Cell Stress and Chaperones (2013) 18:405–414
DOI 10.1007/s12192-012-0393-8

ORIGINAL PAPER

Daily and seasonal changes in heat exposure and the Hsp70 level of individuals from a field population of *Xeropicta derbentina* (Krynicki 1836) (Pulmonata, Hygromiidae) in Southern France

A. Dieterich · U. Fischbach · M. Ludwig · M. A. Di Lellis · S. Troschinski · U. Gärtner · R. Triebskorn · H. -R. Köhler

Received: 17 October 2012 / Revised: 29 November 2012 / Accepted: 30 November 2012 / Published online: 20 December 2012

Abstract The Mediterranean land snail *Xeropicta derbentina* forms huge populations in Southern France. In order to characterize heat exposure and the induction of the 70-kD heat shock protein (Hsp70) response system during the life cycle of this snail, a selected population from the Vaucluse area, Provence, was investigated encompassing the issues of morphological life cycle parameters (shell size and colouration), the daily courses of heat exposure at different heights above the ground, of shell temperature, and that of the individual Hsp70 levels. The study covered all four seasons of the year 2011. Snails were found to be annual, reaching their final size in August. The shell colouration pattern showed high variation in juveniles (spring) with a strong tendency towards becoming uniformly white at old age in autumn. In all seasons, ambient air temperature decreased with increasing distance from the ground surface during daytime while remaining constantly low in the night. Overall, the Hsp70 level of individuals followed the ambient temperature during diurnal and seasonal variations. Correlation analysis revealed a positive association of individual shell temperature and Hsp70 level for the most part of the life cycle of the snails until late summer, whereas a negative correlation was found for aged animals indicating senescence effects on the capacity of the stress response system.

Keywords Heat shock response · Mediterranean land snail · Stress proteins · Temperature · Life cycle

A. Dieterich (✉) · M. A. Di Lellis · S. Troschinski · R. Triebskorn · H. -R. Köhler
Animal Physiological Ecology, University of Tübingen, Konrad-Adenauer-Str. 20, 72072 Tübingen, Germany
e-mail: A-Dieterich@gmx.de

U. Fischbach · M. Ludwig · U. Gärtner
Institute of Applied Research, University of Applied Sciences, Kanalstr. 33, 73728 Esslingen, Germany

Introduction

Climbing vertical structures to avoid lethal ground temperatures is a common and frequently recognized adaptive behaviour of land snails to their environment (Aubry et al. 2006; Kiss et al. 2005; Storey 2002). Besides other behavioural adaptations like burrowing in the soil during the day or hiding beneath fallen leaves, climbing is one of the most obvious responses of snails to adverse conditions in the field during daytime. Measurements of the ground temperature and several centimetres above show a dramatic decrease of the air temperature even a few centimetres above the ground (Köhler et al. 2009). Shifting the activity to the cooler and moister night hours is another common behaviour of land snails in their response to hot environments (Abdel-Rehim 1983; Di Lellis et al. 2012). In the climate of Southern France with hot and dry summers, ground temperatures frequently reach 50 °C and more. For snails that consist of roughly 75 % water (Reuner et al. 2008), such temperatures are lethal (Dittbrenner et al. 2009).

In Southern France, the land snail *Xeropicta derbentina* (Krynicki 1836) (Gastropoda, Hygromiidae) is an introduced species originating from the Eastern Mediterranean. First records in France date from 1949 (Altena 1960; Kiss et al. 2005; Aubry et al. 2006). Adults of *X. derbentina* reach

shell sizes ranging between 10 and 16 mm in diameter, and are generally characterized by a uniformly white shell. Nevertheless, different colour morphs can be found in the field especially in younger stages. Populations of *X. derbentina* may differ in morph composition, and different morphs were also shown to vary slightly in their heat response (Di Lellis et al. 2012). *X. derbentina* is quite often found in areas that are or at least were used for agricultural purposes (Aubry et al. 2005). Especially in open fields with scarce vegetation, at the border of agricultural areas, and along roads *X. derbentina* can be found in large numbers resting at the top of grass-blades or other vegetation—sometimes forming enormous clusters of hundreds of individuals at a single spot. The climbing behaviour protects the snail from potentially lethal temperatures of the soil in summer even though ambient temperatures frequently exceed 40 °C for several hours a day. This climbing behaviour is most likely responsible for the rapid spread of *X. derbentina* in France as snails resting on vehicles disperse rapidly along small roads (Aubry et al. 2006). Apart from the passive means of transport, the movement of these animals is extremely limited during the day. Once they have climbed up vertically, they remain in the sunlight until sunset. Consequently, *X. derbentina* cannot avoid extreme temperatures during hot summer days and, therefore, has to deal with the experienced high temperature in a different way to avoid overheating and desiccation.

Being confronted with thermal stress, almost all organisms investigated so far are able to produce heat shock proteins (=stress proteins, Hsps) to counteract this and other stresses (Feder and Hofmann 1999; Sørensen et al. 2003; Kiang and Tsokos 1998) with the exception of some Antarctic fish (Hofmann et al. 2000). Hsps are considered part of an intracellular defence machinery that also includes other physiological mechanisms protecting the cells from damage and denaturation of proteins. The best investigated Hsp family is that of Hsp70 (Mayer and Bukau 2005; Daugaard et al. 2007). These structurally highly conserved proteins act as molecular chaperones that assist in folding newly produced proteins. Also increasing amounts of misfolded proteins inside the cell due to heat-induced denaturation or other stresses, induce the production of Hsp70 proteins (Daugaard et al. 2007; Sørensen et al. 2003). Therefore, the increased concentration of Hsp70 proteins can be used as a marker of effect for proteotoxicity. This marker is frequently used in studies examining the tolerance of organisms against heat (Tomanek and Sanford 2003; Nakano and Iwama 2002; Dittbrenner et al. 2009; Di Lellis et al. 2012; Köhler 2009; Köhler et al. 2009). As a marker of effect, rising Hsp70 levels can be interpreted as a response to the effects of heat. With respect to proteotoxic stress, Hsp70 induction follows a distinct reaction curve (Eckwert et al. 1997; Tomanek 2002). Starting with a base level that is expressed under "normal" conditions, the curve rises with increasing stress. When proteotoxic stress reaches a distinct (and population specific) level, no further induction is possible (Arts et al. 2004; Köhler et al. 2009). Exceeding this point of stress leads to a collapse of the Hsp70 protection system revealed in a rapid decrease in the Hsp70 level, followed by the death of the organism or, at least serious damage of its inner structures (Eckwert et al. 1997; Scheil et al. 2011).

