Solar radiation stress in climbing snails: behavioural and intrinsic features define the Hsp70 level in natural populations of *Xeropicta derbentina* (Pulmonata)

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Abstract Ectotherms from sunny and hot environments need to cope with solar radiation. Mediterranean land snails of the superfamily Helicoidea feature a behavioural strategy to escape from solar radiation-induced excessive soil heating by climbing up vertical objects. The height of climbing, and also other parameters like shell colouration pattern, shell orientation, shell size, body mass, actual internal and shell surface temperature, and the interactions between those factors may be expected to modulate proteotoxic effects in snails exposed to solar radiation and, thus, their stress response. Focussing on natural populations of *Xeropicta derbentina*, we conducted a ‘snapshot’ field study using the

individual Hsp70 level as a proxy for proteotoxic stress. In addition to correlation analyses, an IT-model selection approach based on Akaike’s Information Criterion was applied to evaluate a set of models with respect to their explanatory power and to assess the relevance of each of the above-mentioned parameters for individual stress, by model averaging and parameter estimation. The analysis revealed particular importance of the individuals’ shell size, height above ground, the shell colouration pattern and the interaction height × orientation. Our study showed that a distinct set of behavioural traits and intrinsic characters define the Hsp70 level and that environmental factors and individual features strongly interact.

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Introduction

In poikilothermic organisms, the interaction of environmental and body temperature requires effective regulation mechanisms to guarantee homeostasis, because metabolism of poikilotherms relates directly to the thermal conditions in their environment. Numerous variables are known to influence the body temperature of ectotherms such as physiological (e.g. behaviour), morphological (size, colour, shape of the body) as well as environmental parameters, like radiation, wind speed and the type of substrate (Stevenson 1985). These variables are often entangled with one another and, therefore, complex interactions among them should be considered. In this context, also the global warming phenomenon which includes a rise in air temperature is consequently affecting the physical

condition, abundance and distribution of organisms and the functioning in their habitat (Helmuth et al. 2010). To understand how the impact of environmental factors influences the persistence of organisms in ecosystems, it is mandatory to consider the protective value of physiological mechanisms and morphological characters of organisms.

The formation of heat shock proteins (Hsps) is part of the physiological response to heat stress accounting for a distinct capacity in the thermal tolerance of biota (Feder and Hofmann 1999; Pörtner and Farrell 2008). Particularly, the members of the Hsp70 family are known as so-called stress markers and are, therefore, important in this context. Hsp70 plays a major role in the cellular stress defense by preventing proteotoxic effects and refolding damaged intracellular proteins. Since the Hsp70 machinery reacts to a variety of stressors and since these stress proteins are phylogenetically highly conserved and ubiquitous throughout all biota, they have been used to monitor the effects of environmental stressors in numerous taxa. In this regard, the Hsp70 level is commonly accepted to reflect the ‘stress status’ (in view of proteotoxicity) of organisms (Köhler et al. 1992; Triebkorn and Köhler 1996; Feder and Hofmann 1999; Lewis et al. 1999; Köhler et al. 2000; Mukhopadhyay et al. 2003).

Stress protein expression in snails of the superfamily Helicoidea from hot climates has been characterized recently (Mizrahi et al. 2009; Arad et al. 2010). This gastropod superfamily occurs in large abundance in the Mediterranean and comprises a number of morphologically very similar species. These snails are known to climb up vertical objects in the beginning of the day and are, therefore, fully exposed to solar radiation for hours, particularly during hot summer days. Even though this behaviour is well-known (Cowie 1985), it has not been investigated so far, whether distinct parameters determine the stress status of an individual in its natural habitat. The following questions are important in this context: Is the position of a snail, its orientation to the sun and its distance from the hot ground surface important? Does the individual size, responsible for the volume-surface ratio, matter? Does the intraspecifically highly variable colouration of the shell play a role? Does

pigmentation contribute to thermoregulation in these snails, as reported for other ectotherms (Clusella Trullas et al. 2006)?

