Biochemische und histologische Untersuchungen physiologischer Anpassungen an Hitzestress bei der mediterranen Landschnecke *Xeropicta derbentina*

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„Ich fragte eine Schnecke, warum sie so langsam wäre.
Sie antwortete, dadurch hätte sie mehr Zeit,
die Welt zu sehen.“

Wolfgang J. Reus (1959 - 2006)

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2.1 Hintergrund der Arbeit

Die Befähigung xerothermophiler Organismen, selbst unter extrem fordernden Umweltbedingungen zu überleben, und die damit verbundenen Verhaltensadaptionen sowie Anpassungen auf physiologischer und morphologischer Ebene sind schon seit langem Gegenstand der Wissenschaft. Besondere Aufmerksamkeit gebührt hierbei terrestrischen Pulmonaten, welche aufgrund ihres hohen Wassergehalts (Reuner *et al.* 2008) und der wasserpermeablen Haut (Machin 1964) leicht durch Austrocknung in Folge von Trockenheit und Hitze gefährdet sind, und die somit eher den Anschein erwecken, geringe Überlebenschancen in ariden und semi-ariden Habitaten zu besitzen. Nichtsdestotrotz konnten sich diese Organsimen dank einer Reihe von Anpassungen diesen Lebensraum zu Nutze machen. Neben Untersuchungen zur Verbreitung und dem Habitat hitzeresistenter Gastropoden (Mazek-Fialla 1934, Aubry *et al.* 2005, Aubry *et al.* 2006) sowie zu adaptiven morphologischen Eigenschaften (Yom-Tov 1971, Riddle 1983, Goodfriend 1986) wurde die Lebensweise bzw. das Verhalten dieser Tiere genauer untersucht. Dabei konnte beobachtet werden, dass sich die Aktivitätsphasen xerothermophiler Schnecken vornehmlich auf die kühleren, feuchten Nachtstunden beschränken, welche der Nahrungsaufnahme und der Fortbewegung dienen (Pomeroy 1968, Yom-Tov 1971). Am Ende dieser aktiven Phase erklettern die Tiere erhöhte Standpunkte wie die vorkommende Vegetation, um während des Tages dem durch Sonneneinstrahlung stark aufgeheizten Boden zu entgehen, da dieser meist selbst für thermophile Gastropoden letale Temperaturen erreicht (Pomeroy 1968, Cowie 1985). Zwar ist bereits wenige Zentimeter über der Bodenoberfläche das Temperaturniveau weitaus geringer (Köhler *et al.* 2009), dennoch sind die Tiere während ihrer inaktiven

Tagesphase gegenüber der Sonneneinstrahlung exponiert. Um längere Perioden von Dürre und Hitze zu überdauern, bedienen sich einige xerothermophile Gastropodenarten der Ästivation: dabei verschließen die Tiere ihre Schalenöffnung mittels eines Epiphragmas und können durch Reduzierung ihrer Stoffwechselrate Wasser und Energie einsparen (Schmidt-Nielsen 1971, Guppy & Withers 1999, Bishop & Brand 2000).

Neben den bereits erwähnten, vornehmlich im Verhalten begründeten Anpassungen, sind Adaptionsmechanismen auf physiologischer Ebene von großer Bedeutung, um schädliche Auswirkungen durch Hitzestress innerhalb des Organismus zu minimieren. Der Hepatopankreas der Gastropoden übernimmt als zentrales Stoffwechselorgan wichtige metabolische Prozesse und ist dadurch stark in die Stressbewältigung involviert (Sumner 1965, Taieb & Vicente 1998). Es ist bekannt, dass durch hohe Temperaturen der Säure-Base-Haushalt, das Gleichgewicht von Ionenkonzentrationen in Folge von Wasserverlust sowie der intrazelluläre pH-Wert beeinflußt wird, was zu osmotischem Stress und metabolischer Azidose führen kann (Barnhart 1986, Heisler 1986, Ryan & Gisolfi 1995). Den Kalkzellen, einem bestimmten Zelltypus im Hepatopankreas, wird eine wichtige Rolle bei der Osmoregulation (Taieb & Vicente 1998) und der Aufrechterhaltung des Säure-Base-Gleichgewichts (Burton 1976) zugeschrieben. Studienergebnisse, in denen eine Hyperplasie sowie Hypertrophie von Kalkzellen in Reaktion auf Hitzestresseinwirkung beobachtet werden konnte (Zaldibar *et al.* 2007, Dittbrenner *et al.* 2009, Scheil *et al.* 2011), können aufgrund der gesteigerten Aktivität dieses Zelltyp als möglich Strategie gedeutet werden, um Temperaturstress erfolgreich entgegen zu wirken.

Ein weiterer physiologischer Mechanismus, der zur Stressbewältigung beiträgt, ist die Induktion von Stressproteinen wie diejenigen der *heat shock protein 70* (Hsp70)-Familie. Diese Proteine blieben im Laufe der Evolution strukturell stark konserviert und sind – bis auf wenige Arten aus arktischen Gewässern - bei allen untersuchten Organismen zu finden (Feder & Hofmann 1999). Unter Bedingungen der Homöostase liegt bereits ein konstitutiv exprimierter Level an Hsp70-Isoformen vor, wobei diese als Chaperone während Proteinfaltungsprozessen agieren oder Polypeptide bei intrazellulären Transportvorgängen stabilisieren (Hendrick & Hartl 1993, Fink 1999, Mayer & Bukau 2005). Eine weitere wichtige Funktion der Stressproteine liegt in ihrer Befähigung, teildenaturierte Proteine zu renaturieren und folglich Proteinschädigungen bis zu einem gewissen Grad zu kompensieren (Köhler *et al.* 1992, Parsell & Lindquist 1993). Durch Einwirkung proteotoxischer

Stressoren wie Hitzestress werden Isoformen des Hsp70 vermehrt induziert (Lindquist & Craig 1988, Feder & Hofmann 1999), wodurch die Ermittlung des Hsp70-Gehalts als Marker für proteotoxischen Stress herangezogen werden kann, um Aussagen über den individuellen Stresszustand eines Organismus machen zu können (Köhler *et al.* 1992, Triebskorn & Köhler 1996, Feder & Hofmann 1999, Köhler *et al.* 2000). In den letzten Jahren konnten bereits einige Studien eine Verbindung von Stressproteinen mit der Thermotoleranz terrestrischer Gastropoden herstellen. Bei den beiden xerothermophilen Arten *Theba pisana* [Müller 1774] und *Xeropicta derbentina* [Krynicki 1836], zwei invasiven Vertretern der Helicoidea im mediterranen Raum, konnten Köhler *et al.* (2009) und Scheil *et al.* (2011) zeigen, dass es nach Hitzeeinwirkung zur gesteigerten Expression von Hsp70 kam. Des Weiteren wurde für Arten der Gattung *Sphincterochila* aus extrem trockenen, ariden Gebieten nachgewiesen, dass die Induktion von Stressproteinen einen essentiellen Beitrag zum Überleben dieser Tiere darstellt (Mizrahi *et al.* 2010, Mizrahi *et al.* 2012). Dabei zeigte sich, dass Hsps stark in die Vorgänge bei Übergängen zwischen Ästivations- und Aktivitäts -Phasen, welche Änderungen in der Stoffwechselrate mit sich bringen, sowie in Phasen der Austrocknung während Dürreperioden involviert sind (Arad *et al.* 2010, Mizrahi *et al.* 2010, Mizrahi *et al.* 2011).

Grundsätzlich ist zu erwähnen, dass die Kinetik der Stressproteininduktion einer von Eckwert *et al.* (1997) beschriebenen Optimumskurve folgt. Dabei kommt es aufgrund von proteotoxischen Stressoren vermehrt zu falsch gefalteten oder denaturierten Proteinen, welche eine triggernde Funktion auf die Transkription von Stressproteinen ausüben, in Folge dessen der Hsp-Level steigt (Kompensationsphase). Nach Erreichen eines Maximallevels und damit einhergehender andauernder oder gesteigerter Stresseinwirkung, kommt es, meist bedingt durch Erschöpfung von Energiereserven sowie durch zunehmende pathologische Effekte in Zellen, zu einem Rückgang des Stressprotein-Levels (Destruktionsphase). Allerdings konnte nachgewiesen werden, dass es bereits im konstitutiven Hsp70-Gehalt sowie in der maximal induzierbaren Höhe des Stressprotein-Levels intraspezifische Unterschiede geben kann (Köhler *et al.* 2009, Scheil *et al.* 2011), welche einen genetischen Hintergrund besitzen können (Sørensen *et al.* 2001, Jensen *et al.* 2009, Bahrndorff *et al.* 2010) und durch mikrohabitatabhängige Selektionsprozesse begründbar sind (Köhler *et al.* 2000) oder andererseits. Außerdem ist die Intensität der Hsp70 Expression vom jeweiligen Energiebudget eines Organismus abhängig (Tomanek & Somero 1999). Zudem ist noch zu bedenken, dass die Stressprotein-Expression generell ein sehr

energieaufwändiger Prozess ist (Sanchez *et al.* 1992, Heckathorn *et al.* 1996, Köhler *et al.* 2000) und daher die gesteigerte Bereitstellung von Hsp70 stetig in einem energetischen *trade-off* Verhältnis zu anderen physiologischen, lebenserhaltenden Prozessen steht. In diesem Zusammenhang bildet die Frage nach notwendigen, effektiven Strategien, welche die Balance zwischen Nutzen und Nachteilen der Bereitstellung hoher Energiekosten für gesteigerte Hsp70-Gehalte herstellen, einen interessanten Forschungsansatz.

Es ist bekannt, dass die Kapazität der Hsp70-Induktion in direkter Abhängigkeit vom jeweiligen Entwicklungsstadium eines Tieres ist. So konnte nachgewiesen werden, dass juvenile oder larvale Stadien in der Lage sind, Hsp70 in einem höheren Grad zu induzieren als ältere Individuen (Mayer & Bukau 2005, Köhler *et al.* 2009). Außerdem existieren bereits einige Studien, in denen saisonale und sogar tageszeitliche Schwankungen im Hsp70-Induktionsmuster bei verschiedenen aquatischen Organismen beobachtet werden konnten (Köhler *et al.* 2001, Nakano & Iwama 2002, Schill *et al.* 2002, Tomanek & Sanford 2003). Bislang gibt es jedoch kaum Untersuchungen zu xerothermophilen Gastropoden, die einen Aufschluss über den Verlauf der Stressprotein-Induktion bezüglich dieser Schwankungen geben.

Äußere Faktoren wie abiotische Umweltparameter haben einen Einfluss auf physiologische Abläufe innerhalb eines Organismus. So sind ektotherme Tiere wie Landschnecken maßgeblich von ihrer Umgebungstemperatur abhängig, da diese stark prägend auf deren Körpertemperatur wirkt und sich dies folglich auch in der Stoffwechselrate niederschlägt (Pörtner 2001). Es konnte gezeigt werden, dass sich neben der Sonneneinstrahlung, den Windverhältnisse oder der Bodenbeschaffenheit auch das Verhalten der Tiere (also oben beschriebene Verhaltensanpassungen) sowie morphologische Eigenschaften wie Größe, Form oder Farbgebung der Tiere einen modellierenden Effekt auf die Körpertemperatur ausüben (Stevenson 1985). Daher ist es nicht überraschend, dass der ausgeprägte Polymorphismus der Schalenfärbung einiger terrestrischer Gastropodenspezies, vor allem in Verbindung mit der Thermotoleranz dieser Schnecken, seit langem Gegenstand der malakologischen Forschung ist. Aufgrund einer Reihe früherer Studien wird angenommen, dass Unterschiede in der thermischen Kapazität verschiedener Schalenmorphen existieren (Jones 1973, Heath 1975, Cook & Freeman 1986, Hazel & Johnson 1990). Demnach sollen sich dunkle Morphen im Gegensatz zu hellen Schalentypen schneller und stärker aufheizen, was einen Selektionsnachteil in wärmeren Gebieten zur Folge haben würde. Dem gegenüber

stehen allerdings aktuelle Forschungsergebnisse, welche nach thermographischer Untersuchung keine signifikanten Unterschiede in der Erwärmung heller vs. dunklerer Gehäuse bei der terrestrischen Schnecke *Theba pisana* feststellen konnten (Scheil *et al.* 2012a). Savazzi & Sasaki (2013) konnten außerdem zeigen, dass die Bänderung von Gehäusen bei einigen Landschneckenarten im nahen Infrarotlicht (NIR; macht circa 45% der Energie durch Sonneneinstrahlung aus) „verschwindet". Es ist bekannt, dass die thermische Kapazität abhängig von der Materialzusammensetzung eines Körpers ist (Eichler *et al.* 2005, Heuberger & Fels 2007), welche bei Schneckenschalen zu 97% aus Calciumcarbonat und zu 3% aus organischem Material, in welches Pigmente eigelagert werden, besteht (Heller & Margitz 1983). Folglich ließe diese Tatsache darauf schließen, dass dem Calciumcarbonat in den Schalen eine bedeutendere Rolle in Aufheizungs- und Abkühlungsprozessen zukommt und diese weniger durch die Pigmentierung begründbar sind. Als weiterer Punkt wird von Savazzi & Sasaki (2013) angeführt, dass die Licht-Reflexion der Schalenoberfläche im NIR-Bereich (und somit eine reduzierte Erwärmung) durchaus relevant für die Thermoregulation dieser Tiere sein könnte.

Dennoch existieren individuelle physiologische Unterschiede verschieden gefärbter Schalenmorphen in Reaktion auf klimatische Bedingungen: So konnten Unterschiede im Verhalten und der Aktivität (Jones, 1982), der Resistenz gegenüber Austrocknung (Arad et al., 1993) und der Toleranz gegenüber Temperatur beobachtet werden, wobei dunklere Phänotypen der Art *Arianta arbustorum* bei Temperaturexperimenten generell höhere Mortalitäten aufwiesen als helle Phänotypen (Abdel-Rehim, 1988). Des Weiteren konnten Staikou *et al.* 1999 bei der Gastropodenart *Cepaea vindobonensis* ebenfalls Variationen hinsichtlich der Aktivitätsmuster und der Resistenz gegenüber Austrocknung mit der Gehäusefärbung in Verbindung bringen. Es ist also offensichtlich, dass die Hintergründe dieser morphenbezogenen Variationen noch nicht vollständig geklärt sind und somit noch Forschungsbedarf besteht.

Neben einer Reihe anderer Einflüsse kommt es vor allem durch Hitzestress im Organismus zur erhöhten Produktion von reaktiven Sauerstoffspezies (*reactive oxygen species*, ROS). Diese ROS werden zwar aufgrund von metabolischen und biochemischen Reaktionen (Zellatmung, Biotransformation) ständig gebildet und stellen daher ein unvermeidbares Nebenprodukt aeroben Lebens dar (Halliwell & Gutteridge, 1989), jedoch wird deren schädlichen Auswirkungen durch effektive Schutzmechanismen entgegengewirkt. Kommt es aufgrund eines Stressors wie

Hitze zu einem Ungleichgewicht zwischen ROS-Bildung und deren „Bekämpfung“, so spricht man von oxidativem Stress (Sies 1994, Sies 1997). Hierbei führen ROS, zu denen hoch reaktive freie Radikale zählen, zur Oxidation von Eiweißen und Lipiden, welche vor allem in Biomembranen lokalisiert sind, verursachen dadurch Schäden an Zellstrukturen und beeinträchtigen somit zelluläre Abläufe (Halliwell & Gutteridge 1989, Halliwell 2006). In biologischen Systemen spielt vor allem die Lipidperoxidation, bei der mehrfach ungesättigte Fettsäuren durch freie Radikale oxidiert werden, eine große Rolle. Dabei werden durch Autoxidation von Lipiden Hydroperoxide als Endprodukt der Lipidperoxidation gebildet (Gutteridge 1995). Die Quantifizierung von entstandenen Lipidperoxiden kann mittels der *ferrous oxidation xylenol orange method* (FOX assay)(Hermes-Lima *et al.* 1995; Monserrat *et al.* 2003) als Maß für den oxidativen Stress eines Organismus herangezogen werden.