As found in helicoid land snails (Dittbrenner et al. 2009; Scheil et al. 2011), Hsp70 clearly increases when the animals heat up. Apart from the intensity of stress affecting the increase of the Hsp70 level in the organism, the exposed life stage also influences the degree of Hsp70 induction. Young or larval stages are especially known to be able to induce Hsp70 to a higher degree than older or senescent organisms (Mayer and Bukau 2005; Köhler 2009). Furthermore, it was shown that Hsp70 levels varied on a seasonal basis, monthly, or even on a daily scale (Nakano and Iwama 2002; Tomanek and Sanford 2003; Schill et al. 2002; Köhler et al. 2001). The induction of Hsp70 was found to vary depending on the environmental conditions the species or a specific population encountered. For example, two closely related *Sphincterochila* species from two different habitats (Mediterranean vs. desert) expressed different levels of Hsp70 when they were exposed to adverse conditions, reflecting a pre-adaptation to their environment (Arad et al. 2010; Mizrahi et al. 2010, 2012).

To date, little is known about the diurnal changes in the Hsp70 level under field conditions in different seasons of a year, particularly for animals living in non-aquatic systems. We investigated a selected population of *X. derbentina* in respect to the daily course of their Hsp70 level in four different months of 2011. Furthermore, we continuously recorded the ambient temperature at different heights over ground during all samplings. According to the known heat-inducibility of stress proteins, we expected the Hsp70 level of the snails to correspond to the external temperature profile recorded in the field. Investigations covered different months and, consequently, different life stages of this annual species. Our aim was to provide a solid data basis to estimate the severity of heat stress and the capacities of the Hsp70 system to counteract this stress during the life-cycle of this annual land snail species.

Materials and methods

Test organism

In this study, *X. derbentina* (Krynicki 1836), a hygromiid land snail, was investigated. All samples of *X. derbentina* were collected from a meadow in the vicinity of Modène,

department Vaucluse, Southern France (N44°4.034′ E5° 11.041′). Samples were taken randomly from this population. The sampling site was not used agriculturally and no pesticides were applied by the owner. Sampling took place during four different months in 2011 to make sure that different climatic conditions were present during sampling and different life stages of *X. derbentina* could be collected. Samples were taken on April 18, June 13, August 30, and October 17, 2011. All samples were taken on sunny days with none to only little cloudiness. In April, ten snails were collected hourly and individually submerged in liquid nitrogen after recording the following parameters: (a) the heights at which individuals were resting, measured with a yard stick, (b) the temperatures at the surface of their shells, in the middle of the first whorl, that was exposed to the sun, using a medical precision thermometer (ELLAB Copenhagen, type DM 825), (c) the shell diameter using a digital calliper, and (d) the patterns of shell colouration as introduced by Köhler et al. (2009). For *X. derbentina*, colour category 1 consisted of white shells only, while in category 2 animals with a single small black or brown band near the umbilicus or a brownish shell colour at the umbilicus side of the shell were grouped. Category 3 snails bore two or more bands near the umbilicus or one large intensely pigmented stripe on the umbilicus side of the shell. Snails that were classified into category 4 showed bands all over the shell as well as on its upper part, in the vicinity of the protoconch. It was avoided to touch snails during steps 1 and 2 of the above-mentioned field measurements to prevent artefacts. All snails taken for the Hsp70 analysis were collected from heights ranging between 5 and 20 cm above ground. In June, samples were taken the same way as in April between 4 am and 11 pm, in August, from 4 am to 10 pm, and in October from 5 am till 12 pm. Morphological species determination of samples from this population were carried out by W. Rähle, University of Tübingen, Germany and E. Gittenberger, University of Leiden, the Netherlands. Genetic determination based on COI gene sequencing was performed by S. Sereda and T. Wilke, University of Giessen, Germany.

Hsp70 analysis

For Hsp70 analysis, only individuals which have been resting between 5 and 20 cm above the ground were taken. The individually frozen samples were homogenized with appropriate volumes of extraction buffer (80 mM potassium acetate, 5 mM magnesium acetate, 20 mM Hepes, and 2 % protease inhibitor at pH 7.5) according to their body weight including the shells. All homogenization steps were performed on crushed ice to prevent degradation of proteins. The samples were centrifuged for 10 min at 13,722 rpm (=20,000 rcf) using an Eppendorf Centrifuge 5804R at 4 °C. The protein content of the supernatants was determined according to Bradford (1976) using 96-well plates and a plate reader (Bio-Tek Instruments, Winooski, VT, USA). Total protein (10–40 μg/sample, depending on the intensity of resulting Hsp70 bands in preliminary analyses) was analysed using minigel SDS-PAGE (12 % acrylamide, 0.12 % bisacrylamide, 30′ at 80 V plus 90′ at 120 V). The proteins were transferred to nitrocellulose membranes by semi-dry electrotransfer. After incubation in blocking solution (50 % horse serum in TBS) for 2 h, the nitrocellulose membranes were incubated with the first monoclonal α-Hsp70 antibody (mouse anti-human Hsp70, Dianova, Hamburg, Germany, dilution 1:5,000 in 10 % horse serum / TBS) on a lab shaker at room temperature overnight. This antibody cross-reacts with all isoforms of the Hsp70 family. To remove surplus Hsp70 antibodies, the nitrocellulose membranes were rinsed in TBS for five minutes. After 2 h of incubation with the secondary antibody (goat anti-mouse IgG conjugated to peroxidase, Jackson Immunoresearch, West Grove, PA, dilution 1:1,000 in 10 % horse serum/TBS) the nitrocellulose membranes were rinsed again for 5 min in TBS. Subsequently, the membranes were stained in a solution containing 1 mM 4-chloro(1)naphthol, 0.015 % H_2O_2, 30 mM Tris pH 8.5 and 6 % methanol. Digitalization of the nitrocellulose membranes was carried out using an Epson Perfection V350 Photo scanner. For each band, the optical volume (= band area × average grey scale value) was calculated with E.A.S.Y. Win 32 (Herolab, Wiesloch, Germany). The optical volumes of the bands were related to a standard sample containing supernatant of full body extract of *Theba pisana* (Müller 1774) snails. In each minigel SDS-PAGE, this standard sample was run in duplicate. All data (means±standard deviations) were calculated by ten individuals.

