Natural annual cycle of heat shock protein expression in land snails: desert versus Mediterranean species of *Sphincterochila*

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SUMMARY

Land snails are subject to daily and seasonal variations in temperature and in water availability, and have evolved annual cycles of activity and aestivation as part of their survival strategy. We tested in the field whether adaptation to different habitats affects the endogenous levels of heat shock proteins (HSPs) in two closely related *Sphincterochila* snail species, a desiccation-resistant desert species, *Sphincterochila zonata*, and a Mediterranean-type, desiccation-sensitive species, *S. cariosa*. We examined HSP levels in various tissues of snails during aestivation and after resumption of activity. Our study shows that, during aestivation, *S. cariosa* had higher standing stocks of Hsp70 in the foot and the hepatopancreas, and of small HSPs (sHSPs) in all the examined tissues, whereas *S. zonata* had higher stocks of Hsp70 in the kidney and of Hsp90 in the kidney and in the hepatopancreas. Arousal induced a general upregulation of HSPs, except for Hsp90, the expression of which in the foot was higher during aestivation. We suggest that the stress protein machinery is upregulated during arousal in anticipation of possible oxidative stress ensuing from the accelerating metabolic rate and the exit from the deep hypometabolic state. Our findings support the concept that, in land snails, aestivation and activity represent two distinct physiological states, and suggest that land snails use HSPs as important components of the aestivation mechanism, and as part of their survival strategy during and after arousal. Our study also indicates that adaptation to different habitats results in the development of distinct strategies of HSP expression with likely consequences for the ecology and distribution of land snails.

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Key words: HSP, land snails, annual cycle, aestivation, arousal, environmental stress.

INTRODUCTION

Land snails are subject to annual cycles of activity and aestivation in relation to seasonal changes in temperature, humidity and water availability. In general, during aestivation, there is a marked decrease in metabolic activity (Pakay et al., 2002; Storey, 2002; Storey and Storey, 1990). This metabolic slow-down 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 (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, 2001; Arad, 2009; Arad et al., 1989; Arad et al., 1992; Arad et al., 1993). 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. Unsurprisingly, based on GIS maps, and using land snails to test their approach, Kadmon and Heller (Kadmon and Heller, 1998) found that patterns of faunal variation were significantly correlated with underlying variation in rainfall in Israel.

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, and these species were previously found to differ in their susceptibility to desiccation stress (Arad et al., 1989). 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. (Arad et al., 1989) showed that *S. zonata* was the most resistant species to desiccation, characterized by the lowest rates of water loss, the thickest epiphragm, the lowest epiphragm area-specific water vapor conductance and the most favorable surface-to-volume ratio, compared with all other congeners, including the Mediterranean-type *S. cariosa*.

Environmental stressors can cause the induction of a selected group of proteins known as stress proteins or heat shock proteins (HSPs) in diverse organisms (Feder and Hofmann, 1999; Lindquist and Craig, 1988; Sørensen et al., 2003). 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). The 70kDa 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 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; Mayer and Bukau, 2005). Small HSPs (sHSPs) comprise the most widespread but also the most poorly conserved family of molecular chaperones. sHSPs share characteristic features, including a conserved α-crystallin domain, formation of large oligomers, induction by stress conditions and chaperone activity (Haslbeck et al., 2005; Sun and MacRae, 2005). It is generally accepted that HSPs protect organisms from the detrimental effects of heat and possibly other stressors, including various chemicals, heavy metals, cold, oxidative stress, osmotic stress and desiccation (Kregel, 2002; Lindquist, 1986; Somero, 1995). HSPs reduce aggregation of stress-damaged proteins and serve to restore their native structures, allowing normal protein functions to continue. sHSPs were also found to interact with and stabilize certain components of the cytoskeleton such as actin, thus preventing disruption of the cytoskeleton resulting from stress.

Recently, our study in the land snail Sphincterochila 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- and tissue-dependent manner (Mizrahi et al., 2010). These findings 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. Other studies found induction of various HSPs during short-term experimental aestivation in the land snail Otala lactea, suggesting that these de novo produced stress proteins are important components of the overall aestivation strategy (Brooks and Storey, 1995; Ramnanan et al., 2009). Stress-induced HSP expression has been well characterized in other hypometabolic systems, including hibernating mammals, anoxia-tolerant turtles and diapausing insects (Epperson et al., 2004; Rinehart et al., 2000; Storey, 2007).