Zu den unter Hitzestress entstehenden ROS zählen unter anderem das hoch reaktive Hydroxylradikal (·OH), das Superoxidanion-Radikal ($\cdot O_2^-$) und Wasserstoffperoxid (H_2O_2), wobei dessen Gefährdungspotential vor allem in der Umwandlung zum Hydroxylradikal liegt (Storey 1996, Pannunzio & Storey 1998). Um die schädlichen Auswirkungen der ROS zu minimieren und einem oxidativen Stressmilieu entgegen zu wirken, besitzen daher alle Zellen einen konstitutiven Gehalt enzymatischer und nicht-enzymatischer antioxidativer Schutzmechanismen (Halliwell & Gutteridge 1989). Zu den primären Schutzenzymen gehören Superoxiddismutase (SOD, welche die Umwandlung von Superoxidanion zu Wasserstoffperoxid katalysiert), Katalase (KAT, welche den Abbau von Wasserstoffperoxid zu Wasser und Sauerstoff katalysiert) und Glutathionperoxidase (GPx, welche neben Wasserstoffperoxid auch durch Lipidperoxidation entstandene Lipidperoxide mittels reduziertem Glutathion entgiftet)(Aebi 1984, Halliwell & Gutteridge 1989, Gutteridge 1995). Neben diesen primären Schutzenzymen existieren bei aeroben Organismen sekundäre Schutzenzyme wie Glutathion-S-Transferase (GST) oder Glutathionreduktase (GR) sowie nicht-enzymatische Antioxidantien, welche als Radikalfänger oder als Substrat bei katalytischen Prozessen agieren, wobei reduziertes Glutathion (GSH) als eine der wichtigsten Verbindungen zu nennen ist (Meister 1988, Pannunzio & Storey 1998, Radwan *et al.* 2010).

Seit langem ist bekannt, dass das System der antioxidativen Abwehr ein essentieller Bestandteil zum Schutz und Erhalt zellulärer Abläufe und Strukturen innerhalb eines Organismus ist, vor allem auch in Anbetracht sich ändernder Umweltfaktoren. So gibt es eine Reihe von Studien, die Reaktionen des

antioxidativen Schutzsystems in Bezug auf anoxische Lebensbedingungen (Hermes-Lima & Storey 1996, 1993, Pannunzio & Storey 1996, Lushchak et al. 2001), beim Kältestress, d.h. beim „Einfrieren“ eines Organismus (Hermes-Lima & Storey 1993, Joanisse & Storey 1996), und beim Hitzestress (Lushchak & Bagnyukova 2006b, Heise *et al.* 2006, Verlecar *et al.* 2007) untersucht haben. Zu Gastropoden existieren diesbezüglich vor allem Studien, die sich mit der Beteiligung der antioxidativen Abwehr bei metabolischen Prozessen während Phasen der Ästivation beschäftigen (Hermes-Lima & Storey 1995, Storey 1996, Hermes-Lima *et al.* 1998, Storey 2002, Ramos-Vasconcelos & Hermes-Lima 2003, Nowakowska *et al.* 2009, Nowakowska *et al.* 2010, Nowakowska *et al.* 2011). Jedoch wurden bislang kaum Untersuchungen zum oxidativen Stress generell und insbesondere *in puncto* Thermotoleranz bei terrestrischen Gastropoden durchgeführt (Scheil *et al.* 2012b). Auch wurde die Beteiligung von antioxidativen Enzymen an diesen Vorgängen bei diesen Tieren bislang nicht beschrieben.

2.2 Durchgeführte Studien und Zielsetzung

Die vorliegende wissenschaftliche Arbeit beschäftigt sich maßgeblich mit den Auswirkungen erhöhter Temperaturen auf physiologische Mechanismen der xerothermophilen Gastropodenart *Xeropicta derbentina*. Neben externen Faktoren (wie Umwelteinflüssen) oder Verhaltensschemata, die den Stresszustand der Tiere modulieren, stehen dabei vor allem biochemische als auch zelluläre Adaptationsmechanismen im Fokus, welche essentiell für das Überleben dieser Organismen unter extremen Umweltbedingungen zu sein scheinen. Ziel dieser Arbeit ist es, diese adaptiven Mechanismen innerhalb der Lebensspanne von *X. derbentina* zu erfassen und dabei aufzudecken, welcher Anpassungsstrategien sich diese Tiere in den jeweiligen Lebensphasen bedienen. Dafür soll der Frage nachgegangen werden, inwieweit erhöhte Temperaturen (im natürlichen Habitat vor allem bedingt durch Sonneneinstrahlung) den individuellen Stresszustand dieser Schnecken beeinflussen und inwiefern sich dies in den unterschiedlichen Lebensphasen sowie in verschiedenen Populationen wiederspiegelt.
Im Rahmen der vorliegenden Arbeit sollen folgende Arbeitshypothesen überprüft werden:

1. Das Verhalten von *X. derbentina* als auch ökologische und morphologische Faktoren haben einen distinkten Einfluss auf den Stresszustand der Tiere.

2. Die Induktion von Stressproteinen korreliert mit dem Alter der Schnecken und unterliegt saisonalen Schwankungen.
3. Es existieren populationsspezifische Unterschiede in biochemischen und histologischen Reaktionsmustern nach Hitzestresseinwirkung bei *X. derbentina*.
4. Dunkler gefärbte Morphen von *X. derbentina* erfahren aufgrund stärkerer Erwärmung mehr Stress und induzieren daher mehr Stressproteine, um dies zu kompensieren. Gleichzeitig kommt es in diesen Individuen zu mehr oxidativem Stress als bei Vertretern mit heller Gehäusefärbung.
5. Antioxidative Enzyme sind bei der Hitzestressbewältigung beteiligt, um den schädlichen Auswirkungen von oxidativem Stress entgegen zu wirken.

In den im Folgenden beschriebenen Studien sollen die dafür notwendigen Teilaspekte genauer betrachtet werden:
Im ersten Teil dieser Arbeit sollte zunächst untersucht werden, inwieweit ökologische und morphologische Parameter einen Einfluss auf die individuelle Stressbelastung xerothermophiler Schnecken besitzen. Dazu wurden sieben verschiedene Populationen der Spezies *Xeropicta derbentina* in Südfrankreich untersucht, wobei die Variation des Hsp70-Levels der Tiere, welcher als Proxy für deren Stressbelastung dient, durch eine Reihe von im Feld erhobenen extrinsischen und intrinsischen Variablen sowie deren Interaktionen modelliert wurde. Dabei sollte ermittelt werden, welcher der gewählten Faktoren im Rahmen dieser „Schnappschusssituation“ den größten Einfluss auf die physiologische Stressreaktion dieser Tiere unter natürlichen Bedingungen ausübt.
Weiterführend sollte die Induktion von Hsp70 in verschiedenen Lebensphasen von *X. derbentina* und somit deren protektives Potential zu diesen Zeitpunkten untersucht werden. Es sollte ermittelt werden, wie sich die Induktion von Stressproteinen abhängig vom tageszeitlichen Temperaturprofil innerhalb eines 24-Stunden Zyklus verhält und ob jahreszeitliche Schwankungen, einhergehend mit dem Alter der Tiere, im Induktionsmuster existieren.
Um neben der Induktion von Stressproteinen die Auswirkungen von Hitzestress auch auf zellulärer Ebene erfassen zu können, wurden zusätzlich zur Ermittlung des Hsp70-Levels histopathologische Untersuchungen des Hepatopankreas von Gastropoden an Individuen aus sieben räumlich voneinander getrennten Populationen vorgenommen, welche zuvor gegenüber erhöhten Temperaturen exponiert wurden. Dabei sollte die Frage geklärt werden, inwieweit

populationsspezifische Unterschiede in der Induktionskapazität von Hsp70 und dem zellulären Gesundheitszustand existieren, ob Hsp70 tatsächlich eine schützende Funktion auf die untersuchten zellulären Strukturen ausübt und ob unterschiedliche Hitzestressbewältigungsstrategien identifiziert werden können. Abschließend soll geprüft werden, ob genetische Parameter (populationsgenetische Indices) mit den physiologischen Stressantworten korrelieren und ob mit Hilfe dieser Informationen zur genetischen Populationsstruktur die Variation der physiologischen Hitzestressantwort erklärt werden kann.

Des Weiteren ist bekannt, dass, wie bereits oben genauer erläutert, erhöhte Temperaturen zu oxidativem Stress in Organismen führen. In weiteren Temperaturexpositionen wurde deshalb mittels FOX-Assay (*ferrous oxidation xylenol orange method*) der Level an Lipidperoxiden, welcher als Maß für den erfahrenen oxidativen Stress im Organismus herangezogen wird, zusätzlich zum Stressproteingehalt bestimmt und in Verbindung mit dem Schalenpolymorphismus von *X. derbentina* genauer betrachtet. Anhand dieser Untersuchungen sollte der Frage nachgegangen werden, ob dunklere Morphen unter erhöhter Temperatureinwirkung mehr Hsp70 exprimieren, um einen eventuellen Selektionsnachteil aufgrund stärkerer Erwärmung im Sonnenlicht auszugleichen, und ob sich dies ebenfalls in einer erhöhten Lipidperoxidation wiederspiegelt.

Um den vorangegangenen Ansatz im Hinblick auf den oxidativen Stress bei *X. derbentina* weiterführend zu bearbeiten, wurde das System der antioxidativen Abwehr, d.h. dessen Reaktion auf Temperaturerhöhung, als weiterer involvierter Mechanismus bei der Hitzestressbewältigung dieser Gastropoden untersucht. Dazu wurden die Aktivitätsprofile der antioxidativen Enzyme Katalase und Glutathionperoxidase nach Hitzestresseinwirkung ermittelt und mit den jeweiligen Ergebnissen zum Lipidperoxid-Gehalt sowie der Induktionskapazität von Hsp70 verglichen. Dabei galt es die Fragen zu klären, ob die gewählten Vertreter aus der Reihe der enzymatischen Antioxidantien eine positive Wirkung auf den Stresszustand der Tiere haben, d.h. ob es zu einer Senkung des Lipidperoxid-Levels und somit zu einer Reduzierung des oxidativen Stresses kommt, ob und gegebenenfalls in welchem Ausmaß diese Enzymaktivitäten von der Umgebungstemperatur beeinflusst werden und ob bzw. wie sich das Stressproteinsystem und die antioxidative Abwehr gegenseitig ergänzen.

3. Material und Methoden

3.1 Testorganismus

In der vorliegenden Arbeit wurden Schnecken der Spezies *Xeropicta derbentina* [Krynicki 1836] untersucht. Diese pulmonaten Gastropoden zählen zur Familie der Hygromiidae (Laubschnecken), welche wiederum der Überfamilie der Helicoidea und der Unterordnung der Stylommatophora (Landlungenschnecken) angehören. Der Verbreitungsraum dieser xerothermophilen Schneckenart bezieht sich ursprünglich auf den osteuropäischen Raum (Kaukasus, Türkei). *X.derbentina* wurde jedoch in den 1940er Jahren im mediterranen Raum eingeschleppt, wo sie sich seitdem invasiv verbreitet (Kiss *et al.* 2005, Aubry *et al.* 2006). Die Art weist einen farblichen Schalenpolymorphismus auf, der von rein weißen bis hin zu dunkel bräunlich gebänderten Gehäusen reicht. Im Adultstadium erreichen die Tiere eine Größe von 10 bis 16 mm und sind meist auf offenen Grasflächen, angeheftet an der dort vorkommenden Vegetation, zu finden (Abb. 1).

Abbildung 1: *Xeropicta derbentina* (Aufnahmen von A. Dieterich, Universität Tübingen).

3.2 Beprobungsgebiet

Da *X. derbentina* sich mittlerweile invasiv im Mittelmeerraum verbreitet hat, wurde der Süden Frankreichs als Beprobungsgebiet gewählt. Dort sind Individuen dieser Gastropodenart in großen Zahlen zu finden. Das Klima in Südfrankreich ist durch heiße, trockene Sommer und dabei auftretenden Lufttemperaturen meist über 30°C charakterisiert. Die Untersuchungen im Feld sowie die Entnahme von Individuen

erfolgten im Département Vaucluse, Provence, etwa 50 km nordöstlich von Avignon. Für das zweite, vierte und fünfte Kapitel dieser Arbeit wurde ausschließlich eine *X. derbentina*-Population in Modène (N 44° 6.055' E 5° 7.937') untersucht, wohingegen für das erste und dritte Kapitel zusätzlich sechs weitere Populationen in der Umgebung betrachtet wurden.

3.3 Morphologische Einteilung

Für das erste, zweite und vierte Kapitel der vorliegenden Arbeit wurden die unterschiedlichen Gehäusefärbungen der Schnecken in vier definierte Kategorien eingeteilt: Kategorie 1 mit rein weißen Gehäuse; Kategorie 2 mit weißen Gehäuse und leicht bräunlicher Bänderung auf der Unterseite; Kategorie 3 mit weißen bis gräulichen Gehäusen und einem dicken oder mehreren dunklen Bändern auf der Unterseite; Kategorie 4 mit vielen schmalen bräunlichen Bändern auf Ober-und Unterseite des Gehäuses (nach Köhler *et al.* 2009). Die übrigen Kapitel erforderten keine Einteilung der Schnecken in die unterschiedlichen Farbmorphen.

3.4 Datenerhebung im Freiland

Für die Untersuchungen im ersten Kapitel wurden im Mai 2010 sieben räumlich voneinander getrennte Populationen von *X. derbentina* ausgewählt und für jeweils 12 Individuen morphologische und ökologische Parameter im Feld bestimmt: die Aufenthaltshöhe über der Erdoberfläche [cm], die Schalenorientierung zur Sonne (Apex, Nabel oder Seite des Gehäuses zur Sonne gewandt), die Oberflächentemperatur des Gehäuses [°C], die Innentemperatur des Gehäuses (Weichkörper der Schnecke)[°C], der Schalendurchmesser [mm] und die Gehäusefärbung (nach o.g. Kategorien). Anschließend wurden die Individuen für spätere Hsp70-Analysen in flüssigem Stickstoff schockgefroren.
Die im zweiten Kapitel dieser Arbeit beschriebenen Untersuchungen erfolgten zu vier verschiedenen Jahreszeitpunkten (April, Juni, August, Oktober) im Jahr 2011. Um die Verteilung der Individuenim Feld zu ermitteln, wurde ein 1 × 3 m großes Wiesenstück in Modène untersucht und daraus von 250 Schnecken die Schalengröße [mm], die Schalenfärbung [Kategorien 1 bis 4] und die Aufenthaltshöhe über dem Grund [cm] notiert. Für spätere Hsp70-Analysen wurden

Schnecken zufällig aus dem Feld entnommen und zusätzlich zu den oben genannten Parametern die Außentemperatur der Schale mit einem Nadelthermometer[°C] bestimmt. Um ein Temperaturprofil zu erstellen, wurde die atmosphärische Temperatur während jeder Beprobung mittels Thermosensoren in verschiedenen Höhen über dem Grund (1, 2, 3, 5, 10, 15, 20, 25, 30 und 40 cm) die Außentemperatur aufgezeichnet.
Für die in den Kapiteln drei bis fünf beschriebenen Studien wurden Schnecken aus dem Feld lebend gesammelt und in Plastikboxen (20,5 × 30 × 19,5 cm) für spätere Temperaturexpositionen im Labor nach Tübingen transportiert.

