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Antioxidant defense and stress protein induction following heat stress in the Mediterranean snail *Xeropicta derbentina* [Pulmonata, Hygromiidae]

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Abstract

The Mediterranean snail *Xeropicta derbentina*, 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. In 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 on the biochemical level by investigation of antioxidant defenses 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 activities: CAT activity was significantly increased in response to high temperatures (43 and 45°C), whereas GPx exhibited a significantly increased activity at 40°C, likely in response to high levels of lipid peroxides which already 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 damaging effects by heat. Furthermore, we could demonstrate that, beside the well documented Hsp70 stress response, the antioxidant defense plays a crucial role in these snails competence to survive extreme temperatures.

Keywords: 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. Especially terrestrial snails with their water-permeable skin (Machin, 1964) and their external shell easily 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 behavioral and physiological adaptations to their habitat:

climbing on vegetation to escape from hot ground temperatures or shifting activity phases to favorable time periods (Pomeroy, 1968; Yom-Tov, 1971) can be seen as behavioral 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 for a physiological mechanism of adaptation. Furthermore, there are different mechanisms acting on the biochemical level, which are known to play an important role in the thermotolerance of animals.

One of these mechanisms is the antioxidant defense, which plays a crucial role in periods of oxidative stress, e.g. caused by 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), functioning as tool to assess the extent of oxidative damage an organism had 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 latter 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 hydrogen peroxide and oxygen, (ii) catalase (CAT), which degrades hydrogen peroxide, and (iii) glutathione peroxidase (GPx), which degrades hydrogen peroxide and also lipid peroxides generated by lipid peroxidation (Aebi, 1984; Halliwell and Gutteridge, 1989; Gutteridge, 1995). Beside these enzymes there are much 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 defenses 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, 1996; Pannunzio and Storey, 1998; Lushchak et al., 2001), freezing (Hermes-Lima and Storey, 1993; Joanisse and Storey, 1996), and also 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 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 weight of about 70kD. Heat shock proteins 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 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 up-regulated 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 up-regulation (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 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 *Xeropicta 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 defense system, the Hsp70 defense system is supposed to form a well-working mechanism that ensures survival in a challenging habitat. However, to date, only little is known about the interaction and the respective role of these two defense 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 an activation of the antioxidant defense machinery. Here, we investigate the effects of different temperatures on the activity of the two enzymes catalase and glutathione peroxidase as representatives of the antioxidant defense 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 70kDa heat-shock protein, Hsp70. Furthermore, we aimed at assessing the role of the antioxidant defense mechanism in this snail's ability to counteract high temperatures. Thus, we investigated, for the first time, the interplay between the antioxidant defense system and the Hsp70 response in this context.

Results

Catalase

The catalase activity was generally very high in our samples (dilution 1:2000). A highly significant increase of catalase activity compared to 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 38°C and highly significantly different from 40°C. A slightly significant and a highly significant increase of catalase activity compared to 38 and 40°C, respectively, was observed after exposure to 45°C.

Glutathione peroxidase

The enzyme glutathione peroxidase showed maximum activity in the 40°C treatment (Fig.2). This elevation was slightly significant vs.25°C and highly significant vs. 38°C. The decrease in activity at higher temperatures (43 and 45°C) was also significant compared to 40°C.

Non-linear regression analysis of catalase and glutathione peroxidase activities vs. temperature illustrates different responses of these enzymes to changes in temperature:

catalase activity has a sigmoidal shaped curve while 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 (vs. 25°C) elevation in lipid peroxidation was followed by a decrease at higher temperature (40 to 45°C). In the 43 and 45° C treatment the levels of lipid peroxidation decreased in a slightly significant way, compared to 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 Hsp70 level decline 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 *Xeropicta derbentina* is a well adapted organism to such unfavorable conditions. Thus, this snail species had become a common object for the investigation of physiological heat stress responses in the last 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 heat-shock proteins in response to heat exposure, the activation of the antioxidant defense system is poorly understood in this context in this terrestrial snail species.

