J. Limnol., 2012; 71(1): 211-215
DOI: 10.4081/jlimnol.2012.e22

Heat shock proteins in encysted and anhydrobiotic eutardigrades

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ABSTRACT

Heat shock proteins (Hsps) can help organisms to survive environmental stresses. Tardigrades are aquatic metazoans able to colonize unpredictable, or "hostile to life", terrestrial habitats entering resting stages such as cysts and anhydrobiotic tuns. In this paper we compared the Hsp70 and Hsp90 expression between resting stages (tuns or cysts) and active hydrated specimens of two eutardigrade species, namely Bertolanius volubilis *and* Ramazzottius oberhaeuseri*. The two species partly differ in the kind of dormant stages utilized and in habitats colonized. In both species desiccation stress did not induce an up-regulation of either Hsps. Our data, together with those from literature, suggest that in tardigrades Hsps are involved in repairing molecular damages after anhydrobiosis, rather than in the stabilization of molecules during the dry state. Finally, the first demonstration of the presence of Hsps in diapausing cysts of* B. volubilis *are reported and discussed.*

Key words: Ramazzottius oberhaeuseri*,* Bertolanius volubilis*, dormancy, desiccation, encystment, Hsps.*

Received: June 2011. Accepted: August 2011.

INTRODUCTION

Dormancy involves a temporary suspension of active life, a reduction or suspension of metabolism and cessation of development. It can have profound ecological and evolutionary implications, affecting rates of adaptation to a varying environment (Hunter, and McNeil 1997) and contributing to biodiversity maintenance and genetic variability within populations (Ellner, and Hairston 1994). Dormancy can be subdivided into quiescence and diapause. Quiescence is under exogenous control, being directly induced and maintained by adverse environmental conditions and immediately reversed by removal of the external stimuli (Hand 1991). In contrast, diapause is under endogenous control, not being directly induced by environmental conditions but maintained by an internal physiological response. Its termination requires a specific cue that may not correspond to favorable environmental conditions (Hand 1991).

Tardigrades, or water bears, although little-known and neglected, are present in many environments. In terrestrial environments they frequently inhabit unstable habitats (*e.g.*, ephemeral aquatic habitats on the surface and interstice of lichens and mosses) and extreme or "hostile to life" habitats (*e.g.*, glaciers and deserts; Bertolani et al. 2004). Their persistence in these habitats is related to a wide variety of life histories and dormancy strategies (Guidetti et al. 2011). Encystment, cyclomorphosis and resting eggs are considered types of diapauses and are well documented in terrestrial and freshwater tardigrades (Hansen, and Katholm 2002; Guidetti et al. 2006, 2008, 2011; Møbjerg et al. 2007; Halberg et al. 2009; Altiero et al. 2010). Encystment involves the synthesis of several cuticular layers, a reduction in metabolism, and the loss of a small amount of body water (Hansen, and Katholm 2002; Guidetti et al. 2006, 2008, 2011). Anhydrobiosis and cryobiosis are types of quiescence and are widespread in terrestrial tardigrades (Wright 2001; Rebecchi et al. 2007; Schill 2010; Guidetti et al. 2011). Under desiccation stress, semiterrestrial tardigrades contract their body longitudinally into a tun, lose most of their free and bound water and reduce or suspend their metabolism. In this anhydrobiotic state tardigrades can persist for months, or even for years, and exhibit a remarkable resilience to physical and chemical extremes (for a review see Rebecchi et al. 2007; Schill 2010; Guidetti et al. 2011), including outer space (Jönsson et al. 2008; Rebecchi et al. 2009a, 2011; Persson et al. 2011).

Activation of the synthesis of heat shock proteins (Hsps) is a ubiquitous adaptive mechanism in organisms ranging from bacteria to plants and animals that helps them survive and adapt to a wide range of environmental stresses. Hsps are rapidly up-regulated in response to environmental insults such as temperature extremes, anoxia, various chemical constraints and desiccation (Feder, and Hofmann 1999). Moreover, Hsps can be important for populations that are exposed to variable environments, including occasional stress exposure (Sørensen et al. 2003). Molecular studies have indicated that the genes encoding Hsps are highly conserved and occur in every species studied. Many of these genes and their products can be as-

signed to families on the basis of sequence homology and typical molecular weight (Feder, and Hofmann 1999). The Hsp70 family is the largest and most conserved group of heat shock proteins and consists of inducible (Hsp) and constitutive (heat shock cognate, Hsc) forms that can be activated and/or increased in response to environmental insults (Feder, and Hofmann 1999). Hsp70 proteins function as molecular chaperones and play a primary role in folding, assembly, intracellular localization, secretion, and degradation of other damaged proteins. Hsp90 proteins are essential molecular chaperones involved in signal transduction, cell-cycle control, stress management, folding, degradation and transports of proteins and have a specific function during recovery from stress, being especially important in the reactivation of stress-inactivated protein (Gkouvitsas et al. 2009). Hsp90 genes are also implicated in developmental regulation and diapause in insects and during the dauer stage of nematodes (Dalley, and Golomb 1992; Marcus 2001; Sonoda et al. 2006).

