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RESEARCH ARTICLE

Antioxidant defence and stress protein induction following heat stress in the Mediterranean snail *Xeropicta derbentina*

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ABSTRACT

The Mediterranean snail *Xeropicta derbentina* (Pulmonata, Hygromiidae), being highly abundant in Southern France, has the need for efficient physiological adaptations to desiccation and over-heating posed by dry and hot environmental conditions. As a consequence of heat, oxidative stress manifests in these organisms, which, in turn, leads to the formation of reactive oxygen species (ROS). In this study, we focused on adaptations at the biochemical level by investigation of antioxidant defences and heat shock protein 70 (Hsp70) induction, both essential mechanisms of the heat stress response. We exposed snails to elevated temperature (25, 38, 40, 43 and 45°C) in the laboratory and measured the activity of the antioxidant enzymes catalase (CAT) and glutathione peroxidase (GPx), determined the Hsp70 level and quantified lipid peroxidation. In general, we found a high constitutive level of CAT activity in all treatments, which may be interpreted as a permanent protection against ROS, i.e. hydrogen peroxide. CAT and GPx showed temperature-dependent activity: CAT activity was significantly increased in response to high temperatures (43 and 45°C), whereas GPx exhibited a significantly increased activity at 40°C, probably in response to high levels of lipid peroxides that occurred in the 38°C treatment. Hsp70 showed a maximum induction at 40°C, followed by a decrease at higher temperatures. Our results reveal that *X. derbentina* possesses a set of efficient mechanisms to cope with the damaging effects of heat. Furthermore, we demonstrated that, besides the well-documented Hsp70 stress response, antioxidant defence plays a crucial role in the snails' competence to survive extreme temperatures.

KEY WORDS: Catalase, Glutathione peroxidase, Hsp70, Stress response, Oxidative stress

INTRODUCTION

In the Mediterranean climate, which is characterized by dry and hot summers, animals need particular adaptations to ensure survival under extreme environmental conditions. Terrestrial snails in particular, with their water-permeable skin (Machin, 1964) and their external shell, face the risk of desiccation and over-heating. One example is the pulmonate land snail *Xeropicta derbentina* (Krynicki 1836), which occurs in high numbers in southern France. These snails possess special behavioural and physiological adaptations to their habitat: climbing on vegetation to escape from hot ground temperatures or shifting activity phases to favourable time periods (Pomeroy, 1968; Yom-Tov, 1971) can be seen as behavioural adaptations, whereas aestivation attended by metabolic depression (Guppy and Withers, 1999; Bishop and Brand, 2000; Storey, 2002) during periods of extreme dry conditions is an example of a physiological mechanism of adaptation. Furthermore, there are different mechanisms acting at the biochemical level that are known to play an important role in the thermotolerance of animals.

One of these mechanisms is antioxidant defence, which plays a crucial role in periods of oxidative stress, caused by, for example, heat overload. This stress status occurs whenever there is an overproduction of reactive oxygen species (ROS) due to an imbalance between ROS formation and ROS detoxification (Sies, 1994; Sies, 1997). These ROS have deleterious effects on DNA, proteins and lipids (Halliwell and Gutteridge, 1989; Halliwell, 2006), leading to functional alterations in cells and tissues. The oxidation of polyunsaturated fatty acids by ROS is known as 'lipid peroxidation' (Gutteridge, 1995), leading to the formation of lipid peroxides and, consequently, to the impairment of biomembranes (Gutteridge and Halliwell, 1990). The lipid peroxidation process can be determined by quantification of lipid peroxides via the ferrous oxidation Xylenol Orange method (FOX assay) (Hermes-Lima et al., 1995; Monserrat et al., 2003), which functions as a tool to assess the extent of oxidative damage an organism has experienced due to oxidative stress.

Aerobic organisms must deal with the continuous generation of ROS as byproducts of metabolism (Halliwell and Gutteridge, 1989). These products are molecules derived from molecular oxygen and include the superoxide anion radical ($\cdot O_2^-$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($\cdot OH$), the last being highly reactive and most destructive (Pannunzio and Storey, 1998). To minimize their destructive action, ROS should be rapidly eliminated. All cells possess constitutive antioxidant defences, which include enzymes and small molecules that detoxify or scavenge ROS (Halliwell and Gutteridge, 1989). Enzymes that directly degrade ROS include: (i) superoxide dismutase (SOD), which catalyses the dismutation of superoxide into H_2O_2 and oxygen, (ii) catalase (CAT), which degrades H_2O_2, and (iii) glutathione peroxidase (GPx), which degrades H_2O_2 and also lipid peroxides generated by lipid peroxidation (Aebi, 1984; Halliwell and Gutteridge, 1989; Gutteridge, 1995). Besides these enzymes, there are many more antioxidants acting as free radical scavengers or substrates (e.g. the most important one is reduced glutathione, GSH) involved in the detoxification of hydroperoxides, or other enzymes like glutathione reductase (GR) 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).