In the present study, we succeed to replicate 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 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 catalase (CAT) and glutathione peroxidase (GPx) activity as two enzymatic representatives of the antioxidant defense 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 have a permanent protection against the cytotoxic action of hydrogen peroxide (H_2O_2) in *X. derbentina*. The same conclusion was proposed by Nowakowska et al. (2011) in the context of relatively high CAT activity during aestivation/arousal cycles in Helicidae. Furthermore, Storey (1996) demonstrated that anoxic-tolerant organisms that experience bursts of ROS generation during the anoxic to aerobic transition (facultative anaerobes as, e.g., freshwater turtles) maintain high levels of antioxidant enzymes and glutathione constitutively. He 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 for a good constitutive ability for dealing with ROS formation. This, in turn, confirms the assumption that a permanent antioxidant defense is a crucial mechanism to counteract repetitive periods of oxidative stress (Storey, 1996).

We used the determination of lipid peroxides via FOX assay as index for oxidative stress. When we compare the levels of lipid peroxides with the observed levels of antioxidant enzyme activity in the different temperature treatments, a clear physiological response is 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. In consequence to 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 for the highly effective work of this enzyme against the reactive oxygen species H_2O_2. Furthermore, the increase of CAT activity was also associated with a decrease in activity of GPx. Our data suggest that, here in our artificial heat exposure experiment, GPx has its activity optimum at 40°C, whereby CAT activity remains unaffected staying on 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, lead to a reduction of damaging effects of H_2O_2 (mirrored by low lipid peroxide levels). This phenomenon reflects a competition between CAT and GPx for the same reactive oxygen species (ROS), since both enzymes degrade H_2O_2. In a study by 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 assumption that the enzyme activity must be stimulated by high levels if lipid peroxides (as the result of oxidative damage) . This implication is supported by a study of Ramos-Vasconcelos and Hermes-Lima (2003) who pointed out that increased levels of lipid peroxides in the hepatopancreas of the pulmonate land snail *Helix aspersa* could be a triggering factor for the activation of signaling pathways leading to the activation of GPx biosynthesis and/or maintenance of other enzymatic antioxidants in general.
In the last years, several studies demonstrated that antioxidants, i.e. catalase 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). Beside this well-documented phenomenon and the role of the antioxidant defense 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 due to elevated environmental temperature was shown in several organisms (Heise et al., 2006; Lushchak and Bagnyukova, 2006b, a; 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 activities of CAT and GPx (beside other antioxidants) were recorded (Verlecar et al., 2007). In the present study, we could demonstrate that terrestrial snails undergo oxidative stress as a result of elevated temperature which suggests the activation of physiological mechanisms to scavenge produced ROS. We could show that CAT and GPx activities were increased as enzymatic antioxidant defenses in a temperature-dependent, serial way of induction, indicating an essential role of antioxidants in the thermotolerance of *X. derbentina*.

Also 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 8h, followed by a rapid Hsp70 decline when ambient temperature exceeded 40°C. Our data support these results, since we found a maximum heat shock protein induction at 40°C. The

significant decrease of the Hsp70 level, especially in the 45°C exposure group, is assumed to be due to an overwhelmed stress protein machinery (destruction phase), which is in accordance with the kinetics of stress protein induction described by Eckwert et al. (1997). Molecular chaperones as the heat shock proteins 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 of 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 the 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, 2012). In this context, heat shock proteins are essential for 'repairing' partly malfolded proteins due to damaging effects of ROS, so an up-regulation of these proteins may be important for an organisms' 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 the antioxidant defense. It could already be shown that both, Hsps as well as the antioxidant defense, 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 reveal 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 can be due to an energetic trade-off between Hsp70 and antioxidants, in a way that, in consequence, energy is spend in biosynthesis of enzymatic antioxidants (here: CAT) instead of Hsp70. As suggested by Giraud-Billoud et al. (2013), antioxidants and chaperone-mediated protective mechanisms as the Hsp70 may work independently, but the activation of different stress response pathways is promoted by reactive metabolites of oxidative stress. 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. Since we found an elevated CAT activity associated with low

levels of lipid peroxides (indicative for reduced ROS levels) and also decreased Hsp70 levels, our observations strengthen this hypothesis.

It has to be mentioned that we just investigated a "snap-shot" of the biochemical heat response after 8 hours of exposure. A previous study showed *X. derbentina* to exhibit a maximum level of Hsp70 after two hours of exposure to 45°C, whereas, in a 25°C treatment, the maximum stress protein induction was reached after four hours of exposure (Scheil et al., 2011). Furthermore, the activity of antioxidant enzymes (CAT and SOD) and levels of glutathione in *Helix aspersa* were measured at different time points during awakening process after aestivation. Results indicated differences in the glutathione levels but none in enzyme activities (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 defense 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 defense mechanisms following heat exposure with different temperature-dependent boosts in activity. More precisely, we could show that CAT as well as GPx activities have different optima related to temperature thus complementing both one another and the Hsp70 response when external temperature increases.