In extremely desiccation-tolerant organisms, Hsps have not been investigated very much, but their strong connection to several stresses and documented association with cell membranes suggests that they may play a role in the desiccation tolerance mechanism. Nevertheless their role in anhydrobiotic tardigrades has not yet been firmly established and some controversy due to a variable pattern of gene expression were found (Ramløv, and Westh 2001; Schill et al. 2004; Jönsson, and Schill 2007; Förster et al. 2009; Rebecchi et al. 2009a; Reuner et al. 2010; Rizzo et al. 2010; Grohme et al. 2011), while their presence in encysted tardigrades has never been analysed. Consequently, in this paper we compare the expression of selected heat shock proteins (Hsp70 and Hsp90) in dormant (both anhydrobiotic and encysted specimens) and in active hydrated animals of two eutardigrade species. Target species were chosen for the difference in their ability to produce various dormant stages and to colonize unpredictable terrestrial habitats.

MATERIALS AND METHODS

The two eutardigrade species studied are the mossdwelling *Bertolanius volubilis* (Durante Pasa, and Maucci, 1975) (Eohypsibiidae) and the lichen-dwelling *Ramazzottius oberhaeuseri* (Doyère, 1840) (Ramazzottiidae). Specimens of *B. volubilis* were extracted from the mosses *Racomitrium sudeticum* (Funck) Brunch and Schimp and Racomitrium elongatum (Ehrh.) ex Frisvoll growing on sandstone located in a post-glacial valley of the Northern Apennines (Rondinaio Mountain, Modena, Italy; N44°7.421 E010°35.222; 1670 m a.s.l.). Specimens of *R. oberhaeuseri* were extracted from patches of the lichen *Xanthoria parietina* (L.) Th. Fr. growing on a large basaltic outcrop located in the Northern Apennines (Sassomorello, Modena, Italy; N44° 25' E10° 44'; 765 m a.s.l.).

Specimens of *B. volubilis* are able to enter anhydrobiosis and to form cysts. Two types of cysts are produced, which show opposite seasonal trends. The type 2 cysts are the most abundant and are produced only during the warm season while the type 1 cysts are very scarce and are present only during the cold season (Guidetti et al. 2006, 2008, 2011). Consequently for *B. volubilis* three different physiological conditions, namely hydrated (active) and desiccated (anhydrobiotic) animals and type 2 cysts (diapause) were used. Since *R. oberhaeuseri* specimens are only able to enter anhydrobiosis (Rebecchi et al. 2006), only hydrated (active) and desiccated (anhydrobiotic) animals were studied.

To extract tardigrades from their substrate, the lichen and mosses were slowly hydrated. Initially substrates were sprinkled with tap water and after 15 min submerged in tap water for 15 min. Then, tardigrades (active or encysted) were extracted from the substrates by means of sieves under running water. Collected material was washed into a Petri dish, where the animals were picked up with a glass pipette under a stereomicroscope.

The type 2 cysts of *B. volubilis* were collected from mosses, maintained in mineral water at 14°C for 24 hours and then used for the biochemical assays. After collection, active (hydrated) specimens of both species were kept in Petri dishes with natural mineral water without any food source for 24 h at 14°C. Then, some of these animals were used to obtain desiccated (anhydrobiotic) animals while others were used for the biochemical assays.

Tardigrades were forced into anhydrobiosis following the protocol used by Rebecchi et al. (2009b). Then, desiccated anhydrobiotic tardigrades are ready for biochemical assays or for evaluation of their survival.

Coordinated and active movements of the body (locomotion behaviour) constituted the criterion to evaluate tardigrade viability. To evaluate tardigrade survival, desiccated animals were slowly hydrated under controlled conditions following the protocol by Rebecchi et al. (2009b). For both species, viability tests were run with 6 replicates, each with 10 desiccated tardigrades.

For both species Hsps level was determined in replicates each with 70 desiccated specimens and in replicates each with 60 hydrated animals. In addition, for *B. volubilis* replicates each with 50 type 2 cysts were used. The expression of Hsp70 was studied in all physiological states of both species, while the expression of Hsp90 was determined only for the three physiological states of *B. volubilis*. For the Hsps expression assay the protocol described in Rebecchi et al. (2009a) and Rizzo et al. (2010) was used. Data on the relative level of Hsp70 and Hsp90 within each species were compared with the Kruskal-Wallis test and pairwise with the Mann-Whitney test. The software program SPSS Version 16.0 was used.

