Phenotypic diversity, population structure and stress protein-based capacitors in populations of *Xeropicta derbentina*, a heat-tolerant land snail species

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Abstract The shell colour of many pulmonate land snail species is highly diverse. Besides a genetic basis, environmentally triggered epigenetic mechanisms including stress proteins as evolutionary capacitors are thought to influence such phenotypic diversity. In this study, we investigated the

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relationship of stress protein (Hsp70) levels with temperature stress tolerance, population structure and phenotypic diversity within and among different populations of a xerophilic Mediterranean snail species (*Xeropicta derbentina*). Hsp70 levels varied considerably among populations, and were significantly associated with shell colour diversity: individuals in populations exhibiting low diversity expressed higher Hsp70 levels both constitutively and under heat stress than those of phenotypically diverse populations. In contrast, population structure (cytochrome *c* oxidase subunit I gene) did not correlate with phenotypic diversity. However, genetic parameters (both within and among population differences) were able to explain variation in Hsp70 induction at elevated but non-pathologic temperatures. Our observation that (1) population structure had a high explanatory potential for Hsp70 induction and that (2) Hsp70 levels, in turn, correlated with phenotypic diversity while (3) population structure and phenotypic diversity failed to correlate provides empirical evidence for Hsp70 to act as a mediator between genotypic variation and phenotype and thus for chaperone-driven evolutionary capacitance in natural populations.

Keywords COI · Eco-devo · Evolutionary capacitance · Hsp70 · *Xeropicta derbentina*

Introduction

Moderate stress is essential for healthy growth and development of organisms (Müller 2003) and, under stressful conditions, organisms tend to increase their phenotypic variation (Waddington 1942; Jablonka et al. 1995; Badyaev 2005). In

contrast to the traditional point of view, that only gene-based differences in organisms determine their fitness, there is now increasing evidence that organisms are able to adaptively modify their developmental program according to the environment (Dusheck 2002; Sultan 2007; Bolker 2012). Stressful environments can influence ontogenetic pathways and therefore may initiate the induction of particular phenotypes. Organisms with a high phenotypic plasticity, that is the capacity to change their phenotype in response to environmental changes (Price et al. 2003), benefit from being more adaptive.

Various authors have investigated in respect to how phenotypic diversity in polymorphic snail populations are influenced by genetic traits and environmental conditions (Jones et al. 1982; Baur 1988; Baur and Raboud 1988; Cowie 1990; Johnson 2011; Di Lellis et al. 2012; Köhler et al. 2013). Shell colour and pattern are highly variable in pulmonate snail species such as *Xeropicta derbentina* (Krynicky, 1836) and *Theba pisana* (Müller, 1774) (Johnson 1981; Cowie 1990; Köhler et al. 2009). They are known for their great range of morphs, the frequency of which can be associated with different microgeographical specifics (Mazek-Fialla 1934). Yet the combination of factors determining this diversity is still not fully understood. Several gene loci primarily control shell colour, presence or absence of shell banding, intensity of pigmentation, et cetera (Jones et al. 1982; Cowie 1984). Habitat structure (such as shaded/exposed) and ambient temperature influence the composition of phenotypes in snail populations (Johnson 2011; Köhler et al. 2013). These conditions, in turn, might also be reflected in their population structure (as inferred from their genotypic diversity). Thus genes that echo a population's phylogeographical and demographic history could be informative, even though not directly coding for shell colour/pattern. This might be particularly true for populations subjected to strong bottle-necks caused by, for example, high ambient temperatures. In *T. pisana*, high temperatures emerged either as a selective force or an epigenetically acting component of the environment (Johnson 2011, 2012; Köhler et al. 2013): organisms possess various mechanisms to deal with high temperatures.

