 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 · Environmental stress

Introduction

The ability of land snails to successfully colonize terrestrial habitats depends on a range of behavioral, physiological, and biochemical adaptations for coping with problems of maintaining water, ionic, and thermal balance. Aestivation during the dry and hot seasons can represent such adaptations. Land snails are subject to annual cycles of activity and aestivation in relation to seasonal changes in temperature, humidity, and water availability. During aestivation, there is a marked decrease in metabolic activity that allows them 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.

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, 1989). 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 Mediterranean environment to the arid desert environment. Recently, our study in two closely related *Sphincterochila* species, a desiccation-resistant desert species, *Sphincterochila zonata*, and a Mediterranean-type, desiccation-sensitive species, *Sphincterochila cariosa*, indicated the involvement of the heat shock protein (HSP) machinery in experimental desiccation and as part of the natural annual cycle of activity and aestivation (Mizrahi et al.

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

2010, 2011; Arad et al. 2010). 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). 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).

In *Sphincterochila* species, both experimental desiccation and arousal from long-term aestivation induced the expression of most tested HSPs (members of the 70 kDa, 90 kDa, and small HSP family), suggesting that land snails use HSPs as part of their survival strategy. However, our studies suggested that the desert-inhabiting *S. zonata*, which is naturally exposed to extreme environmental conditions of heat and aridity, developed the distinct strategy of HSP expression. In field studies, *S. zonata* snails collected after the first rains maintained lower standing stocks of Hsp70 in the foot, kidney, and hepatopancreas tissues compared to the Mediterranean-type species *S. cariosa*. In addition, *S. zonata* displayed delayed induction of Hsp70 and Hsp90 in response to desiccation stress compared to *S. cariosa*. We suggested that in *Sphincterochila* species, evolution in a harsh environment may have resulted in selection for reduced Hsp70 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).

Studies on the cellular and molecular mechanisms underlying the response of land snails to different environmental stressors are sparse, in particular studies examining the HSP response. In the land snail *Otala lactea*, induction of various HSPs was found during short-term experimental aestivation (Brooks and Storey 1995; Ramnanan et al. 2009), and heat stress induction of Hsp70 and Hsp90 was demonstrated in various populations of Mediterranean land snails (Reuner et al. 2008; Scheil et al. 2011; Köhler et al. 2009). However, our knowledge of how adaptation to different habitats in congeneric land snails affects the cellular stress response and on the kinetics of this response is still limited.

The present study aimed at examining the effect of heat stress on the induction of HSPs in two closely related *Sphincterochila* species occupying different habitats, a desert species, *S. zonata*, and a Mediterranean-type species, *S. cariosa*. Specifically, we aimed to test whether desert species differ from their non-desert congeners in their strategies, as recently suggested by us for *Sphincterochila*. Our approach was to examine the expression level of Hsp70 and Hsp90 in the foot and kidney of snails experimentally exposed to heat stress followed by a 48-h recovery period.

Materials and methods

Collection of *Sphincterochila* snails

Adult *S. zonata* (Bourguignat, 1853) and *S. cariosa* (Olivier, 1804) were collected in the Negev desert near Sde-Boquer and in the northern Mediterranean coast of Israel near Atlit, respectively. The snails were collected during the winter months (November–December), brought to the laboratory, maintained in aquaria within a temperature-controlled room at 25 ± 0.3 °C (a temperature within the natural range of both species) and a 12L:12D photoperiod, and were fed lettuce.