Our recent study (Mizrahi et al., 2010) suggested 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. The desert inhabiting, desiccation-resistant S. zonata, which is naturally exposed to extreme environmental conditions, maintained lower standing stocks of Hsp70 under normal conditions compared with the Mediterranean-type, desiccation-sensitive species S. cariosa. In addition, S. cariosa revealed higher inducible synthesis of Hsp72, Hsp74 and Hsp90 in response to desiccation stress, but only a moderate response of sHSPs, compared with S. zonata. The upregulation of HSPs may enhance survival under stress exposure by rescuing crucial proteins and reducing the energetic cost associated with protein damage. However, HSP expression might also incur fitness costs on individuals that regularly experience environmental stress, because of the reduced energy available for growth and reproduction, suggesting that evolution in harsh environments will result in selection for reduced HSP expression. This position is supported by our recent study in Sphincterochila snails and by studies in other organisms; for example, in natural populations of Drosophila from different climates (Sørensen et al., 2001) and in marine snail Tegula species of different biogeographical ranges (Tomanek and Somero, 2002).

The present study aimed to test whether the natural annual activity cycle of land snails involves the stress protein machinery and, if so, whether desert species differ from their non-desert congeners in their strategies, as recently suggested by Mizrahi et al. (Mizrahi et al., 2010) for Sphincterochila. We thus sampled S. zonata and S. cariosa in their respective desert and Mediterranean habitats during aestivation and arousal, and analyzed several tissues for various HSPs.

MATERIALS AND METHODS
Adult S. zonata (Bourguignat 1853) and S. cariosa (Olivier 1804) were collected in the Negev desert near Sde-Boger, and from the northern Mediterranean coast of Israel near Atlit, respectively. Of each species, 25 snails were sampled in the field during aestivation (July–August) and after arousal (October–November, after the first rains). A group of 15 snails were weighed on an analytical balance to the nearest 0.1 mg and sacrificed immediately, and the tissues (hepatopancreas, foot and kidney) were dissected out, weighed and frozen in liquid nitrogen. The extra-pallial fluid was collected from snails during aestivation and after arousal in the field and analyzed for total osmolality. Control groups (N = 10) of snails collected during aestivation were brought to the laboratory and allowed to recover on a damp substrate for 72 h within a temperature-controlled room at 25±0.3°C (a temperature within the natural range of both species) and a 12 h light:12 h dark photoperiod. Active snails were then weighed and sacrificed, the tissues (foot and kidney) were dissected out, weighed and frozen in liquid nitrogen, and the extra-pallial fluid was collected and analyzed for total osmolality.

Sample processing for stress protein analysis
Frozen tissues were homogenized in ice-cold buffer containing 0.1 mol l−1 NaCl, 20 mmol l−1 Tris pH 7.4, 1 mmol l−1 EDTA, 1% Igepal, 1 mmol l−1 dithiothreitol (DTT), Protease Inhibitor Cocktail (Sigma P-8340, St Louis, MO, USA) and 1 mmol l−1 phenylmethylsulfonylfluoride (PMSF). The homogenate was centrifuged (10000 g, 15 min, 4°C) and total protein concentration measured using the BCA assay (Pierce) following the manufacturer’s recommendations. The total protein concentration was determined using the dye-binding assay (Bradford).