The expression of heat shock proteins (Hsps), for example, is part of the cellular heat response and directly associated with thermal tolerance (Feder and Hofmann 1999; Pörtner and Farrell 2008). Particularly, Hsp70 chaperones own abilities that make them indispensable in the physiological stress response (Lindquist and Craig 1988; Bukau and Horwich 1998). In addition to their function in the process of re-folding damaged proteins, compensating for proteotoxic effects (Gething and Sambrook 1992; Köhler et al. 1992; Parsell and Lindquist 1993; Feder and Hofmann 1999; Lewis et al. 1999; Köhler et al. 2000), they assist in folding and stabilizing nascent protein chains. The structure of Hsp70 is phylogenetically highly conserved, even though the capacities for its induction may vary considerably among populations of the

same species (Köhler et al. 2000). Differences in Hsp70 levels can directly account for stress tolerance (Köhler et al. 1992; Dahlgard et al. 1998; Feder and Hofmann 1999; Köhler et al. 2000), which often is influenced by microgeographical selection processes. Since the production of stress proteins, however, is energy consuming, it is reasonable to assume that Hsp induction trades off against other fitness parameters because of constraints in energy allocation (Krebs and Loeschcke 1994; Silberman and Tatar 2000; Kristensen et al. 2008). On the other hand, a high capacity to transiently elevated chaperoning activity may guarantee survival during short periods of extreme heat. In this context, a combination of a low constitutive (basic) level and a high induction capacity would allow low energy expenditure but high short-term stress tolerance. Several studies have revealed selection for reduced base Hsp expression in highly thermo- or metal-tolerant species or populations (Krebs and Feder 1997; Bettencourt et al. 1999; Köhler et al. 1999, 2000; Sørensen et al. 1999, 2001; Zatssepina et al. 2001; Arts et al. 2004; Haap and Köhler 2009). Mizrahi et al. (2009) also showed a delayed induction of Hsp70 and Hsp90 in a highly thermotolerant desert-dwelling snail species, which enhanced small Hsp molecules (sHsps) under thermal stress that are more efficient in production due to their molecular size.

Heat stress can influence phenotypic variation (Child et al. 1940; Imasheva et al. 1997; Royer et al. 2009; Sisodia and Singh 2009; Hansen et al. 2011; Crichigno et al. 2012) and chaperones were suggested to canalize phenotypic development and, therefore, to act as capacitors of phenotypic variation (Rutherford and Lindquist 1998; Roberts and Feder 1999; Queitsch et al. 2002; Rutherford 2003). Thus, it seems reasonable to hypothesize that they also participate in the regulation of phenotypic variation in natural populations of polymorphic land snails. Specifically, Hsp70 can suppress effects of gene-based mutations in proteins in order to preserve their function and, therefore, is thought to function as a capacitor (Roberts and Feder 1999; Mayer and Bukau 2005). Capacitoring, however, has so far almost exclusively been studied in model organisms, and its role in ecology and evolution of natural populations is still unclear. The first indirect evidence for an association of Hsps with capacitoring of phenotypic variation in natural populations was provided by Manitasovic et al. (2007) for Hsp90 and Köhler et al. (2009) for Hsp70.

However, the hypothesis that high stress protein levels confer high capacitoring potential and, therefore, low phenotypic variation has not been tested in a larger number of natural populations. To address this hypothesis, we investigated the correlation of shell colouration diversity, population structure and the capacity to induce Hsp70 in ten populations of the Mediterranean hygromiid snail *X. derbentina*. More specifically, the following questions were asked: Do snail populations differ in their constitutive Hsp70 levels under

stress-free conditions, in their maximum induction levels and in their capacity to induce Hsp70? Do Hsp70 levels correspond to phenotypic variation within and among the investigated populations, and does population structure reflect the variation in shell colour? To what extent does genotypic diversity correspond to the capacity to induce Hsp70?

Material and methods

Test organisms

X. derbentina is a xerophilic species widespread in the circum-Mediterranean region. It originates in the eastern Mediterranean and the Middle East but is also abundant in southern France (Aubry et al. 2005; Kiss et al. 2005). The shell of adult individuals is 10–16 mm in diameter. On hot days, individuals climb up vertical objects to escape the heat near the ground, seal their shell aperture and remain inactive (Mazek-Fialla 1934; Machin 1968; Aubry et al. 2005). During the night when temperatures are lower and humidity higher, they are active and feed.

Overall, ten populations were sampled in the Vaucluse department, Provence, Southern France (Table 1) in May 2010 and June 2012. Identification was based on morphological traits and a fragment of the mitochondrial cytochrome *c* oxidase subunit I (*COI*) gene. Since *X. derbentina* is annual in Southern France and only few individuals (which are easily identified by their size) live for 2 years (Dieterich et al. 2013), all tested individuals had hatched in early spring and, therefore, were of about the same age.