Experimental design

In order to reduce variation in the snail mass resulting from differences in the hydration state, snails were transferred to a damp substrate and allowed to hydrate for 48 h to a stable mass. Active snails were blotted dry and weighed on an analytical balance to the nearest 0.1 mg, and snails of approximately the same body mass were divided into six groups ($n=3$ –5 snails for each group). The control group of each species was not submitted to thermal stress, and the snails were sacrificed in the fully hydrated state. The foot and kidney tissues were dissected out, weighed, and frozen in liquid nitrogen for later analysis of HSP expression. The other groups were placed in plastic containers and transferred into an oven held at 42 °C for 3 h. Immediately thereafter, one group of snails (the heat stress group) was sacrificed, and the tissues were dissected out, weighed, and frozen in liquid nitrogen. The remaining heat-exposed snails were placed in plastic containers laid with moistened filter paper and returned to the temperature-controlled room for 48 h of post-stress recovery. During the recovery period, additional groups of snails were sacrificed after 1, 4, 24, and 48 h. For both species, we conducted three independent experiments. Each time point of the recovery groups was sampled in at least two independent experiments (except for the 48-h recovery group in *S. zonata* that was sampled in only one experiment).

Sample processing for stress protein analysis

Frozen tissues were homogenized in ice-cold buffer containing 0.1 M NaCl; 20 mM Tris, pH 7.4; 1 mM EDTA; 1 % Igepal; 1 mM dithiothreitol (DTT); Protease Inhibitor Cocktail (Sigma, Saint Louis, MO, USA, P-8340); and 1 mM phenylmethylsulfonylfluoride. The homogenate was centrifuged (10 min, $17,000 \times g$ at 4 °C), and total protein concentration in each supernatant was determined by a standard method (Bradford 1976). The calibration curve of the Bradford assay was created using bovine serum albumin standards.

Western blotting

Equivalent amounts of protein (35 µg) from tissue lysates prepared from individual snails were boiled in sample buffer containing DTT and loaded into each lane. Proteins were separated using SDS-PAGE with a 10 or 12 % acrylamide gel and transferred onto nitrocellulose membrane (Pall Gelman Laboratory, Ann Arbor, MI, USA). The membranes were probed with mouse monoclonal antibody against bovine brain Hsp70 (Sigma H-5147), recognizing both the constitutive and inducible forms of mammalian Hsp70 and mouse monoclonal antibody against Hsp90 (Sigma H-1775). Hsp70 antigen (72 kDa, 250 ng) (StressMarq, Victoria, BC, Canada, SPR-115A) and Hsp90 antigen (200 ng) (MBL, Woburn, MA, USA, SR-P770) were simultaneously run. The secondary antibody was goat anti-mouse IgG-HRP (Sigma A-2554).

The proteins were visualized by enhanced chemiluminescence, and the intensity of the bands was quantified using densitometry software (ImageJ). Because all samples were analyzed on a single western blot, no standardization of band intensities was required.

Statistics

The results are expressed as percentage change from control (mean values±SE) and reported as the summation of three independent experiments. Once data were confirmed to fit the validation criteria (homogeneity of variances and normality), the statistical analysis software program SPSS 19.0 was used to perform a one-way ANOVA with Hochberg's GT2 and Gabriel's post hoc tests for multiple comparisons. In case of doubt that the population variances are equal, we used the Games–Howell procedures. In the case of interspecies comparisons, the significance was verified by independent *T* test. In all the cases, $p < 0.05$ was considered significant.

Results and discussion

HSPs can be important for natural populations that are exposed to variable environments, including occasional stress exposures and environmental conditions that appear to us as benign (Sørensen et al. 2003). 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). Our current work in two closely related land snail

Sphincterochila species occupying different habitats follows the same pattern. In *S. cariosa*, a Mediterranean-type species adapted to a more mesic niche, heat exposure at 42 °C caused rapid induction of HSPs in both the foot and kidney tissues, whereas the desert-inhabiting species *S. zonata* displayed limited and delayed upregulation of HSPs.

