Heat shock proteins and resistance to desiccation in congeneric land snails

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Abstract Land snails are subject to daily and seasonal variations in temperature and in water availability and depend on a range of behavioral and physiological adaptations for coping with problems of maintaining water, ionic, and thermal balance. Heat shock proteins (HSPs) are a multigene family of proteins whose expression is induced by a variety of stress agents. We used experimental desiccation to test whether adaptation to different habitats affects HSP expression in two closely related *Sphincterochila* snail species, a desiccation-resistant, desert species *Sphincterochila zonata*, and a Mediterranean-type, desiccation-sensitive species *Sphincterochila cariosa*. We examined the HSP response in the foot, hepatopancreas, and kidney tissues of snails exposed to normothermic desiccation. Our findings show variations in the HSP response in both timing and magnitude between the two species. The levels of endogenous Hsp72 in *S. cariosa* were higher in all the examined tissues, and the induction of Hsp72, Hsp74, and Hsp90 developed earlier than in *S. zonata*. In contrary, the induction of sHSPs (Hsp25 and Hsp30) was more pronounced in *S. zonata* compared to *S. cariosa*. Our results suggest that land snails use HSPs as part of their survival strategy during desiccation and as important components of the aestivation mechanism in the transition from activity to dormancy. Our study underscores the distinct strategy of HSP expression in response to desiccation, namely the delayed induction of Hsp70 and Hsp90 together

with enhanced induction of sHSPs in the desert-dwelling species, and suggests that evolution in harsh environments will result in selection for reduced Hsp70 expression.

Keywords HSP · Land snails · Desiccation · Environmental stress

Introduction

Although Mollusca originally evolved from a marine environment, the order Pulmonata is abundant on land, including arid and semiarid zones, where conditions of high ambient temperature and low humidity prevail. Therefore, the ability of land snails to colonize terrestrial habitats is a result of their development of a range of structural, behavioral, and physiological adaptations for coping with problems of maintaining water, ionic, and thermal balance (Riddle 1983) that will ensure their survival under their specific microhabitat conditions. Aestivation during the dry and hot seasons can represent such adaptations. Land snails are subject to annual cycles of activity in relation to seasonal changes in temperature, humidity, and water availability. In general, during aestivation, there is a marked decrease in metabolic activity (Storey and Storey 1990; Pakay et al. 2002; Ramnanan et al. 2009). This metabolic slowdown is especially evident in land snails and allows them to survive even in extreme arid conditions (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 (Machin 1967). A series of studies on water relations and resistance to experimental desiccation of Israeli land snails (Arad et al. 1989, 1992, 1993; Arad 2001) have

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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. In addition, interspecific differences were found that stem from a variety of morphological, behavioral, and physiological adaptations such as snail size, epiphragm thickness, site selection and lifestyle, and osmoregulatory capacities.

Environmental stressors can cause the induction of a selected group of proteins known as stress proteins or heat shock proteins (HSPs) in diverse organisms (Lindquist and Craig 1988; Feder and Hofmann 1999; Sorensen et al. 2003). Although initially named because their production is induced by heat, there are many other inducers of HSPs including various chemicals, osmotic stress, and desiccation. Generally, HSPs have been categorized into four major families on the basis of their molecular weight and degrees of homology: Hsp90 (83–99 kDa), Hsp70 (68–80 kDa), Hsp60, and a family of small HSPs (15–40 kDa). Among them, the highly conserved families of Hsp70 and Hsp90 are the most studied ones. 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 (Hsc70) and the heat-inducible Hsp70, whereas Hsp90 is one of the most abundant cytosolic proteins in eukaryotes. Under nonstress 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; Mayer and Bukau 2005).

It is generally accepted that HSPs protect organisms from the detrimental effects of heat, and possibly other stressors (Lindquist 1986; Somero 1995). Overall, it appears that thermotolerance depends on the synthesis of HSPs and that intracellular levels of Hsp70 are good indicators of thermotolerance (Moseley 1997; Nollen et al. 1999; King et al. 2002). It should be noted, however, that there are several studies suggesting that thermotolerance can be induced with a delayed HSP response (Shabtay and Arad 2005) or without the induction of HSP synthesis (Widelitz et al. 1986; Boon-Niermeijer et al. 1987) or that induction of HSPs may be uncoupled from acquired thermotolerance (Easton et al. 1987; Smith and Yaffe 1991). Boon-Niermeijer et al. (1986, 1988), for example, characterized two states of thermotolerance in snail larvae, a rapidly appearing transient state that depends on constitutively present HSPs, and a longer, more stable thermotolerant condition that requires the synthesis of new HSPs. The stress needed to induce HSPs is strongly related to the niche of the organism in question (Feder and Hofmann 1999). For example, Edgerly et al. (2005) found that two Australian embiid insect species upregulated Hsp70 at higher temperatures than a rainforest species, implying adaptation to higher habitat temperatures in the former. HSP

levels may change over microevolutionary timescales, and in such cases, environmental temperature is a potentially important selective agent (Dietz and Somero 1992; Norris et al. 1995; Feder et al. 2002).