Snails were brought to the laboratory and kept in plastic boxes (20.5 × 30 × 19.5 cm) on moist terrarium ground-covering material (JBL TerraBasis, Neuhausen, Germany) under a light regime of 12 h/12 h and a constant room temperature of

25 °C. As recommended by Cowie and Cain (1983), they were fed baby porridge (HIPPI *Gute Nacht Bio-Milchbrei, Hafer & Apfel*, Pfaffenhofen, Germany) and cuttlebone.

Heat exposure

After 2 weeks of laboratory acclimation, snails from each of the ten populations were exposed to heat stress. The experiments were carried out in heating cabinets using plastic boxes (6.5 × 18 × 13 cm) with moist tissue paper as ground cover, each box containing ten individuals. Snails were exposed to 25 (control), 33, 38, 40, 43, 45, 48 °C and, in populations 1–7, additionally 50 °C and 52 °C for a duration of 8 h to measure the base (at 25 °C) and maximum level of stress protein induction. Finally, they were individually shock frozen in liquid nitrogen and stored in a freezer (−25 °C) until Hsp70 and genetic analyses were done.

At temperatures ≥50 °C snails became immobile indicating severe detrimental impact. We therefore omitted data from these temperatures from the analysis.

Hsp70 stress protein analysis

Individual snails were mechanically homogenized, centrifuged and the total protein concentration in the supernatant determined according to Bradford (1976). For each sample, 40 µg of total protein were analyzed by minigel SDS-PAGE and semi-dry Western blotting according to Köhler et al. (2005) using a monoclonal α-Hsp70 antibody (Dianova, Hamburg, Germany) that cross-reacted with both constitutive and inducible stress protein isoforms. The optical volumes of the individual Hsp70 bands (a single band per sample, comprising all isoforms) were quantified with the E.A.S.Y. Win 32 densitometric image analysis program (Herolab, Wiesloch, Germany). All samples were quantified relative

Table 1 Investigated *X. derbentina* populations (Pop. no.), respective sampling sites in Provence, France, and recorded data on phenotypic diversity (Hs), constitutive (Hsp70 const.) and maximum (Hsp70 max) stress protein levels and population structure (π , F_{ST} and H_{MH}). Note that

the latter two parameters were obtained by estimating the average pairwise distances between the population of concern and all other populations in the dataset

Pop. no.	Sampling date	Locality	Hs (<i>n</i>)	Hsp70 const.	Hsp70 max	Hsp70 cap%	π	F_{ST}	H_{MH}
1	May 2010	Modène Mazzia	0.72 (282)	0.643	0.851	132	2.24	0.10	0.07
2	May 2010	Modène wine yard	1.19 (282)	0.620	0.779	126	1.67	0.07	0.07
3	May 2010	Modène West	0.80 (282)	0.627	0.978	156	1.64	0.07	0.07
4	May 2010	St Pierre	0.51 (282)	0.567	0.607	107	2.50	0.32	0.32
5	May 2010	Mazan South	1.22 (282)	0.534	0.763	143	2.10	0.13	0.13
6	May 2010	Bon Remède	1.04 (282)	0.532	1.031	194	1.13	0.08	0.08
7	May 2010	Mazan North	0.20 (282)	0.687	0.961	140	0.00	0.17	0.17
8	June 2012	la Roque sur Pernes	0.26 (154)	1.042	1.539	148	2.18	0.07	0.07
9	June 2012	Voie St. Didier	0.00 (142)	1.345	1.615	120	0.40	0.12	0.12
10	June 2012	Malauccène	0.29 (184)	0.769	1.352	176	1.71	0.07	0.10

to a standard prepared from full body homogenate of *T. pisana*, which was run twice on each gel, to ensure comparability among all samples (Fig. 1). This assay displayed a methodological variance of $\pm 2.7\%$ from the mean (Köhler et al. 2005).

Phenotypic diversity

For each snail population, the shell pigmentation pattern was categorized according to the classification system of Köhler et al. (2009) (for the number of replicates see Table 1): 1 = white; 2 = white with a single pale band; 3 = grayish with several light bands; 4 = dark with lots of intense bands (Fig. 2). To assess the level of phenotypic variation within each population, we calculated Shannon-Wiener-Indices (Hs) from the shell colour categories of all available individuals from each locality ($142 \leq n \leq 282$).