Material and Methods

Test organism and sampling

Individuals from a single population of the terrestrial snail, *Xeropicta 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) in a density of approximately 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 two times per week to assure 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 8h, respectively. 25°C was used as control temperature.
After eight hours of exposure, ten randomly selected individuals from each experimental group were taken for the CAT-assay (for catalase activity), the GPx-assay (for glutathione peroxidase activity), and the FOX-assay (for quantification of lipid peroxidation), respectively. The snails were sacrificed and their shells were removed. For the stress protein analyses, ten individuals per group were individually shock-frozen in liquid nitrogen and stored at -20°C until further analysis.

Catalase assay

To measure the catalase activity in the samples, we used Cayman´s Catalase Assay Kit (Item No. 707002, Cayman Chemical Company, Michigan, USA). The method is based on the reaction of catalase with methanol in presence of H_2O_2. Produced formaldehyde 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 colorless to a purple color upon oxidation.
The samples were weighed and homogenized in 5ml of ice-cold buffer (50mM potassium phosphate, pH 7.0, containing 1mM EDTA) per gram tissue, and centrifuged at 10,000 g for 15 minutes at 4°C. Supernatants were removed and stored on ice. The assay was conducted in 96-well plates.

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

To initiate reactions, 20µL of hydrogen peroxide solution was added to all wells and incubated on a shaker for 20 minutes at room temperature. After that, 30µl of potassium hydroxide (10M solution) was added to terminate the reaction. 30µl of purpald (in 0.5M hydrochloric acid) was added to all wells and incubated for 10 minutes. Then, 10µl of potassium periodate (in 0.5M potassium hydroxide) was added and again incubated for 5 minutes. Absorbance was then read at 540nm using a spectrometer (Automated Microplate Reader, Elx8006, Bio Tek Instruments, Bio Tek Germany, Bad Friedrichshall, Germany). Catalase activity was calculated using the following equation:

CAT activity [nmol/min/mg] = [(µM formaldehyde of sample/20 min) x sample dilution]/1000

Glutathion peroxidase assay

Glutathione peroxidase activity was measured by using Cayman´s Glutathione Peroxidase Assay Kit (Item No. 703102, Cayman Chemical Company, Michigan, USA). GPx activity is measured indirectly by a coupled reaction with glutathione reductase (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 340nm. The rate of decrease in 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 5ml of ice-cold buffer (50mM Tris-HCl, pH 7.5, 5mM EDTA, and 1mM DTT) per gram tissue, and centrifuged at 10,000 g for 15 minutes at 4°C. Supernatants were removed and stored on ice. The assay was conducted in 96-well plates. Background wells were filled with 120µl of assay buffer (50mM Tris-HCl, pH 7.6,

containing 5mM EDTA) and 50µl of co-substrate mixture (containing NADPH, glutathione, and glutathione reductase). 100µl of assay buffer, 50µl of co-substrate mixture, and 20µl of diluted GPx (control: bovine erythrocyte GPX) was added to the positive control wells. Sample wells were prepared in triplicate containing 100µl of assay buffer, 50µl of co-substrate mixture, and 20µl of sample.

Reactions were initiated by adding 20µl of cumene hydroperoxide to all wells, and absorbance was read once every minute over a period of five minutes at 340nm 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 was calculated by the following equation:

GPx activity [nmol/min/mg] = [((ΔA_{340}/min)/0.000373µM^{-1}) x (0.19ml/0.02ml) x sample dilution]/1000

FOX-assay (quantification of lipid peroxides)

In this study we conducted a modified FOX assay deriving from the method described by 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 weight of the individual / density of methanol (0.791 g/cm^3)), centrifuged at 15.000 g and 4°C for 5 minutes. Supernatants were stored at -80°C until further analysis. The assay was conducted using 96-well plates. In each well (except for the blank) 50µL of each reagent was added following this order: 0.25mM $FeSO_4$, 25mM H_2SO_4, and 0.1mM xylenol orange. Then, 15µL of sample supernatant was added and the final sample volume adjusted to 200µL with aqua bidest. For each sample, three wells were prepared (3 replicates) and a mean value was calculated. Master blanks contained 200µL of aqua bidest.