Yet, if stress proteins in ectothermic species are involved in adaptation to extreme environmental conditions, we might expect an extensive stress protein response in species that naturally inhabit such environments, and by studying closely related species that differ in the HSP response and in thermotolerance we may identify the underlying adaptive strategies. 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 rainy Mediterranean environment to the arid desert environment and were previously found to differ in their susceptibility to desiccation stress (Arad et al. 1989). Stress conditions associated with lack of water were previously found to induce the expression of HSPs in different organisms. Brooks and Storey (1995) found induction of stress-related proteins (members of the 30-, 50-, and 70-kDa families) in the land snail *Otala lactea* during aestivation and suggested that these de novo produced stress proteins are important components of the overall aestivation strategy. In the Antarctic midge *Belgica antarctica*, the genes of sHSPs, Hsp70, and Hsp90 were upregulated in response to dehydration (Lopez-Martinez et al. 2009). Induction of Hsp70 mRNA was also found in the eutardigrade *Milnesium tardigradum* in the transitional stage between activity and desiccation (cryptobiosis; Schill et al. 2004) and of Hsp70 and Hsp23 in the flesh fly (*Sarcophaga crassipalpis*) pupae following desiccation exposure (Tammariello et al. 1999; Hayward et al. 2004). However, our knowledge of how species that differ in desiccation tolerance vary in expression patterns of HSPs with changing humidity is still limited. In the current study, we examined whether experimental desiccation causes the induction of stress-related proteins in the land snail *Sphincterochila* and whether there are differences in the HSP response patterns to desiccation between genetically desiccation-resistant desert snails *Sphincterochila zonata* and the more desiccation-sensitive Mediterranean-type *Sphincterochila cariosa* snails. Our approach was to test the effect of desiccation on the snails by analyzing the foot, kidney, and hepatopancreas tissues for the presence and quantity of various HSPs.

Materials and methods

Adult *S. zonata* (Bourguignat 1853) and *S. cariosa* (Olivier 1804) were collected in the Negev desert near Sde-Boqer and in the northern Mediterranean coast of Israel near Atlit, respectively. The snails were collected during winter months (November–December), brought to the laboratory,

and maintained in aquaria within a temperature-controlled room at $25 \pm 0.3^\circ\text{C}$ (a temperature within the natural range of both species) and a 12L:12D photoperiod.

Experimental design

The experimental groups of 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 each of the species was divided into two groups of approximately the same body mass. A group of control snails was sacrificed in the fully hydrated state. The second group of each species was placed in small individual plastic vials with perforated caps and exposed to normothermic desiccation (complete dryness) for up to 21 days at an ambient temperature of 25°C , within the temperature-controlled room (see details in Arad et al. 1989). For protein analysis, samples of three to five snails were sacrificed on days 1, 2, 3, 4, 7, and 10 of desiccation. The tissues (hepatopancreas, foot, and kidney) were dissected out and immediately frozen in liquid nitrogen. In a separate experiment that lasted 21 days, the extrapallial fluid was collected and analyzed for total osmolality (as an index of dehydration).

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 ethylenediaminetetraacetic acid (EDTA), 1% Igepal, 1 mM dithiothreitol (DTT), Protease Inhibitor Cocktail (Sigma P-8340), and 1 mM phenylmethylsulfonyl fluoride (PMSF). 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). The remaining pellet (the detergent-insoluble fraction that contains cytoskeletal components, nuclei, and aggregated proteins) was further processed to obtain the cytoskeletal–nuclear proteins. The pellet was solubilized in extraction solution (6 M urea, 0.46 M NaCl, 20 mM Tris pH 7.4, 1 mM EDTA, 1% Igepal, 1 mM DTT, and 1 mM PMSF), vortexed for 15 s, and incubated on ice for 10 min. The procedure was repeated three times; the homogenate was centrifuged (10 min, $17,000 \times g$ at 4°C), and the total protein concentration in the supernatant was determined by a standard method (Bradford).

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 sodium dodecyl sulfate–polyacrylamide gel with a 10% or 12% acrylamide gel and transferred onto nitrocellulose membrane. The membranes were probed with mouse monoclonal antibody against bovine brain Hsp70 (Sigma H-5147), recognizing both the constitutive (Hsc70, 73 kDa) and inducible (Hsp70, 72 kDa) forms of mammalian Hsp70, mouse monoclonal antibody against Hsp90 (Sigma H-1775), mouse monoclonal antibody against actin (Sigma A-4700), and rabbit polyclonal antibody against chicken Hsp25 (a gift from Professor Geiger, Weizmann Institute, Israel). Hsp70 antigen (72 kDa, 250 ng; StressMarq SPR-115A) and Hsp90 antigen (200 ng; Stressgen SR-P770) were simultaneously run to serve as standards for analysis of Hsp70 and Hsp90. The secondary antibodies were goat antimouse immunoglobulin G-horse radish peroxidase (IgG-HRP; Sigma A-2554) or goat antirabbit IgG-HRP (Santa Cruz Biotechnology sc-2004).