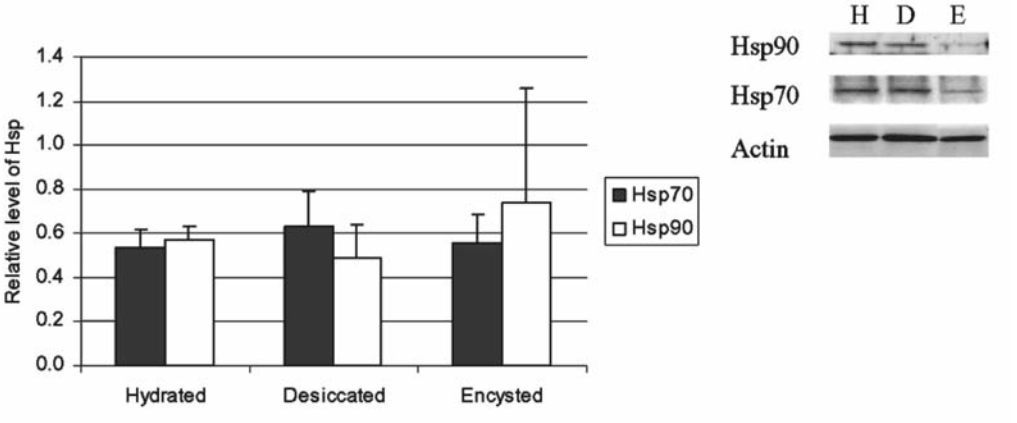

Fig. 1. Representative western blot and relative levels of Hsp70 and Hsp90 in *Bertolanius volubilis*. The bars show the mean of 7 replicates±S.E., except for encysted animals in which the number of replicates was 3.

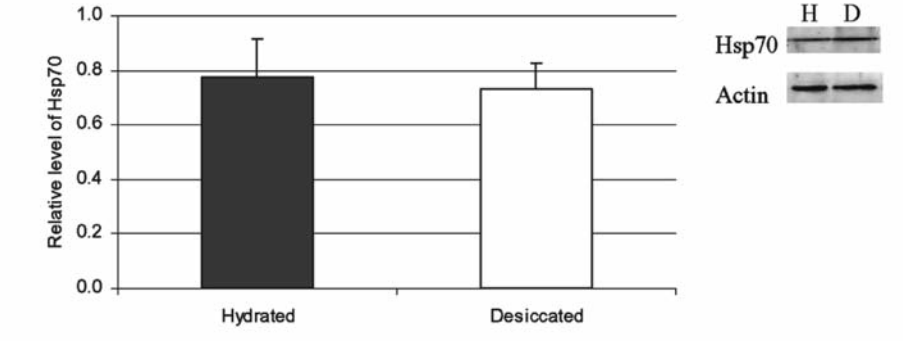

Fig. 2. Representative western blot and relative levels of Hsp70 in *Ramazzottius oberhaeuseri*. The bars show the mean of 7 replicates±S.E. for desiccated specimens and the mean of 6 replicates±S.E. for hydrated ones.

RESULTS

Desiccated specimens of *Bertolanius volubilis* and *Ramazzottius oberhaeuseri* showed a survival rate of 100% at both 1 h and 24 h after rehydration.

Relative levels of Hsp70 and Hsp90 of hydrated, desiccated and encysted (type 2 cyst) specimens of *B. volubilis* and the relative levels of Hsp70 of hydrated and desiccated specimens of *R. oberhaeuseri* are reported, respectively, in Figs 1 and 2. Specimens of *B. volubilis* in all physiological states were analysed (hydrated, desiccated and encysted) and were shown to express both Hsp70 and Hsp90. The relative levels of both Hsp70 and Hsp90 were statistically similar (Hsp70: χ^2=0.011, *df*=2; *p*=0.9994; Hsp90: χ^2=1.154, *df*=2; *p*=0.562) among hydrated, desiccated and encysted ("type 2" cyst) specimens of *B. volubilis*. Similarly, Hsp70 was detected in both physiological states (hydrated and desiccated) of *R. oberhaeuseri*. No statistical difference (*p*>0.05) in the relative levels of Hsp70 expression between hydrated and desiccated specimens of *R. oberhaeuseri* was detected.

DISCUSSION

This study provides further information on Heat shock protein families (70 and 90 kDa) in desiccated (anhydrobiotic) tardigrades and the first demonstration of the presence of Hsps in diapausing encysted tardigrades.

