Hsp70 and lipid peroxide levels following heat stress in *Xeropicta derbentina* (Krynicky 1836) (Gastropoda, Pulmonata) with regard to different colour morphs

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Abstract Terrestrial snails which live under dry and hot conditions need efficient mechanisms of adaptation to counteract the problems of desiccation and over-heating. A profoundly heat tolerant snail species is the Mediterranean *Xeropicta derbentina*, exhibiting different shell colour morphs ranging from pale white to darkly banded. Considering that dark-pigmented snails are believed to have a disadvantage due to faster heating, we investigated possible differences in the stress markers Hsp70 and lipid peroxidation between four pre-defined colour morphs which were exposed to different temperatures for eight hours. The highest Hsp70 levels were observed in response to 38–40 °C. Levels decreased when this temperature was exceeded. Snails of a pre-defined colour category 3 (with a large black band at the umbilicus side of the shell) showed the most prominent Hsp70 response. Lipid peroxidation levels also showed a maximum at 38 °C but displayed a second peak at rather high temperatures at which the Hsp70 level already had decreased (45–48 °C). Particularly pure white snails (category 1) and the most pigmented ones (category 4) were found to have different levels of lipid peroxidation at 38 °C and 45 °C compared to the other morphs. A hypothesis involving a combined two-phase defence mechanism, to which both, the Hsp70 protection system and the antioxidant defence system, may contribute, is discussed.

Keywords FOX assay · Heat stress · Hsp70 level · Lipid peroxidation · Shell colouration

Introduction

Hot and dry conditions, as being common during summer in Southern France, constitute hostile conditions for terrestrial animals with high water content, like snails. Embodying of more than 75 % water (Reuner et al. 2008), snails per se are vulnerable against desiccation and overheating. Nevertheless, *Xeropicta derbentina* Krynicky 1836, a hygromiid land snail species, occurs in high abundance in Southern France, where it was first recorded in 1949 (Regteren 1960) and, in the following, has successfully spread over this area. Its origin lies in the Eastern Mediterranean from where it was presumably introduced during the Second World War. In its adult state, *X. derbentina* reaches shell sizes up to 16 mm in diameter. This annual species (Dieterich et al. 2012; Kiss et al. 2005) can often be found in areas with scarce vegetation, at the borders of agricultural areas and along roads. The ability to climb vertical objects can not only be seen as a way to protect these snails from overheating in consequence of high ground temperatures, as postulated by different authors (Cowie 1985; Pomeroy 1968). Furthermore it can be seen as a way of dispersal (Aubry et al. 2006), as snails are frequently found to be attached on mobile devices like cars.

Most of the yet investigated populations of *X. derbentina* in Southern France were mainly characterized by individuals that carried a pure white shell (Dieterich et al. 2012; Köhler et al. 2009) when they have reached their final size. Among these pale individuals lower percentages of individuals were observed which were characterized by a darkly pigmented banding of the shell and which can be categorized according to the banding pattern described in previous studies (Di Lellis

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et al. 2012; Dieterich et al. 2012; Dittbrenner et al. 2009; Köhler et al. 2009).

Polymorphism in shell colouration is a well-known phenomenon in a number of land snail species. Different morphs of one of the best-studied genus, *Cepaea* sp., were found to differ in their activity, their resistance against desiccation and their shell temperature, partly depending on the natural habitat in which they were collected (Staikou 1999). Moreover, numerous studies on *Cepaea nemoralis* Linnaeus 1758, one of the most polymorphic land snail species in Europe (Cain 1977; Goodhart 1987), revealed differences between shell colour morphs: this was the case for the reflectance of the shell, the internal temperature after solar radiation, and the extent of dehydration and mortality after severe heat exposure (Chang 1991; Heath 1975; Richardson 1974). Another frequently studied snail species is *Theba pisana* Müller 1774, which also shows a highly polymorphic shell banding (Cowie 1984; Köhler et al. 2013) and is found in Southern France as well as in coastal plains of the Mediterranean Sea. The northernmost boundary of its distribution is Southern England and Wales; furthermore *T. pisana* can be found in Northern Africa and in Australia. *T. pisana* was, analogous to *X. derbentina*, observed to climb vertical objects, thereby preventing overheating (McQuaid et al. 1979). Quite frequently, the shell pigmentation of snails has been linked to higher shell temperatures, higher internal temperatures, and a quicker heating of dark banded morphs caused by solar radiation (Hazel and Johnson 1990; Heath 1975). Especially in habitats with high temperatures this should be a great disadvantage for darker individuals. Nevertheless, banded morphs – even though in smaller amounts – are abundant in hot and dry habitats. Hence, some kind of pre-adaptation can be assumed in banded or darker morphs. On the other hand, it has been reported that shells of differently coloured morphs of *T. pisana* did neither differ in heating nor in heat loss when being illuminated by light with a natural spectrum (Scheil et al. 2012a).