Protein quantification

The proteins were visualized by enhanced chemiluminescence, and the intensity of the bands was quantified using densitometry software (ImageJ). In the cases where two consecutive gels were combined, the intensity of the bands was related to the internal Hsp70 or Hsp90 standard to normalize the bands on different gels. In addition, three samples were simultaneously run on both gels in order to confirm the correctness of normalization. The obtained value of Hsp70, Hsp90, or Hsp25 in the cytosolic fraction was normalized to actin for the foot and kidney tissues. In the hepatopancreas, the level of actin significantly increased during desiccation in both species, so the obtained values of different HSPs were not normalized to actin. Relative endogenous levels of Hsp70 and Hsp90 in fully hydrated control snails were calculated by expressing the intensity of HSP bands in control snails relative to the intensity of the internal HSP standards.

Statistics

The results are reported as summation of three to four independent experiments and expressed as percentage change from control (mean values $\pm$ SE). Once data were confirmed to fit the validation criteria, the statistical analysis software program SPSS 16.0 was used to perform a one-way analysis of variance (ANOVA) with Bonferroni post hoc test for multiple comparisons. In case of changes in the expression of cytoskeletal–nuclear proteins and in case of mass and osmolality changes during normothermic

desiccation experiments, the significance was verified by unpaired *t* test. In all the cases, $p < 0.05$ was considered significant.

Results

Normothermic desiccation

During 21 days of normothermic desiccation, *S. zonata* and *S. cariosa* lost $11.8 \pm 1.1\%$ and $15.2 \pm 1.7\%$, respectively, of total mass (Fig. 1a). A major difference was established in the response during the first 2 days where *S. zonata* lost significantly less water than *S. cariosa*, $4.3 \pm 0.3\%$ vs. $8.4 \pm 1.1\%$ ($p < 0.01$), respectively, on day 1 and $6.1 \pm 0.7\%$ vs. $11.3 \pm 0.7\%$ ($p < 0.001$), respectively, on day 2. Thereafter, rate of change was moderated and after day 4 body mass practically stabilized for the rest of the period. The extrapallial fluid was collected and analyzed for total osmolality, as an index of dehydration (Fig. 1b). In both species, osmolality dropped significantly from its peak values during aestivation upon arousal on damp substrate

($p < 0.0001$). The osmolality significantly increased from day 1 of desiccation onward ($p < 0.001$ for *S. cariosa* and $p < 0.01$ for *S. zonata*). The high level reached at the end of 21 days of desiccation was still significantly lower than that in the aestivated snails ($p < 0.05$ for *S. cariosa* and $p < 0.01$ for *S. zonata*). There were no statistical differences between species at any time point.

Expression of Hsp70

In the foot of both species, the monoclonal antibody to Hsp70 detected two bands of approximately 72 and 74 kDa. Both isoforms of Hsp70 were upregulated in *S. cariosa* foot during normothermic desiccation (Fig. 2a–c). Hsp74 was induced already on the first day (increase of $104 \pm 32.4\%$, $p < 0.01$), while the 72-kDa band seems to be induced later, from day 3 onwards ($20.2 \pm 6.3\%$, $p < 0.05$ on day 3). In contrast, there was no induction of both isoforms in *S. zonata* foot in the first 3 days of desiccation. Only on day 7 (Hsp72, $34.8 \pm 12.7\%$, $p < 0.001$) and day 10 (both isoforms, $22.7 \pm 6.8\%$ $p < 0.01$ for Hsp72; $38.8 \pm 20\%$ $p < 0.05$ for Hsp74) was there a significant upregulation of Hsp70 isoforms. Our results show a significant elevation in Hsp72 level in the cytoskeletal–nuclear fraction in *S. cariosa* but not in *S. zonata* foot in the first 3 days of desiccation ($48.7 \pm 17.8\%$, $p < 0.05$ on day 3; Fig. 2d). ANOVA tests between the two species revealed significant differences in the expression of Hsp70 isoforms in both the cytosolic and cytoskeletal–nuclear fraction ($p < 0.001$).

In the kidney and in the hepatopancreas, only the lower 72-kDa band appeared (Fig. 3a). In the kidney tissue, Hsp72 expression remained unchanged during the first 10 days of desiccation in both species. In the hepatopancreas tissue, Hsp72 expression was significantly increased in *S. cariosa* on day 10 ($30.6 \pm 11.8\%$, $p < 0.05$), whereas in *S. zonata* the level of Hsp72 remained unchanged (data not shown).