The activation of antioxidant defences is an essential factor in protecting an organism from cellular damage when environmental conditions become deleterious. Changes in the activities of antioxidant enzymes have been found in many organisms in response to anoxia (Hermes-Lima and Storey, 1993; Hermes-Lima and Storey, 1996; Pannunzio and Storey, 1998; Lushchak et al., 2001), freezing (Hermes-Lima and Storey, 1993; Joanisse and Storey, 1996) and also

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List of symbols and abbreviations

$\cdot O_2^-$	superoxide anion radical
·OH	hydroxyl radical
CAT	catalase
FOX	ferrous oxidation Xylenol Orange method
GPx	glutathione peroxidase
GR	glutathione reductase
GSH	reduced glutathione
GST	glutathione *S*-transferase
H_2O_2	hydrogen peroxide
Hsp70	72 kDa heat shock protein
Hsp	heat shock protein
ROS	reactive oxygen species
SOD	superoxide dismutase

heat stress (Heise et al., 2006; Lushchak and Bagnyukova, 2006a; Verlecar et al., 2007). An increase in antioxidants during aestivation in snails (Hermes-Lima et al., 1998; Ramos-Vasconcelos and Hermes-Lima, 2003; Nowakowska et al., 2009) has also been shown. Furthermore, the application of oxidative stress indices can be used as a biomarker of environmental pollution (Jena et al., 2009; Luna-Acosta et al., 2010; Radwan et al., 2010).

Another efficient mechanism to cope with the action of elevated temperature is the heat shock protein 70 (Hsp70) protection system, comprising chaperones with a molecular mass of about 70 kDa. Hsps are phylogenetically highly conserved and abundant throughout almost all organisms investigated so far (Lindquist and Craig, 1988; Feder and Hofmann, 1999). It is known that Hsps are synthesized in response to a wide range of stressors, not only heat (Lindquist, 1986; Parsell and Lindquist, 1993). Under conditions of homeostasis, Hsp70 is expressed constitutively and mainly functions in assisting newly synthesized proteins in their correct folding. Besides this chaperoning function, Hsp70 plays an essential role in the intracellular trafficking, degradation and localization of proteins (Hendrick and Hartl, 1993; Fink, 1999; Mayer and Bukau, 2005). Under stressful conditions, the Hsp70 level can be upregulated by an intensified expression of the corresponding genes, in the context of which an elevated intracellular level of malfolded or degraded protein is seen as a trigger for this upregulation (Parsell and Lindquist, 1993; Morimoto, 1998; Feder and Hofmann, 1999; Kregel, 2002; Mayer and Bukau, 2005). Hence, Hsp70 has frequently been used as a marker of the proteotoxic effect, as a direct link between the consequences of heat exposure and the resulting Hsp70 level in different organisms (Feder and Hofmann, 1999; Sørensen et al., 2001; Daugaard et al., 2007). In the case of the Mediterranean land snail *X. derbentina*, elevated Hsp70 levels in response to heat exposure have been found in a number of recent studies (Köhler et al., 2009; Scheil et al., 2011; Dieterich et al., 2013; Di Lellis et al., 2014; Troschinski et al., 2014).

Together with the antioxidant defence system, the Hsp70 defence system is supposed to form an efficient mechanism that ensures survival in a challenging habitat. However, to date, little is known about the interaction and the respective role of these two defence mechanisms in the context of heat tolerance in snails, i.e. in *X. derbentina*.

In an earlier study (Dieterich et al., 2014), we conducted heat exposure experiments with the result of a clear decrease of lipid peroxides at a distinct temperature (43°C) in *X. derbentina*, which brought us to the hypothesis that this effect might be due to activation of the antioxidant defence machinery. Here, we investigated the effects of different temperatures on the activity of the two enzymes CAT and GPx as representatives of the antioxidant defence system, by exposing snails of the species *X. derbentina* to different heat exposure regimes (25, 38, 40, 43 and 45°C) in the laboratory. In addition, we also determined lipid peroxidation levels (as a marker for oxidative stress) and the Hsp70 level. Furthermore, we aimed at assessing the role of the antioxidant defence mechanism in this snail's ability to counteract high temperatures. Thus, we investigated, for the first time, the interplay between the antioxidant defence system and the Hsp70 response in this context.