Western blotting
Western blot analysis was used to compare the relative levels of HSPs during aestivation and after arousal within each species and between the two species. 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. Separated gels were run for intraspecific (N = 10 for aestivation and N = 10 for activity) and interspecific (N = 10 for each species) comparisons. Proteins were separated using SDS-PAGE with a 10 or 12% acrylamide gel and transferred onto a nitrocellulose membrane (Pall Gelman Laboratory, Ann Arbor, MI, USA). The membranes were probed with a mouse monoclonal antibody against bovine brain Hsp70 (Sigma H-5147) that recognizes both the constitutive (Hsc70, 73 kDa) and inducible (Hsp70, 72 kDa) forms of mammalian Hsp70, a mouse monoclonal antibody against Hsp90 (Sigma H-1775) and a rabbit polyclonal antibody against chicken Hsp25 (a gift from Professor Geiger, Weizmann Institute, Israel). Hsp70 antigen (72 kDa, 250 ng) (StressMarq SPR-115A; Victoria, BC, Canada) and Hsp90 antigen (200 ng) (MBL, Woburn, MA, USA) were run alongside samples to serve as standards for analysis of Hsp70 and Hsp90. The secondary antibodies were goat anti-mouse IgG-HRP (Sigma A-2554) or goat anti-rabbit IgG-HRP (Santa Cruz Biotechnology sc-2004; Santa Cruz, CA, USA). In all of the westerns, the antibodies detected proteins within the linear range of detection. The proteins were visualized by enhanced chemiluminescence and the intensity of the bands was quantified using densitometry software (ImageJ).
The relative endogenous levels of HSPs during aestivation and after arousal (activity) in the two species are expressed as pixel intensities (mean values ± s.e.m.). Because all samples were analyzed on a single western blot, no standardization of band intensities was required. The significance was verified by unpaired t-test. In all cases P<0.05 was considered significant.

RESULTS

Expression of Hsp70

The relative endogenous level of Hsp70 was measured in the desert-inhabiting species *S. zonata* and the Mediterranean-type species *S. cariosa* during aestivation and after arousal (after the first rains). In the foot tissue of both species, the monoclonal antibody to Hsp70 detected two different forms of Hsp70, a constitutive form of Hsp72 (Fig.1A). The two species differed significantly in the induction of Hsp72 during aestivation (Fig.1B). During aestivation, the level of Hsp74 was significantly lower in *S. zonata* than in *S. cariosa* (<0.01), whereas in *S. cariosa* the levels of Hsp72 were significantly upregulated in both the kidney and the hepatopancreas tissues (P<0.001; Fig.1A,C). In both tissues, the induction of Hsp72 was stronger in *S. cariosa* (P<0.001).

Expression of Hsp90

In the foot tissue, no interspecific differences in the level of Hsp90 were found during aestivation and after arousal (Fig.2B). However, the two species differed significantly in the expression of Hsp90 in the kidney and in the hepatopancreas. In *S. zonata*, the levels of Hsp70 isoforms during aestivation and after arousal, compared with during aestivation (Hsp72, P<0.05 for *S. zonata* and P<0.001 for *S. cariosa*; Hsp74, P<0.001 for both species), but in *S. zonata* the induction of Hsp74 during aestivation was stronger (P<0.001; Fig.1A,C).

The change in HSP level after arousal in each species is expressed as the percentage change from aestivation (mean values ± s.e.m.). The significance was verified by unpaired t-test. In all cases P<0.05 was considered significant.

Statistics

The relative endogenous levels of Hsp70 was measured in the desert-inhabiting species *S. zonata* and the Mediterranean-type species *S. cariosa* during aestivation and after arousal (activity) in the two species are expressed as pixel intensities (mean values ± s.e.m.). Because all samples were analyzed on a single western blot, no standardization of band intensities was required. The significance was verified by unpaired t-test. In all cases P<0.05 was considered significant.

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Hsp90 were significantly higher than in *S. cariosa* in the kidney during aestivation and after arousal (*P*<0.001), and in the hepatopancreas during aestivation (*P*<0.05).

In both species, the level of Hsp90 in the foot tissue was significantly higher during aestivation than during activity (*P*<0.01 for *S. zonata*; *P*<0.001 for *S. cariosa*; Fig. 2A,C). In the kidney and the hepatopancreas, the level of Hsp90 in *S. zonata* remained unchanged after arousal compared with during aestivation, whereas in *S. cariosa*, the levels of Hsp90 were upregulated in the kidney (n.s.) and in the hepatopancreas (*P*<0.01). In both tissues, the induction of Hsp90 was stronger in *S. cariosa* than in *S. zonata* (*P*<0.001).

**Expression of small HSPs**

The tissues of both species expressed inducible low-molecular-mass proteins of approximately 25 and 30 kDa, and in *S. zonata* also of 27 kDa. In the *S. cariosa* foot, an additional band of 26 kDa was revealed. Although not significant, the level of Hsp25 in the *S. zonata* foot during aestivation was lower than that in *S. cariosa* (Fig. 3A). In addition, a strong-staining 26 kDa band was revealed during aestivation in the *S. cariosa* but not in the *S. zonata* foot. In the kidney and hepatopancreas, the endogenous levels of Hsp25 were significantly lower in *S. zonata* than in *S. cariosa* during aestivation and after arousal (*P*<0.001; Fig. 3B,C). The level of Hsp30 was significantly lower in *S. zonata* than in *S. cariosa* only in the hepatopancreas (*P*<0.05 during aestivation and *P*<0.001 after arousal); in the foot and the kidney, no interspecific differences in the level of Hsp30 were found.