3. 5 Temperaturexpositionen im Labor

Um die Auswirkungen von experimentell induziertem Temperaturstress auf ausgewählte physiologische Mechanismen der Schnecken im dritten bis fünften Kapitel dieser Arbeit zu untersuchen, wurden die Tiere zunächst über einen Akklimatisationszeitraum von zwei bis drei Wochen in Plastikbehältern (20,5 × 30 × 19,5 cm), welche mit feuchter Terrarienerde (JBL TerraBasis, Neuhofen Deutschland) gefüllt waren, bei 25°C gehalten. Die Fütterung der Tiere erfolgte mit Babybrei (HIPP Gute Nacht Bio-Milchbrei, Hafer & Apfel, Pfaffenhofen, Deutschland) und Sepiaschulp (nach Cowie & Cain, 1983). Für die anschließenden Temperaturexpositionen im Wärmeschrank wurden Schnecken in mit feuchtem Vliespapier ausgelegte Plastikboxen (6,5 × 18 × 13 cm) überführt und für die jeweiligen Studien wie folgt exponiert und weiterbearbeitet:
Für die Studie des dritten Kapitels wurden insgesamt 22 Schnecken pro Population gegenüber erhöhten Temperaturen (25, 33, 38, 40, 43, 45, 48, 50 und 52°C) über einen Zeitraum von 8 Stunden exponiert. Nach Beendigung der Expositionszeit wurden, nachdem die Größe der Schnecken notiert wurde, zum einen 12 Proben für Hsp70-Analysen schockgefroren und zum anderen 10 Proben für histopathologische Untersuchungen nach Entfernen der Schale in 2% Glutardialdehydlösung (25 % Glutardialdehyd gelöst in 0,01M Cacodylatpuffer, pH 7,4) fixiert.
Für die im vierten Kapitel durchgeführten Untersuchungen wurden für Analysen zum Hsp70-Gehalt und dem Lipidperoxid-Level der Tiere pro Farbkategorie jeweils 22 Schnecken gegenüber 25, 33, 38, 40, 43, 45 und 48°C für 8 Stunden exponiert

und anschließend in flüssigem Stickstoff schockgefroren, wobei für die Proben des FOX-Assays zuvor die Schale der Schnecken präparativ entfernt wurde.
Die Temperaturexpositionen der im fünften Kapitel dieser Arbeit beschriebenen Studie erfolgten für 8 Stunden gegenüber 25, 38, 40, 43 und 45°C mit jeweils insgesamt 40 Individuen für Untersuchungen zum Hsp70-und Lipidperoxid-Level sowie den Aktivitätsmessungen der Enzyme Katalase und Glutathionperoxidase. Im Anschluss wurden die Schalen der Schnecken entfernt (ausgenommen die der Proben für die Stressproteinanalysen) und die Weichkörper in flüssigem Stickstoff schockgefroren.
Alle Proben wurden bis zur weiteren Bearbeitung bei -20°C bzw. die für die histopathologischen Analysen im Kühlschrank bei -4°C gelagert

3.6 Stressproteinanalysen (Hsp70)

In allen Studien der vorliegenden Arbeit wurde der individuelle Hsp70-Gehalt der Schnecken ermittelt. Dazu wurden die gefrorenen Proben mit 2µL Extraktionspuffer (80mM Kaliumacetat, 5mM Magnesiumacetat, 20mM Herpes) pro 1mg Probengewicht versetzt, mittels eines Stößels mechanisch auf Eis zerkleinert und anschließend für 10 Minuten bei 20.000 *g* und 4°C zentrifugiert. Aus dem erhaltenen Überstand wurde zunächst die Gesamtproteinmenge nach Bradford (1976) bestimmt. Anschließend wurden konstante Proteinmengen von 40µg pro Probe mittels SDS-PAGE (SDS-Polyacrylamidgelelektrophorese) aufgetrennt und mittels Semi-Dry Elektrotransfers auf Nitrocellulosemembran transferiert. Um die Proteinbanden mittels einer Peroxidasefarbreaktion sichtbar zu machen, wurden diese zunächst mit Antikörpern (1. Antikörper monoclonal mouse anti-human Hsp; 2. Antikörper goat anti-mouse IgG Peroxidase-Konjugat) inkubiert und im Anschluß über eine 1mM 4-Chlor(1)naphtol-Lösung angefärbt. Die densitometrische Auswertung des optischen Volumens (Pixelintensität × Fläche) jeder Bande erfolgte mit dem Programm E.A.S.Y. Win 32 (Herolab, Wiesloch, Deutschland).

3.7 Histopathologische Untersuchungen

In den im dritten Kapitel beschriebenen Untersuchungen wurde der Hepatopankreas der Schnecken histopathologisch ausgewertet, nachdem die

Individuen gegenüber erhöhten Temperaturen exponiert waren. Nach Exposition wurden zunächst die Schalen entfernt und die Weichkörper der Schnecken für mindestens eine Woche im Fixans (25% Glutardialdehyd gelöst in 0,01M Cacodylatpuffer, pH 7,4) belassen und bei 4°C gelagert. Nach Dekalzifizierung über ein Gemisch aus Ameisensäure und Ethanol (1:2), wurden die Proben über eine aufsteigende Alkoholreihe entwässert und anschließend in Kunstharz (Technovit, Heraeus Kulzer GmbH, Wehrheim, Deutschland) eingebettet. Am Rotationsmikrotom wurden 7 µm dicke Schnitte angefertigt, mittels Hämatoxylin-Eosin-Lösung angefärbt und am Lichtmikroskop qualitativ beschrieben sowie semi-quantitativ ausgewertet. Hierfür wurde das Erscheinungsbild von Verdauungstubuli, Kalk- und Resorptionzellen der Mitteldarmdrüse mittels eines 5-stufigen Bewertungssystems kategorisiert. Dabei stellt Kategorie 1 den Kontrollzustand, Kategorie 3 den Reaktionszustand und Kategorie 5 den Destruktionszustand dar. Kategorie 2 und 4 stehen für Zwischenstadien. Aus den einzelnen individuellen Bewertungen pro Tier wurden Mittelwerte für jede Versuchsgruppe berechnet.

3.8 Bestimmung des Lipidperoxid-Levels mittels FOX (*ferrous oxidation xylenol orange*) Assay

Die in den Kapiteln vier und fünf angewandte Methodik zur Ermittlung des Lipidperoxidlevels wurde anhand einer modifizierten Variante des FOX-Assay (Hermes-Lima *et al.*, 1995) durchgeführt. Die gefrorenen, schalenlosen Proben wurden nach Bestimmung des Gewichtes in Methanol (im Gewichts-Verhältnis 1:2) auf Eis homogenisiert und bei 15.000 *g* und 4°C über 5 Minuten zentrifugiert. Jeweils 15µL des gewonnen Überstandes wurden zusammen mit einem Reaktionsgemisch (0,25mM FeSO4, 25mM H2SO4, 0,1mM Xylenolorange) auf 96-well Platten aufgetragen, über einen Zeitraum von 180 Minuten bei Raumtemperatur inkubiert und anschließend bei 580nm spektralphotometrisch vermessen. Nach Zugabe von 1µL CHP (1mM Cumolhydroperoxid-Lösung) und einer weiteren Inkubationszeit von 30 Minuten wurde die Absorption bei 580nm erneut bestimmt.
Der Gehalt an Lipidperoxiden in der Probe wird als Cumolhydroperoxidäquivalente pro Gramm Naßgewicht (CHPE/g wet weight) ausgedrückt und wie folgt berechnet:

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

wobei 200 = das Gesamtvolumen, V1 = das Probevolumen (15µL) und 2 = den Verdünnungsfaktor mit Methanol (1:2) darstellt.

3.9 Bestimmung der Katalase-Aktivität

Für die im fünften Kapitel beschriebene Ermittlung der Katalaseaktivität wurde *Cayman´s Catalase Assay Kit* (Item No. 707002, Cayman Chemical Company, Michigan, USA) verwendet. Nach Gewichtsbestimmung wurden die Proben zusammen mit 5mL Kaliumphosphatpuffer (50mM Kaliumphosphat, pH 7, versetzt mit 1mM EDTA) pro Gramm Probengewicht homogenisiert und bei 10.000 *g* und 4°C über 15 Minuten zentrifugiert. Der gewonnene Überstand wurde im Verhältnis 1:2000 mit Kaliumphosphatpuffer verdünnt (Verdünnungsverhältnis wurde in Vorversuchen ermittelt) und jeweils 20µL dieses Gemisches zusammen mit 100µL Probenpuffer (100mM Kaliumphosphat, pH 7) und 30µL Methanol auf eine 96-well Platte aufgetragen. Zusätzlich wurden auf der Platte verschiedene Standards mit aufsteigenden Konzentrationen an Formaldehyd sowie Katalase-Positivkontrollen (Katalase aus Rinderleber) angefertigt. Um die Reaktion zu starten, wurden jeweils 20µL Wasserstoffperoxid-Lösung hinzugegeben, für 20 Minuten auf dem Schüttler bei Raumtemperatur inkubiert und anschließend die Reaktion mittels Zugabe von 30µL 10M Kaliumhydroxid-Lösung gestoppt. Danach wurden jeweils 30µL Katalase-Purpalp (gelöst in 0,5M Kaliumhydroxidlösung) hinzu pipettiert, weitere 10 Minuten inkubiert und nach Hinzufügen von 10µL Katalase-Kaliumperjodat (gelöst in 0,5M Kaliumhydroxidlösung) wiederum für 5 Minuten inkubiert. Abschließend wurde die Absorption spektrometrisch bei 540nm gemessen.
Die Katalase-Aktivität wurde mittels folgender Gleichung berechnet:

$$\text{Katalase-Aktivität [nmol/min/mg]} = [(\mu M \text{ Formaldehyd pro Probe}/20min) \times \text{Verdünnungsfaktor der Probe}]/1000$$

3.10 Bestimmung der Glutathionperoxidase-Aktivität

In der im fünften Kapitel dieser Arbeit beschriebenen Studie wurde für die Bestimmung der Glutathionperoxidase-Aktivität das *Cayman´s Glutathione Peroxidase Assay Kit* (Item No. 703102, Cayman Chemical Company, Michigan, USA) verwendet. Nach Bestimmung ihres Gewichtes wurden die gefrorenen Proben in 5mL Assaypuffer (50mM Tris-HCl, pH 7,6, 5mM EDTA, 1mM DTT) pro Gramm Probengewicht homogenisiert und über einen Zeitraum von 15 Minuten bei 10.000 *g* und 4°C zentrifugiert. Jeweils 20µL des erhaltenen Überstandes wurden zusammen mit 100µL Assaypuffer und 50µL Co-Substat-Lösung (NADPH, Glutathion und Glutathionreduktase gelöst in H_2O) auf 96-well Platten aufgetragen. Positivkontrollen (Glutathionperoxidase aus Rinder-Erythrocyten) wurden zusätzlich angefertigt. Durch die Zugabe von 20µL Cumolhydroperoxid wurde die Reaktion gestartet und die Absorption über einen Zeitraum von 5 Minuten alle 60 Sekunden spektrometrisch bei 340nm gemessen.
Für jede Probe wurde die Absorptionsänderung (ΔA_{340}) pro Minute ermittelt und die Enzymaktivität mittels folgender Gleichung berechnet:

GPx-Aktivität [nmol/min/mg] = [((ΔA_{340}/min)/0.000373µM-1) x (0.19ml/0.02ml) x Verdünnungsfaktor der Probe]/1000

3.11 Genetische Untersuchungen

Im dritten Abschnitt dieser Arbeit wurden genetische Untersuchungen zur Populationsstruktur von *X. derbentina* durchgeführt. Genomische DNA wurde hierfür mittels *DNeasy Blood & Tissue Kit* (QIAGEN, Inc., Mississauga, Ontario, USA) aus dem Fußgewebe der gefrorenen Schnecken extrahiert. Über PCR wurde ein Fragment des mitochondrischen Cytochrom-C-Oxidase Untereinheit 1 (CO1)-Gens auf eine Länge von 700 Basenpaaren amplifiziert. Primer für PCR-Amplifizierung und DNA-Sequenzierung waren LCO1490 (Folmer *et al.* 1994) und der neuentwickelte Primer HeliR2 5´-CCTAAAATATGWGAAAYAATACCAAA-3`. Bidirektionale DNA-Sequenzierung wurde mittels eines ABI 3730 XL DNA Analyzers ausgeführt.
Aus allen generierten Sequenzen wurde ein Haplotypen-Netzwerk mittels des Programms TCS 1.21 (Clement *et al.* 2000) konstruiert um sicher zu stellen, dass

alle untersuchten Populationen tatsächlich derselben Art angehören. Anschließend wurden aus dem CO1-Datensatz drei verschiedene Populations-Indices berechnet: die Nukleotid-Diversität π (durchschnittliche Anzahl von Nukleotidunterschieden, basierend auf dem K2P Sequenzevolutionsmodel), deren Berechnung mit dem Programm Arlequin 3.5.1.2 (Excoffier *et al.* 2005) erfolgte, der paarweise Fixationsindex F_{ST} nach Nei (1973) und die Haplotypen-Divergenz H_{MH}, welche auf dem Morisita-Horn Index (Horn 1966) basiert. Sowohl der F_{ST} als auch der H_{MH} Index wurden mit dem Programm R 2.15 (R Development Core Team 2011) kalkuliert.

4. Ergebnisse und Diskussion

4.1 Kapitel 1: MA Di Lellis, M Seifan, S Troschinski, C Mazzia, Y Capowiez, R Triebskorn, H-R Köhler (2012) Solar radiation stress in climbing snails: behavioural and intrinsic features define the Hsp70 level in natural populations of *Xeropicta derbentina* (Pulmonata). Cell Stress and Chaperones, 17(6): 717-727

Die Ergebnisse dieser Studie zeigen, dass einige der untersuchten ökologischen bzw. morphologischen Parameter einen deutlichen Einfluss auf den Stressprotein-Level bei *X. derbentina* haben. Das Kletterverhalten dieser Gastropoden, ermittelt als Sitzhöhe der Tiere über Grund, erwies sich dahingehend als wichtiger Faktor, da der detektierte Hsp70-Level mit zunehmendem Abstand vom Boden abnahm. Um den hohen Bodentemperaturen zu entgehen, welche an sonnigen Sommertagen bis zu 50°C überschreiten können, ist es für die Schnecken bereits von Vorteil, sich wenige Zentimeter über dem Grund aufzuhalten, da dort die Temperaturen bereits weitaus niedriger als direkt an der Bodenoberfläche sind (Cowie 1985, Kempster & Charwa 2003). Durch geringere Außentemperaturen nimmt der proteotoxische Stress ab, folglich auch der Hsp70-Spiegel. Pomeroy (1966) und Köhler *et al.* (2009) konnten bereits zeigen, dass die Höhe über Grund auch in engem Zusammenhang mit der inneren Temperatur der Schnecken steht. In unserer Untersuchung konnten wir tatsächlich nur geringe Temperaturunterschiede zwischen den Individuen feststellen (max. 6°C), folglich hatte dieser Faktor nur wenig Einfluss auf den Hsp70-Gehalt. Jedoch muss hier beachtet werden, dass Hsp70 über längere Zeiträume induziert und somit spontane, kurzfristige Ereignisse wie Änderungen in Windgeschwindigkeit oder Sonneneinstrahlung, wie sie unmittelbar auf die

Körpertemperatur der Tiere wirken, über Messungen des Hsp70-Levels nicht erfasst werden können. Zudem können intraspezifische Unterschiede der Individuen in ihrer genetischen sowie physiologischen Zusammensetzung mit der Innentemperatur interferieren und auf die Hsp70-Induktion wirken.