As mentioned above, confrontation with elevated habitat temperatures leads, like in most animals, to behavioural adaptations. In land snails the most prominent adaptations are climbing (Arad et al. 1993; Aubry et al. 2006; Cowie 1985; Pomeroy 1968) and shifting their activity to the night hours. Besides these, also physiological responses like the up-regulation of protective biochemical systems are common defence mechanisms to cope with the consequences of heat (Jäättelä 1999; Kregel 2002). One of the best known and frequently investigated mechanisms in dealing with elevated temperatures is the heat shock protein (Hsp) protection system (Feder and Hofmann 1999). Hsps are proteins which, beside other functions, assist newly synthesised proteins in their folding. This chaperoning function allows organisms to cope with elevated temperatures and to reduce protein misfolding. Hsps are categorized according to their molecular weight, and best investigated is the 70 kDa family – Hsp70. As a marker of

effect, Hsp70 has been frequently used in characterizing the molecular stress response of different organisms to heat and other stressors (Daugaard et al. 2007; Dieterich et al. 2012; Feder and Hofmann 1999; Köhler et al. 2001; Mayer and Bukau 2005). However, some Hsp70 isoforms are also expressed under non-stress conditions. These constitutively expressed stress proteins have chaperone function in protein folding processes, stabilize proteins in intracellular trafficking, and play an essential role in the assembly, degradation, and intracellular localization of proteins (Fink 1999; Hendrick and Hartl 1993; Mayer and Bukau 2005). It is known that different populations of a species can differ in their Hsp70 content and in their ability to induce Hsp70 as a response to heat, depending on their natural habitat and on the organisms' general ability of Hsp70 induction as, for example, shown in whole body homogenates of *Drosophila* sp flies (Bährndorff et al. 2006; Krebs and Feder 1997; Sørensen et al. 2001). In case of *X. derbentina*, analyses of whole body homogenates have shown that the Hsp70 level of individuals depends on the population (Di Lellis et al. 2014), the life stage, the season, and the intensity of heat exposure (Dieterich et al. 2012) as well as on the total load of heat stress over a given period of time (Köhler et al. 2009; Scheil et al. 2011). Furthermore, it has been shown that several populations of *X. derbentina* deriving from the same area have developed different heat response strategies characterized by different levels of Hsp70 (Troschinski et al. 2014).

Elevated temperatures not only lead to higher amounts of Hsp70, but can also lead to oxidative stress, as higher temperatures are known to generate reactive oxygen species (ROS) that include the superoxide anion radical (O_2^-), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($\cdot\text{OH}$). These ROS have deleterious effects on DNA, proteins, and lipids – the latter affected by peroxidation leading to the formation of lipid peroxides and a disturbance of biomembranes (Gutteridge and Halliwell 1990). In aerobic organisms, ROS are continuously formed as by-products of metabolism and are scavenged or detoxified by antioxidant defence systems (Halliwell and Gutteridge 1989; Sies 1997; Storey 1996). Whenever these systems are overwhelmed by a sudden burst of generated ROS, oxidative damage rapidly manifests in cells (Abele et al. 1998; Pannunzio and Storey 1998).

Measuring the amount of oxidative waste products such as lipid peroxides is a common method to assess an organism's ability to cope with oxidative stress which has been applied to marine (Jena et al. 2009) and terrestrial molluscs (Scheil et al. 2012b) before. Lipid peroxides can be quantified by the ferrous oxidation xylene orange method (FOX-assay) (Hermes-Lima et al. 1995; Monserrat et al. 2003).