To investigate the influence of these intrinsic features on the stress status, or on its proxy, the Hsp70 level, ‘snapshot’ correlation analyses between morphology, the behavioural patterns of organisms, and their physiological stress response were conducted at a given time point. For this analysis, we found the following aspect worth to be considered: The actual internal temperature of the snails may vary transiently according to environmental short-term events, such as wind and shadow, whereas the stress protein level may integrate over time to a greater extend. Consequently, we examined the Hsp70 level of sun-exposed individuals of a Mediterranean pulmonate snail species (*Xeropicta derbentina*) after determining their height above ground, their shell orientation (geographic direction) towards the sun, their shell colouration pattern, their internal and shell surface temperature, their shell diameter and their body mass. The aim of this study was to extract those factors that explain most of Hsp70 level variation in this group of terrestrial snails.

Materials and methods

Study sites

Sampling took place between May 24 and 26, 2010 under the same climatic conditions around noon on these three consecutive days in the Vaucluse department, Provence, Southern France. The prevalent Mediterranean climate is characterized by dry and hot summers with cool and moist winters. The surrounding area of our sampling sites possesses an annual average temperature of 13.8°C and a precipitation rate of 693.6 mm per year (infoclimat.fr, retrieved 02 November 2011). The climate in Provence is dominated by a particulate strong wind, the so-called Mistral, which is responsible for the dry and hot conditions. Overall, we examined eight sampling sites in Provence (Table 1).

Table 1 Sampling sites in Provence, France. Given is the sampling size (n), the difference (Diff) in size, height above ground, internal temperature and body mass of the organisms and Shannon–Wiener

indices (HS) as measure for the variability in shell orientation towards the sun and the shell colouration pattern, within each population, respectively

Pop.	Locality	n	Diff _{size} [mm]	Diff _{height} [cm]	Diff _{temp} [°C]	Diff _{bm} [mg]	HS _{orient}	HS _{shell p}
1	Modène Mazzia	12	4.47	23	3.3	139	0.87	1.20
2	Modène wine yard	12	2.02	25	3.5	70	0.89	1.24
3	Modène West	12	4.20	53	3.4	282	0.96	0.89
4	St Pierre	12	4.93	14	3.3	207	0.82	0.45
5	Mazan South	12	2.29	11	3.6	126	0.72	1.01
6	Bon Remède	12	5.05	34	3.1	233	1.01	0.72
7	Mazan North	12	3.14	26	1.5	175	0.96	0.00

Test organisms

We investigated seven populations of *X. derbentina* (Krynicky, 1836) [Hygromiidae]. This snail species is well-adapted to high temperature regimes. *X. derbentina* is an introduced and well-established snail in the South of France with its origin in the Eastern Mediterranean countries (Aubry et al. 2005). Adult individuals possess a shell size ranging from 10 to 16 mm in diameter. In Provence, this species occurs in huge populations among very diverse landscapes and displays a high variation in shell colouration.

Species determination was conducted on the basis of morphological criteria and verified by *COI* gene sequencing (Thomas Wilke and Sergej Sereda, Giessen University, Germany).

Sampling in the field

Measures were made consecutively for each snail. In all individuals, we determined the residing height on vertical objects above the ground with a yard stick. Then, we classified the shell orientation (geographic direction). For this purpose, we defined three categories (Fig. 1): (1) apex directed to the sun [*x*], (2) umbilicus directed to the sun [*y*], and (3) shell laterally directed to the sun [*z*]. Because the sampling time was always around noon, the orientation of the apex was southwards in [*x*] and northwards in [*y*]. Subsequently, the surface temperature of the shell was

Fig. 1 Code for the factor ‘orientation towards the sun’. *x* Apex directed to the sun; *y* umbilicus directed to the sun; *z* shell laterally directed to the sun. The respective characters symbolize the position of the sun

measured with a thin medical precision thermometer (ELLAB Copenhagen, type: DM 852). Here, we took care not to influence the temperature by touching the snail. For the measurement of the internal body temperature, we removed the snail from its location to allow the pin of the thermometer to penetrate the soft body. Subsequently, the size of the shell was determined by measuring its width by an electronic caliper rule, and each individual was classified regarding its shell pattern (Fig. 2; 1 = white; 2 = white with a single pale band; 3 = greyish with several light bands; 4 = dark with lots of intense bands). Both fresh weight and the relative Hsp70 level were determined in the laboratory afterwards.