We also measured the relative endogenous levels of Hsp72 in the foot, kidney, and hepatopancreas tissues of fully hydrated control *S. cariosa* and *S. zonata* snails. As shown in Fig. 4a, the relative levels of Hsp72 synthesis were significantly higher in *S. cariosa* than in *S. zonata* in all the examined tissues ($p < 0.001$).

Expression of Hsp90

The expression of Hsp90 in the foot and kidney was significantly upregulated during desiccation in *S. cariosa* but remained unchanged or was only mildly induced in *S. zonata* (Fig. 5). In *S. cariosa* foot, Hsp90 was significantly upregulated already on the first day ($55.7 \pm 19.9\%$, $p < 0.05$; Fig. 5a, c). In contrast, in *S. zonata* foot, Hsp90 was mildly increased only on day 10 ($16.7 \pm 10.8\%$, $p < 0.01$). In the

Fig. 1 Changes in mass and in extrapallial fluid osmolality during 21 days of normothermic desiccation in *S. cariosa* and *S. zonata*. **a** Changes in mass. The results are summation of two to three independent experiments ($n=21$ snails for *S. zonata* and 32 snails for *S. cariosa*). All values are expressed as percentage of initial fully hydrated mass (mean $\pm$ SE). Asterisks denote significant differences between the two species (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). **b** Changes in extrapallial fluid osmolality. All values are expressed as mean $\pm$ SE ($n=6$ snails, except for aestivation group where $n=9$ –14 snails and rehydration group $n=4$ –6 snails). Asterisks denote significant differences from hydration group (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$)

Fig. 2 Expression of Hsp70 isoforms (Hsp72 and Hsp74) in the foot tissue of *S. cariosa* and *S. zonata*. Total protein was extracted from fully hydrated control and desiccated snails during 10 days of desiccation and subjected to Western blotting. **a** A representative blot for tissue lysate of three independent experiments is shown. Arrows denote the point of connection, where two consecutive gels were combined. **b** and **c** are graphical presentations of the relative levels of Hsp74 (**b**) and Hsp72 (**c**) in tissue lysates. $n=6-15$ snails. 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 (* $p<0.05$; ** $p<0.01$; *** $p<0.001$). **d** Relative levels of Hsp72 in the foot cytoskeletal–nuclear fraction. $n=7-8$ snails. Asterisks denote significant differences within each species and also between the two species (* $p<0.05$; ** $p<0.01$)

kidney, the expression of Hsp90 in *S. cariosa* was significantly upregulated from day 3 on ($79.8\pm10.2\%$, $p<0.01$ on day 3) but remained unchanged in *S. zonata* (Fig. 5b, d). The two species differed significantly in the expression of Hsp90 in both tissues ($p<0.001$).

In the hepatopancreas, Hsp90 expression was significantly increased during desiccation in both species on day 7 ($65.2\pm10.3\%$, $p<0.05$ for *S. cariosa*; $58.1\pm13.8\%$, $p<0.01$ for *S. zonata*) and in *S. zonata* also on day 10 ($49.6\pm10.4\%$, $p<0.05$; Fig. 5e).

In both species, relative levels of Hsp90 in the kidney of fully hydrated snails were significantly higher than in the foot ($p<0.01$) and also significantly higher than in the hepatopancreas in *S. zonata* ($p<0.001$; Fig. 4b).

Expression of sHSPs

Western blots incubated with the polyclonal anti-Hsp25 antibody demonstrated the presence of four bands of approximately 15, 17, 25, and 30 kDa (Fig. 3b). In the foot tissue, normothermic desiccation affected the expres-

sion of Hsp25 in both the cytosolic and the cytoskeletal–nuclear fractions. In *S. zonata*, the expression of Hsp25 in both fractions was significantly enhanced ($192.7\pm41.0\%$, $p<0.01$, on day 3 in the cytosolic fraction; $120.8\pm43.1\%$, $p<0.05$, on day 3 in the cytoskeletal–nuclear fraction), while in *S. cariosa*, the expression of Hsp25 in the cytosolic fraction was only mildly induced ($49.7\pm11.6\%$, $p<0.05$ on day 3), and in the cytoskeletal–nuclear fraction it remained unchanged (Fig. 6). The two species demonstrated a significant difference in the expression of Hsp25 in both the cytosolic and cytoskeletal–nuclear fractions ($p<0.001$). The expression of Hsp30 in the cytosolic fraction remained unchanged in both species (data not shown).

In the kidney tissue, both Hsp25 and Hsp30 expression significantly decreased in *S. zonata* during desiccation ($-48.4\pm11.4\%$, $p<0.05$, on day 3 for HSP25; $-52\pm7.6\%$, $p<0.05$, on day 7 for Hsp30) whereas in *S. cariosa* kidney Hsp30 expression decreased ($-49.4\pm6.9\%$, $p<0.01$, on day 3), but Hsp25 expression was not affected (Fig. 7). The two species demonstrated a significant difference in the expression of Hsp25 ($p<0.01$) and Hsp30 ($p<0.05$).