Additional sampling for field distribution and colouring

In addition to the samples taken for Hsp70 analysis, 250 individuals were randomly collected from a randomly chosen area of 1×3 m in the same meadow at each sampling event. For each individual, the pattern of shell colouration, the shell diameter, and the position (height above the ground) was recorded. The shell temperature was not recorded here as these additional samples were exclusively used for investigations on the shell growth and colouration patterns.

Recording of temperature at different heights

During the time of each sampling event, the ambient temperatures were recorded in ten different heights simultaneously. For this purpose, Type T thermocouples were placed 1, 2, 3, 5, 10, 15, 20, 25, 30, and 40 cm above the ground using a wooden stand. Each sensor was read out every 15 seconds using a multi-channel data logger (Agilent

34972A). In April, these measurements were carried out by hand using a medical precision thermometer (ELLAB Copenhagen, type DM 825) and a yard stick. In order to condense these data, hourly mean temperatures were calculated for each height.

Statistics

All data were checked for normal distribution using the Shapiro-Wilk W-Test in JMP 9.0.0. (SAS Institute Inc.). Since the data were not normally distributed, non-parametric tests had to be applied. To compare sample sets describing the change of shell diameter during the year, individual Wilcoxon tests were performed between all examined months. Correction for multiple testing was accomplished by adjusting the significance level according to Bonferroni. The resulting α-level was 0.0083. Correlation between the parameters Hsp70 level, shell temperature, shell diameter, and climbing height were performed using SAS JMP 9.0.0. A Spearman's ρ test was performed to check for significance and α-levels were also corrected according to Bonferroni as mentioned above.

Results

Shell growth and colouration

During the 4 days of sampling in 2011, a total number of 1996 individuals were examined. In the course of the year, a significant increase in shell diameter was observed between April (4.62±1.08 mm, n=490), June (9.99±1.30 mm, n=538), and August (13.35±0.98 mm, n=478; all, Wilcoxon, p<0.0001). In October (12.90±0.95 mm, n=490), a slight but significant decrease in shell diameter, compared to August, was found (Wilcoxon, p<0.0001, Fig. 1).

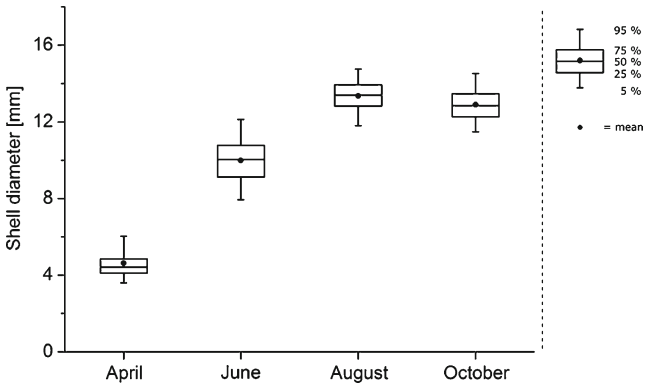

Fig. 1 Increase in shell diameter of samples taken in 2011. *Boxes* indicate 25 %, 50 %, and 75 % percentiles of all samples taken during the corresponding sampling day. *Black dots*=mean shell diameter, *whiskers*=5 % and 95 % percentiles

In addition to the observed increase of the shell diameter, snails tended to have paler shell patterns in the course of the year. Although a mixture of the pre-defined categories could be found in April, where category 3 was the predominant colouration (55 % of the total observed snails), almost the entire population displayed a pure white shell in August (96 %) and October (97 %) which was classified as shell pattern category 1. In June, an intermediate situation was present. Compared to the observations from April, a strong increase in the frequency of category 1 snails (78 % category 1 in June vs. 11 % in April) could be found. On the other hand, the number of snails categorized into category 3 decreased from 55 % in April to 5 % in June. The composition of shell patterns in all four samplings is shown in Table 1.

Hsp70 induction and ambient parameters

In June, the lowest measured temperature 5 cm above the soil surface was 13.2 °C measured at 4 am. The maximum temperature at the same height was 32.9 °C at 2 pm. In August the temperature 5 cm above ground ranged from 10.4 °C (6 am) to 33.7 °C (3 pm). In these 2 months, the temperature exceeded 30 °C during the day which made conditions different from those in April and October. In April the lowest temperature of all samplings was measured. At a height of 5 cm above the soil surface, it was found to be 4.8 °C (4 am). The maximum temperature at this height in April was 27.3 °C (5 pm). In October, the temperature in 5 cm above ground varied from 7.6 °C (7 am) to 23.0 °C (3 pm). In all months, an increase of air temperature after sunrise was observed as well as a decrease after sunset. By comparing the temperature at different heights, a gradient with decreasing temperatures at increasing heights above the ground was found to be established during the day. At night and during sunrise and sunset, only little temperature differences were recorded at different heights. In April, sunrise was roughly at 6:30 am and sunset roughly at 8:30 pm. In June, sunrise took place around 6 am and sunset around 9:30 pm. In August, sunrise took place at approximately 7 am and sunset at 8:30 pm. In October, sunrise took place at roughly 8 am and sunset at 7 pm. On June 13th, a sudden decrease in ambient temperature was recorded at all heights at 4 pm. At this time, clouds temporarily covered the sky and ambient temperature decreased transiently. Five centimetres above the ground the overall mean temperature of the sampling day in April was calculated to be 14.1 °C, in June 22.3 °C, in August 22.9 °C, and in October 14.7 °C. Temperatures at heights between 1 and 30 cm above ground are presented in Fig. 2 for each sampling.

The daily course of shell temperature largely reflects the course of ambient temperature. A daily increase in shell temperature with progressively increasing time of exposure

Table 1 Percentage of colour morphs in the selected *X. derbentina* population in four different months in 2011

Month	Category 1 [%]	Category 2 [%]	Category 3 [%]	Category 4 [%]
April (n=490)	11	30	55	4
June (n=538)	78	15	5	2
August (n=478)	96	3	0	1
October (n=490)	97	3	0	0

to solar irradiation was also recorded in all months, as well as a decrease in shell temperature after sunset (Fig. 3). In general, shell temperatures were higher even at night, in June and August compared to the other months.