To date, only little is known about the influence of a snail's shell colour on the Hsp70 level or the extent of oxidative damage, reflected by lipid peroxidation, at different temperatures. In this study we will therefore address the question of

pre-adaptation of different shell colour morphs of *X. derbentina* to passive heating by analysing their Hsp70 level and their level of lipid peroxidation after exposure to elevated temperature for a fixed period of time in an artificial scenario.

Material and methods

Test organism and sampling setup

Equal sample sizes from a single, annual field population of *Xeropicta derbentina* were taken. To avoid any negative influence of aging, the investigated specimens were all collected in early summer, where the growth of the snails is almost finished but production of eggs has not yet taken place. In former studies, June revealed to be the best time in the year to perform such studies (Dieterich et al. 2012).

Samples were collected in the vicinity of Modène (Vaucluse, Provence, Southern France, N44°6.055' E5°7.937') in June 2012. The sampling site was not used for agricultural purpose, thus, no pesticides were applied. Individual snails were sorted according to their colour category as predefined in other studies (Di Lellis et al. 2012; Dieterich et al. 2012; Köhler et al. 2009). Colour category 1 snails were defined as snails which carry a uniformly white shell. In colour category 2 snails with only a narrow light pigmented single band on the umbilicus side of the shell or with a light brownish shell colour on the umbilicus side of the shell were grouped. Category 3 snails bore a dark pigmented thick band on the umbilicus side of the shell or more than one light pigmented band. In colour category 4 snails with multiple bands on the umbilicus side of the shell and pigmentation on the apical side of the shell were grouped (Fig. 1). The snails were allowed to acclimatise to laboratory conditions (25 °C) for three weeks until further processing. They were kept

in plastic containers (20.5 x 30 x 19.5 cm) filled with a layer of ground cover material (JBL, Terra Basis, Neuhofen, Germany). Snails were fed organic milk mash (Hipp, Pfaffenhofen, Germany) *ad libitum* twice a week. Every other day boxes were cleaned and sprayed with water to keep humidity.

For experimental purpose 22 snails of each colour morph were randomly chosen and transferred into separate plastic boxes (18 x 13 x 6.5 cm) with a moist paper towel used as ground cover. The boxes were sealed with plastic foil to prevent the escape of snails during the experiment and to ensure a water saturated atmosphere. This was done to prevent fluctuations in the results that might have appeared as there was no possibility to control the humidity during the experiment. To ensure air circulation, the foil was perforated with nine small holes with 2 mm in diameter. Subsequently, the snails were exposed for eight hours in heating cabinets to temperatures of 25, 33, 38, 40, 43, 45, and 48 °C. After heat exposure, the snails were immediately and individually frozen in liquid nitrogen for further analyses. The shell of the specimens taken for the FOX assay was cracked between two glass slides and removed prior to freezing. Samples were stored at −25 °C until further analyses. To ensure comparability of biochemical data, snails of similar size were chosen for analysis. To avoid effects that might be addressed to senescent animals, only snails between 0.7 and 1.1 cm representing late juveniles or young adults were used for the experiment. Only individuals that survived the exposure phase were used in the experiments. To check for the snails' survival, individuals were tabbed with a blunt needle. Retraction movement of the foot was seen as a sign of survival. As no mortality was detected during the experiments, all treated snails were used.

Hsp70 Analysis

For Hsp70 analysis twelve out of the above-mentioned twenty-two individuals from each experimental setup were taken. While two individuals of them were kept as a backup and stored at −20 °C, ten individuals were subsequently analysed as follows: The individually frozen snails were homogenized as a whole on crushed ice in 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 mass including the shell (2 µl buffer each mg snail weight). After ten minutes of centrifugation at 13722 rpm (=20000 rcf) in an Eppendorf Centrifuge 5804R at 4 °C, the resulting supernatant was divided into two portions. The first portion was used to calculate the total protein content using a standard procedure (Bradford 1976) in 96-well plates and a plate reader (Bio-Tek Instruments, Winooski, VT, USA). The second portion (40 µg of total protein) was processed for the minigel SDS-PAGE (12 % acrylamide, 0.12 % bisacrylamide, 30' at 80 V plus 90' at 120 V). The proteins were transferred to a nitrocellulose membrane by semi-dry