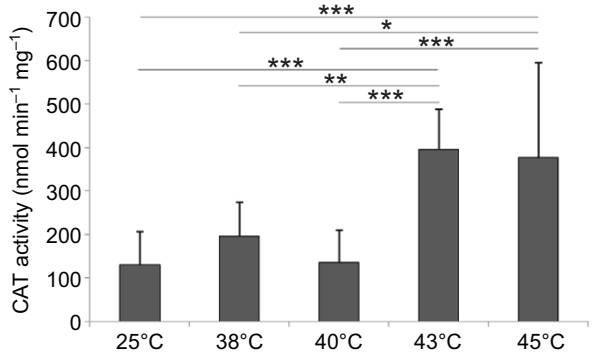

Fig. 1. Catalase activity in *Xeropicta derbentina* after different temperature treatments. Data are means + s.d., *N*=10. Asterisks indicate significant differences between the groups: *0.01<*P*≤0.05, **0.001<*P*≤0.01, ***$P \leq 0.001$.

RESULTS

CAT

CAT activity was generally very high in our samples (dilution 1:2000). A highly significant increase of CAT activity compared with the control level (25°C) was detected after exposure to 43 and 45°C (Fig. 1). This increase in the 43°C group was also significantly different from CAT levels at 38°C and highly significantly different from those at 40°C. A slightly significant and a highly significant increase of CAT activity compared with 38 and 40°C, respectively, was observed after exposure to 45°C.

GPx

The enzyme GPx showed maximum activity in the 40°C treatment (Fig. 2). This elevation was slightly significant versus activity at 25°C and highly significant versus that at 38°C. The decrease in activity at higher temperatures (43 and 45°C) was also significant compared with that at 40°C. Non-linear regression analysis of

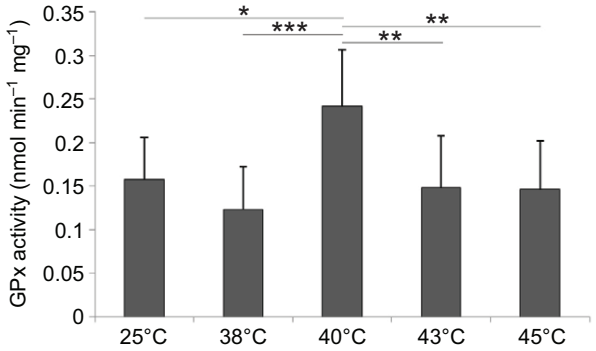

Fig. 2. Glutathione peroxidase (GPx) activity in *X. derbentina* after different temperature treatments. Data are means + s.d., *N*=10. Asterisks indicate significant differences between the groups: *0.01<*P*≤0.05, **0.001<*P*≤0.01, ***$P \leq 0.001$.

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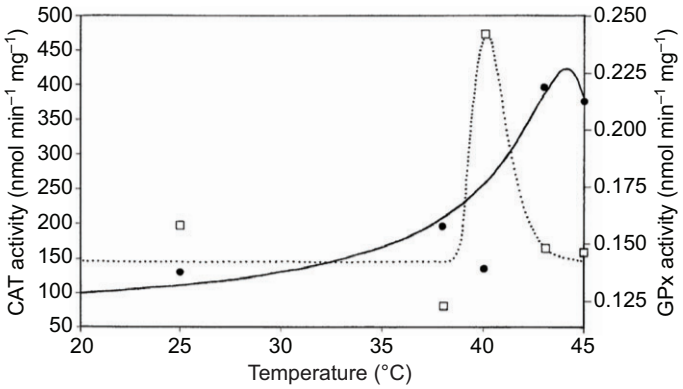

Fig. 3. Non-linear regression analysis of catalase (black dots, solid line, left scale, CAT) and glutathione peroxidase (squares, dotted line, right scale, GPx) activities vs temperature (*T*). $(CAT)^{-1}=0.011-(7.369\times10^{-7})T^{2.5}+3.757e^{T}$ with $r^2=0.773$; $GPx=\{0.142\exp[(T/31.370)+1.274\}-\{31.371\exp[(T/40.063)+0.242]/31.370)\}$ with $r^2=0.924$.

catalase and glutathione peroxidase activities vs temperature illustrates different responses of these enzymes to changes in temperature: catalase activity has a sigmoidal shaped curve whereas glutathione peroxidase activity has a clear peak at 40°C (Fig. 3).

Lipid peroxidation

We found the highest level of lipid peroxides after exposure to 38°C (Fig. 4). This slightly significant (versus 25°C) elevation in lipid peroxidation was followed by a decrease at higher temperatures (40–45°C). In the 43 and 45°C treatments, the levels of lipid peroxidation decreased in a slightly significant way, compared with the exposure at 38°C.

Hsp70

We observed a distinct stress protein response in the snails after exposure to elevated temperature (Fig. 5). The levels of Hsp70 increased up to their maximum induction at 40°C followed by a decrease at higher temperatures, particularly at 45°C where the decline in Hsp70 level became significant.