Stress protein analysis

After determining the above-mentioned parameters, the snails ($n=12$ for each population) were frozen individually in liquid nitrogen and stored in a freezer at -25°C before biochemical analysis. We homogenized the snails on the whole, without separating it into various tissues, according to their body mass in extraction buffer (2 μl buffer/mg snail), containing 80 mM potassium acetate, 5 mM magnesium acetate, 20 mM Hepes, and 2 % protease inhibitor at pH 7.5. In the following, the supernatant was separated from the remaining cell debris via centrifugation for 10 min at $2,000\times g$ and 4°C . Subsequently, we determined the total protein concentration of each sample by use of a protein-dye binding assay (Bradford 1976). We analysed constant protein weights of 40 μg total protein from each sample by minigel SDS-PAGE (12 % acrylamide, 0.12 % bisacrylamide, 30 min at 80 V, and 75–90 min at 120 V) and semi-dry Western blotting on nitrocellulose membranes. The membranes were blocked with a 50 % horse serum/tris-buffered saline (TBS) solution for 2 h, before they were incubated in a solution of monoclonal α -Hsp70 antibody which was cross-reacting with all isoforms of Hsp70 and detected both constitutive and inducible stress protein molecules (mouse anti-human Hsp70, Dianova, Hamburg, Germany, dilution 1:5,000 in 10 % horse serum in TBS) on a lab shaker overnight. Subsequently, the membranes were

Fig. 2 Code for the factor ‘shell colouration pattern’, according to Köhler et al. (2009) modified. *Cat. 1* = white; *cat. 2* = white with a single pale band; *cat. 3* = greyish with several light bands; *cat. 4* = dark with lots of intense bands

washed in TBS for 5 min to remove dispensable Hsp70 antibodies, before we applied a second antibody (goat anti-mouse IgG conjugated to peroxidase, Jackson Immuno-research, West Grove, PA, dilution 1:1,000 in 10 % horse serum/TBS) for 1 h. After short washing in TBS, the membranes were developed in a staining solution, containing 1 mM 4-chloro(1)naphthol, 0.015 % H_2O_2 , 30 mM Tris pH 8.5, and 6 % methanol. The optical volume of the individual bands was calculated by multiplication of the area of the bands (number of pixels) with the average grey scale value after background subtraction. For this purpose, we used the densitometric image analysis program E.A.S.Y. Win 32 (Herolab, Wiesloch, Germany). All sample data were normalized against a standard sample (prepared from *Theba pisana*) to ensure comparability between all samples. Figure 3 displays representative Western blot bands for Hsp70 in snail individuals of featuring different size.

Statistical analysis

The aim of our analysis was to identify which of the observed variables (height above ground, shell orientation, shell colouration pattern, internal temperature and body mass) have an effect on the Hsp70 level and are consequently contributing to better explanations in terms of the variance in the data set. The traditional way to analyse such a data set employs a selection process of the various variables out of one regression model (such as stepwise, backward or forward regressions). This traditional statistic framework makes use of only one null hypothesis, which typically and intentionally has a low biological meaning (e.g. the explanatory variable has no effect on the measurement; Anderson et al. 2000). The null hypothesis is then tested in the light of an arbitrary significance threshold (usually $p < 0.05$) in favour of an alternative hypothesis (Johnson and Omland 2003). Additionally to common correlation analyses, we chose to use an information theory (IT) approach, a relatively new method which was introduced to the biological sciences by Burnham and Anderson (2002, 2004). According to information theory, all statements in science are approximations of reality and

it is the scientist's responsibility, to evaluate, how well these statements fulfil these approximations. Model selection in this approach takes account of multiple competing hypotheses and allows inferences through the whole set of models, thus takes into account the fact that no single model (or variable composition) can perfectly reflect nature.

The IT-model selection approach we used is based on Akaike's Information Criterion (AIC). In general, this criterion is based on the estimation of information loss when a model is used to approximate the truth (Anderson et al. 2000). The better the model is reflecting nature, the less information is lost. Using this approach, a researcher can estimate what is the relative precision of several models created from the same data set. Model-based inference has three general advantages (Johnson and Omland 2003). First, as mentioned above, no single relatively meaningless null hypothesis is employed. Instead, there are several well-grounded models, the amount of support of which can be evaluated by the data set. Second, models can be ranked according to their data fit and, third, all single factors and interactions can be estimated and predicted by model averaging.