As seen in Fig. 4, arousal induced a general upregulation of sHSPs in the foot, kidney and hepatopancreas tissues. In the foot tissue, the levels of Hsp25 and Hsp30 of both species and of Hsp27 in *S. zonata* were upregulated after arousal compared with during aestivation (Hsp25, *P*<0.05 for *S. zonata* and *P*<0.01 for *S. cariosa*; Hsp30, *P*<0.001 for both species; Hsp27, *P*<0.001 for *S. zonata*). In *S. cariosa*, the level of Hsp26 during aestivation was higher than the level of Hsp25 (about 3.6-fold) and it was downregulated after arousal (*P*<0.05). In the kidney, the levels of Hsp25 and Hsp27 in *S. zonata*, and of Hsp30 in both species, were upregulated after arousal compared with during aestivation (Hsp25, *P*<0.05 for *S. zonata*; Hsp27 and Hsp30, *P*<0.001). The induction of Hsp25 and of Hsp30 in the kidney was stronger in *S. zonata* (*P*<0.01). In the hepatopancreas, the levels of Hsp27 in *S. zonata* and of Hsp25 in *S. cariosa* were significantly upregulated after arousal compared with during aestivation (*P*<0.001). Both species expressed only in
The critical elements for long-term survival during aestivation are the hepatopancreas an additional band of approximately 17 kDa. In both species, the levels of Hsp30 and of Hsp17 were downregulated after arousal compared with during aestivation (P<0.001).

**Extra-pallial fluid osmolality**

The extra-pallial fluid was collected from snails in the field during aestivation and after arousal, and was analyzed for total osmolality. In both species, osmolality dropped significantly upon arousal from aestivation and after arousal, and was analyzed for total osmolality. The extra-pallial fluid was collected from snails in the field during both species, the levels of Hsp30 and of Hsp17 were downregulated on a damp substrate for 72h. After recovery, the levels of Hsp72 in the foot and kidney remained unchanged in S. zonata but increased significantly in S. cariosa (P<0.05), whereas Hsp74 sharply increased in S. zonata (P<0.01) yet only moderately increased (n.s.) in the S. cariosa foot (see supplementary material Fig.S1A). In both species, the level of Hsp90 after recovery was significantly upregulated in the kidney (P<0.05; see supplementary material Fig.S1B), but remained unchanged in the foot (data not shown). None of the small HSPs responded in S. cariosa. However, in S. zonata there was an upregulation of Hsp30 in the foot (P<0.05) and kidney (P<0.001), and of Hsp27 in the kidney (P<0.01; see supplementary material Fig.S1C).

**DISCUSSION**

Land snails are subject to annual cycles of activity and aestivation. The critical elements for long-term survival during aestivation are water retention and sufficient fuel reserves. Conservation of fuel reserves results from metabolic rate depression, one of the most important adaptations that support aestivation. The mechanisms of metabolic depression include the coordinated suppression of protein synthesis and degradation, and the enhancement of defense 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 (Brooks and Storey, 1995) suggested that, in *Otala lactea*, aestivation specific proteins (members of the 30, 50, 70 and 90kDa families) might be involved in the transition to a depressed metabolic state. Recently, Ramnanan et al. (Ramnanan et al., 2009) showed that the expression of some HSPs increased following two 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. (Reuner et al., 2008) found that, in the Mediterranean Grunt Snail (*Sphincterochila*), long-term laboratory aestivation had no effect on the level of both Hsp70 protein and mRNA in the foot tissue, although there was a trend that hsp70 mRNA was less abundant in aestivating specimens. 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 (Brooks and Storey, 1995), might be limited to the transition from an active to a depressed state, and might not be apparent in animals in the dormant state.