Genotyping

Genomic DNA from the foot tissue of 15–20 specimens of each population was isolated using the DNeasy Blood & Tissue Kit (QIAGEN, Inc., Mississauga, Ontario, Canada). Amplification of a 700 base pair long fragment (excluding primer sequence) of the mitochondrial COI gene was carried out using the universal forward primer LCO1490 (Folmer et al. 1994) and a newly developed reverse primer HeliR2 5'-CCTAAAATATGWGAAAYAATACCAAA-3'. Sequencing was performed in both directions by LGC Genomics (Berlin, Germany) using an ABI 3730 XL DNA Analyzer. Consensus sequences were aligned in BioEdit 7.0.9.0 (Hall 1999) and deposited in GenBank (KF734452-KF734589 and 426529-KJ426573).

Statistical analyses

For testing whether genetic parameters of the populations studied significantly reflect mean differences in cellular and biochemical heat stress response, three population indices were calculated

Fig. 1 Representative Western blot for Hsp70 in snails exposed to either 25 °C (5 bands on the left side of the blot) or 33 °C (5 bands on the right side). All samples were quantified relative to a standard sample, which was run twice on each gel. The α -Hsp70 antibody was cross-reactive with all isoforms of Hsp70 and detected both constitutive and inducible proteins within a single band per sample

Fig. 2 Classification system to determine the shell coloration pattern, according to Köhler et al. (2009) modified

from the COI dataset. They comprised within-site ('diversity') and between-site ('divergence') parameters. The first parameter was nucleotide diversity π (average number of nucleotide differences within populations based on the K2P model of sequence evolution), estimated in Arlequin 3.5.1.2 (Excoffier et al. 2005). The two divergence parameters were Nei's (1973) pairwise fixation index (F_{ST}) and haplotype divergence (H_{MH}) based on the Morisita-Horn index (Horn 1966), both calculated in the R 2.15 statistical environment (R Development Core Team 2011). For the former index, we used the adegenet package (version 1.3-6, Jombart 2008); for the latter, we treated haplotypes as species (Helmus et al. 2007; Schrader et al. 2013) and estimated the dissimilarity between the haplotype structures of two groups with the vegan package (version 2.0-5, Oksanen et al. 2012).

To detect differences in constitutive Hsp70 levels at 25 °C and maximum induction levels among populations, we conducted analyses of variance (Welch-ANOVA) subsequent to checking data for normal distribution (visual inspection of histograms in addition to Shapiro-Wilk tests with $\alpha=0.05$) and for homoscedasticity (Levene's test). Subsequently, linear least squares regression analyses (basic Hsp70 levels vs. Hs, maximum Hsp70 levels vs. Hs, π vs. Hs) were conducted. For all these statistical procedures, we used JMP, version 9 (SAS Institute Inc., Cary, NC).

We then used generalized additive models (GAM) in order to examine the relationship between Hsp70 level, temperature and population structure. GAMs are often used in case of non-linear relationships between the dependent and independent variables by fitting a polynomial function. Moreover, being an extension of generalized linear models, they allow the use of a gamma error distribution, which in turn prevents the occurrence of any modelled Hsp response below 0. All regression analyses were conducted using the R 2.15 and the mgcv 1.7.22 package (Wood 2011). Hsp70 data consisted of expression measurements from ten individuals for each of seven temperatures (25, 33, 38, 40, 43, 45 and 48 °C). Preliminary analyses indicated $k=6$ (basis dimensions) to be optimal for temperature. To facilitate interpretation, three basis dimensions were chosen for the other independent variables.

Finally, we applied structural-equation modelling using lavaan (Rosseel 2012) 0.5-13 for R 2.15 in order to test simultaneously our hypothesized causal assumptions that Hsp70 levels depend on ambient temperature and population structure, and that phenotypic diversity is driven by Hsp70 levels.

Results

Hsp70 induction profiles

With the exception of population 4, all populations showed stress protein induction kinetics to follow an optimum curve in response to elevated temperature regimes. At moderately elevated temperatures, Hsp70 was induced, followed by a decline of the Hsp70 level at high temperatures, when the maximum response level had been surpassed. Most populations (Figs. 3P1-3, P5-10) showed

a clear stress protein induction with a maximum induction capacity of 120–194 % compared to the control level at 25 °C. The highest mean Hsp70 induction was always at either 38 or 40 °C, except in population 4 (Fig. 3), in which the Hsp70 level reached a maximum of just 107 % of the constitutive level, at 33 °C.