Heat-induced HSP expression in the foot

The Mediterranean coastal temperate habitat of *S. cariosa* is characterized by rather mild temperatures along the coast. However, despite the small annual temperature range, the extreme maximum temperatures are very high, exceeding 40 °C during heat waves in the transition seasons (Jaffe 1988). Thus, *S. cariosa* snails are likely to be exposed to temperatures exceeding 40 °C in nature, although not regularly but rather as isolated events during the transition seasons that may induce the HSP response. Because the foot is directly facing the environment, any change in HSP level may affect the snail's ability to cope with external stress and indicate their sensitivity to the imposed stress. In the present study, heat exposure at 42 °C induced rapid expression of HSPs in the foot of *S. cariosa* snails (Fig. 1). The monoclonal antibody to Hsp70 detected in the foot tissue of both species two bands of approximately 72 and 74 kDa. In *S. cariosa*, the level of Hsp74 was initially reduced after 1 h of recovery compared to control ($-40 \pm 11.7\%$), followed by a significant increase after 4 and 24 h of recovery compared to the 1-h recovery group ($78.2 \pm 23.3\%$, $p < 0.05$, and $80.7 \pm 20.7\%$, $p < 0.01$, respectively) (Fig. 1a). The induction of Hsp72 seems to be delayed compared to Hsp74, and a significant increase was detected after 24 h of recovery ($30.6 \pm 8.2\%$, $p < 0.05$). Hsp90 was upregulated already after heat stress ($60.5 \pm 18.6\%$, compared to control) (Fig. 1b), and the level remained significantly higher throughout the recovery period ($59.3 \pm 8.6\%$, $p < 0.01$, after 4 h of recovery; $60.5 \pm 9.1\%$, $p < 0.01$, after 24 h of recovery; $109.1 \pm 20.2\%$, $p < 0.01$, after 48 h of recovery).

Other 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 the grunt snail (*Cantareus 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). In contrast, our present study shows that in the desert-adapted species, *S. zonata*, heat exposure at 42 °C failed to induce Hsp90 in the foot, and the induction of Hsp70 isoforms was delayed compared to *S. cariosa*. The level of both Hsp70 isoforms remained unchanged in the foot of *S. zonata* up to 4 h after recovery (Fig. 1a). Thereafter, the kinetics of Hsp70 isoforms resembled the one observed for *S. cariosa*.

Fig. 1 Expression of Hsp70 isoforms (Hsp72 and Hsp74) and Hsp90 in the foot of *S. cariosa* and *S. zonata* following heat stress. Total protein was extracted from the foot of the control and heat-stressed snails immediately after heat stress (42 °C for 3 h) and after 1, 4, 24, and 48 h of recovery, and subjected to western blotting. Levels of Hsp70 isoforms (a) and Hsp90 (b) in tissue lysates are shown. All values are expressed as percentage of control snails (mean±SE). $n=7-12$ snails for all data points, except for the 48-h recovery group in *S. zonata* ($n=4$). Different letters above the bars denote significant differences within each species (lower case, *S. cariosa*; upper case, *S. zonata*). Asterisks denote significant differences between the two species (* $p<0.05$; ** $p<0.01$; *** $p<0.001$). C control, HS immediately after heat stress, Rec after recovery period

The Hsp74 level was initially reduced after 24 h of recovery (-67.5 ± 21.3 %, $p<0.05$, compared to 1 h of recovery) followed by a significant increase after 48 h of recovery (97.7 ± 28.6 %, $p<0.05$, compared to 24 h of recovery), whereas the first induction in Hsp72 level appeared only after 48 h of recovery (53.4 ± 15.4 %).

The observed limitation in the HSP response to heat in the foot of *S. zonata* compared to *S. cariosa* resembles the variation in the HSP response to heat between species that differ in their resistance to heat. 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). The results from heat stress experiments in the present study are also in agreement with our previous work in *Sphincterochila* species demonstrating delayed expression of HSPs in *S. zonata* snails in response to desiccation stress compared to *S. cariosa*. In this context, Shabtay and Arad (2005) demonstrated that heat-resistant fowl strains are characterized by a delayed induction of the HSP machinery (including activation of heat shock transcription factor (HSF) and

transcription and translation of HSPs) compared to heat-sensitive strains.