The analysis of our samples revealed differences between the 4 months of sampling, and even during a single day, changes in Hsp70 induction were found (Fig. 4). Our study showed that, in general, hot months lead to higher Hsp70 levels in *X. derbentina*. In April, a slight increase in the Hsp70 level was revealed from sunrise until noon. The highest relative Hsp70 level in April, however, was just 1.2. In June, the course of the Hsp70 level followed the increase of ambient temperatures in the morning and the decrease of ambient temperatures in the evening (Fig. 5). In addition, a secondary peak of Hsp70 expression was found at night, which decreased again at around midnight. The highest relative Hsp70 level in June was 2.7. In the samples taken in August, the highest Hsp70 level was 4.4 which was also the maximum recorded for the entire year. Again, an increase of Hsp70 was recorded at sunrise and in the morning when ambient temperatures rose. Except for a relatively low value at 1 pm, a steady increase of Hsp70 levels could be observed till sunset. After sunset, the Hsp70 levels decreased again. In contrast to the other months, samples taken in October did not show any increase in the Hsp70 level during the day. Instead of an increase in the Hsp70 level that follows the ambient temperature, a slight decrease was observed particularly from sunrise until noon. Subsequently, Hsp70 levels rose again at the end of the day until midnight. The highest measured relative Hsp70 level in October was 0.7, even lower than in spring.

In April, June, and August, a significant positive correlation between Hsp70 level and shell temperature was found (Spearman's ρ; April, rho=0.3380, n=236, p<0.0001; June, rho=0.5339, n=209, p<0.0001; August, rho=0.3143, n=190, p<0.0001) whereas in October a negative correlation between these two factors was found (Spearman's ρ; rho=−0.3328, n=200, p<0.0001). Compared to the other months of sampling, the majority of the Hsp70 levels measured in October were below those of the other months (Fig.6).

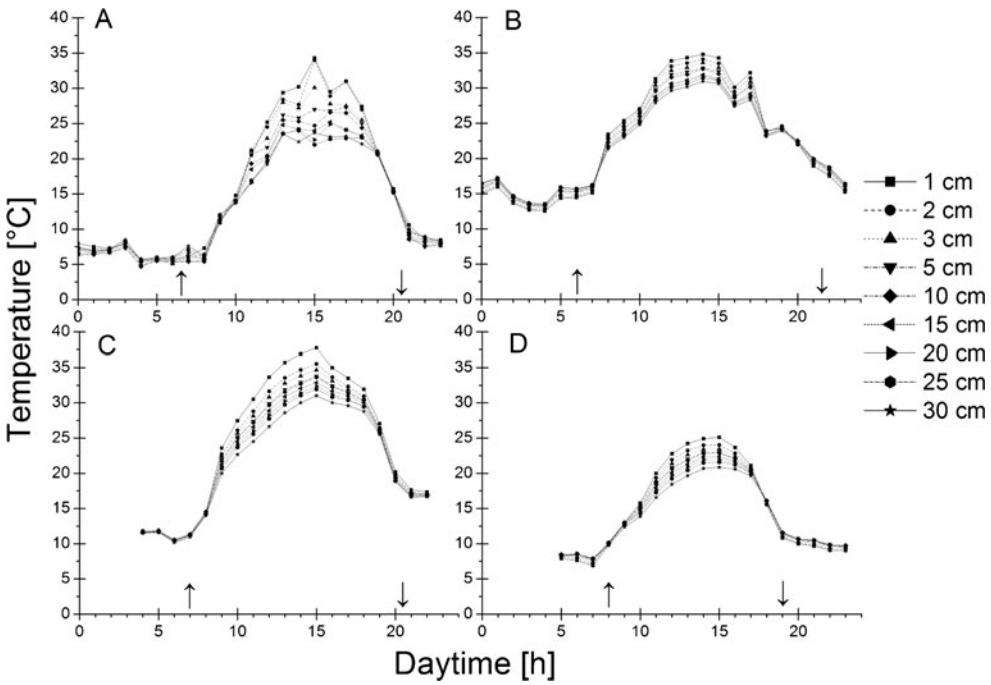

Fig. 2 Daily course of the air temperatures at different heights above the ground in Modène, France, during samplings in 2011. **a** April 18. **b** June 13. **c** August 30. **d** October 17. Sunrise is indicated by an *up arrow* and sunset by a *down arrow*

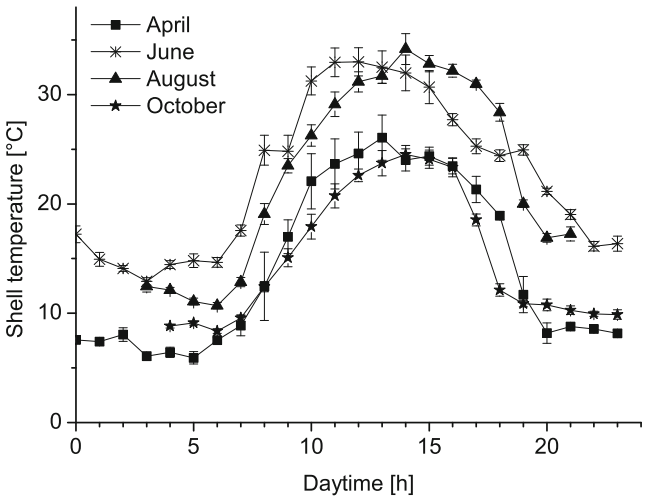

Fig. 3 Daily course of the shell temperature in four different months in 2011. *Error bars* indicate the standard deviation of ten samples taken per hour. Each *data point* represents the mean value of ten individuals

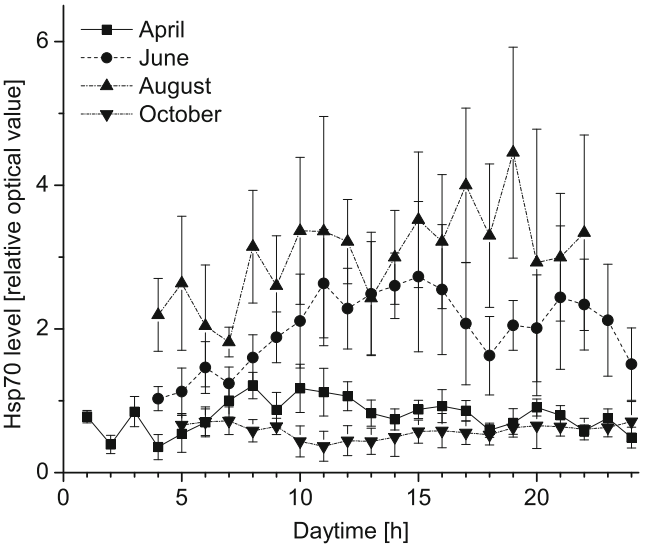

Fig. 5 Daily course of mean Hsp70 levels (n=10) obtained from samples taken in 2011. *Error bars* indicate the standard deviation of ten samples taken per hour

In addition to these results, a negative correlation between the Hsp70 level and the shell diameter was found for snails collected in June (Spearman's ρ; rho=−0.3596, n=209, p <0.0001). For all other samples taken, no significant correlation between these two factors was found. Furthermore, in April a positive correlation between shell diameter and shell temperature was revealed (Spearman's ρ; rho=0.2558, n=236, p<0.0001). No such findings were observed for the samples which were taken in the other months. The factor "climbing height" was recorded for every snail, but no correlation was found between this parameter and any other factor. However, since no general trend was visible for the other months, these occasional differences must be attributed to stochastic effects and should not lead to further interpretation. Considering that more than 95 % of the population was found to belong to category 1, no statistics were applicable to find correlations between the colouration of the shell and other factors. Only few or no snails were found to contribute to category 3 or 4 in these months.