In both highly desiccation-tolerant eutardigrades *B. volubilis* and *R. oberhaeuseri*, the dehydration stress did not induce an increase in Hsp70 expression. This datum is in line with those recorded in two other highly desiccation-tolerant eutardigrades, namely *Richtersius coronifer* (Richters, 1903) and *Paramacrobiotus richtersi* (Murray, 1911) (Ramløw, and Westh 2001; Jönsson, and Schill 2007; Rizzo et al. 2010). In the moss-dwelling *R. coronifer*, desiccated animals exhibited a lower level of Hsp70 with respect to hydrated ones, with a clear induction of Hsp70 only one hour after rehydration of the animals (Jönsson, and Schill 2007). In another desiccation-tolerant eutardigrade, *Milnesium tardigradum* Doyère, 1840, only the gene transcript of one of the three detected hsp70 isoforms was significantly up-regulated in the transitional

stage between active and anhydrobiotic stages (Schill et al. 2004; Reuner et al. 2009). In regard to Hsp90, our data on *B. volubilis*, are in line with those obtained by Rizzo et al. (2010) on *P. richtersi*, and indicate that dehydration also does not induce an increase of Hsp90. In *M. tardigradum* a high level of hsp90 mRNA was detected during the permanence in the anhydrobiotic state, which decreased during the rehydration phase because this hsp90 mRNA could be translated into proteins (Reuner et al. 2009). Similarly, in the flesh fly *Sarcophaga crassipalpis* MacQuart, 1839, both Hsp90 and Hsc70 were up-regulated when the pupae were rehydrated following a period of dehydration (Hayward et al. 2004). Data collected till now on tardigrades suggest that Hsps may be involved in the biochemical mechanism utilized during rehydration rather than that connected to the stabilization of molecules in a dry state (Guidetti et al. 2011). During rehydration, Hsps could be involved in repairing molecular damages that occurred during the anhydrobiotic process as demonstrated by Neumann et al. (2009) and Rebecchi et al. (2009b). Similarly, in the moss *Tortula ruralis* [Hedw.] Gaerten., Meyer, and Scherb. most of the molecular repair mechanisms are thought to be initiated during rehydration rather than desiccation (Oliver 1991; Bewley, and Oliver 1992). A similar pattern was detected for antioxidant metabolism in which the high level of antioxidant enzyme systems detected in desiccated specimens of *P. richtersi* are accumulated during the entering phase of the anhydrobiotic process and then used during the rehydration phase when the metabolism is active (Rizzo et al. 2010).

Encystment represents one of the enigmatic dormancy states of tardigrades and the causes of its induction and evolution remain still unclear (Guidetti et al. 2008). Even though several morphological changes occur during the encystment process (Guidetti et al. 2006) almost nothing is known about its physiology. For the first time, our data revealed that both Hsp70 and Hsp90 are not up-regulated in encysted specimens of *B. volubilis* with respect to desiccated or hydrated specimens. This absence could be due to the fact that tardigrades undergo encystment in advance to environmental adversities (Guidetti et al. 2008) so they probably are not in a stressed state facing stressful conditions that need high level of Hsps. To protect themselves from the coming environmental stresses (related to summer period), and therefore to maintain their homeostasis, the tardigrade cysts minimize exchange with their environment by remaining within their old cuticles (exuvia) and producing further cuticular layers (Guidetti et al. 2006).

Finally, considering the importance of Hsps in biochemical systems (Sørensen et al. 2003), their constant presence in hydrated tardigrades allow them to withstand the changing conditions of their unpredictable habitats promptly, providing an important role in the maintenance of homeostasis across environmental regimes.

CONCLUSIONS

The functional role of Hsps in tardigrade dormant states is still enigmatic and requires more studies towards the understanding the biochemical mechanisms involved in the production and maintenance of resting stages evolved in organisms able to survive in "hostile to life" habitats. However, as it has become more and more evident in other desiccation-tolerant organisms (Goyal et al. 2005), the biochemistry of anhydrobiosis is bound to a complex system involving many different molecular components (*e.g.*, sugar, Hsps and LEA proteins, see Wełnicz et al. 2011) working together.

ACKNOWLEDGMENTS

Tiziana Altiero and Roberto Guidetti contributed equally to the work. The authors wish to thank Roberto Bertolani (University of Modena and Reggio Emilia, Italy) for critical discussion of the manuscript and Diane Nelson (East Tennessee State University, USA) for great courtesy in revising the English of the manuscript. This research is supported by a grant from University of Modena and Reggio Emilia (ex 60% to L.R.) and by a grant from the "Fondazione Cassa di Risparmio di Modena", Modena, Italy.

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