Both constitutive and maximum induction Hsp70 levels differed significantly among populations (Welch-ANOVA; $P < 0.0001$, $F_{1,9}$: 13.3387 and $P < 0.0001$, $F_{1,9}$: 24.5448, respectively) but were positively correlated with one another ($P = 0.0009$; $R^2 = 0.7650$) (Fig. 3).

Fig. 3 Hsp70 responses in *X. derbentina* to elevated temperatures. Means and SD. P1 to P10 refer to the respective population numbers. Bottom right Correlation analyses for *X. derbentina* populations.

Constitutive Hsp70 level vs. maximum Hsp70 level. Dotted lines indicate 95 % confidence interval of the linear regression curve

In addition to the maximum of induction, the majority of all populations (1–5, 7, 8) exhibited a second, smaller Hsp70 peak or at least a plateau (6, 9) after the stress protein level had decreased from maximal induction. This phenomenon occurred always in response to 45–48 °C treatment. No mortality was recorded in the experiments ≤ 48 °C.

Phenotypic diversity and population structure

Phenotypic diversity differed remarkably among populations. While population 9 was uniformly white, populations 2 and 5 showed rather high Shannon-Wiener indices of about 1.2 (Table 1, Fig. 4).

Genetic diversity within populations (π) was generally low (all < 3.0), with the highest diversity in populations 4 and 1; population 7 was homogeneous (Table 1). Genetic divergences among populations, expressed by F_{ST} and H_{MH} , were also low. The only exception was population 4, which had relatively high values for both F_{ST} and H_{MH} , indicating that it was most dissimilar from all other populations (Table 1).

Correlation of Hsp70 levels and phenotypic diversity

Regression analyses revealed significant negative correlations between both constitutive and maximum induced Hsp70 levels and phenotypic diversity (Hs) ($P=0.0139$, $R^2: 0.5515$; and $P=0.0324$, $R^2: 0.4552$, respectively) (Fig. 4).

Correlation of population structure and phenotypic diversity

Neither the diversity parameter π ($P=0.25$, $R^2=0.16$) nor the two divergence parameters F_{ST} and H_{MH} ($P=0.59$, $R^2=0.004$ and $P=0.86$, $R^2=0.004$, respectively) were significantly correlated with Hs values.

Correlation of Hsp70 levels and population structure

Based on the corrected Akaike information criterion (AICc), selecting the best generalized additive model of all possible combinations of temperature, mean population divergence

(F_{ST} and H_{MH}) and population diversity (π), resulted in a GAM incorporating all four variables (Table 2). For the seven temperature treatments with ten individuals each for each of the ten populations, 37.4 % of the variance in Hsp70 level was explained by this model. The analysis also showed that Hsp70 expression depends mostly on temperature: expression increased up to 40 °C and subsequently declined (Fig. 5). Hsp70 levels first decrease with increasing genetic diversity and increase again at π values > 1.4 . In contrast, mean genetic divergence (F_{ST}) shows the opposite pattern with intermediate differentiation coinciding with the highest Hsp70 levels (figure not shown here). For the second divergence index, mean H_{MH} , low values caused an increase in Hsp70 expression. Overall, the fit of the prediction varied for the temperature treatments (Table 3). However, the best fit was observed for non-harmful temperatures (i.e., up to 40 °C).

Structural-equation modelling

Our model, used to simultaneously test the causal assumptions that Hsp70 levels depend on ambient temperature and population structure, and that phenotypic diversity is driven by Hsp70 levels, is not rejected ($\chi^2=4.7$, $df=3$, $P=0.20$). This indicates a reasonable approximation of the causal pathways (Fig. 6).

Discussion

Our study revealed differences in phenotypic diversity, population structure and in susceptibility of the Hsp70 system to heat stress among populations of *X. derbentina*. In this species, high constitutive and high maximum levels of Hsp70 were significantly associated with low phenotypic variation in shell colouration, which is in accordance with the capacitating concept, while population structure was not correlated with phenotypic diversity but with Hsp70 expression levels. This main finding of the study strongly supports the assumption that evolutionary capacitance, originally found in model species and laboratory studies, also applies to natural populations.