Die Schalengröße der Schnecken stellte sich als weiterer einflussreicher Faktor auf die Höhe des Hsp70-Spiegels heraus. Große Schnecken wiesen dabei einen geringeren Hsp70-Level auf, wobei kleinere Schnecken viel Hsp70 induzierten. Das Alter der Tiere könnte dafür als mögliche Erklärung bereit stehen: da juvenile Tiere sich noch in Entwicklungsprozessen befinden, wird viel konstitutives Hsp70 für dessen Chaperon-Funktion in Faltungs-, Transport- und Aggregierungsprozessen von Proteinen benötigt (Mayer & Bukau 2005). Des Weiteren kann Wasserverlust, bedingt durch erhöhte Evaporation bei höheren Temperaturen und ein ungünstigeres Oberflächen-Volumen-Verhältnis bei kleineren Individuen, zur erhöhten Hsp70-Induktion führen (Mizrahi *et al.* 2010).

Die Schalenorientierung zur Sonne stellte sich als einzelner Faktor als wenig bedeutsam für die Stressproteininduktion heraus. In Kombination mit der Aufenthaltshöhe über Grund zeigte sich jedoch ein interessanter Effekt: Wenn der Apex der Gastropodenschale zur Sonne gewandt war und zusätzlich der Abstand zum Boden zunahm, schlug sich dies in einer Steigerung des Hsp70-Levels nieder. Wenn jedoch das Gehäuse lateral oder mit der Nabelseite in Richtung Sonne zeigte, führte dies mit erhöhter Aufenthaltshöhe über Grund zu einer Senkung des Stressprotein-Levels. Dies könnte mit der Lokalisierung der inneren Organe zusammenhängen, die durch Variation in der Ausrichtung unterschiedlich stark der Sonnenbestrahlung ausgesetzt waren. Gewebespezifische Unterschiede in der Hsp70-Expression konnten bereits für Mollusken nachgewiesen werden (Lyons *et al.* 2003, Mizrahi *et al.* 2010, Arad *et al.* 2010).

Des Weiteren konnte festgestellt werden, dass der Hsp70-Level mit zunehmender Pigmentierung der Gehäuse stieg. Einerseits wurde bisher angenommen, dass sich aufgrund von Reflektion bzw. Absorption dunkel gefärbte Gehäuse von Gastropoden schneller aufheizen als helle Morphen (Yom-Tov 1971, Dittbrenner *et al.* 2009), andererseits konnte nachgewiesen werden, dass Pigmentierungsunterschiede keinen Einfluss auf die Wärmekapazität besitzen (Scheil *et al.* 2012a). Da eine zunehmende Pigmentierung stark negativ mit der Größe korrelierte, könnte das Alter der Tiere hier ein entscheidender Faktor sein. Von Hoshino *et al.* (2010) konnte in Melanomzellen von Mäusen beobachtet werden, dass ein hoher Hsp70-Level zu einer Unterdrückung der Melaninsynthese führte und bringen dieses

Phänomen mit einer Hemmung der Genexpression und Aktivität von Tyrosinase (ein Enzym zur Melaninsynthese) sowie einer Beeinflußung des intrazellulären Melanosomentransportes in Verbindung. Daher könnte es aufgrund anhaltend hoher Hsp70-Spiegel in heranwachsenden Schnecken zu einer Hemmung der Melaninsynthese gekommen sein (Hoshino *et al.* 2010).
Auf der Basis dieser Ergebnisse lässt sich festhalten, dass kleine (juvenile) Schnecken sich eher in Bodennähe aufhalten, dunkler gefärbt sind und einen höheren Hsp70-Gehalt aufweisen. Große (adulte) Tiere hingegen klettern höher, sind eher weiß gefärbt und induzieren weniger Stressproteine, wobei es dabei von Vorteil ist, nicht den Apex zur Sonne auszurichten. Da sowohl das Klettern als auch die Produktion von Stressprotein energieaufwendige Prozesse sind, stehen diese vermutlich in einem energetischen *Trade-off* Verhältnis zueinander.

4.2 Kapitel 2: A Dieterich, U Fischbach, M Ludwig, MA Di Lellis, S Troschinski, U Gärtner, R Triebskorn, H-R Köhler (2012) Daily and seasonal changes in heat exposure and the Hsp70 level of individuals from a field population of *Xeropicta derbentina* (Krynicki 1836) (Pulmonata, Hygromiidae) in Southern France. Cell Stress and Chaperones, 18(4): 405-414

Durch diese Untersuchungen konnte gezeigt werden, dass bei *X. derbentina* die Hsp70-Induktion einen jahreszeitlichen Verlauf zeigt. Im April, Juni und August zeigte sich jeweils eine positive Korrelation des Hsp70-Levels mit der Schalenaußentemperatur, wobei im Oktober die Stressproteininduktion negativ mit diesem Parameter korrelierte, d.h. trotz erhöhter Temperaturen sank der Gehalt an Hsp70. Gründe dafür können zum einen darin liegen, dass die Tiere aufgrund ihres Alters und einem damit einhergehenden energetischen *Trade-off* zwischen Stressproteinproduktion und reproduktiven Vorgängen, die im Herbst stattfinden, wenig Hsp70 induzieren (Sørensen & Loeschcke 2002, Mayer & Bukau 2005, Köhler *et al.* 2009, Mizrahi *et al.* 2011). Zum anderen können pathologische Effekte durch exzessive Hitzebelastung über den Sommer hinweg oder auch eine Verringerung von Stoffwechselvorgängen im Zuge einer zuweilen temporären Ästivation eine Rolle spielen (Dittbrenner *et al.* 2009, Scheil *et al.* 2011, Storey 2002, Reuner *et al.* 2008).
Neben einem jahreszeitlichen Hsp70-Induktionsmuster konnte auch eine Tagesrhythmik, welche in engem Zusammenhang mit dem Temperaturprofil steht,

ermittelt werden. Im April stieg der Hsp70-Wert nur auf ein moderates Level bei Maximaltemperaturen von bis zu 27,3°C, wohingegen im Juni und August eine deutliche Zunahme des Stressproteins in Reaktion auf hohe Umgebungstemperaturen (über 30°C) erfolgte. Im Oktober wiederum blieb eine deutliche Induktion von Hsp70 gänzlich aus (Temperaturen unter 25°C): hier konnte lediglich der konstitutive Basislevel des Hsp70 detektiert werden. Zusätzlich zu Alter und reproduktiven Prozessen führen die vergleichsweise niedrigen Temperaturen im April und Oktober, welche unter der stressproteininduzierenden Temperatur von 30°C liegen (Köhler *et al.* 2009), zu diesem Induktionsmuster.
Bezüglich der Schalengröße und –färbung bei *X. derbentina* konnte beobachtet werden, dass die Tiere im Verlauf des Jahres stetig an Größe zunahmen, um schließlich im August ihr Adultstadium zu erreichen, wobei sie mehrheitlich eine weiße Schalenfärbung aufwiesen. Die zu Beginn des Jahres häufiger vorkommende dunkle Bänderung wird dabei als Juvenilfärbung interpretiert, welche vermutlich im Zuge des Wachstums oftmals durch neugebildetes, rein weißes Schalenmaterial überlagert wird und somit von außen nicht mehr sichtbar ist. Aufgrund der erzielten Resultate kann ein einjähriger Lebenszyklus von *X. derbentina* in Südfrankreich angenommen werden, wie er bereits in anderen Studien postuliert wurde (Staikou & Lazaridou-Dimitriadou 1991, Kiss *et al.* 2005).

4.3 Kapitel 3: S Troschinski, MA Di Lellis, S Sereda, T Hauffe, T Wilke, R Triebskorn, H-R Köhler (2014): Intraspecific variation in cellular and biochemical heat response strategies of Mediterranean *Xeropicta derbentina* [Pulmonata, Hygromiidae]. PloS one 9 (1):e86613

In dieser Studie konnten nach Exposition gegenüber hohen Temperaturen diverse pathologische Effekte im Hepatopankreas der Gastropoden beobachtet werden: lytische Zellapizes, vornehmlich an den Resorptionszellen, sprechen für eine Freisetzung lysosomaler Enzyme (Moeller *et al.* 1976, Poste 1971), wohingegen geweitete Lumina der Tubuli auf einen erhöhten Metabolismus und einen damit verbunden gesteigerten Nährstoffbedarf hindeuten (Gillooly *et al.*, 2001). Vermutlich führte osmotischer Stress und Azidose, welche bekanntlich durch Hitzebelastung ausgelöst werden können (Heisler 1986, Ryan & Gisolfi 1995, Avwioro 2011, Barnhart 1986) in den Kalkzellen zu einer gestörten Zellkompartimentierung, einer reduzierten Dichte des Zytoplasmas und zu dunkel gefärbten Zellkernen. Generell

konnte beobachtet werden, dass die Resorptionszellen sensitiver auf Hitzestress reagieren als Kalkzellen.

Zusätzlich zu den histologischen Analysen wurde der Stressproteingehalt ermittelt, welcher in jeder der untersuchten Populationen dem typischen Induktionsmuster einer Optimumskurve folgte (Eckwert *et al.* 1997). Diese wies einen Maximalwert bei 40°C auf, wobei sich die unterschiedlichen Populationen in der Höhe dieses Maximalwertes teils beträchtlich unterschieden. Aufgrund dieser populationspezifischen Unterschiede war es durch eine Analyse der histologischen und biochemischen Reaktionsmuster möglich, verschiedene Strategien zur Hitzestressbewältigung zu identifizieren:

- Strategie 1: Investition in eine moderate Hsp70-Induktion mit mäßigen zellulären Reaktionen (in 3 Populationen beobachtet)
- Strategie 2: Investition in hohe Hsp70-Level mit zellulären Reaktionen auf einem moderaten Niveau (in 3 Populationen beobachtet)
- Strategie 3: Keine signifikante Erhöhung des Hsp70-Spiegels mit dem Erhalt zellulärer Funktionen bis zu einer gewissen Hitzestresseinwirkung, darüber hinaus mit dem Risiko eines rapiden zellulären Zerfalls (in 1 Population beobachtet)

Bei dieser Analyse fiel auf, dass die unterschiedlichen Sensitivitäten von Resorptions- und Kalkzellen bezüglich Hitzestress in engem Zusammenhang mit der Hsp70-Induktion stand: Hohe Hsp70-Level waren meist mit guten Zuständen der Kalkzellen assoziiert. Aufgrund der wichtigen metabolischen Funktionen der Kalkzellen unter Stress, könnte eine schützende Funktion durch Stressproteine von Vorteil sein. Spekulativ bleibt hierbei, ob die Synthese von Hsp70 vornehmlich in diesem Zelltyp des Hepatopankreas stattfindet.

Da die Proteinbiosynthese vor allem unter erhöhten Temperaturen ein energieaufwendiger Prozess ist (Tomanek & Somero 1999), kann eine übermäßige Induktion von Hsp70 zu einer reduzierten Fitness der Tiere führen (Feder *et al.* 1992, Krebs & Loeschke 1994, Krebs & Feder 1998). Folglich kann es durch eher niedrige Stressproteinlevel zu Energieeinsparungen kommen und – parallel hierzu - eine Toleranz gegenüber Stressoren evolutiv auf anderem Wege erreicht werden (Köhler *et al.* 2000, Feder & Krebs 1998, Arts *et al.* 2004, Mizrahi *et al.* 2010). Außerdem können demographische Effekte zu populationsspezifischen Unterschieden in der Hsp70-Expression führen (Sørensen *et al.* 2001, Jensen *et al.* 2009, Bahrndorff *et al.* 2010). In den genetischen Untersuchungen der sieben Populationen stellte sich in diesem Zusammenhang heraus, dass genetisch

ähnliche Populationen eher hohe Hsp70-Level mit guten zellulären Zuständen unter Temperaturstress (40°C) aufwiesen, wohingegen die Population mit einer geringen maximalen Hsp70-Induktion sich aus genetischer Sicht stark von Nachbarpopulationen unterschied. Dabei könnte aufgrund mikrohabitatabhängiger Unterschiede die Energieeinsparung durch niedrige Hsp70-Induktionsmuster selektiert worden sein.

4.4 Kapitel 4: A Dieterich*, S Troschinski*, S Schwarz, MA Di Lellis, A Henneberg, U Fischbach, M Ludwig, U Gärtner, R Triebskorn, H-R Köhler (2014): Hsp70 and lipid peroxide levels following heat stress in *Xeropicta derbentina* (Krynicki 1836) (Gastropoda, Pulmonata) with regard to different colour morphs. Cell Stress and Chaperones, DOI 10.1007/s12192-014-0534-3. * gleichberechtigte Erstautorenschaft

Untersuchungen der unterschiedlichen Schalenmorphen von *X. derbentina* zeigten, dass Individuen der zuvor definierten Gehäusefärbung „Kategorie 3“ zwar befähigt sind Hsp70 bis zu einer gewissen Temperatur (43°C) stärker zu induzieren als andere Phänotypen und folglich effektiv an diesen Temperaturbereich angepasst sind, jedoch aufgrund des damit verbundenen hohen Energieverbrauchs bei steigenden Temperaturen schneller überfordert zu sein scheinen (reflektiert durch einen Rückgang des Hsp70-Levels bei höheren Temperaturen). In sehr heißen Sommern könnte dies schneller zu zellulärer Schädigung führen (Dittbrenner *et al.* 2009, Scheil *et al.* 2011). Unklar bleibt jedoch, ob sich bei *X. derbentina* die populationsspezifische Morphenzusammensetzung über die Jahre hinweg ändert, wie für andere Vertreter der Helicoidea gezeigt wurde (Cowie 1992, Johnson 2011, Silvertown *et al.* 2011), und ob lokale Temperaturunterschiede einen Einfluss darauf nehmen.

Des Weiteren zeigte sich ein 2-phasiger Verlauf der Lipidperoxidgenerierung mit steigenden Temperaturen: Zum einen konnte ein geringer Level nach Exposition gegenüber niedrigen Temperaturen (25 und 33°C) nachgewiesen werden, zum anderen sank der Gehalt an Lipidperoxiden bei stärkerer Hitzeeinwirkung (43°C) nach einem vorherigen Anstieg (bei 38 und 40°C) auf ein geringeres Niveau ab. Ein effektives antioxidatives Abwehrsystem, welches verschiedene Enzyme und kleine Moleküle beinhaltet (Aebi 1984, Meister 1988, Halliwell & Gutteridge 1989, Gutteridge 1995), könnte aufgrund temperaturspezifischer Aktivitätsmaxima von antioxidativen Enzymen als Erklärung dienen. Hinsichtlich der vier

Schalenmorphen ist bei rein weiß gefärbten Individuen („Kategorie 1“) diese Abwehr in „niedrigen“ Temperaturbereichen (38°C) am effektivsten. Bei hohen Temperaturen (45 und 48°C) zeigte sich, dass dieses antioxidative System bei den am dunkelsten gefärbten Individuen („Kategorie 4“) am schlechtesten ausgeprägt ist. Aufgrund des antagonistischen Verlaufs von Hsp70-Induktion und Aktivität der antioxidativen Abwehr (gemessen am Lipidperoxid-Level, welcher bei maximalem Hsp70-Gehalt am höchsten ist) ist ein energetisches *Trade-off* Verhältnis dieser beider Mechanismen zueinander anzunehmen.