Using this model selection method, we do not assess the statistical power of single models, but rank their relative importance (Irvine et al. 2009). In general, models with a high number of parameters involved are penalized more heavily to avoid overfitting of models (law of simplicity and parsimony) and, therefore, disadvantaged in favour of competing models with less parameters.

As a first step, we formulated a set of competing models. Here, we took care to choose all models which had a relevance to our study aims, to ensure an ecophysiological sense of our model collection. Secondly, we fitted each model to the observed data by conventional univariate statistical methods (i.e. regressions, ANOVA). During this process we square root-transformed our dependent variable (Hsp70 level) to fulfil the univariate methods requirements. This way we generated a set of relevant models, each with its variance and coefficient estimation. For each of the models we calculated the AIC_C , a modified variation of the AIC which is adequate for

Fig. 3 Representative Western blot bands for Hsp70 in snails featuring different size. The α -Hsp70 antibody was sensitive to all isoforms of Hsp70 and detected both constitutive and inducible stress protein molecules

small sample sizes, according to the following equation (Symonds and Moussalli 2010; Burnham and Anderson 2002):

$$AIC_C = n \times \ln(MSS) + 2k + \frac{2k(k+1)}{(n-k-1)}$$

where n is the sample size ($n=84$); MSS is the mean sum of squares of the specific model and k is the number of parameters including the intercept used in the specific model.

The model with the lowest AIC_C value was considered as the model with the best explanatory power, and the best fit to the data set. To assess the relative strength of the candidate models, we calculated the difference between the AIC_C values of each respective model and the best ranked one (Δ_i). This allowed us to judge the lower ranked models (i.e. the models which were considered worse) compared to the prime one, instead of arbitrarily ignoring them. From the Δ_i parameters we further calculated the Akaike weight (ω_i) by dividing the appropriate model likelihood $\exp(-0.5\Delta_i)$ by the sum of all values across the model set. This likelihood weight is an assessment of the specific model probability to be the best ranked one in a repeated data collection. The Akaike weight of all models sum up to 1 and can be translated into percentage values. Therefore, the Akaike weight (ω_i) for each model in our set can be easily interpreted as the probability of the model to fit the data. Finally, we estimated parameter coefficients (i.e. their relative importance and trend of effect) by calculating a weighted average across all models. This process strongly reduces model selection bias and ensures that we took into account as many possible scenarios as the biological logic of the system dictates, instead of constraining ourselves due to a limited statistical analysis (Symonds and Moussalli 2010; Johnson and Omland 2003).

Because of strong correlations between the factors ‘height above ground’, ‘shell colouration pattern’, ‘internal’ and ‘surface temperature’, ‘size’ and ‘body mass’, the factors ‘body mass’, ‘shell colouration pattern’ and ‘surface temperature’ were excluded from the model selection analysis to prevent multicollinearity (Table 2). We decided to omit these factors for the following reasons: Compared to the internal temperature, the shell surface temperature is even stronger affected by environmental short-term events and the body mass is more sensitive to desiccation than the shell size. The shell colouration pattern was highly correlated with the shell size and might be an effect of the individual age in this species.

Because both factors ‘height above ground’ and ‘size’ showed indication to own a high importance in explaining Hsp70 variability in snails, we conducted two separate AIC_C analyses, with the inclusion of either factor, respectively.

For the statistical data analysis, we used JMP, version 9 (SAS Institute Inc., Cary, NC).

Results

Correlation analyses

Significant correlation at a threshold of $\alpha=0.05$ was found between the Hsp70 level and, respectively, the factors ‘height above ground’ (negatively correlated, $p=0.006$), ‘shell colouration pattern’ ($p=0.022$) and ‘size’ (negatively correlated, $p<0.0001$). There was no correlation of the Hsp70 level and ‘orientation towards the sun’ ($p=0.526$) and ‘internal temperature’ ($p=0.801$). Correlations among the independent variables are displayed in Table 2.