Fig. 3 Expression of Hsp70 and sHSPs in the foot (F), kidney (K), and hepatopancreas (H) tissues of *S. cariosa* and *S. zonata*. Total protein was extracted from snail tissues and subjected to Western blotting using an anti-Hsp70 (a) and anti-Hsp25 (b) antibodies. Lanes contain equivalent amounts of protein from tissue extracts prepared from individual snails

In the hepatopancreas, Hsp25 expression was not affected by desiccation (data not shown). The expression of Hsp30 in *S. cariosa* and in *S. zonata* was upregulated on day 1 ($67.3 \pm 26.2\%$, $p < 0.05$ and $91.9 \pm 30.7\%$, ns, respectively) and from day 7 on ($79.7 \pm 23.7\%$, $p < 0.05$ and $230.3 \pm 45.2\%$, $p < 0.001$, respectively; Fig. 8a, b). The two species demonstrated a significant difference in the expression of Hsp30 ($p < 0.05$). In both species, a band of approximately 17 kDa was significantly increased during desiccation from day 7 on ($114.3 \pm 20.5\%$, $p < 0.05$, in *S. cariosa*; $71.5 \pm 14.4\%$, $p < 0.05$ in *S. zonata*; Fig. 8c, d).

Discussion

The desert-inhabiting *S. zonata* seems to have developed effective regulatory mechanisms to withstand extreme environmental conditions (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 species, including the Mediterranean-type *S. cariosa*. The present study was designed to examine the cellular stress response machinery of *Sphincterochila* snails that allow them to cope with desiccation. Specifically, we aimed to find whether the difference in desiccation resistance between *S. cariosa* and *S. zonata* is correlated with differential upregulation of the HSP response. Our study suggests that species that differ in their resistance to

Fig. 4 Relative endogenous levels of Hsp72 and Hsp90 in the foot, kidney, and hepatopancreas tissues of *S. cariosa* and *S. zonata*. Total protein was extracted from fully hydrated, control snails and subjected to Western blotting. Levels of Hsp72 (a) and Hsp90 (b) are expressed relative to an internal Hsp70 protein (250 ng) or Hsp90 protein

(200 ng) standards. $n = 8-14$ snails. 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 (** $p < 0.01$)

Fig. 5 Expression of Hsp90 in the foot, kidney, and hepatopancreas tissues of *S. cariosa* and *S. zonata*. Total protein was extracted from fully hydrated control and desiccated snails during 10 days of desiccation and subjected to Western blotting. A representative blot of three independent experiments in the foot (a) and kidney (b) is shown. Arrows denote the point of connection where two consecutive

gels were combined. Relative levels of Hsp90 in the foot (c), kidney (d), and hepatopancreas (e) are shown. $n=6-15$ snails. 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 (* $p<0.05$; ** $p<0.01$; *** $p<0.001$)

desiccation developed distinct strategies of HSP expression in response to desiccation that reflect the difference in aridity encountered in their natural habitats.

In the present study, the relative endogenous levels of Hsp72 in all the tissues examined were significantly higher in *S. cariosa* than in *S. zonata*. These results suggest that the desiccation-sensitive *S. cariosa* snails maintain larger pools of Hsp72 compared to the desiccation-resistant *S. zonata* and are in line with other interspecific studies demonstrating higher endogenous levels of Hsp70 in the more thermally sensitive species compared to the heat-resistant ones. Thus, Tomanek and Somero (2002) studied three congeneric marine snails of the marine subtidal to intertidal gastropod *Tegula* differing in their heat tolerance and found higher endogenous levels of Hsp74 (yet lower levels of Hsp72) in the heat-sensitive species compared to the heat-tolerant ones in field-acclimatized specimens. Likewise, studies in northeastern pacific *Nucella* species revealed lower levels of Hsp70 in the more thermotolerant *Nucella emarginata* than in the less thermotolerant species (Sorte and Hofmann 2005). It is important to note, however, that other interspecific studies revealed correlations between a greater heat tolerance and higher constitutive levels of HSPs (Evgen'ev et al. 2007). Zatssepina et al

(2000), for example, found higher levels of both Hsp70 mRNA and protein in desert-dwelling lizard species than in nondesert species. Similarly, in study in 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. We are currently performing field experiments in order to determine if *Sphincterochila* snails in the natural habitat reveal similar HSP responses as those held under laboratory conditions. Preliminary results (unpublished) in snails collected after the first rains indicate that also in nature *S. cariosa* is characterized by higher pools of Hsp70 in both the foot and kidney tissues.