DISCUSSION

Terrestrial snails inhabiting dry and hot habitats experience daily periods of high temperatures due to the absorbance of solar radiation. The pulmonate *X. derbentina* is well adapted to such unfavourable conditions. Thus, this snail species has become a

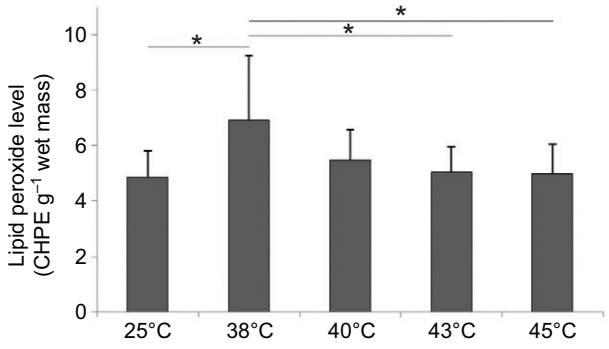

Fig. 4. Levels of lipid peroxides in *X. derbentina* after different temperature treatments. CHPE, cumene hydroperoxide equivalents per gram wet mass. Data are means + s.d., *N*=10. Asterisks indicate significant differences between the groups: *0.01<*P*≤0.05.

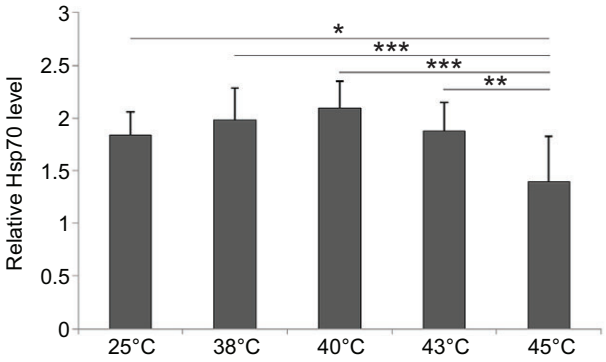

Fig. 5. Relative Hsp70 levels in *X. derbentina* after different temperature treatments. Data are means + s.d., *N*=10. Asterisks indicate significant differences between the groups: *0.01<*P*≤0.05, **0.001<*P*≤0.01, ***$P\leq0.001$.

common subject for the investigation of physiological heat stress responses in recent years (Dittbrenner et al., 2009; Köhler et al., 2009; Scheil et al., 2011; Di Lellis et al., 2012; Dieterich et al., 2013; Di Lellis et al., 2014; Troschinski et al., 2014). In contrast to the well-documented induction of Hsps in response to heat exposure, activation of the antioxidant defence system is poorly understood in this context in this terrestrial snail species.

In the present study, we succeeded in replicating our results from our previous investigation (Dieterich et al., 2014). We found an increase in lipid peroxides (as an index for oxidative stress) after exposure of the snails to 38°C followed by an unexpected decrease at higher temperatures. To test the hypothesis that an activation of antioxidant mechanisms is responsible for this effect, we measured CAT and GPx activity as two enzymatic representatives of the antioxidant defence system.

In general, we found a very high CAT activity in all treatments, particularly in contrast to the overall activity of GPx, which was quite low. We suggest that this generally high CAT activity can be seen as a constitutive base level of this enzyme, which might provide permanent protection against the cytotoxic action of H_2O_2 in *X. derbentina*. The same conclusion was proposed by Nowakowska et al. (Nowakowska et al., 2011) in the context of relatively high CAT activity during aestivation/arousal cycles in Helicidae. Furthermore, Storey (Storey, 1996) demonstrated that anoxic-tolerant organisms that experience bursts of ROS generation during the anoxic to aerobic transition (facultative anaerobes such as freshwater turtles) maintain high levels of antioxidant enzymes and glutathione constitutively. Storey described this phenomenon as a strategy to face any stress effectively. In addition, he found generally high antioxidant enzyme activities in tissues of the land snail *Otala lactea*, which is indicative of a good constitutive ability for dealing with ROS formation. This, in turn, confirms the assumption that a permanent antioxidant defence is a crucial mechanism to counteract repetitive periods of oxidative stress (Storey, 1996).