4.5 Kapitel 5: S Troschinski, A Dieterich, S Krais, R Triebskorn, H-R Köhler (zur Publikation angenommen bei: The Journal of Experimental Biology): Antioxidant defense and stress protein induction following heat stress in the Mediterranean snail *Xeropicta derbentina* [Pulmonata, Hygromiidae]

In dieser Studie zeigte sich, dass neben der Induktion von Stressproteinen auch das antioxidative Abwehrsystem zur Thermotoleranz von *X. derbentina* beiträgt. Generell wurde in allen Proben eine relativ hohe Aktivität des Enzyms Katalase nachgewiesen, welche als konstitutiver Level zum permanenten Schutz gegen reaktive Sauerstoffspezies wie H_2O_2 gedeutet werden kann (Nowakowska *et al.* 2011). Von Storey (1996) wurde in diesem Zusammenhang postuliert, dass dauerhaft hohe Grundlevel von Antioxidantien Teil einer Strategie zu sein scheinen, um repetitiven Perioden oxidativen Stresses entgegenzuwirken (Storey 1996).
Des Weiteren konnte beobachtet werden, dass in Folge gesteigerter Aktivitäten von sowohl Katalase als auch Glutathionperoxidase es zu einer Verminderung der Lipidperoxidation kam, welche als Maß für den erfahrenen oxidativen Stress herangezogen wurde. Interessanterweise zeigte sich dabei eine temperaturabhängige Steigerung dieser Enzymaktivitäten, bei welcher immer nur eines der Enzyme stimuliert wurde: Das Aktivitätsmaximum für Glutathionperoxidase lag hier bei 40°C, für Katalase wiederum bei 43°C. Da sowohl Katalase als auch Glutathionperoxidase dasselbe ROS, H_2O_2, umsetzen können, kann hier eine Konkurrenzsituation beider Enzyme angenommen werden, welche auch schon zuvor von Nowakowska *et al.* (2011) bei Gastropoden der Gattung *Helix* beobachtet wurde.
Für Hsp70 konnte ein relativ hoher konstitutiver Level nachgewiesen werden, welcher zunächst bis zu einem Maximalwert zunahm und schließlich, einhergehend

mit einem Anstieg in der Katalase-Aktivität, zurück ging. Zum einen kann es hier zu einem energetischen *trade-off* gekommen sein, wobei an dieser Stelle die gesteigerte Bereitstellung von Katalase favorisiert wurde, andererseits wurde bereits von Giraud-Billoud *et al.* (2013) postuliert, dass die Aktivierung verschiedener Stressantworten, einschließlich Hsp70, durch reaktive Metabolite des oxidativen Stresses gefördert werden, was wiederum die Hypothese von Gorman *et al.* (1999), dass die Induktion von Hsp70 durch ROS stimuliert wird, bestätigt. Folglich würde ein Rückgang von ROS zu einer Senkung der Hsp70-Induktion beitragen.
Zusammenfassend lässt sich festhalten, dass Katalase als auch Glutathionperoxidase sich gegenseitig durch die temperaturspezifischen Aktivitätsoptima ergänzen und gemeinsam mit der Hsp70-Antwort ein effektives System in der Hitzestressbewältigung bilden.

5. Abschließende Betrachtung

In der vorliegenden Arbeit konnten eine Reihe neuer Erkenntnisse über adaptive physiologische Mechanismen in der Hitzestressbewältigung der xerothermophilen Schneckenart *Xeropicta derbentina* gewonnen und dabei für die jeweiligen Lebensphasen der Tiere Anpassungsstrategien abgeleitet werden.
Aufgrund der vorliegenden Ergebnisse kann ein weitgehend annueller Lebenszyklus von *X. derbentina* in Südfrankreich angenommen werden, d.h. die Tiere schlüpfen zu Beginn und sterben zum Ende des Jahres nach der Eiablage. Nur wenige Individuen scheinen im Adultstadium zu überwintern. Im Frühjahr (April) weisen die Tiere daher noch eine geringe Schalengröße auf. Zusätzlich konnte in diesem Lebensstadium häufig eine dunkle Bänderung der Tiere detektiert werden, welche als Juvenilfärbung interpretiert werden kann und im Laufe des Schalenwachstums über das Jahr hinweg durch verschiedene Schalenfärbungen (Morphen) abgelöst wird. Trotz noch eher geringen Temperaturen mit Maximalwerten bis 27,3°C (5 cm über Grund), welche unter der bei Adulti Stressprotein-induzierenden Temperatur von 30°C liegen (Köhler *et al.* 2009), konnten vor allem bei kleinen, juvenilen Individuen hohe Hsp70-Level nachgewiesen werden, welche jedoch einer erhöhten Beteiligung dieser Proteine als Chaperone während Entwicklungsprozessen zugeschrieben werden können (Mayer & Bukau 2005). Durch Korrelation des Hsp70-Gehaltes mit dem Temperatur-Tagesprofil zeigte sich, dass die Induktion von Hsp70 zwar mit steigenden Umgebungstemperaturen zunimmt, jedoch, vermutlich

aufgrund des bereits hohen konstitutiven Basis-Levels von Hsp70, die Induktion weniger stark ausfällt als bei adulten Tieren.

Im späten Frühling (Mai/Juni) führen die bereits recht hohen Temperaturen (ermittelte Maximaltemperatur von 32, 9°C, 5 cm über Grund) zu einer deutlich gesteigerten Induktion von Hsp70, welche positiv mit der Außentemperatur korrelierte. Dabei konnte folgendes Schema identifiziert werden: Große Schnecken mit vornehmlich heller Gehäusefärbung halten sich tagsüber in größerer Höhe über Grund auf und weisen dabei einen niedrigen Hsp70-Level auf, wohingegen kleine Vertreter mit dunkleren Gehäusen eher in Grundnähe verweilen, dabei jedoch mehr Hsp70 induzieren. Durch bereits geringe Zunahmen in der Aufenthaltshöhe über Grund reduziert sich der Stress, da dort die Temperaturverhältnisse weitaus 'angenehmer' werden (Cowie 1985, Kempster & Charwa 2003). Zusätzlich stellte sich die Gehäuseorientierung zur Sonne in Interaktion mit der Kletterhöhe als bedeutend heraus, was auf eine gewebespezifische Induktion von Hsp70 schließen lässt (Lyons *et al.* 2003, Mizrahi *et al.* 2010, Arad *et al.* 2010).

Außerdem konnte nach experimentell induziertem Temperaturstress beobachtet werden, dass dunklere Morphen befähigt waren, Hsp70 bis zu einem gewissen Grad höher zu induzieren als hellere Morphen. Neben der Induktion von Stressproteinen zum Schutz vor zellulärer Schädigung führen die bereits hohen Temperaturen in dieser Lebensphase vermehrt zur Generierung von Lipidperoxiden – hervorgerufen durch oxidativen Stress – wodurch wiederum die Notwendigkeit zur Induktion antioxidativer Mechanismen besteht. Bezüglich der verschiedenen Farbmorphen offenbarte sich in diesem Zusammenhang, dass helle Morphen vor allem in „niedrigen" Temperaturbereichen einen geringeren Gehalt an Lipidperoxiden aufwiesen als dunkler gefärbte Individuen und daher scheinbar ein wirkungsvolleres antioxidatives Abwehrsystem besitzen. Weiterführende Untersuchungen konnten außerdem offenlegen, dass bei *X. derbentina* bereits ein hoher konstitutiver Level an Katalase vorliegt, welcher als permanenter Schutz gegen reaktive Sauerstoffspezies bzw. als Strategie um wiederholenden Perioden oxidativen Stresses entgegenzuwirken, interpretiert werden kann (Storey 1996).

Die heißen, trockenen Sommermonate stellen aufgrund der hohen Temperaturen, welche in Bodennähe letale Temperaturen von bis zu 50°C erreichen können, die größte Herausforderung im Lebenszyklus von *X. derbentina* dar, welche in dieser Zeit ihr Adultstadium erreichen und mehrheitlich eine weiße Schalfärbung aufweisen. Im Feld konnten hier Maximaltemperaturen von 33,7°C (5 cm über Grund) einhergehend mit einer deutlichen Hsp70-Induktion im Tagesverlauf

ermittelt werden. Untersuchungen des Hepatopankreas wiesen diesen als ein stark in die Hitzestresssituation involviertes Organ aus, wobei sich vor allem die Kalkzellen als resistenter gegen Hitzestress herausstellten als die Resorptionszellen. In Verbindung mit der Induktionskapazität von Hsp70 zeigte sich, dass eine gute Integrität der Kalkzellen mit höheren Hsp70-Gehalten assoziiert war, was die wichtige Funktion dieses Zelltyps in der Stressbewältigung und somit die Notwendigkeit dessen Erhalts unterstreicht (Burton 1976, Taieb & Vicente 1998), zugleich kann dies als eine protektive Schutzfunktion von Hsp70 auf diesen Zelltyp interpretiert werden. Aufgrund von intraspezifischen Variationen im Hsp70-Induktionsmuster konnten bei Vergleichen mit der jeweiligen populationsspezifischen Integrität des Hepatopankreas drei verschiedene (populationsspezifische) Strategien zur Hitzestressbewältigung bei *X. derbentina* identifiziert werden.

Im Zuge weiterer Hitzeexpositionen wurde deutlich, dass Hsp70 bis zu einem Maximallevel bei 40°C induziert wird, dieser Level bei höheren Temperaturen aber rasch abnimmt. Gleichzeitig konnte jedoch mit einem Abfall des Hsp70-Gehaltes bei hohen Temperaturen eine Steigerung des antioxidativen Enzyms Katalase verzeichnet werden. Die Aktivität von Katalase sowie die schützende Funktion der Stressproteine werden außerdem noch durch die enzymatische Aktivität von Glutathionperoxidase ergänzt, welche ihr Aktivitätsmaximum ebenfalls bei 40°C besitzt. Zusammenfassend lässt sich hier festhalten, dass all diese Mechanismen ein effektives Schutzsystem unter physiologisch ungünstigen Temperaturbedingungen bei *X. derbentina* bilden.

Gegen Ende des Jahres fällt die Kapazität der Schnecken zur Induktion von Stressproteinen deutlich ab bzw. bleibt gänzlich aus (negative Korrelation von Hsp70 mit den Außentemperaturen), was mit dem Alter der Tiere in Verbindung gebracht werden kann. Möglicherweise stehen die Stressproteinproduktion sowie reproduktive Vorgänge, die im Herbst stattfinden, in einem energetischen *trade-off* Verhältnis zueinander, und eventuell spielen zusätzlich eine Überbelastung des Stressabwehrsystems und zunehmende pathologische Effekte durch intensive Stressbelastung über den Sommer hinweg eine Rolle (Sørensen & Loeschcke 2002, Mayer & Bukau 2005, Dittbrenner *et al.* 2009, Mizrahi *et al.* 2011, Scheil *et al.* 2011). Vermutlich bedingt durch Seneszenz sterben die meisten Individuen nach der Eiablage zu Ende des Jahres.

Durch die im Rahmen der vorliegenden Arbeit gewonnenen Erkenntnisse konnten die anfangs formulierten Arbeitshypothesen größtenteils verifiziert werden. Lediglich Arbeitshypothese vier konnte nur teilweise bestätigt werden.

6. Literatur

Abdel-Rehim AH (1988) Influence of shell colour on the mortality rate of the land snail *Arianta arbustorum* under different microclimatic regimes. *Biological Journal of the Linnean Society* 35: 29-35.

Aebi H (1984) Catalase in vitro. *Methods in Enzymology* 105:121-126.

Arad Z, Goldenberg S & Heller J (1993) Intraspecific variation in resistance to desiccation and climatic gradients in the distribution of the bush-dwelling land snail *Trochoidea simulata*. *Journal of Zoology* 229: 249-265.

Arad Z, Mizrahi T, Goldenberg S & Heller J (2010) Natural annual cycle of heat shock protein expression in land snails: desert versus Mediterranean species of *Sphincterochila*. *Journal of Experimental Biology* 213: 3487-3495.

Arts MJSJ, Schill RO, Knigge T, Eckwert H, Kammenga JE & Köhler H-R (2004) Stress proteins (hsp70, hsp60) induced in isopods and nematodes by field exposure to metals in a gradient near Avonmouth, UK. *Ecotoxicology* 13: 739-755.

Aubry S, Labaune C, Magnin F & Kiss L (2005) Habitat and integration within indigenous communities of *Xeropicta derbentina* (Gastropoda: Hygromiidae), a recently introduced land snail in South-Eastern France. *Diversity and Distributions* 11: 539–547.

Aubry S, Labaune C, Magnin F, Roche P & Kiss L (2006) Active and passive dispersal of an invading land snail in Mediterranean France. *Journal of Animal Ecology* 75(3): 802–813.

Avwioro G (2011) Histochemical Uses Of Haematoxylin - A Review. *Journal of Pharmacy and clinical Sciences* 1: 24-34.

Bahrndorff S, Marien J, Loeschcke V & Ellers J (2010) Genetic variation in heat resistance and HSP70 expression in inbred isofemale lines of the springtail *Orchesella cinta*. *Climate Research* 43: 41-47.

Barnhart MC (1986) Control of acid-base status in active and dormant land snails, *Otala lacteal* (Pulmonata, Helicidae). *Journal of Comparative Physiolgy B* 156: 347-354.

Bishop T & Brand MD (2000) Processes contributing to metabolic depression in hepatopancreas cells from the snail *Helix aspersa*. *Journal of Experimental Biology* 203: 3603–3612.

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities utilizing the principle of protein dye binding. *Analytical Biochemistry* 72: 248-254.

Burton RF (1976) Calcium metabolism and acid-base balance in *Helix pomatia*. *Perspectives in experimental biology* 1: 7-16.

Clement M, Posada D & Crandall KA (2000) TCS: a computer program to estimate gene genealogies. *Molecular Ecology* 9: 1657–1659.

Cook L M & Freeman P M (1986) Heating properties of morphs of the mangrove snail *Littoraria pallescens*. *Biological Journal of the Linnean Society* 29: 295–300.

Cowie RH & Cain AJ (1983) Laboratory maintenance and breeding of land snails, with an example of *Helix aspersa. Journal of Molluscan Studies* 49: 176-17.

Cowie RH (1985) Microhabitat choice and high temperature tolerance in the land snail *Theba pisana* (Mollusca: Gastropoda). *Journal of Zoology* 207: 201-211.

Cowie RH (1992) Shell pattern polymorphism in a 13-year study of the land snail *Theba Pisana* (Müller) (Pulmonata : Helicidae), vol 34. vol 1-2. Institute of Malacology, Ann Arbor, MI, ETATS-UNIS.

Dittbrenner N, Lazzara R, Köhler H-R, Mazzia C, Capowiez Y & Triebskorn (2009) Heat tolerance in Mediterranean land snails: histopathology after exposure to different temperature regimes. *Journal of Molluscan Studies* 75: 9-18.

Eckwert H, Alberti G & Köhler H-R (1997) The induction of stress proteins (hsp) in *Oniscus asellus* (isopoda) as a molecular marker of multiple heavy metal exposure: I. Principles and toxicological assessment. *Ecotoxocology* 6: 249-262.