Table 2 Determined factors with their correlation to each other (lower part; p values, identified by correlation analysis) and their correlation trends (upper part)

	Height	Orientation	Shell pattern	Surface temperature	Internal temperature	Size	Body mass
Height	X	—	—	—	—	Positive	Positive
Orientation	0.8605	X	—	—	—	—	—
Shell pattern	0.8430	0.6234	X	—	— ^a	— ^b	— ^c
Surface temperature	0.6683	0.2808	0.5283	X	Positive	—	—
Internal temperature	0.9960	0.4942	0.0175	0.0001	X	—	—
Size	0.0165	0.8758	0.0001	0.7374	0.4980	X	Positive
Body mass	0.0105	0.9806	0.0171	0.6102	0.4784	0.0001	X

The significance threshold was set to $\alpha=0.05$

^a Significantly higher internal temperature in snails with cat. 2 compared with snails attendant to cats. 1, 3 and 4

^b Significantly larger shell size in snails of cat. 1 compared with the darker morphs (cats. 2–4)

^c Significant decrease in body mass with an increase in shell pigmentation

Model selection

The first model selection procedure for the factors ‘size’, ‘orientation towards the sun’ and ‘internal temperature’ revealed that, of a total of 15 potentially relevant models, only one showed high empirical support, because $\Delta_i \leq 2$ (the difference in AIC_C between the best ranked model and the respective one of interest) was not fulfilled for any other model (Table 3). The model with the lowest AIC_C value and, correspondingly, the highest explanatory power for Hsp70 level variation was built by the single factor ‘size’. This parameter had a very high probability value (97.8 %), representing the chance to be part of the best model and, therefore, clearly contributed to the variation in the Hsp70 level. Mentionable are also the factors ‘orientation towards the sun’ (21.8 %) and ‘internal temperature’ (18.5 %) with a moderate influence on the Hsp70 level, whereas the interactions between those factors have shown to be redundant in this analysis and own no explanatory power for the Hsp70 level. Another important indication to the robustness of our results is the fact that the null model is ranked relatively low (place 9), implying that our chosen measurements clearly improved our ability to predict the change in Hsp70 values.

The second model selection procedure, performed with the factors ‘orientation towards the sun’, ‘internal temperature’ and ‘height above ground’ (instead of ‘size’), showed that two models exhibit high empirical support with $\Delta_i \leq 2$ (Table 4). The best model with the highest explanatory power comprised the interaction term ‘height above ground’ and ‘orientation towards

the sun’, with a probability value of 48.8 %. The second model with comparable empirical support is built by the factor ‘height above ground’ which had an expectation of 51.9 % to be part of the best model. The factors ‘orientation towards the sun’ (16.8 %) and ‘internal temperature’ (15.8 %) had low impact in explaining Hsp70 level variation, and the interactions ‘height $\times$ temperature’ and ‘temperature $\times$ orientation’ (both < 1.6 %) had no impact at all.

Specific effect of the model parameters

The standardized regression coefficients (β) for all model parameters are presented in Table 5 for the first model selection analysis and in Table 6 for the second testing. For a better understanding of the specific effect of these parameters a visual overview is given in Figs. 3 and 4 for the influential factors (probability ≥ 0.1). As predicted, the Hsp70 levels decreased with an increase in the size of the shell (Fig. 4a) as well as with increasing ‘height above ground’ at which the snails were located on plants (Fig. 4b), while the shell orientation towards the sun (Fig. 4c), as a single factor, had no explanatory effect for the Hsp70 level of the individuals, just as the actual internal temperature (Fig. 4d).

However, snails with a shell orientation towards the south (x) slightly increased their Hsp70 level with an increase in their height above ground (Fig. 5a), whereas snails with an orientation towards y or z showed a distinct decline in their Hsp70 content with an increase in height (Fig. 5b, c).

Table 3 Tested models including the parameter ‘size’ for explaining the relative Hsp70 level in Mediterranean land snails, listed in decreasing succession, the best ranked model at the top

The rank of a model, the model constitution, the AIC_C , the Akaike weight (ω), and the number of parameters involved are listed. The probability reveals the importance of each factor to be part of a model
S shell size, *O* shell orientation towards the sun, *T* internal temperature