We used the determination of lipid peroxides via the FOX assay as an index for oxidative stress. When we compared the levels of lipid peroxides with the observed levels of antioxidant enzyme activity in the different temperature treatments, a clear physiological response was obvious: after exposure to 38°C, we detected an increase in lipid peroxides, which was followed by an increased activity of GPx in the 40°C treatment. As a consequence of this elevated enzyme activity, the level of lipid peroxides decreased. After exposure to 43 and 45°C, we measured a significant increase

of CAT activity associated with low lipid peroxide levels, which is indicative of the highly effective work of this enzyme against the ROS H_2O_2. Furthermore, the increase of CAT activity was also associated with a decrease in the activity of GPx. Our data suggest that, here in our artificial heat-exposure experiment, GPx has its activity optimum at 40°C, whereas CAT activity remains unaffected, staying at its 'base level'. But when exceeding this temperature, reaching 43 and 45°C, a boost in CAT activity, associated with a decrease in GPx activity, leads to a reduction of the damaging effects of H_2O_2 (mirrored by low lipid peroxide levels). This phenomenon reflects a competition between CAT and GPx for the same ROS, as both enzymes degrade H_2O_2. In a study by Nowakowska et al. (Nowakowska et al., 2011), this competing action between CAT and GPx could also be demonstrated in two molluscan species (*Helix aspersa* and *Helix pomatia*): here, extremely low levels of CAT activity were usually associated with extremely high activities of GPx.

Our data show that GPx activity was elevated in response to increased levels of lipid peroxides, leading us to the conclusion that the enzyme activity must be stimulated by high levels of lipid peroxides (as the result of oxidative damage). This is supported by a study of Ramos-Vasconcelos and Hermes-Lima (Ramos-Vasconcelos and Hermes-Lima, 2003), who pointed out that increased levels of lipid peroxides in the hepatopancreas of the pulmonate land snail *H. aspersa* could be a triggering factor for the activation of signalling pathways leading to the activation of GPx biosynthesis and/or maintenance of other enzymatic antioxidants in general.

In recent years, several studies have demonstrated that antioxidants, i.e. CAT and GPx, play an important role during aestivation as a mechanism of preparation for the oxidative stress that accompanies arousal in snails (Hermes-Lima and Storey, 1995; Storey, 1996; Hermes-Lima et al., 1998; Storey, 2002; Ramos-Vasconcelos and Hermes-Lima, 2003; Nowakowska et al., 2009; Nowakowska et al., 2010; Nowakowska et al., 2011). Besides this well-documented phenomenon and the role of the antioxidant defence system in this context, it is generally known that heat can induce oxidative stress. An increase in temperature stimulates all metabolic processes; for example, it elevates oxygen consumption, which can result in oxidative stress due to an increase in ROS as by-products during intensified metabolism (Storey, 1996; Lushchak, 2011). The induction of oxidative stress as a result of elevated environmental temperature was shown in several organisms (Heise et al., 2006; Lushchak and Bagnyukova, 2006a; Lushchak and Bagnyukova, 2006b; Bagnyukova et al., 2007b; Verlecar et al., 2007; Bocchetti et al., 2008) and was associated with an increase in antioxidants (Bagnyukova et al., 2006; Lushchak and Bagnyukova, 2006a; Bagnyukova et al., 2007a). For example, in the mussel *Perna virdis*, increased activity of CAT and GPx (in addition to other antioxidants) was recorded (Verlecar et al., 2007). In the present study, we demonstrated that terrestrial snails undergo oxidative stress as a result of elevated temperature, which suggests the activation of physiological mechanisms to scavenge produced ROS. We showed that CAT and GPx activities were increased as an enzymatic antioxidant defence in a temperature-dependent, serial induction, indicating an essential role of antioxidants in the thermotolerance of *X. derbentina*.

Additionally, the Hsp70 induction kinetics recorded here were in accordance with previous findings (Köhler et al., 2009; Di Lellis et al., 2014; Troschinski et al., 2014). In these studies, the maximum Hsp70 level was observed at temperatures around 38 and 40°C applied for 8 h, followed by a rapid Hsp70 decline when ambient temperature exceeded 40°C. Our data support these results, as we found a maximum Hsp induction at 40°C. The significant decrease of the Hsp70 level at higher temperatures, especially in the 45°C exposure group, is assumed to be due to the stress protein machinery becoming overwhelmed (destruction phase), which is in accordance with the kinetics of stress protein induction described by Eckwert et al. (Eckwert et al., 1997).

Molecular chaperones as Hsps are primary sensors of misfolded proteins and assist in refolding processes. Some isoforms of Hsp70 are stress-inducible proteins that repair damaged proteins and prevent protein aggregation. The regulation of the expression of Hsp70 in gastropods has been linked to different factors at the developmental or ecological level (Tomanek and Somero, 2002; Arad et al., 2010; Mizrahi et al., 2010). Furthermore, in *X. derbentina*, seasonal and intraspecific variations in Hsp70 induction could be found, leading to different survival strategies in *X. derbentina* populations (Dieterich et al., 2013; Troschinski et al., 2014). Generally, it is known that Hsp induction is used as an important survival strategy in land snails living under extreme environmental conditions (Mizrahi et al., 2010; Mizrahi et al., 2012). In this context, Hsps are essential for 'repairing' partly malfolded proteins due to damaging effects of ROS, so an upregulation of these proteins may be important for an organism's cellular fitness (de Oliveira et al., 2005).