Samples were incubated at room temperature for 180 minutes and absorbance was then read at 580nm (A_{580nm}) using a photospectrometer (Automated Microplate Reader, Elx8006, Bio Tek Instruments, Bio Tek Germany, Bad Friedrichshall, Germany). After that time, 1µL of 1mM cumenehydroperoxide (CHP) solution was added to the samples, incubated for 30 minutes at room temperature and again read at 580nm ($A_{580nm+CHP}$).

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):

$CHPE/g_{wet\ weight} = (A_{580nm}/A_{580nm+CHP})*1\mu L\ CHP_{1nmol}*200/V1*2$

where 200= total sample volume, V1=added sample supernatant volume (15 µL) and 2= dilution factor with methanol (1:2).

Hsp70 analysis

Frozen individuals were homogenized on ice in extraction buffer (80mM potassium acetate, 5mM magnesium acetate, 20mM Hepes and 2% protease inhibitor at pH 7.5) according to their body mass (2µL buffer/mg snail) and centrifuged for 10 minutes at 20,000 *g* and 4°C. To determine the total protein content of each sample, the protein-dye binding assay of Bradford (1976) was used. Constant protein weights (40µg per sample) were separated by minigel SDS-PAGE (12% acrylamide, 0.12% bisacrylamide, 30 minutes at 80 V, and 75-90 minutes 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 (50mM Tris, pH 5.7, 150 mM NaCl) for 2 hours. 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. After washing for 5 minutes in TBS, membranes were incubated in the second antibody solution (goat anti-mouse IgG conjugated to peroxidase, Jackson Immunoresearch, West Grove, PA, dilution 1:1000 in 10% horse serum/ TBS) on a lab shaker for 2 hours at room temperature. Following another washing step in TBS, the developed antibody complex was detected by staining with a solution of 1mM 4-chloro(1)naphthol, 0.015% H_2O_2, 30mM 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 assure comparability.

Statistics

All data were checked for normality and homogeneity of variance using the D'Agostino Omnibus Test and Levene's test. Data from catalase 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 analyzed using JMP 9 (SAS Institute Inc., Cary, NC) and Microsoft Excel 2007 (Microsoft Corporation, Redmond, USA).

Levels of significance were defined as: $0.01 < P \leq 0.05$: * (slightly significant); $0.001 < P \leq 0.01$: ** (significant); $P \leq 0.001$: *** (highly significant).

Non-linear regression analysis of catalase and glutathione peroxidase activities vs. temperature was performed with Table Curve 2D 5.1 (Systat Software Inc., San José, USA).

List of symbols and abbreviations

CAT	catalase
DNA	deoxyribonucleic acid
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
Hsps	heat shock proteins
$^{\cdot}O_2^-$	superoxide anion radical
$^{\cdot}OH$	hydroxyl radical
ROS	reactive oxygen species
SOD	superoxide dismutase

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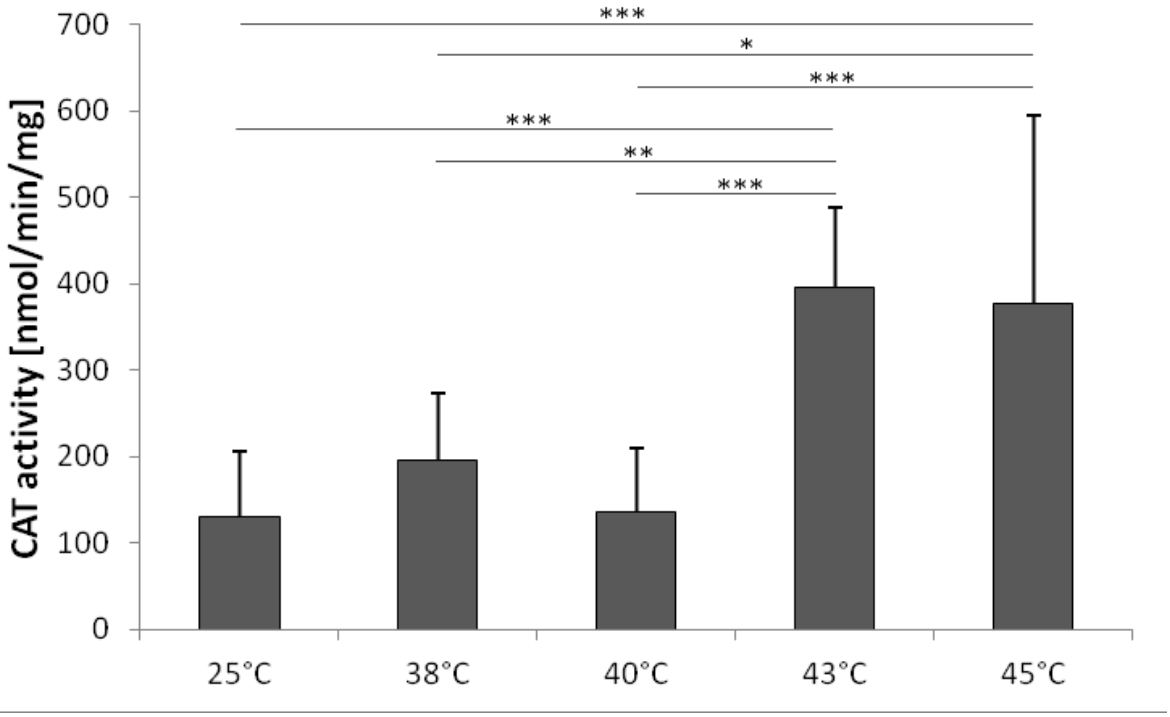