Rank	<i>S</i>	<i>O</i>	<i>T</i>	<i>S</i> $\times$ <i>O</i>	<i>S</i> $\times$ <i>T</i>	<i>T</i> $\times$ <i>O</i>	AIC_C	ω	Parameters
1	•						-326.203	0.6319	2
2	•	•					-323.474	0.1614	3
3	•		•				-323.022	0.1288	3
4	•	•	•				-320.228	0.0318	4
5				•			-319.259	0.0196	2
6	•	•	•			•	-317.677	0.0089	5
7	•	•	•	•			-317.384	0.0077	5
8	•	•	•		•		-317.302	0.0074	5
9							-313.343	0.0010	1
10	•	•	•	•	•	•	-311.667	0.0004	7
11					•		-311.228	0.0004	2
12		•					-310.526	0.0002	2
13			•				-310.291	0.0002	2
14						•	-309.953	0.0002	2
15		•	•				-307.502	0.0001	3
Probability	0.9783	0.2180	0.1853	0.0278	0.0082	0.0095			

Table 4 Tested models including the parameter ‘height above ground’ for explaining the relative Hsp70 level in Mediterranean land snails, listed in decreasing succession, the best ranked model at the top

Rank	H	O	T	H*O	H*T	T*O	AICc	ω	Parameters
1				•			-318.722	0.4228	2
2	•						-318.010	0.2963	2
3	•	•					-315.197	0.0726	3
4	•	•	•	•			-314.901	0.0626	5
5	•		•				-314.899	0.0625	3
6							-313.343	0.0287	1
7	•	•	•				-312.118	0.0156	4
8					•		-311.198	0.0098	2
9		•					-310.526	0.0070	2
10			•				-310.291	0.0062	2
11						•	-309.953	0.0053	2
12	•	•	•		•		-309.332	0.0039	5
13	•	•	•			•	-308.847	0.0030	5
14	•	•	•	•	•	•	-308.158	0.0021	7
15							-307.502	0.0015	3
Probability							0.5186	0.1684	0.1575
							0.4875	0.0158	0.0105

The rank of a model, the model constitution, the AIC_C, the Akaike weight (ω), and the number of parameters involved are listed. The probability reveals the importance of each factor to be part of a model. Models with high empirical support are arranged above the dashed line ($\Delta_i \leq 2$)

H height above ground, *O* shell orientation towards the sun, *T* internal temperature

Discussion

There are no ways for snails to avoid solar radiation stress, but several strategies to limit its degree of strength. Our study showed that among a number of biologically plausible parameters, predominantly, a distinct set of behavioural and intrinsic features define the stress protein level of Mediterranean snails in their habitat.

Table 5 Correlation coefficients (β) for the model selection analysis with the factors ‘size’, ‘orientation towards the sun’, ‘internal temperature’ and their interactions

		β
Orientation	Size	-0.0562
	Temperature	-0.0001
	$O(x)$	-0.0067
	$O(y)$	0.0058
	$O(z)$	0.0009
Size $\times$ orientation	$S \times O(x)$	0.0003
	$S \times O(y)$	0.0010
	$S \times O(z)$	-0.0012
	Size $\times$ Temperature	0.0001
Temperature $\times$ orientation	$T \times O(x)$	-0.0001
	$T \times O(y)$	-0.0002
	$T \times O(z)$	0.0002

According to the conducted correlation and model selection analyses, we could reveal a distinct pattern of features influencing the Hsp70 level in individuals of the Mediterranean snail species *X. derbentina*: large snails predominantly exhibit primarily plain white shells and climb rather high while holding a comparatively low Hsp70 level. Their small sized conspecifics with a more intense shell pigmentation remained rather low in their residing height above ground

Table 6 Correlation coefficients (β) for the model selection analysis with the factors ‘height’, ‘orientation towards the sun’, ‘internal temperature’ and their interactions

		β
Orientation	Height	-0.0013
	Temperature	0.0010
	$O(x)$	-0.0058
	$O(y)$	0.0049
	$O(z)$	0.0009
Height $\times$ orientation	$H \times O(x)$	0.0020
	$H \times O(y)$	-0.0010
	$H \times O(z)$	-0.0011
	Height $\times$ Temperature	0.0000
Temperature $\times$ orientation	$T \times O(x)$	-0.0001
	$T \times O(y)$	-0.0001
	$T \times O(z)$	0.0002

Fig. 4 All single factors used for model selection analysis plotted against sqrt Hsp70 level. Because the applied model selection approach does not determine significance levels but likelihood estimates, these graphs lack confidence intervals and p levels. These figures serve as completion for the correlation coefficients (β) and shall alleviate their interpretation by giving a visual overview. The 'orientation towards the sun' is shown as means $\pm$ SE

and, in contrast, had a higher Hsp70 level. So, what can a snail do to keep proteotoxicity as low as possible?