For a better understanding of the processes involved in the heat tolerance of *X. derbentina*, we investigated the interplay of Hsp70 and antioxidant defence. It has already been shown that both Hsps and antioxidant defence are included in the response to stress during cycles of aestivation and arousal in gastropods (Storey and Storey, 2011; Giraud-Billoud et al., 2013). However, protein biosynthesis is a costly process, especially under stressful conditions, and it is thought that Hsp70 expression is very energy costly (Sanchez et al., 1992; Heckathorn et al., 1996; Köhler et al., 2000). Thus, it should be expected that only proteins relevant to the maintenance of life would show increased levels under extreme conditions. Our data show that these snails already have a rather high constitutive Hsp70 level, which was elevated up to 40°C, but declined upon exposure to higher temperature treatments (43 and 45°C). Here, first of all, the CAT activity was significantly elevated. One may argue that this effect may be due to an energetic trade-off between Hsp70 and antioxidants, in the same way that, in consequence, energy is spent in the biosynthesis of enzymatic antioxidants (here CAT) instead of Hsp70. As suggested by Giraud-Billoud et al. (Giraud-Billoud et al., 2013), antioxidants and chaperone-mediated protective mechanisms such as Hsp70 may work independently, but the activation of different stress-response pathways is promoted by reactive metabolites of oxidative stress. Gorman et al. (Gorman et al., 1999) examined the hypothesis that ROS contribute to the induction of Hsps during stress response and found that the tested antioxidants caused a reduction or complete inhibition of Hsp induction. As we found an elevated CAT activity associated with low levels of lipid peroxides (indicative of reduced ROS levels) and also decreased Hsp70 levels, our observations strengthen this hypothesis.

It should be noted that we just investigated a 'snap-shot' of the biochemical heat response after 8 h of exposure. A previous study showed *X. derbentina* to exhibit a maximum level of Hsp70 after 2 h of exposure to 45°C, whereas, at 25°C, the maximum stress protein induction was reached after 4 h of exposure (Scheil et al., 2011). Furthermore, the activity of antioxidant enzymes (CAT and SOD) and levels of glutathione in *H. aspersa* were measured at different time points during the awakening process after aestivation. The results indicated differences in glutathione levels but none in

enzyme activity (Ramos-Vasconcelos and Hermes-Lima, 2003). For further studies, it might be interesting to investigate different time points during heat exposure to get a more detailed picture of the physiological processes, especially of the antioxidant defence system, involved in the thermotolerance of terrestrial snails.

Conclusions

In the present study, we found support for our assumption that antioxidants are responsible for the decrease in lipid peroxides at high temperature. A boost of GPx activity at 40°C (associated with moderate CAT activity levels) followed by an increase of CAT activity at 43 and 45°C (associated with a decrease in GPx activity) is likely to be responsible for this effect. These findings demonstrate efficient antioxidant defence mechanisms following heat exposure with different temperature-dependent boosts in activity. More precisely, we showed that CAT activity and GPx activity have different optima related to temperature, thus complementing both one another and the Hsp70 response when external temperature increases.

MATERIALS AND METHODS

Test organism and sampling

Individuals from a single population of the terrestrial snail *X. derbentina* were collected in the last week of May 2013 in Modène, Provence, Southern France. The sampling site was dry, open and sun-exposed. Snails were collected and kept in plastic containers (20.5×30×19.5 cm) at a density of ~200 individuals per box.

Experimental setup

In the laboratory, the snails were acclimatized to 25°C for 3 weeks. The plastic containers were filled with a layer of ground-cover material for terrariums (JBL, Terra Basis, Neuhofen, Germany). The snails were fed organic milk mash (Hipp, Pfaffenhofen, Germany) *ad libitum* and sprayed with water twice a week to ensure an appropriate level of humidity.

The temperature experiments were conducted in heating cabinets using smaller plastic boxes (6.5×18×13 cm) lined with moist paper towels and covered with perforated plastic sheets. Forty individuals were exposed as a group in individual plastic containers to temperatures of 25, 38, 40, 43 and 45°C for 8 h: 25°C was used as a control temperature.

After 8 h of exposure, 10 randomly selected individuals from each experimental group were taken for the CAT assay (for CAT activity), the GPx assay (for GPx activity) and the FOX assay (for quantification of lipid peroxidation), respectively. The snails were killed and their shells were removed. For the stress protein analyses, 10 individuals per group were individually shock-frozen in liquid nitrogen and stored at −20°C until further analysis.

CAT assay

To measure the CAT activity in the samples, we used Cayman's Catalase Assay Kit (item no. 707002, Cayman Chemical Company, MI, USA). The method is based on the reaction of CAT with methanol in the presence of H_2O_2. The formaldehyde produced is measured calorimetrically with purpald (4-amino-3-hydrazino-5-mercapto-1,2,4-triazole) as the chromogen, which forms a bicyclic heterocycle with aldehydes and changes from colourless to a purple colour upon oxidation.