Fig. 1. **Catalase activity in *X. derbentina* after different temperature treatments** (means + s.d., n=10). Asterisks indicate significant differences between the groups: $0.01 < p \leq 0.05$ (*), $0.001 < p \leq 0.01$ (**); $p \leq 0.001$ (***).

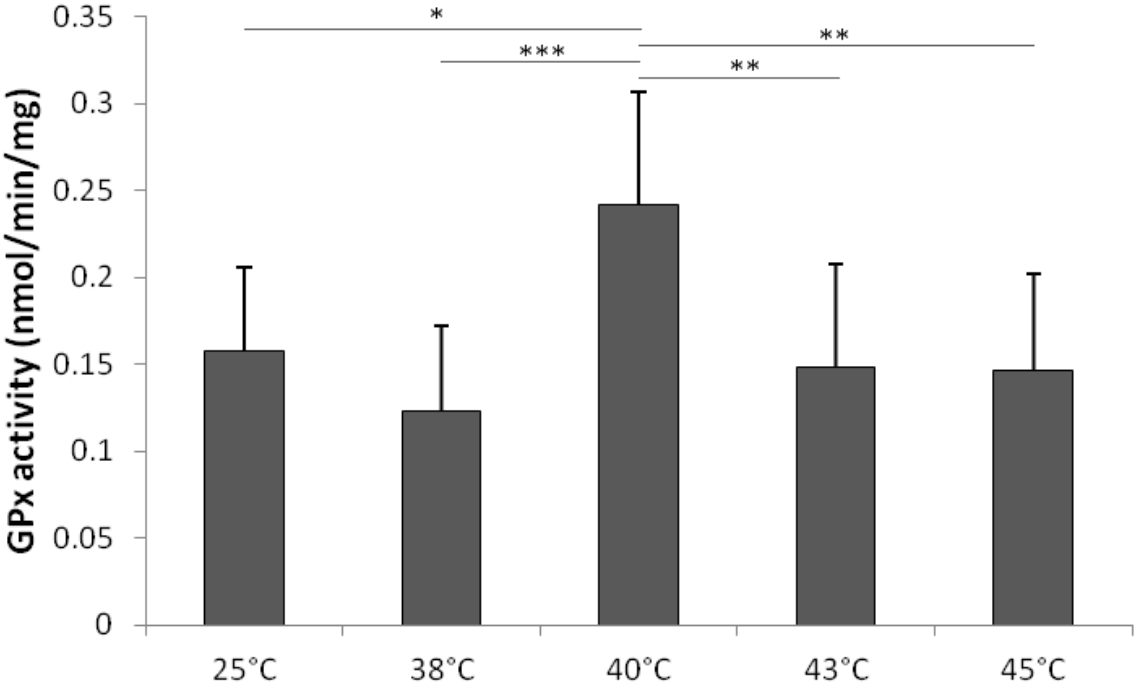

Fig. 2. **Glutathione peroxidase activity in *X. derbentina* after different temperature treatments** (means + s.d., n=10). Asterisks indicate significant differences between the groups: $0.01 < p \leq 0.05$ (*), $0.001 < p \leq 0.01$ (**); $p \leq 0.001$ (***).