Interestingly, in both species, the induction of Hsp74 in the foot was preceded by an initial decrease in its level. Since the expression of HSPs was measured in the cytosol, it is possible that this reduction is due to translocation into the cytoskeletal–nuclear fraction. Other studies demonstrated translocation of Hsp70 from the cytosolic fraction into the nucleus during heat stress and oxidative stress (Dastoor and Dreyer 2000; Adhikari et al. 2004; Wang et al. 2008). Alastalo et al. (2003) proposed that this regulated subcellular distribution of Hsp70 is an important regulatory mechanism of the HSF-1-mediated heat shock response. Other studies proposed that Hsp70 translocates to the nucleus in order to protect the DNA from single-strand breaks (Kotoglou et al. 2009). We suggest that Hsp70 proteins may play important roles in cellular processes that take place in the foot of *Sphincterochila* snails following stress in both the cytoplasmic and nuclear compartments. Our previous study in *Sphincterochila* snails supports this suggestion, demonstrating increased levels of Hsp70 in both the cytosolic and cytoskeletal–nuclear fraction in the foot of *S. cariosa* following desiccation stress (Mizrahi et al. 2010).

Heat-induced HSP expression in the kidney

While the foot is directly facing environmental stress, the kidney is an internal organ that is highly specialized for ionic and osmotic regulation. As such, the kidney is important for the physiological adaptations in land snails that defend the body from water loss during aestivation. Generally, Hsp72 appears to participate in the adaptation of medullary cells to hyperosmolality, probably by acting as a molecular chaperone when cellular proteins are structurally damaged. Hsp90, on the other hand, contributes critically to the maturation of signal-transducing proteins participating in the osmoregulatory processes such as steroid hormone receptors or protein kinases (Beck et al. 2000; Borkan and Gullans 2002). In our previous studies, the level of Hsp90 in the kidney of *Sphincterochila* species was significantly higher than in the foot and in the hepatopancreas, and higher stocks of Hsp72 and Hsp90 were found in the kidney of *S. zonata* snails during aestivation and of Hsp90 also after arousal compared to *S. cariosa*. These data suggest that Hsp90 may have important roles in processes that take place in the kidney of *Sphincterochila* species and that the desert-adapted snails developed a distinct strategy of HSP expression in the kidney to improve their water economy.

In the present study, Hsp72 was significantly upregulated in the kidney of *S. cariosa* after 4 h of recovery ($40.7 \pm 9\%$, $p < 0.01$), and the level remained significantly higher after 24 and 48 h of recovery ($36.6 \pm 6.6\%$, $p < 0.01$, and $31.2 \pm 5.4\%$, $p < 0.05$, respectively) (Fig. 2a). In contrast, in *S. zonata*, the level of Hsp72 remained unchanged following heat stress and throughout the recovery period. The two species differed significantly in the expression of Hsp72 after 4, 24, and 48 h

of recovery ($p < 0.01$, $p < 0.05$, and $p < 0.05$, respectively). Hsp90 was upregulated in the kidney of both species immediately after heat stress ($56.5 \pm 14.4\%$ for *S. cariosa*, $33.7 \pm 11.4\%$ for *S. zonata*) (Fig. 2b). However, while in *S. cariosa* the increase in Hsp90 level was significant already after 1 h of recovery ($52.9 \pm 12.4\%$, $p < 0.05$) and the level remained higher throughout, in *S. zonata*, Hsp90 was only mildly induced during the first 4 h of recovery, and a significant increase was detected only after 24 h of recovery ($57.9 \pm 6.9\%$, $p < 0.01$). While the rapid induction of both HSPs in *S. cariosa* compared to only Hsp90 in *S. zonata* further supports the assumption that heat exposure at 42°C is more stressful for Mediterranean species, the finding that in *S. zonata* even mild stress induced the expression of Hsp90 supports the importance of this HSP for kidney function in these snails. The dual function of Hsp90 as a molecular chaperone and in signal transduction pathways gives Hsp90 the role of mediation between environmental conditions and their consequential physiological and biochemical changes and may explain its importance for kidney function in land snails.