Fig. 1 Illustration of the four different colour morphs of *X. derbentina*, modified version based on Köhler et al. 2009

electro blotting. Subsequently, the membranes were transferred into blocking solution (50 % horse serum in TBS) for two hours. After blocking, the membranes were incubated with a monoclonal α -Hsp70 antibody, cross reacting with all isoforms of the Hsp70-family, (mouse anti-human Hsp70, Dianova, Hamburg, Germany, dilution 1:5000 in 10 % horse serum/TBS) on a lab shaker at room temperature overnight. The following day, the membranes were rinsed in TBS for five minutes to remove surplus antibody. After that step, the second antibody (goat anti-mouse IgG conjugated to peroxidase, Jackson ImmunoResearch, West Grove, PA, dilution 1:1000 in 10 % horse serum/TBS) was applied for two hours. Following another five minutes of rinsing in TBS, the membranes were stained in staining solution (1 mM 4-chloro(1)naphthol, 0.015 % H_2O_2 , 30 mM Tris pH 8.5 and 6 % methanol). Digitalisation was done using an Epson Perfection V350 Photo scanner. For each band, the optical volume (=band area $\times$ average grey scale value) was calculated with E.A.S.Y. Win 32 (Herolab, Wiesloch, Germany). The achieved optical volumes of the samples were related to a standard (full body extracts of *Theba pisana* (Müller 1774)) which was run in duplicate on every single gel. All stained membranes showed a single band of Hsp70 protein for each sample separated in the minigel SDS-PAGE. No broken bands were observed during the whole experiment (exemplarily shown in Fig. 2). All given data were calculated as a mean of ten individuals.

FOX assay

In this study we conducted a modified FOX assay according to the method described by Hermes-Lima et al. (1995). Ten of the stored samples per exposure group (without shell) were used for this assay. The individuals were weighed and homogenized in ice-cold HPLC grade methanol (dilution 1:2; the required amount of methanol is calculated by: wet weight of the individual / density of methanol (0.791 g/cm³)), centrifuged at 15.000 rpm (=21130 rcf) at 4 °C for 5 min in an Eppendorf Centrifuge 5804R. Supernatants were stored at -80 °C. The assay was conducted using 96-well plates and a plate reader (Bio-Tek Instruments, Winooski, VT, USA). In each well (except for the blank) 50 μ l of each reagent was added following this order: 0.25 mM $FeSO_4$, 25 mM H_2SO_4

and 0.1 mM xylene orange. Then, 15 μ l of sample supernatant was added and the final sample volume adjusted to 200 μ l with aqua bidest. For each sample, three wells were prepared (3 replicates) and a mean value was calculated. Master blanks contained 200 μ l of aqua bidest.

Samples were incubated at room temperature for 180 min and absorbance was read at 580 nm (A_{580nm}). Subsequently, 1 μ l of 1 mM cumenehydroperoxide (Chp) solution was added to the samples, incubated for 30 min at room temperature and again read at 580 nm ($A_{580nm+Chp}$).

The content of lipid hydroperoxides in the samples is expressed as cumenehydroperoxide-equivalents per gram wet weight (ChpE / g wet weight) and was calculated according to the equation by Hermes-Lima et al. (1995):

$$ChpE/g_{wet\ weight} = (A_{580nm}/A_{580nm+Chp}) * Chp_{1nmol} * V/V1 * DF$$

where V=total sample volume (200 μ l), V1=added sample supernatant volume (15 μ l) and DF=dilution factor with methanol (2).

Statistics

All data were checked for normality using the Pearson-D'Agostino Omnibus Test. The Levene's test was used to check for homogeneity of variance. In both sample sets normal distribution and homogeneity of variance was present, therefore, parametric test statistics could be applied. Because of a highly significant ($p < 0.001$) interaction between the factors 'temperature' and 'colour category' in both sample sets, the interpretation of a two way ANOVA was avoided. Instead, we performed one way ANOVAs on our data, sorted by temperature, followed by Tukey-Kramer-HSD tests to reveal the differences in Hsp70 and lipid peroxidation levels among the colour categories for each temperature tested and between the tested temperatures ignoring the shell colouration. For statistics we used SAS Jmp10 (SAS Institute Inc. 2012). The Pearson-D'Agostino Omnibus Test was carried out using the SolverStat Plugin (Comuzzi et al. 2003) for Excel. Levels of significance were set to: $0.01 < p \leq 0.05$: * (slightly significant); $0.001 < p \leq 0.01$: ** (significant); $p \leq 0.001$: *** (highly significant).