Interspecific comparisons of ectothermic species from different latitudes have also shown that, typically, species adapted to higher temperature niche were more heat tolerant, induced synthesis of HSPs such as Hsp70 and Hsp90 at higher temperatures, and had higher upper thermal limits of protein synthesis (Hofmann and Somero 1996; Tomanek and Somero 1999; Nakano and Iwama 2002; Evgen'ev et al. 2007). 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

Fig. 6 Expression of Hsp25 in the foot tissue of *S. cariosa* and *S. zonata*. Total protein was extracted from fully hydrated control and desiccated snails during 10 days of desiccation and subjected to Western blotting. **a** A representative blot for tissue lysate of three independent experiments is shown. Arrows denote the point of connection where two consecutive gels were combined. **b** Relative levels of Hsp25 protein in tissue lysate. $n=6-15$ snails. 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** Relative levels of Hsp25 protein in the cytoskeletal-nuclear fraction. $n=3-8$ snails. Asterisks denote significant differences within each species and also between the two species (* $p<0.05$; ** $p<0.01$)

factor (HSF), transcription and translation of HSP) compared to heat-sensitive strains. Thus, the observed delay in the Hsp70 and Hsp90 response to desiccation in the foot tissue 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. The finding that in the

kidney tissue Hsp90 was induced only in *S. cariosa* further supports this assumption. In contrast to *S. zonata* foot, Hsp70 and Hsp90 were already upregulated in *S. cariosa* foot during the first days of desiccation. The first 3 days of aestivation in land snails are critical to the transition from active state to dormancy. At this time window, changes in

Fig. 7 Expression of sHSPs in the kidney tissue of *S. cariosa* and *S. zonata*. Total protein was extracted from fully hydrated control and desiccated snails during 10 days of desiccation and subjected to Western blotting. **a** Relative levels of Hsp25. **b** Relative levels of

Hsp30. $n=6-15$ snails. 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 (* $p<0.05$; ** $p<0.01$)

Fig. 8 Expression of sHSPs in the hepatopancreas tissue of *S. cariosa* and *S. zonata*. Total protein was extracted from fully hydrated control and desiccated snails during 10 days of desiccation and subjected to Western blotting. **a, c** A representative blot of three independent experiments is shown. Arrows denote the point of connection where

two consecutive gels were combined. **b** Levels of Hsp30 protein. **d** Levels of Hsp17 protein. $n=6-15$ snails of both species. 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 (** $p<0.01$)

the degree of enzyme phosphorylation occur (Storey 2002; Ramnanan et al. 2007), respiration falls, and the metabolic rate decreases. Our study shows that the major difference in water loss between the two species was established also during the first 3 days of desiccation. Thus, the rapid increase in Hsp70 and Hsp90 synthesis in *S. cariosa* during the early stages of desiccation suggests that these proteins play a role not only in protecting proteins from desiccation stress but might also be important components of the aestivation mechanism. Further support for this assumption came from the finding that the level of Hsp72 in the cytoskeletal-nuclear fraction in *S. cariosa* foot increased during the first 3 days of desiccation, due to either de novo synthesis and/or translocation of Hsp70 from the cytoplasm. Previous studies demonstrated translocation of Hsp70 into the nucleus during heat shock and oxidative stress (Dastoor and Dreyer 2000; Adhikari et al. 2004). Alastalo et al. (2003) proposed that this regulated subcellular distribution of Hsp70 is an important regulatory mechanism of HSF-1-mediated heat shock response. We suggest that in resemblance to heat stress, in desiccation stress, Hsp70 proteins may play important roles in cellular processes that take place in both the cytoplasmic and nuclear compartments. Another observation concerning Hsp70 expression in *S. cariosa* foot was that although both isoforms of Hsp70 were upregulated in response to desiccation, Hsp74 was induced earlier and to a higher magnitude than Hsp72. These results, together with

the finding that Hsp72 had higher constitutive level than Hsp74 in the foot of control snails, suggest that, in *Sphincterochila* snails, Hsp74 is the inducible form of Hsp70 and Hsp72 is the constitutive form.

While desiccation stress induced Hsp72 and Hsp74 in the foot tissue, in the kidney and in the hepatopancreas the level of Hsp72 remained unchanged or only mildly induced. Tissue-specific variation in the expression of Hsp70 proteins is known to occur in both vertebrates and invertebrates. In the land snail *O. lactea*, Hsp70 protein was induced by aestivation in the foot tissue but not in the hepatopancreas (Brooks and Storey 1995). Scott et al. (2003) found a tissue-specific pattern of Hsp70 isoforms expression in the western painted turtle during both unstressed and stressed conditions. Similarly, the levels of Hsp70, Hsp72, and Hsp78 in the gill, mantle, and adductor tissues in *Mytilus edulis* showed variation in both the magnitude of induction by heat stress and in their basal level in unstressed tissues (Chapple et al. 1997). Other studies suggested that the Hsp70 response is specific to those organs undergoing stress (Flanagan et al. 1995; Krebs and Feder 1997b). One reason for the presence of two members of the Hsp70 family in the foot tissue and for the observed upregulation of Hsp70 proteins in the foot and not in the kidney and hepatopancreas during the first 7 days of desiccation may be related to its being “in the front,” directly facing environmental stress. Thus, the synthesis of both