Fig. 4 Correlation analyses for *X. derbentina* populations. **a** Constitutive Hsp70 level vs. phenotypic diversity. **b** Maximum Hsp70 level vs. phenotypic diversity (Hs). Dotted lines indicate 95 % confidence interval of the linear regression curve

Table 2 Estimated degrees of freedom (edf), F statistic and respective P values of the GAM model for Hsp70 expression based on temperature and genetic parameters. Population divergence was estimated by the mean of F_{ST} and H_{MH} indices, and population diversity measured by genetic diversity (π). Adjusted R^2 of the full model was 37.4 % and corrected AICc 127.4. $\Delta AICc$ shows the variable importance by the decrease in quality of the regression model when the respective variable is omitted

	edf	F	P value	$\Delta AICc$
Temperature	4.965	42.95	<2e-16	204.76
π	1.973	45.00	<2e-16	81.48
Mean F_{ST}	1.954	29.36	5.64e-13	54.56
Mean H_{MH}	1.999	24.03	7.83e-11	47.94

In comparison to earlier field-oriented work of Manitasovic et al. (2007) and Köhler et al. (2009), our present study provides a much larger dataset and, therefore, stronger evidence for a contribution of stress proteins to the capacitating of phenotypic variation under natural conditions (Fig. 6).

While Manitasovic et al. (2007) and the present study have focused on actual levels of stress proteins, Köhler et al. (2009) used the ‘Hsp70 induction capacity’ (ratio between the constitutive Hsp70 level at 25 °C and the highest measured Hsp70 level at elevated temperature) as a proxy for potential capacitating and provided data for four snail populations from different species. Supplementing the dataset of Köhler et al. (2009) by data on ‘induction capacities’ calculated from the present ten *X. derbentina* populations plus another 6 French helicoid snail populations (A.P., S.L., P.A., unpublished) revealed also negative but just weakly significant correlation between the phenotypic diversity of snail populations and the ‘Hsp70 induction capacity’ ($P=0.0474$, $df=18$, $R^2=0.2010$)

Fig. 5 GAM based prediction for the relationship between Hsp70 expression levels, genetic diversity (π), and ambient temperature

Table 3 “Goodness of fit” for the GAM model for Hsp70 expression including temperature and genetic parameters. Pseudo R^2 are given for temperatures of 25–48 °C based on comparisons of measured vs. GAM predicted Hsp70 data

Temperature (°C)	Pseudo R^2
25	0.327
33	0.402
38	0.409
40	0.394
43	−0.130
45	0.166
48	−0.058

irrespective of species and habitat. In this context, the positive association between constitutive and maximum stress protein levels, however, results in a loss of information when data on constitutive and maximum stress protein levels are transformed into ‘induction capacities’. On the basis of the results of the present study, we therefore give preference to actual constitutive and maximum Hsp70 levels as measures of capacitating capability.

This contrasts with what should be expected for the hypothetical, ideal strategy (a low constitutive Hsp70 level, but a high potential to induce Hsp70). Presumably based on mechanistic constraints, independent selection for low constitutive Hsp70 levels on the one hand and high Hsp70 induction capacities on the other does not seem to be possible here and, consequently, selection for low constitutive Hsp levels seems to confer also a low potential of Hsp induction. Consequently, *X. derbentina* populations either follow the strategy of expressing high constitutive and maximum Hsp70 levels, resulting in high energy expenditure and the consequent low phenotypic variation, or reduce constitutive and maximum Hsp70 levels for the benefit of low energy requirements and at the consequence of high phenotypic variation. While the first strategy requires a continuous and sufficient energy supply (probably reducing the stressfulness of extreme environmental conditions), the latter strategy leads to limitations in

Fig. 6 Structural-equation model for causal assumptions of Hsp70 levels, ambient temperature, population structure, and phenotypic diversity. For each path the significant ($\alpha \leq 0.05$) standardized regression coefficients are given. Population structure is a latent variable (shown as ellipse) because it cannot be measured directly and is estimated by its indicators population divergence and diversity. Measured variables are displayed in rectangles including the coefficient of determination for the dependent variables. Dashed lines indicate non-significant relationships

the biochemical response to stressful conditions and increases evolvability resulting from display of previously hidden phenotypic diversity, which is particularly evolutionarily appropriate for populations under stress. Our results are in accordance with the findings of Norris and Hightower (2000), who found a direct correlation between Hsp70 levels and survival at elevated temperature as a test of phenotypic diversity in acquired thermotolerance in *Poeciliopsis*, a genus of subtropical fish. The analogy between helicoid snails and poeciliid fish complements our findings and suggests they may be extended to other organisms than Mediterranean pulmonates.