Fig. 2 Representative picture of a stained nitrocellulose membrane. The standard (S) was run in duplicate. Ten randomly chosen samples are shown. The amount of total protein used for analysis was 40 μ g for each sample. Arrows indicate two representative samples with a high (lane 5) and a low (lane 4) Hsp70 content

Results

Hsp70 analyses

Generally, snails showed a distinct response in their Hsp70 levels after exposure to different temperatures. As shown in Fig. 3, the overall Hsp70 level (ignoring the shell colouration)

Fig. 3 Hsp70 levels of *X. derbentina* after exposure to different temperatures for 8 hours, irrespective of shell colouration (means $\pm$ SD; n=40). Asterisks show significant differences between the different exposure temperatures: 0.01 < p $\leq$ 0.05 (*), 0.001 < p $\leq$ 0.01 (**); p $\leq$ 0.001 (***)

was found to be slightly significantly ($p=0.0117$) elevated in those snails exposed to 38 °C and highly significantly ($p<0.001$) reduced in snails exposed to 45 °C compared to laboratory conditions at 25 °C. A maximum Hsp70 level was observed in snails exposed to 38 °C. The measured Hsp70 level of these snails was found to be highly significantly ($p<0.001$) elevated compared to those of individuals exposed to 45 °C and 48 °C and significantly ($p=0.002$) elevated compared to individuals exposed to 43 °C. The lowest Hsp70 level was found in snails exposed to 45 °C. The observed Hsp70 level of these snails was found to be slightly significantly ($p=0.0179$) lower compared to snails exposed to 48 °C, significantly lower compared to the measured Hsp70 level in snails exposed to 43 °C and highly significantly ($p<0.001$) lower compared to the Hsp70 levels of snails exposed to the other temperatures tested. While the Hsp70 levels were found to be almost identical at 25 °C among the four colour categories, the individuals of the different categories responded differently to elevated temperatures. Particularly the heat response of individuals of colour category 3 diverged from the other morphotypes (Fig. 4). At 33 °C, snails from category 3 started to express a tentatively higher Hsp70 level than the other morphs. After exposure to 38 °C and 40 °C, the resulting Hsp70 levels of category 3 snails were significantly higher than those of the other colour categories (at 38 °C: category 3 differed from category 1 with $p=0.0382$, from category 2 with $p<0.001$ and from category 4 with $p=0.002$. At 40 °C: category 3 differed from category 1 with $p=0.0050$, from category 2 with $p<0.001$ and from category 4 with $p<0.001$). Exposure to 43 °C led to a remarkable breakdown of the Hsp70 level in category 3 snails. At 45 °C, not even half the Hsp70 level was measurable compared to the findings at 40 °C. In the exposure

Fig. 4 Hsp70 levels of each morph category after exposure to elevated temperatures (means $\pm$ SD; n=10). Asterisks show significant differences between the categories within an exposure group: 0.01 < p $\leq$ 0.05 (*), 0.001 < p $\leq$ 0.01 (**); p $\leq$ 0.001 (***)

groups of 45 °C and 48 °C, the category 3 snails were found to express the lowest measured Hsp70 level, compared to the other colour categories. Comparing the Hsp70 level of category 3 with category 1 snails at 48 °C, a slightly significant ($p=0.0412$) lower Hsp70 level was found (Fig. 4).

FOX assay

Generally, the amount of ChpE / g wet weight increased after exposure to 38 and 40 °C (compared to control condition at 25 °C) followed by a decrease after exposure to 43 °C with a level even lower as the control level (25 °C). A second increase of ChpE / g wet weight was observed after exposure to very high temperatures (45 and 48 °C).