The samples were weighed and homogenized in 5 ml of ice-cold buffer (50 mmol l^{-1} potassium phosphate, pH 7.0, containing 1 mmol l^{-1} EDTA) per gram tissue, and centrifuged at 10,000 ***g*** for 15 min at 4°C. Supernatants were removed and stored on ice. The assay was conducted in 96-well plates.

Formaldehyde standard wells were prepared containing 100 µl of assay buffer (100 mmol l^{-1} potassium phosphate, pH 7.0), 30 µl of methanol and 20 µl of standard (concentrations 0, 5, 15, 30, 45, 60 and 75 µmol l^{-1} formaldehyde) per well. Two positive control wells were filled with 100 µl of assay buffer, 30 µl of methanol and 20 µl of CAT (control: bovine liver CAT). Sample wells were prepared in duplicate containing 100 µl of assay buffer, 30 µl of methanol and 20 µl of sample. Because the amount of CAT added to the well should result in an activity level between 2 and 35 nmol min^{-1} ml^{-1}, it was necessary to dilute the samples with sample buffer (1:2000).

To initiate reactions, 20 µl of H_2O_2 solution was added to all wells and the plates were incubated on a shaker for 20 min at room temperature. Afterwards, 30 µl of potassium hydroxide (10 mol l^{-1} solution) was added to terminate the reaction; 30 µl of purpald (in 0.5 mol l^{-1} hydrochloric acid) was added to all wells and incubated for 10 min. Then, 10 µl of potassium periodate (in 0.5 mol l^{-1} potassium hydroxide) was added and again incubated for 5 min. Absorbance was read at 540 nm using a spectrometer (Automated Microplate Reader, Elx8006, Bio Tek Instruments, Bio Tek Germany, Bad Friedrichshall, Germany).

CAT activity (nmol min^{-1} mg^{-1}) was calculated using the following equation:

$$\text{CAT activity} = [(\text{Formaldehyde} / t) \times \text{Sample dilution}] / 1000 , \quad (1)$$

where formaldehyde refers to the concentration in the sample (in µmol l^{-1}) and t is time (in this case, 20 min).

GPx assay

GPx activity was measured by using Cayman's Glutathione Peroxidase Assay Kit (item no. 703102, Cayman Chemical Company). GPx activity is measured indirectly by a coupled reaction with GR: oxidized glutathione (GSSG), which is produced upon reduction of hydroperoxide by GPx, is reconverted to its reduced state (GSH) by GR and NADPH. The oxidation of NADPH to $NADP^+$ in this reaction is accompanied by a decrease in absorbance at 340 nm. The rate of decrease in absorbance at 340 nm (A_{340}) is directly proportional to the GPx activity in the sample. This assay integrates the activity of all glutathione-dependent peroxidases in the sample.

Samples were weighed and homogenized in 5 ml of ice-cold buffer (50 mmol l^{-1} Tris-HCl, pH 7.5, 5 mmol l^{-1} EDTA and 1 mmol l^{-1} DTT) per gram tissue, and centrifuged at 10,000 ***g*** for 15 min at 4°C. Supernatants were removed and stored on ice. The assay was conducted in 96-well plates. Background wells were filled with 120 µl of assay buffer (50 mmol l^{-1} Tris-HCl, pH 7.6, containing 5 mmol l^{-1} EDTA) and 50 µl of co-substrate mixture (containing NADPH, glutathione and GR); 100 µl of assay buffer, 50 µl of co-substrate mixture and 20 µl of diluted GPx (control: bovine erythrocyte GPx) was added to the positive control wells. Sample wells were prepared in triplicate containing 100 µl of assay buffer, 50 µl of co-substrate mixture and 20 µl of sample.

Reactions were initiated by adding 20 µl of cumene hydroperoxide (CHP) to all wells, and absorbance was read once every minute over a period of 5 min at 340 nm using a microplate reader (Infinite M200, TECAN, Männedorf, Switzerland).