This suggestion is confirmed by our studies in the land snail *Sphincterochila*, which provide a wider insight into the aestivation process, indicating the involvement of the HSP machinery in both experimental desiccation and as part of the natural annual cycle of activity and aestivation (Mizrahi et al., 2010) (present study). However, in contrast to experimental desiccation, in which desiccation induced the expression of most tested HSPs (Mizrahi
et al., 2010), there was a general downregulation of HSP levels in aestivating compared with in active snails. The levels of Hsp70 and sHSPs in the foot, kidney and hepatopancreas, and of Hsp90 in the kidney and hepatopancreas were differentially upregulated after arousal compared with during aestivation, in a species-dependent pattern. These findings suggest that the observed upregulation of HSPs during experimental desiccation in Sphincterochila snails was transient and might be limited to the early stages following desiccation stress, as part of the survival strategy and also as an important component of the aestivation mechanism in the transition from activity to depressed metabolism. Under natural conditions, however, it seems that the transition from aestivation to activity might be more stressful than dormancy, leading to the activation of the HSP machinery. Similarly, Epperson et al. (Epperson et al., 2004) found lower levels of Hsp60, Hsp70 and Grp75 in golden-mantled ground squirrels during entrance into hibernation than during summer activity. The authors suggested that the animals were not under stress conditions during entrance into hibernation, and it might be that the early arousal period would find higher levels of these proteins that can dually function as protein folding chaperones and as defenders against oxidative stress.

In contrast to the general increase in the level of HSPs after arousal, the levels of Hsp90 in the foot were higher during aestivation, implying a role for this protein in stabilizing the aestivation state. Like the other major classes of HSPs, Hsp90 exhibits general protective chaperone activities, such as reducing the unspecific aggregation of unfolded proteins and the unspecific refolding of stress-denatured proteins. However, Hsp90 is distinguished from other chaperones in that it preferentially interacts with a specific subset of client proteins, including kinases, transcription factors (including steroid hormone receptors) and cell cycle regulators (Pratt and Toft, 2003), and has highly selective functions in normal metabolism. These dual functions enable Hsp90 to mediate between environmental conditions and their consequential physiological and biochemical changes. The involvement of Hsp90 in protein kinase activation and in steroid hormone signaling suggests an important role for Hsp90 in the control of metabolic depression in aestivating snails and perhaps in other hypometabolic states. The study of Brooks and Storey (Brooks and Storey, 1995) in O. lactea supports this suggestion, showing an increase in the 91 kDa stress protein in the hepatopancreas after two weeks of aestivation, and suggesting that it is specific to long-term aestivating animals.

**HSPs and oxidative stress**

The induction of the HSP response after arousal might come not only as a result of exposure to environmental stressors such as heat and aridity, but also because of the increase in metabolic rate and in oxygen consumption. The resulting rise in the rates of oxyradical production might increase oxidative stress and thus the need for HSPs for cytoprotection. Previous studies demonstrated the induction of Hsp70 and sHSPs in a number of conditions leading to oxidative stress, including renal ischemia, intestinal hypoxia-reoxygenation experiments (Gebhardt et al., 1999; Smoyer et al., 2000) and hypoxia exposure in Lymnaea stagnalis snails (Fei et al., 2007). In the surf clam Donax variabilis, hyperoxia induced the expression of Hsp70, whereas sulfide-induced oxidative damage induced the expression of sHSPs (Joyner-Matos et al., 2006). Hsp70 and Hsp27 have potential roles against cellular damage induced by oxidative stress (An et al., 2008; Giffard et al., 2004; Huot et al., 1995; Mehlen et al., 1995), Hsp70 probably by acting as a molecular chaperone, whereas the protective effects of sHSPs were related to...
Fig. 5. Extra-pallial fluid osmolality of *S. zonata* and *S. cariosa* during aestivation and after arousal. The extra-pallial fluid was collected from snails during aestivation and after arousal in the field and analyzed for total osmolality. Control groups of snails collected during aestivation were brought to the laboratory and allowed to recover on a damp substrate for 72 h. (A) Extra-pallial fluid osmolality during aestivation (Aest) and after arousal (Act) in the field. (B) Extra-pallial fluid osmolality during aestivation (Aest) and after recovery (Rec) in the laboratory. All values are expressed as means ± s.e.m. for *S. cariosa*, N=9–11 snails; for *S. zonata*, N=15 snails for the aestivation group and N=5 for the recovery group. Asterisks denote significant differences (**P<0.01; ***P<0.001).