The kinetics of thermally induced Hsp70 expression as a function of stress, temperature, and time, as well as post-stress recovery time, has typically two sequential concentration peaks (Diller 2006). For example, heat exposure of the land snail *Xeropicta derbentina* at 45°C resulted in the appearance of the first peak in Hsp70 expression at 2 h of recovery and the second peak at 16 h of recovery (Scheil et al. 2011). The stress response is elicited primarily in response to the presence of damaged proteins, and elevated HSPs assist in refolding and repair of denatured proteins and facilitate the synthesis of new proteins to repair damage. Thus, the observed increase in

Fig. 2 Expression of Hsp72 and Hsp90 in the kidney of *S. cariosa* and *S. zonata* following heat stress. Total protein was extracted from the kidney of the control and heat-stressed snails immediately after heat stress (42°C for 3 h) and after 1, 4, 24, and 48 h of recovery, and subjected to western blotting. Levels of Hsp72 (**a**) and Hsp90 (**b**) in tissue lysates are shown. All values are expressed as percentage of

control snails (mean $\pm$ SE). $n = 7$ –12 snails for all data points, except for the 48-h recovery group in *S. zonata* ($n = 4$). Different letters above the bars denote significant differences within each species (lower case, *S. cariosa*; upper case, *S. zonata*). Asterisks denote significant differences between the two species (* $p < 0.05$; ** $p < 0.01$). C control, HS immediately after heat stress, Rec after recovery period

Hsp70 expression in the foot and kidney in *S. cariosa* after 4 and 24 h of recovery may indicate that the heat stress was sufficient to cause cellular damage. On the other hand, the delay in Hsp70 response in the foot of *S. zonata* may indicate the need for Hsp70 during later post-heat stages, maybe in facilitating the synthesis of new proteins needed for the recovery process, and suggests that heat exposure at 42 °C does not represent an extreme heat stress event for this species. This possibility is reasonable when referring to the environmental conditions in the natural habitat of *S. zonata*, a semi-arid region characterized by lower humidity and precipitation, higher solar radiation and temperatures, and larger daily and annual temperature ranges compared with the temperate region. In a study in the central Negev desert, Schmidt-Nielsen et al. (1972) found that aestivating *S. zonata* snails encounter high temperatures in their natural habitat, ranging from 48 °C on a limestone surface to 65 °C on a sandy surface, and measured high temperatures between 45 and 47 °C within their shell. Since *S. zonata* snails in nature regularly experience higher temperatures than 42 °C during aestivation, it is not surprising that heat exposure at 42 °C did not cause intensive induction of HSPs.

Upregulation of HSPs may enhance survival under stress exposure by rescuing critical proteins and reducing the energetic cost associated with protein damage. However, HSP expression may also incur fitness costs on individuals that regularly experience environmental stress, because of reduced energy available for growth and reproduction (Krebs and Bettencourt 1999; Krebs and Feder 1997; Sørensen et al. 1999). Sørensen et al. (2003) proposed that the expression level of HSPs in each species and population is a balance between benefits and costs. Our study supports the concept that the pattern of the HSP response may have been modified during adaptation to different thermal habitats and that evolution in harsh environments will result in selection for reduced HSP expression. Thus, *S. cariosa* snails which adapted to milder Mediterranean conditions responded to heat exposure at 42 °C with rapid induction of HSPs, whereas the desert-inhabiting species *S. zonata* that regularly experiences higher temperatures displayed limited and delayed induction of HSPs. *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 vapor conductance, and the most favorable surface-to-volume ratio, compared to all other congeners, including *S. cariosa*. 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. Additional studies of the relative contribution of

each mechanism will contribute to our understanding of the factors that limit species' distributions.

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