Regarding their biochemical stress response, our microallopatric populations of *X. derbentina* differed not only in their stress protein induction profiles, but also in their Hsp70 levels under stress-free control conditions and in their maximum induction levels under elevated heat stress. Despite this substantial variation in the intensity of Hsp70 basic levels and induction capacities, the general pattern of a maximum stress response at about 40 °C and, in most cases, a second, smaller peak or saddle point at 48 °C, was the same in all populations. Whereas the Hsp70 maximum at 40 °C corresponds to a high but environmentally relevant temperature in the Mediterranean, the 48 °C peak may be the result of a last rearing up of individuals against extreme heating. Our study showed the Hsp70 variation at $T \leq 40$ °C to be reasonably well explained by population structure. The stress response patterns probably resulted from selection of efficient strategies to meet the challenges resulting from elevated and nontransient environmental temperatures (up to 40 °C) on the one hand and from occasional heat pulses, exerted by, e.g. accidental contact to the soil surface (48 °C) on the other hand. For these snails, it remains unknown how the capacity to upregulate stress proteins (by Hsp induction) and the constitutive level of these proteins (eventually dominated by cognate gene products) correspond mechanistically. Nevertheless, the population structure preferentially explained Hsp70 levels, when they were high (indicating selection pressure for high induction capacities in some habitats, e.g. those of populations 6 and 10) and constitutive Hsp70 levels to a much lesser extent (Table 3).

The only snail population, which displayed high π , F_{ST} and H_{MH} values for population structure indicating a high dissimilarity to the other populations and a correspondingly low Hsp70 induction from the constitutive level was population 4. This population was abundant close to a glass containerization site and, therefore, may be subjected to frequent specimen exchange by container replacement. On the basis of a consistent interchange of individuals and the observation that selection has favored at least a moderate Hsp70 induction capacity in all other investigated populations, an incomplete adaption to its microhabitat can be proposed for this population.

Phenotypic shell variation among populations was correlated with both the constitutive and maximum Hsp70 induction level. In contrast, population structure as represented by diversity and divergence parameters was not significantly correlated with phenotypic diversity. Whereas p-distances (π values) of COI are a reliable and readily interpretable proxy for genetic diversity (Kartavtsev and Lee 2006), the power of COI genotypic diversity to explain phenotypic variation in the shell pattern of *X. derbentina* is rather limited (Fig. 6). Consequently, epigenetic mechanisms that can modify the genotype within the range of phenotypic plasticity have to be proposed for these snails.

Populations of *X. derbentina* in the Vaucluse area with low phenotypic (but not necessarily low genotypic) variation are dominated by plain white (category 1) morphs. The question therefore arises of how high Hsp70 levels may be able to favour the emergence of non-melanized individuals. Since melanin synthesis, the ultimate cause of dark shell colouration, involves multi-step pathways with pleiotropic impact (Rebeiz et al. 2009), Hsp70 might operate in various ways. In mouse melanoma cells, in vitro as well as in vivo increased Hsp70 levels led to suppression in melanin production with two possible explanations (Hoshino et al. 2010). Firstly, Hsp70 might affect intracellular trafficking of melanosomes, since it affects intracellular traffic of vesicles (Bukau and Horwich 1998). Second, it influences the expression and activity of tyrosinase, a rate-limiting enzyme in melanin synthesis, by binding to microphthalmia-associated transcription factors (MITF) with subsequent inhibition of the promoter of the tyrosinase gene in the nucleus. Even though mechanisms of gene activation have not been investigated in *X. derbentina*, it cannot be excluded that the snail populations with predominantly white shells and, therefore, comparably low phenotypic variation are influenced by similar Hsp70 action on melanin synthesis as mammals.

Conclusion

In *X. derbentina*, genetic analyses that reflect the demographic structure of populations could not directly be related to phenotype assemblages in these populations. The observations that, on the contrary, haplotype-based genetic parameters could well explain variation in Hsp70 responses to elevated temperature and that, in parallel, constitutive and, partly, induced levels of Hsp70 correlate significantly with phenotypic variation, substantiates the theory of stress proteins acting as environmentally driven capacitors of morphological development.

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