Discussion

In the present study, a field population of *X. derbentina* from Southern France (Modène, Vaucluse) was used to investigate the molecular stress response to ambient temperature. This was accomplished during four different snapshots of a single day, each of these in four different months of one year. In addition to the Hsp70 analysis, we notice the development of colouration and growth in individuals of this population.

Snail growth and colouring

During our samplings in April, June, and August 2011, an increase in shell diameter was found. In April, most of the individuals of this population were around 4.5 mm in diameter; only few were larger than 6 mm. These small snails can most likely be regarded as juveniles that had hatched in

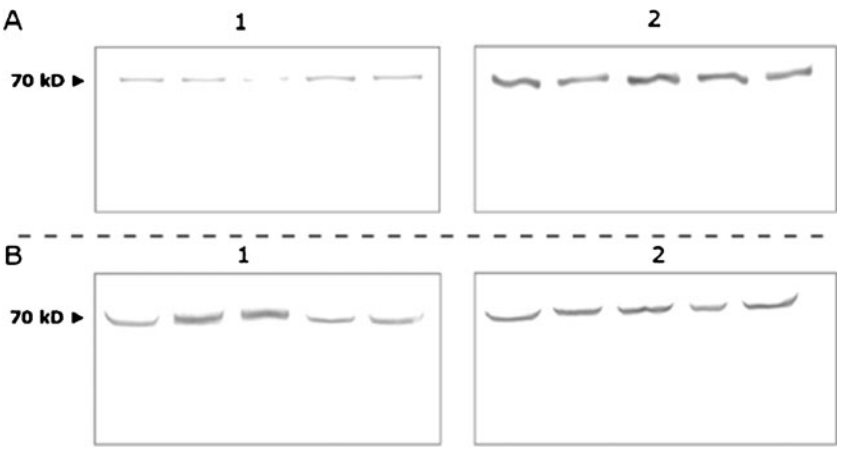

Fig. 4 Western blots for two different seasons and two different sampling times. **a** Samples from June 2011; *1*= 0 h, *2*=15 h daytime. Ten micrograms of total protein was separated per lane. **b** Samples from October 2011; *1*=0 h, *2*= 15 h daytime. Forty micrograms of total protein per lane were separated. *Each band* represents a single individual

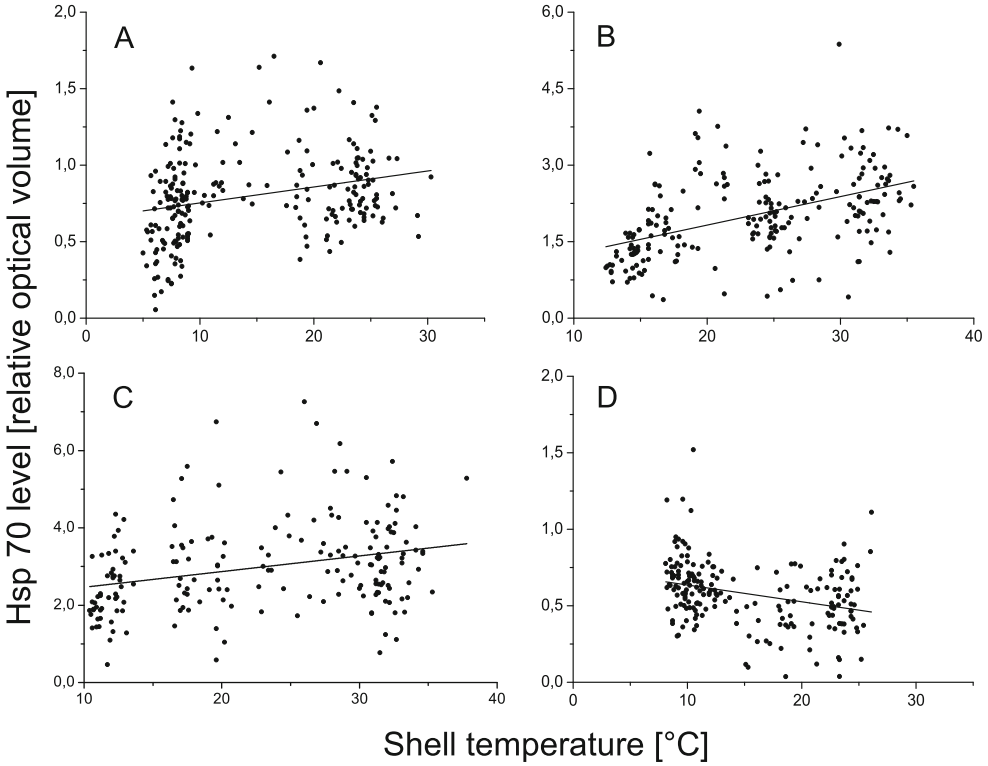

Fig. 6 Correlation between Hsp70 level and external shell temperature in the four different months of sampling. **a** April. **b** June. **c** August. **d** October. In April, June, and August, a significant positive correlation between Hsp70 level and shell temperature was present. In October, a significant negative correlation of these factors was found. For visualization purposes, linear regression lines were added to the figures

spring of 2011. Occasionally found snails of ≥9 mm in size were regarded as survivors from 2010. Similar findings were previously reported for the semelparous annual species *Xeropicta arenosa* (Staikou and Lazaridou-Dimitriadou 1991) in northern Greece as well as by Kiss et al. (2005) for French populations of *X. derbentina* [as long as there is no clarity as to whether *X. derbentina* (Krynicki 1836) and *X. arenosa* (Ziegler) are actually the same species, we treat them as two different ones]. Also for the population in focus of this study, an annual life cycle must be proposed according to the findings of Kiss et al. (2005) and Staikou and Lazaridou-Dimitriadou (1991). In both cases, as well as in our findings, the growth of the snails was continuous from spring until autumn. In our samples the population reached its final mean shell size in August 2011. Even the observed slight decrease in mean shell diameter in October compared to that of August does not support a biennial lifecycle. If hatching of the next generation would have taken place until October, or if snails would have entered aestivation, the mean shell diameter of the sampled population in October would have been much smaller than observed. During the entire sampling in October, no juvenile snails were found in the field.