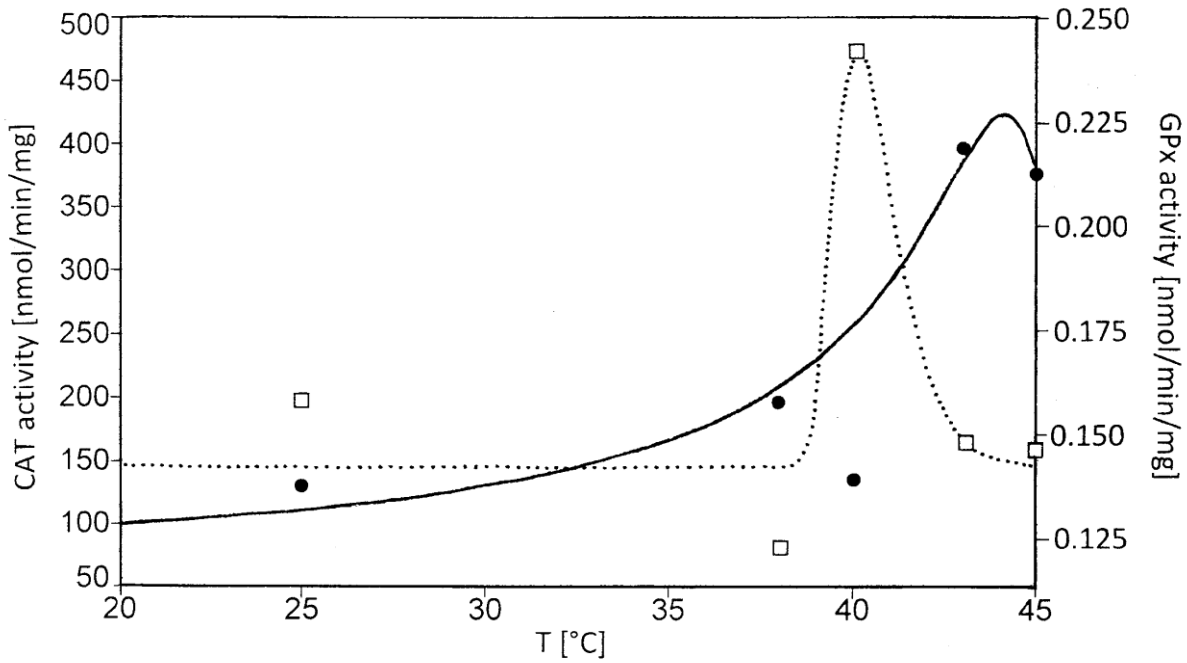

Fig. 3. **Non-linear regression analysis of catalase (black dots, solid line, left scale, CAT) and glutathione peroxidase (squares, dotted line, right scale, GPx) activities *vs.* temperature (T).** $(CAT)^{-1} = 0.011 - (7.369 \cdot 10^{-7}) T^{2.5} + 3.757\ e^{T}$ with $r^2 = 0.773$ $GPx = 0.142 \exp [(T / 31.370) + 1.274 - (31.371 \exp ((T / 40.063) + 0.242) / 31.370)]$ with $r^2 = 0.924$