The observed differences between the colour morphs are displayed in Fig. 5. After exposure to 33, 38, and 45 °C significant differences were found to be present. At 33 °C, a slightly significant difference was observed between categories 2 and 3 ($p=0.0485$) whereby category 2 snails showed a lower level of ChpE / g wet weight. A highly significant difference between category 1 and all other colour categories was observed after exposure to 38 °C (all comparisons with $p<0.001$). Here, snails of category 1 had the lowest level of lipid peroxides measured at this temperature. This level almost mirrored the control level, and thus showed a 'delayed' reaction to increasing temperatures compared to the other categories. After exposure to 45 °C, snails of category 4 showed an increase in ChpE / g wet weight differing from the response of the other colour categories: the level of lipid peroxides in colour category 4 was slightly significantly elevated compared to category 1 ($p=0.0270$), significantly elevated compared to category 2 ($p=0.0010$), and highly significantly

Fig. 5 Lipid peroxide levels (ChpE per gram wet weight) of each colour category after exposure to elevated temperatures (means $\pm$ SD; n=10). Asterisks show significant differences between the colour categories within an exposure group: $0.01 < p \leq 0.05$ (*), $0.001 < p \leq 0.01$ (**); $p \leq 0.001$ (***)

elevated compared to category 3 ($p < 0.001$). Furthermore, category 3 snails tended to exhibit a lower level of ChpE / g wet weight in response to extreme temperature exposures at 45 °C and 48 °C, compared to the other categories (Fig. 5).

Discussion

Hsp70 analysis

As reported in other studies related to adaptations of land snails from Southern France to elevated temperatures (Di Lellis et al. 2012; Dieterich et al. 2012; Köhler et al. 2009; Scheil et al. 2011; Troschinski et al. 2014), *X. derbentina* reacts to heat stress with a clear Hsp70 induction when being confronted with increasing temperatures. A maximum induction of Hsp70, the intensity of which depended on the identity of the population, was regularly observed between 38° and 40 °C in different populations (Di Lellis et al. 2014; Troschinski et al. 2014). Exceeding these temperatures, a decrease in Hsp70 level was found, with the lowest measured values at 45 °C, followed by a second, minor increase in Hsp70 at 48 °C (Di Lellis et al. 2014; Troschinski et al. 2014). Both, qualitative and quantitative aspects of these heat stress response kinetics were also recorded for the *X. derbentina* specimens investigated in the present study. Therefore it can be considered as a general response of the snails to artificially induced heat stress.

In this study we focused on possible differences among the four colour categories found in the field and their ability to induce Hsp70 as a response to passive heating. As revealed by our data, category 3 snails showed a higher capacity to induce Hsp70 compared to snails of any other category. In the study of Dieterich et al. (2012) category 3 snails were mainly found

during spring. Thus, it is possible that category 3 shell colouration may be regarded predominantly as a 'juvenile' colouration pattern that disappears when the next shell whorl is formed with proceeding growth. Particularly shells with more than two small brown bands could mainly be observed in young snails with a size of 3 to 5 mm. Snails with a single large black or brown band, as those used in this study, mainly correspond to larger size and could be observed during the entire year. To prevent effects that may arise from working with very young or senescent snails, our experiments only used individuals collected in June. Nevertheless, differences in the induction of Hsp70 were found. Category 3 snails, for a reason we do not yet understand, were able to induce higher levels of Hsp70 than the other categories when heated up to a maximum of 43 °C, indicating that a particular heat response strategy may be associated with a distinct phenotype. In the temperature range between 33 °C and 43 °C category 3 snails were found to be pre-adapted to elevated temperatures in a better way, as indicated by higher Hsp70 levels. In contrast, category 3 snails were shown to exhibit the lowest Hsp70 content of all categories when the temperature exceeds 43 °C. Apparently, the Hsp70 protection system of the category 3 snails seems to be more effective below 43 °C, compared to the other categories, and seems to get easily overwhelmed when temperatures exceed 43 °C. It is likely that the maintenance of a superior protection system is very cost-intensive. Therefore, it seems that snails of the colour category 3 are less able to cope with temperatures higher than 43 °C, probably as they are no longer able to invest these high energy costs in this protection system. Consequently, at very high temperatures, category 3 snails may show lesions on the cellular level, as described in different studies (Dittbrenner et al. 2009; Scheil et al. 2011; Troschinski et al. 2014), earlier in comparison to the other morphs. In years with very hot summers, this may be a disadvantage for category 3 snails. It is not yet known if X.