Eichler HJ, Kronfeldt HD & Sahm J (2005) Das Neue Physikalische Grundpraktikum. 2. Auflage, Springer, Berlin, Deutschland; Heidelberg, Deutschland; New York, USA.

Excoffier L, Laval G & Schneider S (2005) Arlequin (version 3.0): an integrated software package for population genetics data analysis. *Evolutionary Bioinformatics Online* 1: 47–50.

Feder JH, Rossi JM, Solomon J, Solomon N & Lindquist S (1992) The consequences of expressing Hsp70 in *Drosophila* cells at normal temperatures. *Genes & Development* 6: 1402–1413.

Feder ME & Hofmann GE (1999) Heat-shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology. *Annual Review of Physiology* 61: 243–282.

Fink AL (1999) Chaperone-mediated protein folding. *Physiological Reviews* 79: 425–449.

Folmer O, Black M, Hoeh W, Lutz R & Vrijenhoek R (1994) DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology* 3: 294–299.

Gillooly JF, Brown JH, West GB, Savage VM & Charnoy EL (2001) Effects of size and temperature on metabolic rate. *Science* 293: 2248-2252.

Giraud-Billoud M, Vega IA, Tosi ME, Abud MA, Calderon ML & Castro-Vazquez A (2013) Antioxidant and molecular chaperone defences during estivation and arousal in the South American apple snail *Pomacea canaliculata*. *The Journal of Experimental Biology* 216 (Pt 4):614-622.

Gorman AM, Heavey B, Creagh E, Cotter TG & Samali A (1999) Antioxidant-mediated inhibition of the heat shock response leads to apoptosis. *FEBS letters* 445 (1):98-102

Goodfriend GA (1986) Variation in land-snail shell form and size and its causes: a review. *Systematic Zoology* 35: 204-223.

Guppy M & Withers P (1999) Metabolic depression in animals: physiological perspectives and biochemical generalizations. *Biological Reviews* 74: 1–40.

Gutteridge J (1995) Lipid peroxidation and antioxidants as biomarkers of tissue damage. *Clinical chemistry* 41 (12):1819-1828.

Halliwell B & Gutteridge J (1989) Free radicals in biology and medicine. 2nd edn. Clarendon Press, Oxford, UK.

Halliwell B (2006) Reactive species and antioxidants. Redox biology is a fundamental theme of aerobic life. *Plant physiology* 141 (2):312-322.

Hazel WN & Johnson MS (1990) Microhabitat choice and polymorphism in the land snail *Theba pisana* (Müller). *Heredity* 65: 449–454.

Heath DJ (1975) Color, sunlight and internal temperatures in the land-snail *Cepaea nemoralis* (L.). *Oecologia* 19: 29-38.

Heckathorn SA, Poeller GJ, Coleman JS & Hallberg R (1996) Nitrogen availability alters the patterns of accumulation of heat stress-induced proteins in plants. *Oecologia* 105: 413–418.

Heise K, Puntarulo S, Nikinmaa M, Abele D & Pörtner H-O (2006) Oxidative stress during stressful heat exposure and recovery in the North Sea eelpout *Zoarces viviparus* L. *Journal of experimental biology* 209 (2):353-363.

Heisler N (1986) Acid-base regulation in animals. Elsevier Science, Amsterdam.

Heller J & Magaritz M (1983) From where do land snails obtain the chemicals to build their shells? *Journal of Molluscan Studies* 49: 116- 121.

Hendrick JP & Hartl FU (1993) Molecular chaperone functions of heat-shock proteins. *Annual Review of Biochemistry* 62: 349-384.

Hermes-Lima M & Storey KB (1993) Antioxidant defenses in the tolerance of freezing and anoxia by garter snakes. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology* 265 (3):R646-R652.

Hermes-Lima M & Storey KB (1995) Antioxidant defenses and metabolic depression in a pulmonate land snail. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology* 268 (6):R1386-R1393.

Hermes-Lima M, Willmore WG & Storey KB (1995) Quantification of lipid peroxidation in tissue extracts based on Fe(III)xylenol orange complex formation. *Free Radical Biology and Medicine* 19 (3):271-280.

Hermes-Lima M & Storey KB (1996) Relationship between anoxia exposure and antioxidant status in the frog *Rana pipiens*. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology* 271 (4):R918-R925.

Hermes-Lima M, Storey JM & Storey KB (1998) Antioxidant defenses and metabolic depression. The hypothesis of preparation for oxidative stress in land snails. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology* 120 (3):437-448.

Heuberger M & Fels H (2007) Wärmelehre, in: Schatz, J., Tammer, R. (Eds.), Erste Hilfe- Chemie und Physik: für Mediziner. Springer, Berlin, Deutschland, pp. 153- 191.

Horn HS (1966) Measurement of "overlap" in comparative ecological studies. *The American Naturalist* 100: 419–424.

Hoshino T, Matsuda M, Yamashita Y, Takehara M, Fukuya M, Mineda K, Maji D, Ihn H, Adachi H, Sobue G, Funasaka Y & Mizushima T (2010) Suppression of Melanin Production by Expression of Hsp70. *The Journal of Biological Chemistry* 285: 13254-13263.

Jensen LT, Cockerell FE, Kristensen TN, Rako L, Loeschke V, McKechnie SW & Hoffmann AA (2009) Adult heat tolerance variation in *Drosophila melanogaster* is not related to Hsp70 expression. *Journal of Experimental Zoology* 313: 35-44.

Joanisse DR & Storey KB (1996) Oxidative damage and antioxidants in *Rana syvatica*, the freeze-tolerant wood frog. *American Journal of Physiology* 271:R545-R553.

Johnson MS (2011) Thirty-four years of climatic selection in the land snail *Theba pisana*. *Heredity* 106 (5):741-748.

Jones JS (1973) Ecological Genetics and Natural Selection in Molluscs. *Science* 182: 546-552.

Jones JS (1982) Genetic differences in individual behaviour associated with shell polymorphism in the snail *Cepaea nemoralis*. *Nature* 298: 749-750.

Kempster V & Charwa S (2003) Soil surface temperature and mortality in land snails: Implications for successful management. *Australian Journal of Agriculture* 43: 1351-1356 .

Kiss L, Labaune C, Magnin F & Aubry S (2005) Plasticity of the life cycle of *Xeropicta derbentina* (Krynicki, 1836), a recently introduced snail in mediterranean France. *Journal of Molluscan Studies* 71(3): 221–231.

Köhler H-R, Triebskorn R, Stocker W, Kloetzel PM & Alberti G (1992) The 70kD heat shock protein (Hsp 70) in soil invertebrates: a possible tool for monitoring environmental toxicants. *Archives of Environmental Contamination and Toxicology* 22:334–38.

Köhler, H.-R., Zanger, M., Eckwert, H. & Einfeldt, I. (2000) Selection favours low Hsp70 levels in chronically metal-stressed soil arthropods. *Journal of Evolutionary Biology* 13: 569-582.

Köhler H-R, Bartussek C, Eckwert H, Farian K, Gränzer S, Knigge T & Kunz N (2001) The hepatic stress protein (hsp70) response to interacting abiotic parameters in fish exposed to various levels of pollution. *Journal of Aquatic Ecosystem Stress and Recovery* 8: 261–279.

Köhler H-R, Lazzara R, Dittbrenner N, Capowiez Y, Mazzia C & Triebskorn R (2009) Snail Phenotypic Variation and Stress Proteins: do Different Heat Response Strategies Contribute to Waddington's Widget in Field Populations? *Journal of Experimental Zoology Part B – Molecular and Developmental Evolution* 312B: 136–147.

Krebs RA & Feder ME (1998) Hsp70 and larval thermotolerance in *Drosophila melanogaster*: How much is enough and when is more too much? *Journal of Insect Physiology* 44: 1091-1101.

Krebs RA & Loeschcke V (1994) Costs and benefits of activation of the heat-shock response in *Drosophila melanogaster*. *Functional Ecoloy* 8: 730–737.

Lindquist S & Craig EA (1988) The heat-shock proteins. *Annual Reviews Genetics* 22: 631-77.

Lushchak VI, Lushchak LP, Mota AA & Hermes-Lima M (2001) Oxidative stress and antioxidant defenses in goldfish *Carassius auratus* during anoxia and reoxygenation. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology* 280 (1):R100-R107.

Lushchak VI & Bagnyukova TV (2006) Temperature increase results in oxidative stress in goldfish tissues. 2. Antioxidant and associated enzymes. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology* 143 (1):36-41.

Lyons C, Dowling V, Tedengren M, Gardestrom J, Hartl MJ, O'Brien N, Van Pelt F, O'Halloran J & Sheehan D (2003) Variability of heat shock proteins and glutathione S-transferase in gill and digestive gland of blue mussel, *Mytilus edulis*. *Marine Environmental Research* 56: 585-597.

Machin J (1964) The evaporation of water from *Helix aspersa* I. The nature of the evaporating surface. *Journal of Experimental Biology* 41: 759-769.

Mayer MP & Bukau B (2005) Hsp70 chaperones: cellular functions and molecular mechanism. *Cellular and Molecular Life Sciences* 62: 670-684.

Mazek-Fialla K (1934) Die Lebensweise xerophiler Schnecken Syriens, Griechlands, Dalmatiens und der Türkei und die Beschaffenheit ihrer subepithelialen Drüsen. *Zeitschrift zur Morphologie und Ökologie der Tiere* 28: 445-468.

Meister A (1988) Glutathione metabolism and its selective modification. *The Journal of Biological Chemistry* 263 (33):17205-17208.

Mizrahi T, Heller J, Goldenberg S & Arad Z (2010) Heat shock proteins and resistance to desiccation in congeneric land snails. *Cell Stress and Chaperones* 15: 351-363.

Mizrahi T, Heller J, Goldenberg S & Arad Z (2011) Heat shock protein expression in relation to reproductive cycle in land snails: Implications for survival. *Comparative Biochemistry and Physiology Part A – Molecular & Integrative Physiology* 160(2): 149–155.

Mizrahi T, Heller J, Goldenberg S & Arad Z (2012) The heat shock response in congeneric land snails (*Sphincterochila*) from different habitats. *Cell Stress and Chaperones* 17: 639-645.

Moeller PW, Fields PA, Dutson TR, Landmann WA & Carpenter ZL (1976) Effects of high temperature conditioning on subcellular distribution and levels of lysosomal enzymes. *Journal of Food Science* 41: 216-217.

Monserrat JM, Geracitano LA, Pinho GLL, Vinagre TM, Faleiros M, Alciati JC & Bianchini A (2003) Determination of Lipid Peroxides in Invertebrates Tissues Using the Fe(III) Xylenol Orange Complex Formation. *Archives of Environmental Contamination and Toxicology* 45 (2):177-183.

Nakano K & Iwama GK (2002) The 70-kDa heat shock protein response in two intertidal sculpins, Oligocottus maculosus and O. snyderi: relationship of hsp70 and thermal tolerance. *Comparative Biochemistry and Physiology - Part A: Molecular & Integrative Physiology* 133 (1):79–94.

Nei M (1973) Analysis of Gene Diversity in Subdivided Populations. *Proceedings of the National Academy of Sciences U. S. A.* 70: 3321–3323.

Nowakowska A, Caputa M & Rogalska J (2010) Natural aestivation and antioxidant defence in *Helix pomatia*: effect of acclimation to various external conditions. *Journal of Molluscan Studies* 76 (4):354-359.

Nowakowska A, Caputa M & Rogalska J (2011) Defence against oxidative stress in two species of land snails (*Helix pomatia* and *Helix aspersa*) subjected to estivation. *Journal of Experimental Zoology Part A - Ecological genetics and physiology* 315 (10):593-601.

Nowakowska A, Świderska-Kołacz G, Rogalska J & Caputa M (2009) Antioxidants and oxidative stress in *Helix pomatia* snails during estivation. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology* 150 (4):481-486.

Pannunzio TM & Storey KB (1998) Antioxidant defenses and lipid peroxidation during anoxia stress and aerobic recovery in the marine gastropod *Littorina littorea*. *Journal of Experimental Marine Biology and Ecology* 221 (2):277-292.

Parsell DA & Lindquist S (1993) The function of heat-shock proteins in stress tolerance: Degradation and reactivation of damaged proteins. *Annual Reviews Genetics* 27: 437–496.

Pörtner HO (2001) Climate change and temperature-dependent biogeography: oxygen limitation of thermal tolerance in animals. *Naturwissenschaften* 88: 137–146.

Pomeroy DE (1966) The ecology of *Helicella virgata* and related species of snails in South Australia. PhD thesis, University of Adelaide

Pomeroy DE (1968) Dormacy in the land snail *Helicella virgata* (Pulmonata: Helicidae). *Australian Journal of Zoology* 16: 857-869

Poste G (1971) Sub-lethal autolysis: Modification of cell periphery by lysosomal enzymes. *Experimental Cell Research* 67: 11-16.

Radwan MA, El-Gendy KS & Gad AF (2010) Biomarkers of oxidative stress in the land snail, *Theba pisana* for assessing ecotoxicological effects of urban metal pollution. *Chemosphere* 79 (1):40-46.

Ramos-Vasconcelos GR & Hermes-Lima M (2003) Hypometabolism, antioxidant defenses and free radical metabolism in the pulmonate land snail *Helix aspersa*. *Journal of Experimental Biology* 206 (4):675-685.

R Development Core Team (2011) R: A Language and Environment for Statistical Computing. Vienna: R foundation for Statistical Computing. 409 p.

Reuner A, Bruemmer F & Schill RO (2008) Heat shock proteins (Hsp70) and water content in the estivating Mediterranean Grunt Snail (*Cantareus apertus*). *Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology* 151(1): 28–31.

Riddle WA (1983) Physiological ecology of land snails and slugs. *The Mollusca: Ecology* 6: 431-461.

Ryan AJ & Gisolfi CV (1995) Responses of rat mesenteric arteries to norepinephrine during exposure to heat stress and acidosis. *Journal of Applied Physiology* 78: 38–45.

Sanchez Y, Taulien J, Borkovich KA & Lindquist S (1992) Hsp104 is required for tolerance to many forms of stress. *EMBO Journal* 11: 2357–2364.

Savazzi E & Sasaki T (2013) Observation on land snail shells in near-ultraviolet, visible and near-infrared radiation. *Journal of Molluscan Studies*. doi:10.1093/mollus/eys039

Scheil AE, Köhler H-R & Triebskorn R (2011) Heat tolerance and recovery in Mediterranean land snails after pre-exposure in the field. *Journal of Molluscan Studies* 77: 165-174.

Scheil AE, Gärtner U & Köhler H-R (2012a) Colour polymorphism and thermal capacities in *Theba pisana* (O.F. Müller 1774). *Journal of Thermal Biology* 37 (6):462-467.

Scheil AE, Scheil V, Triebskorn R, Capowiez Y, Mazzia C & Köhler H-R (2012b) Shell colouration and antioxidant defence capacity in *Theba pisana* (O.F. Müller, 1774). *Molluscan Research* 32 (3): 132-136.

Schill RO, Gayle PMH & Köhler HR (2002) Daily stress protein (hsp70) cycle in chitons (*Acanthopleura granulata* Gmelin, 1791) which inhabit the rocky intertidal shoreline in a tropical ecosystem. *Comparative Biochemistry and Physiology Part C-Toxicology & Pharmacology* 131(3):253–258.

Schmidt-Nielsen K, Taylor CR & Shkolinik A (1971) Desert snails: Problems of heat, water and food. *Journal of Experimental Biology* 55: 385-398 .