members of the Hsp70 family in the foot is necessary for survival under environmental stress conditions, while the increased synthesis of only one Hsp70 protein is sufficient for a stress response in the inner organs maintained at a rather constant environment. Similarly, Ulmasov et al. (1993) found that the lymphocytes of the desert camel *Camelus dromedarius* are able to respond to an increased temperature by the increased synthesis of only a constitutive member of the Hsp70 family, whereas the skin fibroblasts which are exposed to the environment can also synthesize the inducible form. However, the observed tissue-specific variability in Hsp70 induction during desiccation may also reflect a differential involvement of Hsp70 in the transition processes from active to dormant states in the different tissues.

In both species, the relative endogenous levels of hsp90 in the kidney were significantly higher than in the foot and in the hepatopancreas, suggesting that Hsp90 may have important roles in processes that take place in the kidney. In addition to participating in the cell stress response in refolding denatured proteins, Hsp90 proteins have specific roles in signal transduction pathways such as cell growth, differentiation, and survival. Hsp90 client proteins include, for example, steroid hormone receptors and several protein kinases (Pratt and Toft 2003). In previous studies, Hsp90 was found to be induced in the kidney by stressors such as nephrotoxic agents and heat (Beck et al. 2000) and in the liver and hepatopancreas by heat and desiccation stress (Brooks and Storey 1995; Jiang et al. 2009). The dual involvement of Hsp90 in stress response and in signal transduction raises the possibility that Hsp90 may participate in several specific processes during desiccation stress. For example, the involvement of Hsp90 in regulation of protein phosphorylation and in regulation of sodium (Ramirez et al. 2004) is consistent with the changes in enzyme phosphorylation and the increase in extrapallial fluid osmolality observed in aestivating snails and further supports this assumption.

Our study also highlights the importance of sHSPs in processes accompanying desiccation. sHSPs comprise the most widespread but also the most poorly conserved family of molecular chaperones (Haslbeck et al. 2005). Their variability in sequence and size was demonstrated in our study, as we found considerable variation in the size of proteins detected by the polyclonal antibody to Hsp25, not only between the different tissues but also between the two species. Most sHSPs share characteristic features, including a conserved α -crystallin domain, formation of large oligomers, induction by stress conditions, and chaperone activity in suppressing protein aggregation (Jakob et al. 1993; Morrow et al. 2006). Hsp27 and Hsp30 were found to be involved in development, cell cycle, and cell differentiation (Heikkilä 2004; Parcellier et al. 2005), and their expression was induced by different stressors including heat shock (Mirkes et al. 1996; Norris et al. 1997; Mulligan-Tuttle and Heikkilä 2007) and hyper-

tonic stress (Dasgupta et al. 1992; Neuhofer et al. 1998). Overexpression of sHSPs including Hsp27 and α -crystallin has been shown to confer resistance to heat-shock as well as to oxidative stress in a number of organisms and cell types (Rollet et al. 1992; Mehlen et al. 1995).

As discussed earlier in the text, previous studies demonstrated upregulation of sHSPs during stress conditions associated with lack of water in different organisms. The observed increase in sHSPs level during desiccation in our study is in line with these studies and further emphasizes the importance of sHSPs during organismal stress response. One of the most intriguing observations in the present study is the finding that the response of both Hsp25 and Hsp30 during desiccation was more pronounced in *S. zonata* compared to *S. cariosa*, in opposite to the more prominent response of both Hsp70 and Hsp90 in *S. cariosa*. In the foot of *S. zonata*, Hsp25 was induced during the first days of desiccation and to a greater extent compared to the mild induction detected in *S. cariosa*. Furthermore, desiccation stress also induced the expression of Hsp25 in the cytoskeletal–nuclear fraction in *S. zonata* foot. Previous studies found that heat shock, ischemia, and other stressors induce the translocation of Hsp27 and Hsp25 (rodent homolog of human Hsp27) to the nucleus and to the cytoskeletal fraction (Lavoie et al. 1993; Bryantsev et al. 2002; Adhikari et al. 2004). Hsp25/27 were found to interact with and stabilize certain components of the cytoskeleton such as actin, thus preventing disruption of the cytoskeleton resulting from stresses. Taken together, these findings suggest a biological function of Hsp25 in the foot tissue during desiccation stress, either in the mechanism regulating cytoskeleton stabilization and/or in chaperoning activity in the cytoplasm and in the nucleus. Previous study in the crustacean *Artemia* supports this assumption. Clegg et al. (1994) detected massive amounts (about 15% of the total nonyolk embryo protein) of the sHSP p26 in the stress-resistant encysted embryos of *Artemia*. They also found that p26 undergoes translocation to the nucleus during anoxia, heat shock, and in diapause embryos and suggested that this protein may play the role of a metabolic regulator and/or a protective molecular chaperone.