derbentina populations change their composition of colour morphs during the years and how different local temperatures may influence the morph frequencies within a population of these snails. However, the phenomenon of morph frequency fluctuations throughout the years has been reported for other helicoid land snails before (Cowie 1992; Johnson 2011; Silvertown et al. 2011). As summarized by Ozgo and Schilthuizen (2012), the shell colour of *Cepaea nemoralis* was found to be associated with a gene locus coding for the different background shell colours in this species, while the banding was found to be associated with another locus, linked to the colour coding one. *Cepaea nemoralis*, as reviewed in Goodhart (1987), was often found to adapt its shell banding and colouration to the habitat. Particularly in warmer regions and in more sun-exposed habitats, yellow unbanded or at least 'effectively unbanded' (snails with at least the top two bands missing and appearing unbanded in the most views) specimens were found to be more abundant than specimens with all five bands expressed on the shell or with a darker background colour. This indicates a natural selection of morphotypes by climate. On the other hand, other examples are given which rather point to a local area effect, as, in some studies cited in this review, snails from one predominant colour morph were found to inhabit differently structured habitats and no change in morph frequencies was found. Further, a change in the colouration frequency of *Cepaea nemoralis* over more than 40 years was reported in the study of Ozgo and Schilthuizen (2012). The authors speculated on anthropogenic change of environment and the increase in temperature in the sampling region to be possible reasons for these morph frequency changes. For the investigated population of *X. derbentina* in this study, as well as for other populations of this species in the vicinity investigated so far (Di Lellis et al. 2014; Köhler et al. 2009; Troschinski et al. 2014), no historic data about the change in morph frequencies are available. Without long term studies like the above mentioned ones, a possible area effect that may explain the predominant white coloured category 1 snails, remains speculative. In our study not the predominant pale category 1 did express the highest Hsp70 level and, therefore, may be best protected against the consequences of heat. Compared to *Cepaea nemoralis*, the 'effectively unbanded' category 3 snails were found to have an increased Hsp70 level when being exposed to 38 °C and 40 °C. A possible explanation for this may lie in a varying adaptation to the climatic conditions in the different colour categories over the years. As mentioned in Sørensen et al. (1999), adaptation to heat over several generations can lead to a decreased Hsp70 level in *Drosophila buzzatii* lines. If this would also be the case in the investigated *X. derbentina* population, the better adapted categories 1 and 2 would show reduced Hsp70 inducibility as a matter of an energetic trade-off with the possible advantage of a more successful reproduction. The significantly higher Hsp70 levels in category 3 snails may

point to a weaker adaptation to the local climate. To date it is not known if *X. derbentina* shows a similar genetically controlled mechanism of shell colouration and shell banding as found in *Cepaea nemoralis* before. The change in morph frequency distribution over a long period of time as well as the genetics of this species needs further investigations to clarify these aspects. In contrast to other publications dealing with Hsp70 induction or shell colouration, we excluded solar radiation as a heat source by heating the snails in heating cabinets. Different heating of shells caused by different shell colouration intensity, as it has often been proclaimed (Heath 1975; Moreno-Rueda 2008; Richardson 1979), cannot be taken to explain the differences in Hsp70 levels in this experiment. Therefore, some kind of pre-adaptation may have been evolved for the different colour categories of the investigated *X. derbentina* population.

To be consistent with the methodology applied in earlier studies on Mediterranean land snails (Dittbrenner et al. 2009; Köhler et al. 2009; Scheil et al. 2011; Troschinski et al. 2014) we exposed the snails for eight hours in a heating cabinet. The principles of Hsp70 induction in *X. derbentina* have been studied in relation to the heat load (Köhler et al. 2009), and also the daily Hsp70 level kinetics in different seasons was reported before (Dieterich et al. 2012). However, data on the temporal kinetics of the Hsp70 system in *X. derbentina* in response to different temperatures are still lacking. Scheil et al. (2011) found that exposure of *X. derbentina* to a very high but still sub-lethal temperature of 45 °C led to a maximum induction of Hsp70 after two hours, followed by a subsequent decline. After exposure to temperatures of about 25 °C, a maximum Hsp70 level was observed after four hours in the same snail species. These results indicate an interrelation of exposure time and temperature, two parameters which are likely not linked in a linear way. No such data are yet available for temperatures in between these two extremes and it is not known whether different colour morphs of these snails induce their maximum Hsp70 level after the same period of experienced heat stress or, possibly, differ in this respect. The latter may explain the divergent '8 h snapshot' Hsp70 data recorded in the present study.