Annual cycle of HSP expression in land snails

Vasconcelos and Hermes-Lima, 2003). A wide range of stress-tolerant animals were also found to display coordinated changes in antioxidant defenses that allow them to deal with the oxidative stress that occurs as part of natural cycles of stress/recovery. Hermes-Lima and Zenteno-Savin (Hermes-Lima and Zenteno-Savin, 2002) proposed that the activation of antioxidant defenses in organs of aestivating land snails and other animal species is a preparative mechanism against an oxidative stressful situation arising from tissue reoxygenation. It seems that, in this respect, *S. cariosa* is more sensitive than *S. zonata*, and therefore higher standing stocks of Hsp70 and of sHSPs are evident in its foot and hepatopancreas during aestivation. We have no data to confirm an upregulation of intracellular antioxidant enzymes in the examined species of the present study. However, we assume that these might be involved, together with the HSP machinery, in the cellular adaptive response recruited in the adaptation to environmental stress and in the annual transitions between aestivation and activity (Kultz, 2003).

**HSPs and osmoregulation**

Aestivators express physiological adaptations that defend the body from water loss during dormancy, including the elevation of body fluid osmolality via the production of high concentrations of solutes. In snail species, urea appears to be the dominant osmolyte that serves to aid in the retention of body water during aestivation and desiccation experiments (Arad, 2001; Horne, 1971; Rees and Hand, 1993). Upon rehydration, the high urea concentration in the soft body tissue facilitates water uptake. Arad and Avivi (Arad and Avivi, 1998) suggested that in land snails the transition from activity to aestivation activates the water-preserving mechanisms to their full capacity until the aestivation metabolism sets in. Arad (Arad, 2001) concluded that land snails use two distinct set points for body hydration: one is the level of osmotic and urea concentration in the body fluid after desiccation, and the other is the body hydration state after rehydration. Thus, during long-term aestivation, a new set point of water economy is established, in association with metabolic depression, and an extended period of moist contact is needed to change this set point. In the present study, the desert-adapted *S. zonata* exhibited higher extra-pallial fluid osmolality during aestivation than did *S. cariosa*. In addition, the endogenous levels of Hsp70 and Hsp90 in the kidney during aestivation were higher in *S. zonata* than in *S. cariosa*. The kidney is highly specialized for ionic and osmotic regulation, and the cells in the renal medulla are exposed to high levels of NaCl and urea. High NaCl and urea concentrations are stressful to cells, altering their function or even killing them by apoptosis (Burg et al., 2007).

The adaptive response of kidney cells to hyperosmolality includes the synthesis of different HSPs (e.g. Hsp72, sHSPs and the osmotic stress protein 94) (Beck et al., 2000; Borkan and Gullans, 2002). The increase in Hsp72 level correlates with cell survival (Neuhofer et al., 2001; Neuhofer et al., 1999; Santos et al., 1998), and constitutive expression of Hsp70 is associated with cell adaptation to chronic hyperosmolality (Santos et al., 2003). Hsp90, by contrast, is implicated in the signal transduction processes accompanying osmoregulation; for example, Hsp90 was found to be involved in sodium excretion by regulating the activity of mineralocorticoid receptors (Ramirez et al., 2004). Thus, the higher osmolality observed in aestivating *S. zonata* might explain the relatively high standing stocks of Hsp70 and Hsp90 in the kidney tissue of this species during aestivation. We suggest that these HSPs participate differentially in osmoregulatory processes, Hsp70 probably by acting as a molecular chaperone when cellular proteins are structurally damaged and Hsp90 possibly by its role in signal transduction. The
high standing stocks of Hsp70 and Hsp90 in the S. zonata kidney could probably suffice this species upon transition to the active state, as arousal induced the upregulation of Hsp70 and Hsp90 in the kidney of S. cariosa but not in S. zonata snails.

In both species the levels of sHSPs in the kidney were higher after arousal. sHSPs are abundant in renal medullas, and hypertonicity increases the expression of these proteins in cell culture. Neuhof et al. (Neuhof et al., 2005; Neuhof et al., 1998b) found differential expression of Hsp72 and Hsp27 in kidney cells exposed to osmotic stress, which suggested an alternative pathway for protection against high urea concentrations by Hsp27, possibly by cytoskeleton stabilization (Neuhof et al., 1998a; Smoyer et al., 2000). These findings suggest possible osmoregulatory roles for sHSPs also in the kidney of aestivating land snails.