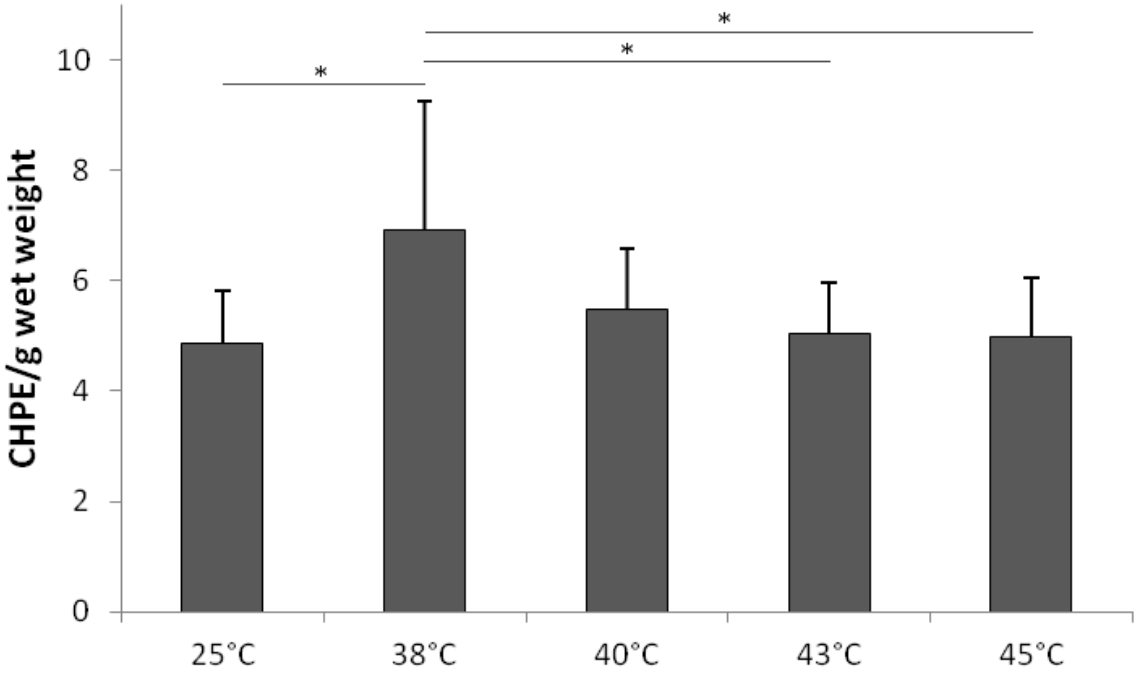

Fig. 4. **Levels of lipid peroxides (expressed as CHPE per gram wet weight) in *X. derbentina* after different temperature treatments** (means + s.d., n=10). Asterisks indicate significant differences between the groups: $0.01 < p \leq 0.05$ (*).

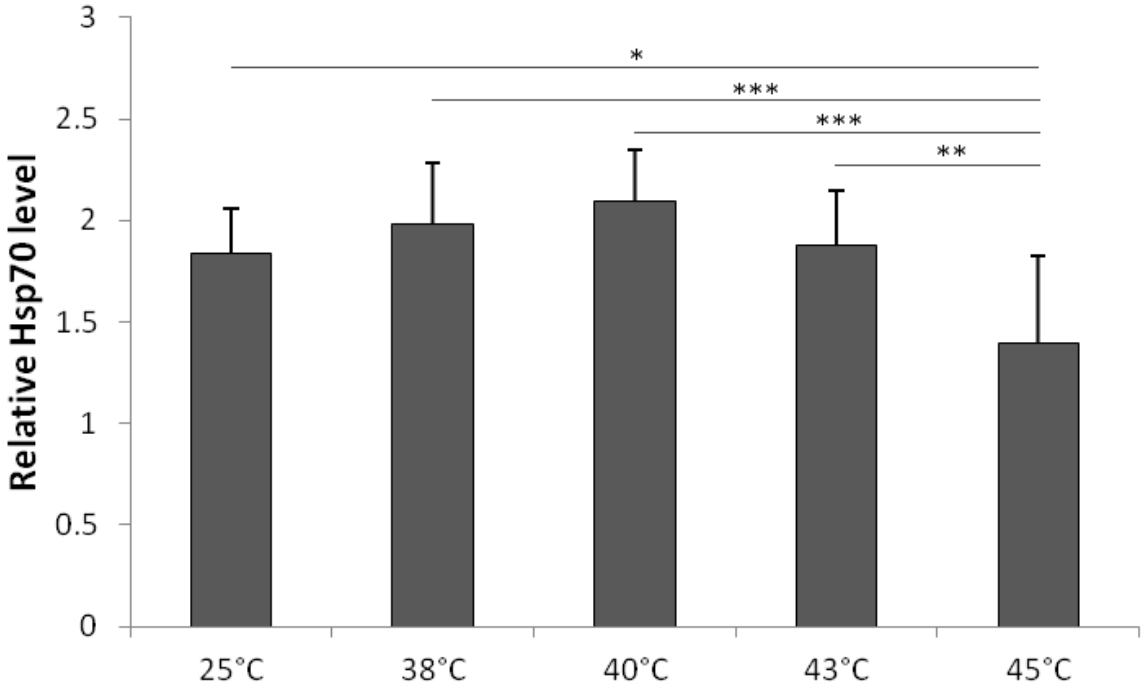

Fig. 5. **Relative Hsp70 levels in *X. derbentina* after different temperature treatments** (means + s.d., n=10). Asterisks indicate significant differences between the groups: $0.01 < p \leq 0.05$ (*), $0.001 < p \leq 0.01$ (**); $p \leq 0.001$ (***).