FOX assay

An assumed time dependency of maximum levels, as it was discussed for Hsp70, does not have to be considered for the measurement of lipid peroxides: Scheil et al. (2012b) exposed snails of *Theba pisana* for 8 h at 43 °C, took samples at four time points (0, 2, 4 and 8 h), and observed a significant increase of lipid peroxides after 4 h which stayed constantly high until the end of the experiment. It became evident that long heat exposure elevated the lipid peroxide level, but without any subsequent decline.

Our results on temperature-induced oxidative damage, as reflected by the relative amount of lipid peroxides, in general revealed a clear increase of lipid peroxidation after 8 h, primarily after exposure to 38 °C and 40 °C and, secondly after exposure to 45 °C and 48 °C. The elevated levels of lipid peroxides indicate cellular damage as a consequence of oxidative stress which was caused by heat exposure. This 'two-peak' pattern is particularly remarkable in view of the low lipid peroxide level at 43 °C. A possible explanation may lie in the activity of the antioxidant defence system which includes both a number of enzymes and also small molecules. Enzymes of this defence system that directly degrade ROS include: superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) (Aebi 1984; Gutteridge 1995; Halliwell and Gutteridge 1989). Aside of these enzymes, there are much more antioxidants acting as free radical scavengers or substrates. For example, most importantly reduced glutathione or other enzymes like glutathione reductase (GSR) or glutathione-S-transferase (GST), which additionally need reduced glutathione as a cofactor for their activity (Meister 1988; Pannunzio and Storey 1998; Radwan et al. 2010). In the context of our results, we assume that one or several enzymes of the antioxidant defence system may have an activity optimum or are expressed in a higher amount at low temperatures (25 and 33 °C), in the following called 'defence mechanism 1'. Whereas another enzyme (or a complex of enzymes), in the following called 'defence mechanism 2', displays either its optimum or at least a high level at 43 °C, thus limiting lipid peroxidation at this temperature. In addition to these enzymes, also a higher amount of scavenger molecules of the non-enzymatic defence system could cause this effect.

It has been shown in aquatic invertebrates (Zhou et al. 2010) that antioxidant protection systems, as well as the stress proteins Hsp60 and Hsp70, are induced by heat. It is likely that both, the heat shock proteins and the antioxidant protection system, are responsible for the snails' survival in the heat. With respect to the interrelation of these two protection systems, one may speculate as follows: The 'defence mechanism 1' may counteract slight oxidative stress and may be expressed in a rather constitutive way with a slight induction potential above 38 °C. Whenever a more severe stress factor (in this case heat) challenges one of the sub-systems of this 'defence mechanism 1' (e.g. the Hsp70 system) to react, the other sub-system may be silenced because of an energetic trade-off. When the first defence mechanism starts getting overwhelmed with increasing stress intensity (e.g. at temperatures above 43 °C), the 'defence mechanism 2' may take over to ensure further survival. When we apply this hypothesis to our results, the high level of Hsp70 observed at 38 °C and 40 °C was probably indicative of Hsp70 being a sub-system of 'defence mechanism 1', associated with an increased extent of oxidative damage, reflected by increased levels of lipid peroxides,

because of an energetic trade-off. Thus, the antioxidant defence was probably at a minimum at these temperatures. The 'defence mechanism 2' protection system may become of importance when the energy-intense Hsp70 protection system is getting overwhelmed between 40 °C and 43 °C. Such a protective system may lead to lower lipid peroxide levels at 43 °C and to the observed reduction in Hsp70 level at 43 °C and 45 °C. Such an interpretation, however, remains speculative in respect to the biochemical antioxidant components involved in 'defence mechanism 1' and 'defence mechanism 2', even though it helps to explain the different patterns observed for Hsp70 and lipid peroxidation following increasing heat stress.

Regarding the different morphs of *X. derbentina*, 'defence mechanism 1' seems to be more efficient in colour category 1, whereas 'defence mechanism 2' is poorly pronounced in colour category 4, respectively compared to the other morphs.

This hypothesis should be addressed by further studies in which the focus should lie on different component enzymes of the antioxidant defence system. Nevertheless in our present study the suitability of the measurement of lipid peroxides via the FOX assay as a biomarker for heat-induced oxidative stress in terrestrial snails could be demonstrated. In combination with the well established marker for proteotoxicity, Hsp70, a more detailed statement about the health conditions of *X. derbentina* individuals in Southern France and their possibility to react to harsh environmental conditions can be given.

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