With respect to the change of the shell colouration pattern of the snails during the course of the year, it was obvious that almost all individuals of the population carried a uniformly white shell when snails have grown to their adult body size in late summer. Particularly morphotypes that fit the pre-defined "category 3" disappeared during the year. Our data suggest that colouration pattern category 3 is typical for at least part of the juvenile snails. The banding may "disappear" when newly produced parts of the shell are forming the next whorl of the shell. Alternatively, the shell pigmentation may fade because of bleaching in ultraviolet light. Our study, however, did not yield information to clarify this question. In other studies (Köhler et al. 2009; Di Lellis et al. 2012) hints on this phenomenon are already given. In their studies, samplings in May revealed partly phenotypic "mixed" populations of *X. derbentina* as well.

Hsp70 induction

Since another study (Di Lellis et al. 2012) has revealed influence of the factor "climbing height" on the Hsp70 level, we have only used snails that were taken from a pre-defined range of height for stress protein analysis. Within this range, no significant effect of the climbing height or correlation between this factor and another parameter was found. This enabled us to relate the stress protein response to the factors "temperature" and "season".

In Southern France, *X. derbentina* snails that consist of 78 % water [including shell, measured as a mean of 15 fully hydrated snails dried to the nearest 0.01 g body weight, measurements performed by A.D. and U.F. in July 2011; similar results were found in *Cantareus apterus* (Born 1778) by Reuner et al. (2008)], have to face comparatively hot conditions during the day. Due to their inactivity during the day, they are not able to take up water from food or from their environment to cool down or to prevent desiccation.

During all samplings, activity of snails was found to take place in the cooler night until the early hours of the morning when the sun has not yet heated up the ground. No activity was observed during the day, thus escaping higher temperatures by moving into shaded regions is not an option for *X. derbentina*. Rising ambient temperature results in higher temperature on the surface of the shell and, consequently, also in higher temperature inside the body (Di Lellis et al. 2012). To prevent misfolding of proteins and to counteract consequences of heat stress and desiccation, Hsp70 is usually up-regulated (Sørensen et al. 2003; Mayer and Bukau 2005; Köhler 2009; Kiang and Tsokos 1998; Feder and Hofmann 1999). In our study, a positive correlation between the Hsp70 level and the temperature at the shell surface could be observed for April, June, and August only. In the samples taken in October, a negative correlation for these two variables was found. When comparing the temperature–stress response relationships from April and October, it became obvious that snails lost their ability to react properly to heat stress in October, even though ambient temperature in these 2 months was almost the same. These findings may have occurred for the following reasons.

It is known that older, senescent individuals have reduced Hsp70 levels compared to younger ones (Sørensen and Loeschcke 2002; Mayer and Bukau 2005; Köhler 2009). This may be due to an energetic trade-off between the maintenance of the stress response system and reproduction (Mizrahi et al. 2011). Furthermore, continuously repeated exposure to high temperature during summer, accompanied by a shortage in energy supply may have reduced the ability of the snails' cells to fully express the energy-costly Hsp system. Moreover, the overwhelming of this stress response machinery in turn could have resulted in cellular pathology as shown by Dittbrenner et al. (2009), Scheil et al. (2011), and S. Troschinski, University of Tübingen (unpublished data) for Mediterranean land snails. The limitation of the stress protein system by environmental parameters resulting in a reduced capacity of organisms to overcome environmental stressors has already been postulated by Nakano & Iwama (2002) and Tomanek (2002). In cases of "overwhelmed" stress physiology, additional stressor action will not result in an induction but rather in a decrease of Hsp70 levels (Eckwert et al. 1997; Tomanek 2002). It is likely that the present results obtained for the October snails should be seen as a consequence stemming from an exhaustion of the stress response system as it was shown before by Scheil et al. (2011) for *X. derbentina*.

Another assumption that could explain the absence of Hsp70 induction in the October snails is, as reported in many studies, that snails, especially in the Mediterranean area, are often able to enter an aestivating phase when conditions turn unfavourable. During this phase metabolism is reduced and the internal milieu of the snails changes (Herreid II 1977; Riddle 1981; Umezurike and Iheanacho 1983; Storey 2002). In our French field population snails did not enter the aestivation phase in April, June, and August as they were foraging on the ground in the night hours during sampling. In October, snails were almost exclusively found resting on the vegetation and only very few snails were active during the night. If snails had entered a temporal aestivation phase due to physiological exhaustion, the low level and limited induction of Hsp70 in snails collected in October could be explained according to the findings of Reuner et al. (2008) who found dormant snails not to express much Hsp70 compared to heat-shocked active ones. Kiss et al. (2005) have shown that populations of *X. derbentina* may be able to change their survival strategy and shift from an annual to a biennial life-cycle, and some of these populations were found to aestivate. In our population, it is more likely that snails entered a short-term aestivation-like phase to temporarily cope with a prolonged phase of dry conditions during autumn 2011. Equivalent to the findings in 2011, predominately small snails were found in spring 2012 on the same sampling ground (personal communication C. Mazzia, University of Avignon). Therefore, it is highly unlikely that large parts of the population had entered a prolonged aestivation phase. In this case, snails with intermediate shell sizes would have been found in spring 2012. As no aestivation was observed, apart from some periods in autumn, we conclude that aestivation is not part of the survival strategy of the investigated population.