**Cost, benefits and survival strategy**

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). Interspecific comparisons of ecotrophic species from different latitudes have shown that, typically, species adapted to higher temperature niches were more heat tolerant, had higher standing stocks of HSPs, induced the synthesis of HSPs at higher temperatures and had higher upper thermal limits of protein synthesis (Dong et al., 2008; Evgen’ev et al., 2007; Nakano and Iwama, 2002; Tomanek and Somero, 1999; Ulmasov et al., 1992). These studies suggest that organisms occupying extreme environments will employ a ‘preparative defense’ strategy involving maintenance of high constitutive levels of HSPs in their cells as a mechanism for protection against periods of extreme and unpredictable stress. However, according to Sørensen et al. (Sørensen et al., 2003), the expression level of HSPs in each species and population is a balance between benefits and costs, i.e. a negative impact on growth, development rate and fertility as a result of overexpression of HSPs (Feder, 1999; Krebs and Bettencourt, 1999; Krebs and Feder, 1997; Krebs and Loschcke, 1994; Sørensen et al., 1999). Thus, because HSP expression might incur fitness costs on individuals that regularly experience environmental stress, evolution in harsh environments might result in selection for reduced HSP expression.

Indeed, there are studies demonstrating lower levels of standing stocks of Hsp70 in the more thermally resistant species compared with the heat-sensitive ones (Bettencourt et al., 1999; Sørensen et al., 2001; Tomanek and Somero, 2002; Zatsepina et al., 2001). Our previous work (Mizrahi et al., 2010) supports this approach, demonstrating lower endogenous levels of Hsp70 in the desiccation-resistant S. zonata compared with the desiccation-sensitive S. cariosa. Notably, our current findings in naturally active and aestivating snails of S. cariosa and S. zonata verify their distinctive expression of Hsp70 previously found under non-stress laboratory conditions, suggesting that the desert-inhabiting S. zonata snails maintain smaller pools of Hsp70 (and also of sHSPs) also in nature, as part of their survival strategy.

The fitness consequences associated with maintenance of high standing stocks of HSPs could play a role in setting species’ distribution limits, and therefore species that regularly experience environmental stresses might adapt alternative mechanisms to cope with harsh conditions. Indeed, the desert inhabiting S. zonata seems to have developed a variety of effective regulatory mechanisms to withstand extreme environmental conditions (see Introduction). Our current work suggests that S. zonata developed a distinct strategy of HSP expression in the kidney that enhances the osmotic tolerance of kidney cells, facilitating their ability to withstand hyperosmotic conditions. This adaptation can enhance the ability of desert snails to retain water during aestivation and improve water uptake upon arousal.

In summary, to the best of our knowledge, this is the first study of natural variation in the heat-shock machinery in land snails. Our present study shows that HSP expression is definitely distinct between aestivation and activity, thus supporting the concept that in land snails, aestivation and activity represent two distinct physiological states. We suggest that land snails use HSPs as important components of the aestivation mechanism, and as part of their survival strategy after arousal. Our findings suggest that the stress protein machinery is upregulated during arousal in anticipation of possible oxidative stress ensuing from the accelerating metabolic rate and the exit from the deep hypometabolic state that characterizes aestivation. We suggest that Hsp70 and Hsp90 have different roles during aestivation and after arousal, Hsp70 probably by acting as a molecular chaperone when cellular proteins are structurally damaged and Hsp90 possibly by its role in signal transduction. Our study also indicates that adaptation to different habitats results in the development of distinct strategies of HSP expression. We suggest that desert-adapted snails developed distinct strategy of HSP expression in the kidney to improve their water economy, while maintaining lower standing stocks of sHSPs and Hsp70 in other tissues. This differential HSP response might reflect the relative vulnerability and the physiological function of different tissues, with likely consequences for the ecology and distribution of different land snails. In future studies, we intend to investigate the HSP response of S. cariosa and S. zonata to heat stress, and to a combination of heat stress and desiccation, in order to simulate seasonal natural fluctuations of environmental conditions.

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