One important factor to explain the variance in the Hsp70 level of *X. derbentina* proved to be the height above the

ground. Most xerophilous land snails exhibit an active behaviour during the cool and moist conditions at night when they feed and roam while they stay inactive during the hot and dry day hours. By the end of the night, the organisms

Fig. 5 Interaction 'height above ground $\times$ orientation towards the sun', each category is plotted against sqrt Hsp70 level. Because the applied model selection approach does not determine significance levels but likelihood estimates, these graphs lack confidence intervals and p levels. These figures serve as completion for the correlation coefficients (β) and shall alleviate their interpretation by giving a visual overview

climb up vertical objects, seal their shell aperture with an epiphragm and reduce their metabolism to save water and energy (Mazek-Fialla 1934; Machin 1968). It seems to be generally advisable to climb up vertically, independent of the assumption that climbing eventually modifies their exposures to predators from ground-living beetles to birds. Evolution has selected this behavioural trait in all snail species from the Mediterranean but, apparently, there is no optimal height a snail should reach. Both climbing and stress protein induction is energy-costly and, thus both of these two responses to solar radiation likely trade-off against one another. Besides the necessity to escape from lethal soil surface temperatures, it seems that the amount of climbing quite often is limited by random factors such as the height of the vegetation or hindering conspecifics. On sunny summer days in Vaucluse, the soil surface reaches temperatures of up to 50°C or higher, which are above the lethal limit for the snails (M.A. Di Lellis and S. Troschinski, unpublished data). Only a few centimeters above the soil, the temperatures decline and conditions hence become more comfortable for the snails (Cowie 1985; Kempster and Charwa 2003). With lower temperatures, the cellular stress is decreasing and the Hsp70 level is expected to adjust at a moderate level (Feder and Hofmann 1999; Mukhopadhyay et al. 2003). However, internal temperature as one of the factors within our model set emerged not to be meaningful, with a probability of 15.8–18.5 % only, in regard to explain the Hsp70 level variation. However, Köhler et al. (2009) and Pomeroy (1966) showed the height above ground to be predominantly relevant for the body temperature. Several considerations may explain this lack of coherence: in our analysis we were looking on a temperature variation within a span of only 6°C, an exiguous span in terms of the large temperature fluctuations the snails experience through the whole day and are adapted to during the whole summer. Furthermore, it is known that stress protein levels do not solely reflect immediate conditions but rather integrate temporally over the effects experienced within some time span. Therefore, in the field, spontaneously fluctuating parameters like wind speed or sun shading may instantaneously influence the temperature of a snail but not its actual Hsp70 level. In addition, intraspecific variation in the heat stress response which can be due to genetic and physiological differences between the individuals might interfere with a possible effect of the internal temperature on the Hsp70 level. Experiments in the laboratory revealed an Hsp70 maximum level in these *X. derbentina* populations at around 40°C after 8 h of this elevated temperature (M.A. Di Lellis, unpublished). Therefore, the examined snails in this study most probably did not reach their thermal stress limit in the field. Besides this, Mizrahi et al. (2009) showed a delayed induction of Hsp70 and Hsp90 in a desert-dwelling snail species (*Sphincterochila zonata*)

together with an enhanced synthesis of small Hsp molecules (sHsps), which are also induced under stressful conditions. It has been shown that highly thermotolerant species adapted to environments with hot temperature regimes induced Hsp70 and other stress proteins at higher thermal limits (Hofmann and Somero 1996; Nakano and Iwama 2002; Evgen'ev et al. 2007). For highly thermotolerant species in particular, this strategy might save fitness costs and high temperature regimes might act as a microevolutionary effective agent (Feder and Hofmann 1999). Addressing the entirety of Hsp classes in this context might be advisable for further studies.