Our results reveal not only a seasonal change of Hsp70 level as reported in several other studies (Nakano and Iwama 2002; Tomanek 2002; Tomanek and Sanford 2003; Arad et al. 2010), but, for one of the few times (Ulmasov et al. 1999), also a daily change in Hsp70 expression in the field. Regarding this daily course it is obvious that Hsp70 levels follow the increase in ambient temperature. In April, where temperatures were lower than in June or August, only a slight increase in the Hsp70 level could be shown during the day. This slight increase indicates that ambient temperatures at that time seemed to generally be below the threshold temperature at which *X. derbentina* starts to up-regulate Hsp70 for their survival. According to Köhler et al. (2009) this threshold temperature should be estimated to be around 30 °C. In experiments where *X. derbentina* was exposed to different temperatures, 24–25 °C was used as a control (Dittbrenner et al. 2009; Köhler et al. 2009; Scheil et al. 2011). On the day of data collection in April, temperatures >25 °C occurred for 5 h only with a measured maximum of 27.3 °C. In June, the Hsp70 levels followed the rise of ambient temperatures till early afternoon and decreased again with sunset in the evening. Additionally at night, a slight elevation of the Hsp70 level was found. This additional Hsp70 peak most likely corresponds to the activity period of the snails that typically starts a few hours after

sunset, when temperatures had decreased. During this period, snails were often found on the ground, eating, moving around, and probably being in a phase where the snails have to deal with balancing their internal milieu and producing new proteins (Herreid II 1977; Riddle 1981; Umezurike and Iheanacho 1983; Storey 2002). This happens at a time of the year when snails have not yet reached their final body size, as shown by our results on shell size. Hence, the induction of Hsp70 during the night could be seen as a consequence of the need to chaperone newly synthesized proteins necessary for the animals' growth (Köhler 2009; Mayer and Bukau 2005). In August, the daily course of Hsp70 was shown to remain at a high level but with high standard deviations. A possible reason for this effect could be the interaction of high temperature at that time of the year and the energy-costly proceeding maturation of reproductive organs. This may have led to a beginning collapse of the Hsp70 protection system in some individuals. Particularly those snails that are still growing and have not entered maturation seemed to produce high levels of Hsp70 to counteract heat stress; others, which have grown to their final size, may have started with egg production which poses an additional stress on them, overcharging the capacity of the molecular stress response and resulting in a sub-optimal Hsp70 level. In October temperatures did not reach 25 °C. The recorded maximum in October was 23.0 °C. Given the fact that such a temperature is not high enough to induce Hsp70, only a "base level" of constitutional Hsp70 should remain which was the case in our investigation.

The negative correlation of Hsp70 level and temperature with the rather small range of this "base level" supports the above mentioned assumption of a "physiological exhaustion" of most *X. derbentina* individuals by the long-term heat exposure plus reproduction effects that they have experienced in late summer and particularly autumn.

Our study showed growth and stress response of *X. derbentina* to be in accordance with the requirements posed on an annual population of invertebrates. In spring and early summer, the Hsp70 response remains adequate to counteract possible heat effects, as the strong positive association of ambient temperature and Hsp70 level indicates. This situation seems to continue for a number of individuals also until the late summer, while others already show symptoms of exhaustion of the stress response system. In autumn, the limited capacity to induce Hsp70 suggests senescence. Most individuals die at the end of the year.

Acknowledgments The authors would like to thank Wolfgang Rähle, Edmund Gittenberger, Sergej Sereda and Thomas Wilke for their contribution in snail species determination and discussion. Furthermore, we would like to thank Stefanie Krais and Katharina Graf for assistance in field work, Simon Schwarz for his help with the statistics and Nishrin Schacherbauer for proofreading. The study was financed by the German Research Council (DFG Ko 1978/5-3) and the University of Tübingens's twinning project with the University of Applied Sciences Esslingen under the supervision of H.-R.K., U.G., Stefan Rösler, and David Wharam.

References

Abdel-Rehim AH (1983) The effects of temperature and humidity on the nocturnal activity of different shell colour morphs of the land snail *Arianta arbustorum*. Biol J Linn Soc 20(4):385–395. doi:10.1111/j.1095-8312.1983.tb01599.x

Altena COR (1960) On the occurrence of a species of *Xeropicta* in France. Basteria 24:21–25

Arad Z, Mizrahi T, Goldenberg S, Heller J (2010) Natural annual cycle of heat shock protein expression in land snails: desert versus Mediterranean species of *Sphincterochila*. J Exp Biol 213 (20):3487–3495. doi:10.1242/jeb.047670

Arts M, Schill RO, Knigge T, Eckwert H, Kammenga JE, Köhler HR (2004) Stress proteins (hsp70, hsp60) induced in isopods and nematodes by field exposure to metals in a gradient near Avonmouth, UK. Ecotoxicology 13(8):739–755. doi:10.1007/s10646-003-4473-5

Aubry S, Labaune C, Magnin F, Kiss L (2005) Habitat and integration within indigenous communities of *Xeropicta derbentina* (Gastropoda: Hygromiidae) a recently introduced land snail in south-eastern France. Divers Distrib 11(6):539–547. doi:10.1111/j.1366-9516.2005.00189.x

Aubry S, Labaune C, Magnin F, Roche P, Kiss L (2006) Active and passive dispersal of an invading land snail in Mediterranean France. J Anim Ecol 75(3):802–813. doi:10.1111/j.1365-2656.2006.01100.x

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Anal Biochem 72(1–2):248–254. doi:10.1006/abio.1976.9999

Daugaard M, Rohde M, Jäättelä M (2007) The heat shock protein 70 family: Highly homologous proteins with overlapping and distinct functions. FEBS Lett 581(19):3702–3710. doi:10.1016/j.febslet.2007.05.039

Di Lellis M, Seifan M, Troschinski S, Mazzia C, Capowiez Y, Triebskorn R, Köhler H-R (2012) Solar radiation stress in climbing snails: behavioural and intrinsic features define the Hsp70 level in natural populations of *Xeropicta derbentina* (Pulmonata). Cell Stress and Chaperones:1–11 IN PRESS. doi:10.1007/s12192-012-0344-4

Dittbrenner N, Lazzara R, Köhler H-R, Mazzia C, Capowiez Y, Triebskorn R (2009) Heat tolerance in Mediterranean land snails: histopathology after exposure to different temperature regimes. Journal of Molluscan Studies 75(1):9–18. doi:10.1093/mollus/eyn033

Eckwert H, Alberti G, Köhler H-R (1997) The induction of stress proteins (hsp) in *Oniscus asellus* (Isopoda) as a molecular marker of multiple heavy metal exposure: I. Principles and toxicological assessment Ecotoxicology 6(5):249–262. doi:10.1023/a:1018682928839

Feder ME, Hofmann GE (1999) Heat-shock proteins, molecular chaperones, and the stress response: Evolutionary and ecological physiology. Annual Reviews of Physiology 61:243–282. doi:10.1146/annurev.physiol.61.1.243

Herreid CF II (1977) Metabolism of land snails (*Otala lactea*) during dormancy, arousal, and activity. Comparative Biochemistry and Physiology Part A: Physiology 56(2):211–215. doi:10.1016/0300-9629(77)90187-6