Sies H (1994) Strategies of antioxidant defense. In: EJB Reviews 1993, vol 1993. EJB Reviews. Springer Berlin Heidelberg, pp 101-107.

Sies H (1997) Oxidative stress: oxidants and antioxidants. *Experimental Physiology* 82 (2):291-295.

Silvertown J, Cook L, Cameron R, Dodd M, McConway K, Worthington J, Skelton P, Anton C, Bossdorf O, Baur B, Schilthuizen M, Fontaine B, Sattmann H, Bertorelle G, Correia M, Oliveira C, Pokryszko B, Ożgo M, Stalažs A, Gill E, Rammul Ü, Sólymos P, Féher Z & Juan X (2011) Citizen Science Reveals Unexpected Continental-Scale Evolutionary Change in a Model Organism. PLoS ONE 6 (4):e18927.

Sørensen JG, Dahlgaard J & Loeschcke V (2001) Genetic variation in thermal tolerance among natural populations of *Drosophila buzzatii*: down regulation of Hsp70 expression and variation in heat stress resistance traits. *Functional Ecology* 15: 289-296.

Sørensen JG & Loeschcke V (2002) Decreased heat-shock resistance and down-regulation of Hsp70 expression with increasing age in adult *Drosophila melanogaster*. *Functional Ecology* 16(3): 379–384.

Staikou A & Lazaridou-Dimitriadou M (1991) The life-cycle, population-dynamics, growth and secondary prouction of the snail *Xeropicta arenosa* Ziegler (Gastropoda, PulmonataI in northern Greece. *Zoological Journal of the Linnean Society* 101(2): 179–188.

Staikou A (1999) Shell temperature, activity and resistance to desiccation in the polymorphic land snail *Cepaea vindobonensis*. *Journal of Molluscan Studies* 65: 171-184.

Stevenson RD (1985) The relative importance of behavioral and physiological adjustments controlling body temperature in terrestrial ectotherms. *The American Naturalist* 126/3:362-386

Storey KB (1996) Oxidative stress: animal adaptations in nature. *Brazilian journal of medical and biological research = Revista brasileira de pesquisas medicas e biologicas / Sociedade Brasileira de Biofisica [et al]* 29 (12):1715-1733.

Storey KB (2002) Life in the slow lane: molecular mechanisms of estivation. *Comparative Biochemistry and Physiology - Part A: Molecular & Integrative Physiology* 133(3): 733–754.

Sumner AT (1965) The cytology and histochemistry of the digestive gland cells of *Helix*. *Quarterly Journal of Microscopical Science* 106: 173-192.

Taieb N & Vicente N (1998) Histochemistry and ultrastructure of the crypt cells in the digestive gland of *Aplysia punctata* (Cuvier, 1803). *Journal of Molluscan Studies* 65: 385-398.

Tomanek L & Somero GN (1999) Evolutionary and acclimation-induced variation in the heat-shock responses of congeneric marine snails (genus *Tegula*) from different thermal habitats: implications for limits of thermotolerance and biogeography. *The Journal of Experimental Biology* 202: 2925–2936.

Tomanek L & Sanford E (2003) Heat-Shock Protein 70 (Hsp70) as a Biochemical Stress Indicator: an Experimental Field Test in Two Congeneric Intertidal Gastropods (Genus: *Tegula*). Biology Bulletin 205 (3):276–284.

Triebskorn R & Köhler H-R (1996) The impact of heavy metals on the grey garden slug, *Deroceras reticulatum* (Müller): metal storage, cellular effects and semi-quantitative evaluation of metal toxicity. *Environmental Pollution* 93:327–343.

Verlecar X, Jena K & Chainy G (2007) Biochemical markers of oxidative stress in *Perna viridis* exposed to mercury and temperature. *Chemico-biological interactions* 167 (3):219-226.

Yom-Tov Y (1971) Body temperature and light reflectance in two desert snails. *Proceedings of the Malacological Society of London* 39: 319-326.

Zaldibar B, Cancio I & Marigomez I (2007) Reversible alterations in epithelial cell turnover in digestive gland of winkles (*Littorina littorea*) exposed to cadmium and their implications for biomarker measurements. *Aquatic Toxicology* 81: 183-196.

Eigenanteil an den durchgeführten Arbeiten bei den in der vorliegenden Dissertation integrierten Publikationen

Kapitel 1: MA Di Lellis, M Seifan, S Troschinski, C Mazzia, Y Capowiez, R Triebskorn, H-R Köhler (2012) Solar radiation stress in climbing snails: behavioural and intrinsic features define the Hsp70 level in natural populations of *Xeropicta derbentina* (Pulmonata). Cell Stress and Chaperones, 17(6): 717-727

S. Troschinski: Probengewinnung im Feld gemeinsam mit M.A. Di Lellis, Prof. Dr. R. Triebskorn und Prof. Dr. H.-R. Köhler (Universität Tübingen) nach Hinweisen von Dr. C. Mazzia und Dr. Y. Capowiez (Université d'Avignon et des Pays de Vaucluse). Aufarbeitung der Proben sowie statistische Auswertung durch M.A. Di Lellis nach Anweisungen von Dr. M. Seifan (Universität Tübingen). Anfertigung des Manuskripts durch M.A. Di Lellis. Fachliche Betreuung durch Prof. Dr. H.-R. Köhler.

Kapitel 2: A Dieterich, U Fischbach, M Ludwig, MA Di Lellis, S Troschinski, U Gärtner, R Triebskorn, H-R Köhler (2012) Daily and seasonal changes in heat exposure and the Hsp70 level of individuals from a field population of *Xeropicta derbentina* (Krynicki 1836) (Pulmonata, Hygromiidae) in Southern France. Cell Stress and Chaperones, 18(4): 405-414

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S. Troschinski: Versuchsdurchführung gemeinsam mit M.A. Di Lellis (Universität Tübingen). Kompletter Eigenanteil an der Gewinnung und Aufarbeitung der histologischen Proben. Bearbeitung der Hsp70-Proben durch M.A. Di Lellis. Kompletter Eigenanteil an der statistischen Auswertung und der Anfertigung des Manuskripts. Genetische Analysen und deren statistische Auswertung durch S. Sereda, T. Hauffe und Prof. Dr. T. Wilke (Universität Gießen). Fachliche Betreuung durch Prof. Dr. R. Triebskorn (Universität Tübingen) und Prof. Dr. H.-R. Köhler (Universität Tübingen).

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Kapitel 1: Solar radiation stress in climbing snails: behavioural and intrinsic features define the Hsp70 level in natural populations of *Xeropicta derbentina* (Pulmonata)

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Abstract

Ectotherms from sunny and hot environments need to cope with solar radiation. Mediterranean land snails of the superfamily Helicoidea feature a behavioural strategy to escape from solar radiation-induced excessive soil heating by climbing up vertical objects. The height of climbing, and also other parameters like shell colouration pattern, shell orientation, shell size, body mass, actual internal and shell surface temperature, and the interactions between those factors may be expected to modulate proteotoxic effects in snails exposed to solar radiation and, thus, their stress response. Focussing on natural populations of *Xeropicta derbentina* we conducted a 'snapshot' field study using the individual Hsp70 level as a proxy for proteotoxic stress. In addition to correlation analyses, an IT-model selection approach based on Akaike's Information Criterion was applied to evaluate a set of models with respect to their explanatory power and to assess the relevance of each of the above-mentioned parameters for individual stress, by model averaging and parameter estimation. The analysis revealed particular importance of the individuals' shell size, height above ground, the shell colouration pattern and the interaction height*orientation. Our study showed that a distinct set of behavioural

traits and intrinsic characters define the Hsp70 level and that environmental factors and individual features strongly interact.

Keywords: stress proteins, *Xeropicta derbentina*, proteotoxic stress, environmental factors, Akaike Information Criterion

Introduction

In poikilothermic organisms, the interaction of environmental and body temperature requires effective regulation mechanisms to guarantee homeostasis, because metabolism of poikilotherms relates directly to the thermal conditions in their environment. Numerous variables are known to influence the body temperature of ectotherms such as physiological (e.g. behavior), morphological (size, colour, shape of the body) as well as environmental parameters, like radiation, wind speed and the type of substrate (Stevenson 1985). These variables are often entangled with one another and, therefore, complex interactions among them should be considered. In this context also the global warming phenomenon which includes a rise in air temperature is consequently affecting the physical condition, abundance and distribution of organisms and the functioning in their habitat (Helmuth et al. 2010). To understand how the impact of environmental factors influences the persistence of organisms in ecosystems, it is mandatory to consider the protective value of physiological mechanisms and morphological characters of organisms.

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

Feder and Hofmann 1999, Lewis et al. 1999, Köhler et al. 2000, Mukhopadhyay et al. 2003).

Stress protein expression in snails of the superfamily Helicoidea from hot climates has been characterized recently (Mizrahi et al. 2009, Arad et al. 2010). This gastropod superfamily occurs in large abundance in the Mediterranean and comprises a number of morphologically very similar species. These snails are known to climb up vertical objects in the beginning of the day and are, therefore, fully exposed to solar radiation for hours, particularly during hot summer days. Even though this behaviour is well-known (Cowie 1985), it has not been investigated so far, whether distinct parameters determine the stress status of an individual in its natural habitat. The following questions are important in this context: Is the position of a snail, its orientation to the sun and its distance from the hot ground surface important? Does the individual size, responsible for the volume-surface ratio, matter? Does the intraspecifically highly variable colouration of the shell play a role? Does pigmentation contribute to thermoregulation in these snails, as reported for other ectotherms (Clusella Trullas et al. 2007)?

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

Materials and Methods

Study sites

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

Table 1. Sampling sites in Provence, France. Given is the sampling size (n), the difference (Diff) in size, height above ground, internal temperature and body mass of the organisms and Shannon-Wiener indices (HS) as measure for the variability in shell orientation towards the sun and the shell colouration pattern, within each population, respectively

Pop.	**Locality**	**n**	**$Diff_{size}$ [mm]**	**$Diff_{height}$ [cm]**	**$Diff_{temp}$ [°C]**	**$Diff_{bm}$ [mg]**	**HS_{orient}**	**HS_{shellp}**
1	Modène Mazzia	12	4,47	23	3,3	139	0,87	1,20
2	Modène wine yard	12	2,02	25	3,5	70	0,89	1,24
3	Modène West	12	4,20	53	3,4	282	0,96	0,89
4	St Pièrre	12	4,93	14	3,3	207	0,82	0,45
5	Mazan South	12	2,29	11	3,6	126	0,72	1,01
6	Bon Remède	12	5,05	34	3,1	233	1,01	0,72
7	Mazan North	12	3,14	26	1,5	175	0,96	0,00

Test organisms

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

this species occurs in huge populations among very diverse landscapes and displays a high variation in shell colouration.

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

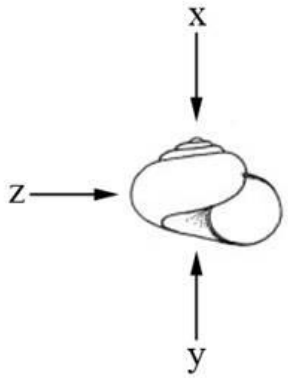

Figure 1. Code for the factor 'orientation towards the sun'. [x] Apex directed to the sun; [y] umbilicus directed to the sun; [z] shell laterally directed to the sun. The respective characters symbolize the position of the sun.

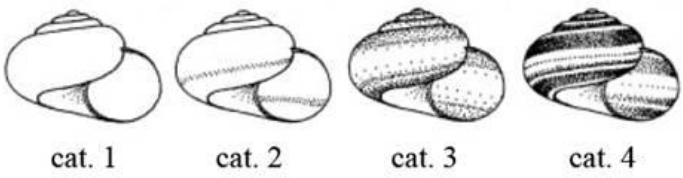

Figure 2. Code for the factor 'shell colouration pattern', according to Köhler et al. (2009) modified. Cat. 1 = white; cat. 2 = white with a single pale band; cat.3 = greyish with several light bands; cat. 4 = dark with lots of intense bands

Sampling in the field

Measures were made consecutively for each snail. In all individuals, we determined the residing height on vertical objects above the ground with a yard stick. Then, we classified the shell orientation (geographic direction). For this purpose, we defined three categories (Fig.1): (1) apex directed to the sun [x], (2) umbilicus directed to the sun [y], and (3) shell laterally directed to the sun [z]. Because the sampling time was always around noon, the orientation of the apex was southwards in [x] and northwards in [y]. Subsequently, the surface temperature of the shell was measured with a thin medical precision thermometer (ELLAB Copenhagen, type: DM 852). Here, we took care not to influence the temperature by touching the snail. For the measurement of the internal body temperature, we removed the snail from its location to allow the pin of the thermometer to penetrate the soft body. Subsequently, the size of the shell was determined by measuring its width by an

electronic caliper rule, and each individual was classified regarding its shell pattern (Fig.2; 1 = white; 2 = white with a single pale band; 3 = grayish with several light bands; 4 = dark with lots of intense bands). Both fresh weight and the relative Hsp70 level were determined in the laboratory afterwards.

Stress protein analysis

After determining the above-mentioned parameters, the snails (n=12 for each population) were frozen individually in liquid nitrogen and stored in a freezer at -25°C before biochemical analysis. We homogenized the snails on the whole, without separating it into various tissues, according to their body mass in extraction buffer (2µl buffer/mg snail), containing 80mM potassium acetate, 5mM magnesium acetate, 20mM Hepes, and 2% protease inhibitor at pH 7.5. In the following, the supernatant was separated from the remaining cell debris via centrifugation for 10 minutes at 2000*g* and 4°C. Subsequently, we determined the total protein concentration of each sample by use of a protein-dye binding assay (Bradford 1976). We analysed constant protein weights of 40 µg total protein from each sample by minigel SDS-PAGE (12% acrylamide, 0.12% bisacrylamide, 30 minutes at 80 V, and 75-90 minutes at 120 V) and semi-dry Western blotting on nitrocellulose membranes. The membranes were blocked with a 50% horse serum/tris-buffered saline (TBS) solution for 2 hours, before they were incubated in a solution of monoclonal α-Hsp70 antibody which was cross-reacting with all isoforms of Hsp70 and detected both constitutive and inducible stress protein molecules (mouse anti-human Hsp70, Dianova, Hamburg, Germany, dilution 1:5000 in 10% horse serum in TBS) on a lab shaker overnight. Subsequently, the membranes were washed in TBS for five minutes to remove dispensable Hsp70 antibodies, before we applied a second antibody (goat anti-mouse IgG conjugated to peroxidase, Jackson Immunoresearch, West Grove, PA, dilution 1:1000 in 10% horse serum/ TBS) for two hours. After short washing in TBS, the membranes were developed in a staining solution, containing 1mM 4-chloro(1)naphthol, 0.015% H_2O_2, 30mM Tris pH 8.5, and 6% methanol. The optical volume of the individual bands was calculated by multiplication of the area of the bands (number of pixels) with the average grey scale value after background subtraction. For this purpose, we used the densitometric image analysis program E.A.S.Y. Win 32 (Herolab, Wiesloch, Germany). All sample data were normalized against a standard sample (prepared from *Theba pisana*) to ensure comparability between all samples. Figure 3 displays

representative Western blot bands for Hsp70 in snail individuals of featuring different size.