The observed decrease in cytosolic Hsp25 and Hsp30 levels in the kidney during desiccation is in line with other studies demonstrating reduction of soluble Hsp25 and the migration of Hsp25 into the cytoskeletal–nuclear fraction during renal ischemia (Schober et al. 1997; Aufricht et al. 1998). The involvement of Hsp25/27 in cytoskeleton stabilization following stresses such as renal ischemia and high concentration of NaCl (Aufricht et al. 1998; Neuhofer et al. 1998; Beck et al. 2000) and in protecting medullary cells against the deleterious effects of high urea concentrations (Ohno et al. 1997; Neuhofer et al. 2005) suggests possible roles for sHSPs in protecting kidney tissue from the

deleterious effects of high urea concentration and osmolality accompanying desiccation in land snails (Arad 2001).

In the hepatopancreas, in addition to Hsp30, desiccation stress induced also the expression of a protein of approximately 17 kDa. sHSPs in the size range of 15–18 kDa were previously shown to be induced by heat shock in fungi extracts (*Pisolithus* sp.; Ferreira et al. 2005) and implicated not only in chaperoning of proteins but also in the development of thermotolerance and in stabilization of lipid membranes (Lee et al. 2000; Torok et al. 2001). Although the question whether the observed 17 kDa protein in the present study belongs to the HSP family is still open, its tissue-specific expression and significant induction in the hepatopancreas of both species suggest an important role of this protein in cellular processes following desiccation. In the case that this protein belongs to the HSP family, we suggest that it is needed in the hepatopancreas not only as chaperone but also in stabilizing the cell membrane.

The findings that, in all the tissues examined, sHSP responses were more pronounced in *S. zonata* compared to *S. cariosa* suggest a distinct strategy of activating sHSPs in the desert snail. The suggestion that organisms use sHSPs to adapt to extreme environmental conditions is supported by other interspecific studies. Sanders et al. (1991) studied two limpets that occupy different intertidal habitats and found in the heat-tolerant species, but not in the more temperature-sensitive one, an induction of a complex group of low molecular weight stress proteins in response to heat shock. Similarly, a study in three *Drosophila* species showed that the most thermotolerant species surpassed thermosensitive species in the level of sHSPs throughout the temperature range tested (Shilova et al. 2006), and study in the blue mussel *Mytilus* showed induction of a 30-kDa stress protein in response to heat stress in the heat-resistant *Mytilus galloprovincialis* but not in the more thermally-sensitive *Mytilus trossulus* (Hofmann and Somero 1996). Furthermore, not only resistance to heat but also resistance to dehydration was correlated with the presence of sHSPs (Ravindran et al. 2005).

In summary, this study has demonstrated differences in the HSP response to experimental desiccation stress in nonmodel species of land snails that differ in their resistance to desiccation. The desert-inhabiting *S. zonata*, which is naturally exposed to extreme environmental conditions, exhibits a delayed response of Hsp72, Hsp74, and Hsp90 to desiccation stress, together with an enhanced response of sHSPs. In contrast, the Mediterranean-type, desiccation-sensitive species *S. cariosa* maintains larger pools of Hsp72 and has high inducible synthesis of Hsp72, Hsp74, and Hsp90, but only a moderate response of sHSPs. Upregulation of HSPs may enhance survival under stress exposure by rescuing critical proteins and reducing the energetic cost associated with protein damage but can also be deleterious for growth and development. For example,

Krebs and Feder (1997a) found deleterious effects of Hsp70 overexpression in *Drosophila melanogaster* larvae. Thus, HSP expression may incur fitness costs on individuals that regularly experience environmental stress, suggesting that evolution in harsh environments will result in selection for reduced HSP expression. Indeed, other studies found reduced expression and slower kinetics of Hsp70 in strain of *D. melanogaster* adapted to constant comparatively high temperature than in a standard wild-type strain and suggested that the reduction in Hsp70 expression may stem from insertion of transposable elements in the hsp70 gene promoter (Bettencourt et al. 1999; Zatsepina et al. 2001; Lerman et al. 2003). Feder (1999) summarizes that the deleterious consequences of overexpression of HSPs “are clear and apparently trade off against the benefits in the evolutionary sense, either constraining directional selection for increased HSP expression or necessitating especially effective autoregulation of Hsp expression.” Our results support this position, showing a lower expression of Hsp70 and a limited use of Hsp70 and Hsp90 in desert snails in response to desiccation stress. Our study also indicates that these energy costs vary for different tissues and under different physiological conditions. We suggest that this differential HSP response reflects the relative vulnerability and the physiological function of different tissues, with likely consequences for the ecology and distribution of different land snails.

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