Hofmann GE, Buckley BA, Airaksinen S, Keen JE, Somero GN (2000) Heat-shock protein expression is absent in the antarctic

fish *Trematomus bernacchii* (family Nototheniidae). J Exp Biol 203(15):2331–2339

Kiang JG, Tsokos GC (1998) Heat Shock Protein 70 kDa: Molecular Biology, Biochemistry, and Physiology. Pharmacol Ther 80 (2):183–201. doi:10.1016/s0163-7258(98)00028-x

Kiss L, Labaune C, Magnin F, Aubry S (2005) Plasticity of the life cycle of *Xeropicta derbentina* (Krynicki, 1836), a recently introduced snail in mediterranean France. Journal of Molluscan Studies 71(3):221–231. doi:10.1093/mollus/eyi030

Köhler H-R, Bartussek C, Eckwert H, Farian K, Gränzer S, Knigge T, Kunz N (2001) The hepatic stress protein (hsp70) response to interacting abiotic parameters in fish exposed to various levels of pollution. Journal of Aquatic Ecosystem Stress and Recovery (Formerly Journal of Aquatic Ecosystem Health) 8(3):261–279. doi:10.1023/a:1012935931161

Köhler HR (2009) Die Rolle von Stressproteinen bei der Anpassung an Umweltbedingungen: Ökophysiologische, ökotoxikologische und evolutionsbiologische Implikationen. Umweltwissenschaften und Schadstoff-Forschung 21(2):150–159. doi:10.1007/s12302-009-0041-9

Köhler H-R, Lazzara R, Dittbrenner N, Capowiez Y, Mazzia C, Triebskorn R (2009) Snail phenotypic variation and stress proteins: do different heat response strategies contribute to Waddington's widget in field populations? Journal of Experimental Zoology Part B: Molecular and Developmental Evolution 312B(2):136–147. doi:10.1002/jez.b.21253

Mayer M, Bukau B (2005) Hsp70 chaperones: Cellular functions and molecular mechanism. Cellular and Molecular Life Sciences 62 (6):670–684. doi:10.1007/s00018-004-4464-6

Mizrahi T, Heller J, Goldenberg S, Arad Z (2010) Heat shock proteins and resistance to desiccation in congeneric land snails. Cell Stress & Chaperones 15(4):351–363. doi:10.1007/s12192-009-0150-9

Mizrahi T, Heller J, Goldenberg S, Arad Z (2011) Heat shock protein expression in relation to reproductive cycle in land snails: Implications for survival. Comp Biochem Physiol A-Mol Integr Physiol 160(2):149–155. doi:10.1016/j.cbpa.2011.05.031

Mizrahi T, Heller J, Goldenberg S, Arad Z (2012) The heat shock response in congeneric land snails (*Sphincterochila*) from different habitats. Cell Stress and Chaperones 17(5):639–645. doi:10.1007/s12192-012-0340-8

Nakano K, Iwama GK (2002) The 70-kDa heat shock protein response in two intertidal sculpins, *Oligocottus maculosus* and *O. snyderi*: relationship of hsp70 and thermal tolerance. Comparative Biochemistry and Physiology - Part A: Molecular & Integrative Physiology 133 (1):79–94. doi:10.1016/s1095-6433(02)00115-0

Reuner A, Bruemmer F, Schill RO (2008) Heat shock proteins (Hsp70) and water content in the estivating Mediterranean Grunt Snail (*Cantareus apertus*). Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology 151(1):28–31. doi:10.1016/j.cbpb.2008.05.004

Riddle WA (1981) Hemolymph osmoregulation and urea retention in the woodland snail, *Anguispira alternata* (Say) (Endodontidae). Comparative Biochemistry and Physiology Part A: Physiology 69 (3):493–498. doi:10.1016/0300-9629(81)93009-7

Scheil AE, Köhler H-R, Triebskorn R (2011) Heat tolerance and recovery in Mediterranean land snails after pre-exposure in the field. Journal of Molluscan Studies 77(2):165–174. doi:10.1093/mollus/eyr003

Schill RO, Gayle PMH, Kohler HR (2002) Daily stress protein (hsp70) cycle in chitons (*Acanthopleura granulata* Gmelin, 1791) which inhabit the rocky intertidal shoreline in a tropical ecosystem. Comp Biochem Physiol C-Toxicol Pharmacol 131(3):253–258. doi:10.1016/s1532-0456(02)00012-1

Sørensen JG, Kristensen TN, Loeschcke V (2003) The evolutionary and ecological role of heat shock proteins. Ecol Lett 6(11):1025–1037. doi:10.1046/j.1461-0248.2003.00528.x

Sørensen JG, Loeschcke V (2002) Decreased heat-shock resistance and down-regulation of Hsp70 expression with increasing age in adult *Drosophila melanogaster*. Funct Ecol 16(3):379–384. doi:10.1046/j.1365-2435.2002.00639.x

Staikou A, Lazaridou-Dimitriadou M (1991) The life-cycle, population-dynamics, growth and secondary prouction of the snail *Xeropicta arenosa* Ziegler (Gastropoda, PulmonataI in northern Greece. Zool J Linn Soc 101(2):179–188. doi:10.1111/j.1096-3642.1991.tb00892.x

Storey KB (2002) Life in the slow lane: molecular mechanisms of estivation. Comparative Biochemistry and Physiology - Part A: Molecular & Integrative Physiology 133(3):733–754. doi:10.1016/s1095-6433(02)00206-4

Tomanek L (2002) The Heat-Shock Response: Its Variation, Regulation and Ecological Importance in Intertidal Gastropods (genus *Tegula*). Integr Comp Biol 42(4):797–807. doi:10.1093/icb/42.4.797

Tomanek L, Sanford E (2003) Heat-Shock Protein 70 (Hsp70) as a Biochemical Stress Indicator: an Experimental Field Test in Two Congeneric Intertidal Gastropods (Genus: *Tegula*). Biol Bull 205 (3):276–284

Ulmasov K, Zatsepina O, Molodtsov V, Evgen'ev M (1999) Natural body temperature and kinetics of heat-shock protein synthesis in the toad-headed agamid lizard *Phrynocephalus interscapularis*. Amphibia-Reptilia 20(1):1–9. doi:10.1163/156853899X00015

Umezurike GM, Iheanacho EN (1983) Metabolic adaptations in aestivating giant African snail (*Achatina achatina*). Comparative Biochemistry and Physiology Part B: Comparative Biochemistry 74 (3):493–498. doi:10.1016/0305-0491(83)90216-x