Another factor with a high validity turned out to be the size of the shell. With an increase in size, the Hsp70 level decreased. This might be due to several reasons. Small snails are developing in growth and maturity. Constitutive Hsp70 molecules own crucial features like chaperoning proteins during folding processes, intracellular protein trafficking, and assembly of proteins besides acting as inducible proteotoxic defense (Mayer and Bukau 2005). Since we used an α -Hsp70 antibody which was sensitive to all isoforms of Hsp70 and detected both constitutive and inducible stress protein molecules, small snails could have had a higher base level of Hsp70 caused by their development at stage. Another important point to consider is desiccation due to shell size. In general, larger specimens possess a more favourable surface-to-volume ratio than smaller individuals and therefore, water loss is minimized. As suggested by Mizrahi et al. (2009), land snails living under harsh environmental circumstances and suffering from desiccation use Hsp induction as important survival strategy. Hence, high Hsp70 levels in small individuals can be evoked by increased desiccation processes. Furthermore, we found coherence in shell size and the residing height above the ground. On the basis of the present dataset it is impossible to decide which of these two correlating parameters is aetiologically defining the Hsp70 level in *X. derbentina*. Lab exposure experiments in which individuals of *X. derbentina* were kept at defined temperature, however, did not reveal any influence of body size on Hsp70 induction (Köhler et al. 2009, M.A. Di Lellis, unpublished data).

The shell orientation in relation to the position of the sun as a single factor, turned out to be rather neglectable, since this parameter alone could explain only a minuscule part of the Hsp70 level variation. Nevertheless, in combination with the height above ground, we obtained an interesting effect. When orientated to the South (x), the residing height above the ground has rather an increasing effect on the Hsp70 level. When orientated in other directions (y or z), elevated heights decreased the Hsp level in the snails. The reason for this effect remains unclear, and its elucidation requires a thermodynamic computer model of the snail's shell and its inner organs which allows to simulate the

thermal effects of virtual illumination from different sides of an individual. Currently, such a computer model is envisaged to be built up in our group. Whether the difference in Hsp70 expression in different body tissues, which has been reported for mollusks (Lyons et al. 2003; Mizrahi et al. 2009; Arad et al. 2010), plays a role in this context, can therefore be addressed in the future.

In our correlation analyses, the shell colouration pattern turned out to have an effect on the Hsp70 level. Since the colouration pattern correlated with other continuous variables such as the internal temperature and the size of the snail, we had to exclude this parameter from the models. However, correlation analyses revealed the Hsp70 level to rise with increasing shell pigmentation. Due to sunlight reflectance, it was postulated that brighter coloured shells generally heat up slower than darker shell patterns (Yom-Tov 1971; Dittbrenner et al. 2009). However, current results of our group show that there are no differences in the thermal capacity of the different morphs in *T. pisana*, another Mediterranean helicoid snail species living in the same habitat as *X. derbentina* (Scheil et al. 2012). Since in *X. derbentina* the increase in shell pigmentation is strongly and negatively correlated with size, we assume that shell colouration pattern distribution could depend on the individual's age. Hoshino et al. (2010) showed that melanin production in cultured mouse melanoma cells was suppressed in cells with a high Hsp70 induction. The long and sun-intense summer time in Provence might lead to shell bleaching by suppression of the melanin synthesis via long term Hsp70 action in the growing snail. Since the shell pattern apparently is connected to the trait 'size' in *X. derbentina*, selection cannot work on it independently. But also in other species the intrinsic character 'bright shell' cannot be unequivocally favoured by evolution—otherwise the often-reported high variation in shell colouration in Helicoidea like *T. pisana*, and *Cepaea* species, would not be maintained in natural populations (Cowie 1990; Silvertown et al. 2011).

The results of this study did not reveal a universal 'best' strategy to effectively limit proteotoxic stress caused by solar radiation in terrestrial snails, even though distinct crucial criteria were discovered. Small (young) individuals apparently need to locate themselves closer to the soil surface at higher costs for Hsp70 production. For larger individuals, however, it is certainly advantageous to climb up as high as possible, particularly, if they do not expose their shell apex to the South. The latter, however, seems not to be a matter of active choice: our data suggest the geographical orientation of the shell to be rather random. Since climbing itself also is energy-costly, the actual position of *X. derbentina* presumably always is a compromise in an energetic trade-off continuum between vertical movement and stress protein production.

The present data revealed environmental factors and individual traits to strongly interact. The Hsp70 level of *X. derbentina* reflects these interactions and gives a good measure of the proteotoxic stress experienced by the snails. Our study highlights that even a single aspect of the physiological stress response requires consideration of multi-factorial action to explain its biological variation.

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