For each sample, the change in absorbance (ΔA_{340}) per minute was determined and GPX activity (nmol min^{-1} mg^{-1}) was calculated by:

$$\text{GPx activity} = \{[(\Delta A_{340} / \text{min}) / 0.000373\ \mu\text{mol}\ l^{-1}] \times (0.19\ \text{ml} / 0.02\ \text{ml}) \times \text{Sample dilution}\} / 1000 . \quad (2)$$

FOX assay

In this study we conducted a modified FOX assay (for quantification of lipid peroxides) derived from a previously described method (Hermes-Lima et al., 1995). The individuals were weighed and homogenized in ice-cold HPLC grade methanol [dilution 1:2; the required amount of methanol is calculated by: wet mass of the individual/density of methanol (0.791 g cm^{-3})], and centrifuged at 15,000 ***g*** and 4°C for 5 min. Supernatants were stored at −80°C until further analysis. The assay was conducted using 96-well plates. In each well (except for the blank), 50 µl of each reagent was added in the following order: 0.25 mmol l^{-1} $FeSO_4$, 25 mmol l^{-1} H_2SO_4 and 0.1 mmol l^{-1} Xylenol Orange. Then, 15 µl of sample supernatant was added and the final sample volume adjusted to 200 µl with aqua bidest. For each sample, three wells were prepared (three replicates) and a mean value was calculated. Master blanks contained 200 µl of aqua bidest.

Samples were incubated at room temperature for 180 min and absorbance was then read at 580 nm (A_{580}) using a photospectrometer (Automated

Microplate Reader, Elx8006, Bio Tek Instruments). Then, 1 µl of 1 mmol l^{-1} CHP solution was added to the samples, incubated for 30 min at room temperature and again read at 580 nm ($A_{580+CHP}$).

The content of lipid hydroperoxides in the samples is expressed as CHP equivalents per gram wet mass (CHPE g^{-1} wet mass) and was calculated according to the equation (Hermes-Lima et al., 1995):

$$\text{CHPE g}^{-1} = (A_{580} / A_{580+\text{CHP}}) \times 1\ \mu\text{l CHP}_{1\ \text{nmol}} \times 200 / V \times 2\,, \qquad (3)$$

where 200 is the total sample volume, V is the added sample supernatant volume (15 µl) and 2 is the dilution factor with methanol (1:2).

Hsp70 analysis

Frozen individuals were homogenized on ice in extraction buffer (80 mmol l^{-1} potassium acetate, 5 mmol l^{-1} magnesium acetate, 20 mmol l^{-1} Hepes and 2% protease inhibitor at pH 7.5) according to their body mass (2 µl buffer mg^{-1} snail) and centrifuged for 10 min at 20,000 ***g*** and 4°C. To determine the total protein content of each sample, the protein–dye binding assay of Bradford (Bradford, 1976) was used. Constant protein weights (40 µg per sample) were separated by minigel SDS-PAGE (12% acrylamide, 0.12% bisacrylamide, 30 min at 80 V and 75–90 min at 120 V) and transferred to nitrocellulose membranes by semi-dry blotting. The membranes were blocked in a 1:2 mixture of horse serum and TBS (50 mmol l^{-1} Tris pH 5.7, 150 mmol l^{-1} NaCl) for 2 h. Subsequently, the membranes were incubated in the first antibody solution containing a monoclonal α-Hsp70 antibody (mouse anti-human Hsp70, Dianova, Hamburg, Germany; dilution 1:5000 in 10% horse serum in TBS) on a lab shaker at room temperature overnight. Membranes were washed for 5 min in TBS, then incubated in the second antibody solution (goat anti-mouse IgG conjugated to peroxidase, Jackson ImmunoResearch, West Grove, PA, USA; dilution 1:1000 in 10% horse serum/TBS) on a lab shaker for 2 h at room temperature. Following another washing step in TBS, the developed antibody complex was detected by staining with a solution of 1 mmol l^{-1} 4-chloro(1)naphthol, 0.015% H_2O_2, 30 mmol l^{-1} Tris pH 8.5 and 6% methanol. The optical volume [area of the bands (number of pixels)×average grey scale value after background subtraction] of the western blot protein bands was quantified using a densitometric image analysis system (E.A.S.Y. Win 32, Herolab, Wiesloch, Germany). For each sample, data were related to an internal Hsp70 standard (extracted from *Theba pisana* snails) to ensure comparability.

Statistics

All data were checked for normality and homogeneity of variance using the D'Agostino Omnibus Test and Levene's test. Data from the CAT and FOX assay were transformed (square root; log) to guarantee a normal distribution of the data. To detect significant differences within the treatments, we used ANOVA followed by the Tukey–Kramer HSD *post hoc* test. Data were analysed using JMP 9 (SAS Institute Inc., Cary, NC, USA) and Microsoft Excel 2007 (Microsoft Corporation, Redmond, WA, USA). Levels of significance were defined as: $*0.01<P\leq0.05$: slightly significant; $**0.001<P\leq0.01$: significant; $***P\leq0.001$: highly significant.

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Competing interests

The authors declare no competing financial interests.

Author contributions

S.T.: conception, design and execution of experiments, interpretation of findings, drafting and revising article. A.D., S.K.: conception, design and execution of experiments, interpretation of findings. R.T.: conception of experiments. H.K.: conception of experiments, interpretation of findings.

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