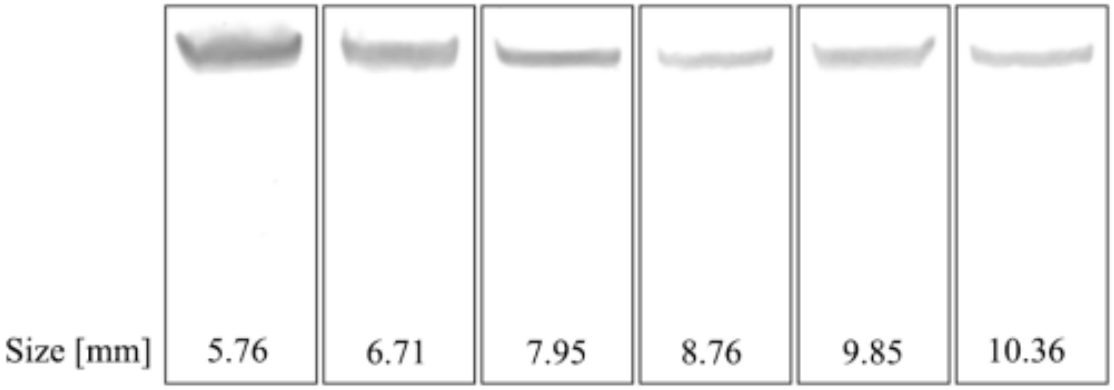

Figure 3. Representative Western blot bands for Hsp70 in snails featuring different size. The α-Hsp70 antibody was sensitive to all isoforms of Hsp70 and detected both constitutive and inducible stress protein molecules.

Statistical Analysis

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

and allows inferences through the whole set of models, thus takes into account the fact that no single model (or variable composition) can perfectly reflect nature.

The IT-model selection approach we used is based on Akaike's Information Criterion (AIC). In general, this criterion is based on the estimation of information loss when a model is used to approximate the truth (Anderson et al. 2000). The better the model is reflecting nature, the less information is lost. Using this approach, a researcher can estimate what is the relative precision of several models created from the same data set. Model-based inference has three general advantages (Johnson and Omland 2004). First, as mentioned above, no single relatively meaningless null hypothesis is employed. Instead, there are several well-grounded models, the amount of support of which can be evaluated by the data set. Second, models can be ranked according to their data fit and, third, all single factors and interactions can be estimated and predicted by model averaging.

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

As a first step, we formulated a set of competing models. Here, we took care to choose all models which had a relevance to our study aims, to ensure an ecophysiological sense of our model collection. Secondly, we fitted each model to the observed data by conventional univariate statistical methods (i.e. regressions, ANOVA). During this process we squareroot-transformed our dependent variable (Hsp70 level) to fulfill the univariate methods requirements. This way we generated a set of relevant models, each with its variance and coefficient estimation. For each of the models we calculated the AIC_C, a modified variation of the AIC which is adequate for small sample sizes, according to the following equation (Symonds and Moussalli 2011, Burnham and Anderson 2002):

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

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

The model with the lowest AIC_C value was considered as the model with the best explanatory power, and the best fit to the data set. To assess the relative strength of the candidate models, we calculated the difference between the AIC_C values of each respective model and the best ranked one (Δ_i). This allowed us to judge the lower ranked models (i.e. the models which were considered worse) compared to the prime one, instead of arbitrarily ignoring them. From the Δ_i parameters we further calculated the Akaike weight (ω_i) by dividing the appropriate model likelihood exp ($-0.5\Delta_i$) by the sum of all values across the model set. This likelihood weight is an assessment of the specific model probability to be the best ranked one in a repeated data collection. The Akaike weight of all models sum up to 1 and can be translated into percentage values. Therefore, the Akaike weight (ω_i) for each model in our set can be easily interpreted as the probability of the model to fit the data. Finally, we estimated parameter coefficients (i.e. their relative importance and trend of effect) by calculating a weighted average across all models. This process strongly reduces model selection bias and ensures that we took into account as many possible scenarios as the biological logic of the system dictates, instead of constraining ourselves due to a limited statistical analysis (Symonds and Moussalli 2011, Johnson and Omland 2004).

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

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

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

Results

Correlation analyses

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

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

	Height	**Orientation**	**Shell pattern**	**Surface temperature**	**Internal temperature**	**Size**	**Body mass**
Height	X	-	-	-	-	positive	positive
Orientation	0.8605	X	-	-	-	-	-
Shell pattern	0.8430	0.6234	X	-	*	**	***
Surface temperature	0.6683	0.2808	0.5283	X	positive	-	-
Internal temperature	0.9960	0.4942	**0.0175**	**0.0001**	X	-	-
Size	**0.0165**	0.8758	**0.0001**	0.7374	0.4980	X	positive
Body mass	**0.0105**	0.9806	**0.0171**	0.6102	0.4784	**0.0001**	X

The significance threshold was set to α=0.05.* significantly higher internal temperature in snails with cat. 2 compared with snails attendant to cat. 1,3 and 4. ** significantly larger shell size in snails of cat. 1 compared with the darker morphs (cat. 2-4). *** significant decrease in body mass with an increase in shell pigmentation.

Model selection

The first model selection procedure for the factors 'size', 'orientation towards the sun' and 'internal temperature' revealed that, of a total of 15 potentially relevant models, only one showed high empirical support, because $\Delta_i \leq 2$ (the difference in AIC_C between the best ranked model and the respective one of interest) was not fulfilled for any other model (Tab.3). The model with the lowest AIC_C value and, correspondingly, the highest explanatory power for Hsp70 level variation was built by the single factor 'size'. This parameter had a very high probability value (97.8%),

representing the chance to be part of the best model and, therefore, clearly contributed to the variation in the Hsp70 level. Mentionable are also the factors 'orientation towards the sun' (21.8%) and 'internal temperature' (18.5%) with a moderate influence on the Hsp70 level, whereas the interactions between those factors have shown to be redundant in this analysis and own no explanatory power for the Hsp70 level. Another important indication to the robustness of our results is the fact that the null model is ranked relatively low (place 9), implying that our chosen measurements clearly improved our ability to predict the change in Hsp70 values.

The second model selection procedure, performed with the factors 'orientation towards the sun', 'internal temperature' and 'height above ground' (instead of 'size'), showed that two models exhibit high empirical support with $\Delta_i \leq 2$ (Tab.4). The best model with the highest explanatory power comprised the interaction term 'height above ground' and 'orientation towards the sun', with a probability value of 48,8%. The second model with comparable empirical support is built by the factor 'height above ground' which had an expectation of 51.9% to be part of the best model. The factors 'orientation towards the sun' (16.8%) and 'internal temperature' (15.8%) had low impact in explaining Hsp70 level variation, and the interactions 'height * temperature' and 'temperature * orientation' (both < 1.6%) had no impact at all.

Specific effect of the model parameters

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

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

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

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

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

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

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

The rank of a model, the model constitution, the AIC_C, the Akaike weight (ω), and the number of parameters involved are listed. The probability reveals the importance of each factor to be part of a model. Models with high empirical support are arranged above the dashed line ($\Delta_i \leq 2$). H: height above ground, O: shell orientation towards the sun, T: internal temperature.

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

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

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

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

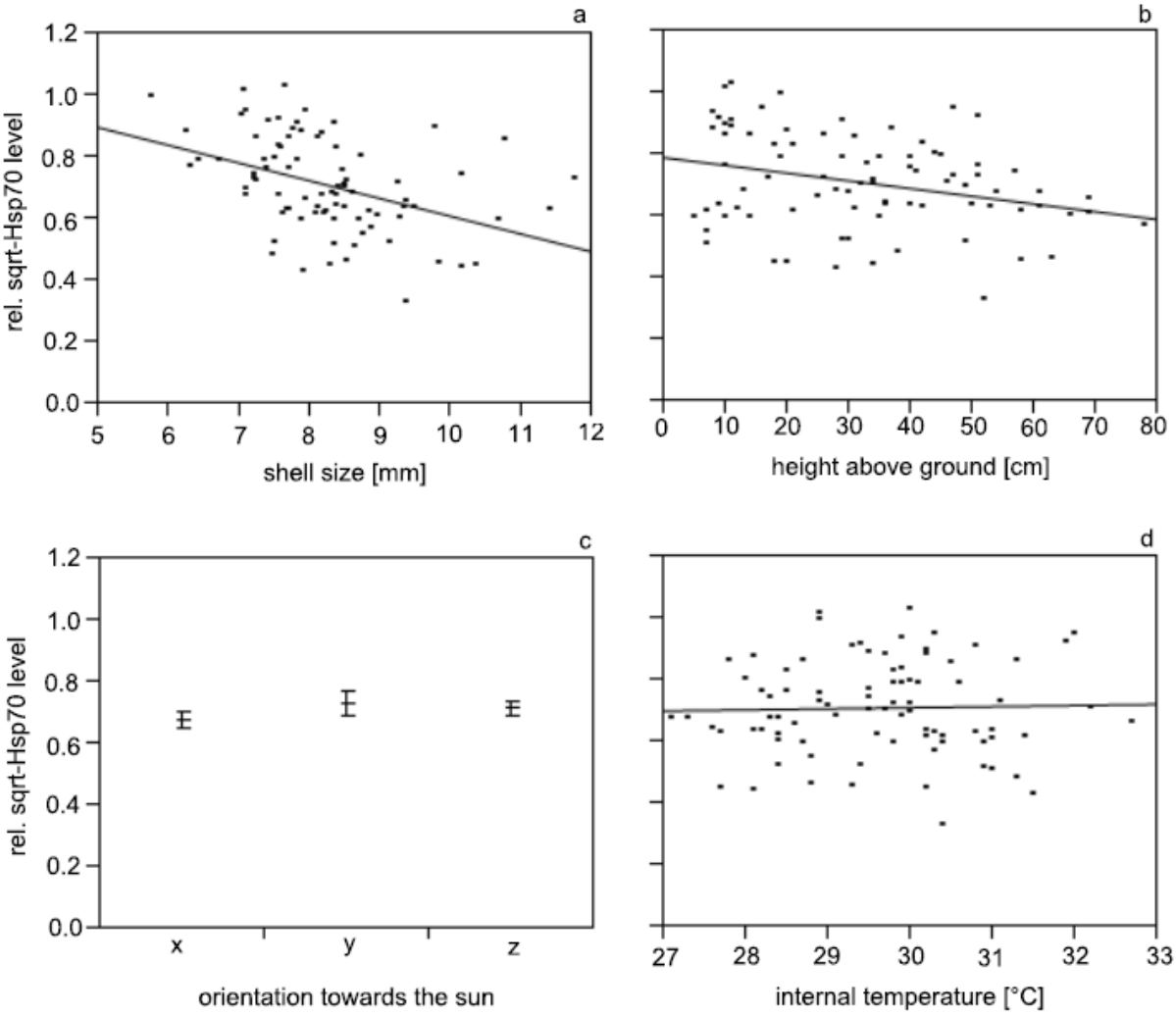

Figure 4. All single factors used for model selection analysis plotted against sqrt Hsp70 level. Because the applied model selection approach does not determine significance levels but likelihood estimates, these graphs lack confidence intervals and p-levels. These figures serve as completion for the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

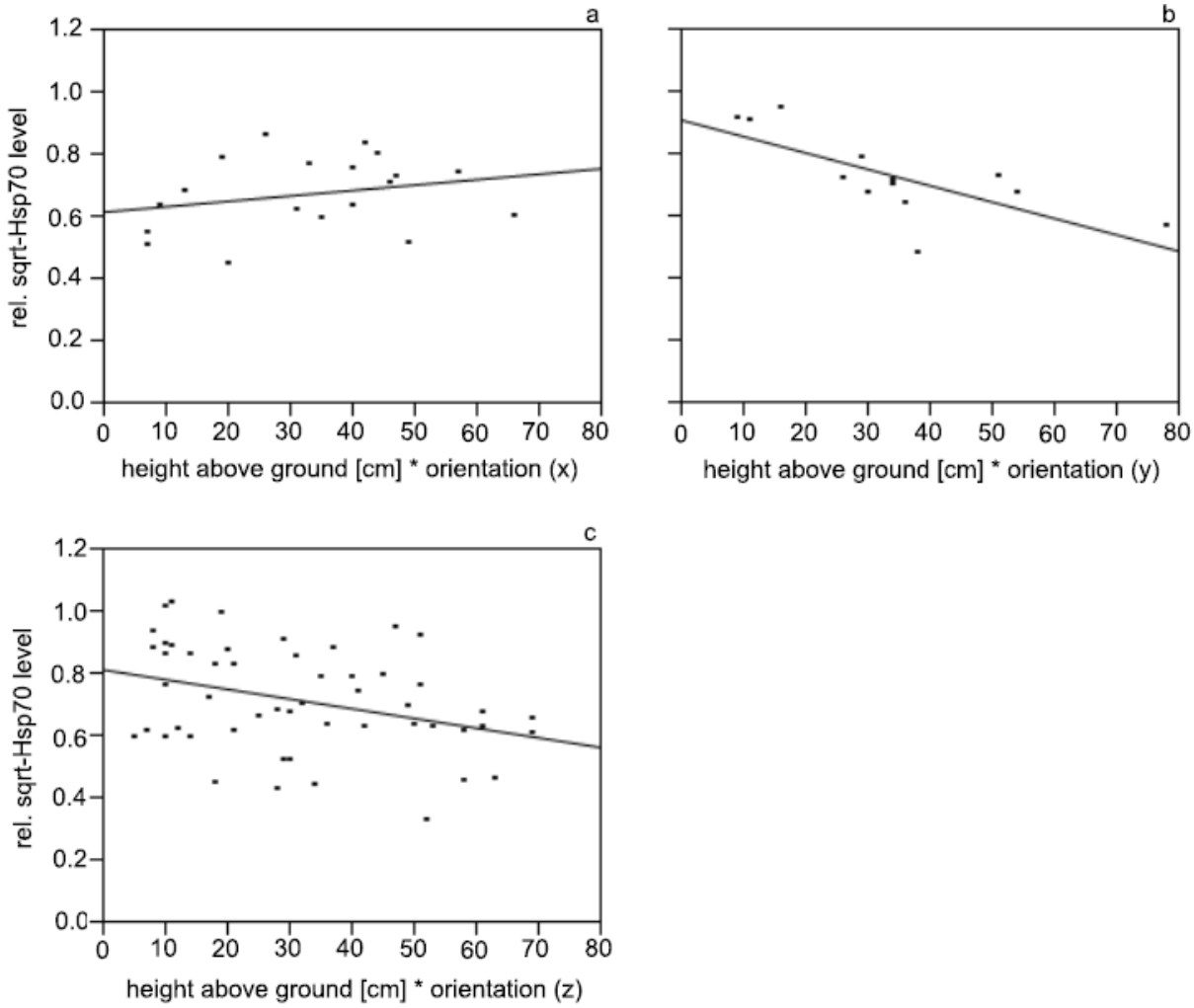

Figure 5. Interaction 'height above ground * orientation towards the sun', each category is plotted against sqrt Hsp70 level. Because the applied model selection approach does not determine significance levels but likelihood estimates, these graphs lack confidence intervals and p-levels. These figures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

Discussion

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

According to the conducted correlation and model selection analyses, we could reveal a distinct pattern of features influencing the Hsp70 level in individuals of the Mediterranean snail species *Xeropicta derbenina*: large snails predominantly exhibit primarily plain white shells and climb rather high while holding a comparatively low

Hsp70 level. Their small sized conspecifics with a more intense shell pigmentation remained rather low in their residing height above ground and, in contrast, had a higher Hsp70 level. So, what can a snail do to keep proteotoxicity as low as possible?

One important factor to explain the variance in the Hsp70 level of *X. derbentina* proved to be the height above the ground. Most xerophilous land snails exhibit an active behavior during the cool and moist conditions at night when they feed and roam while they stay inactive during the hot and dry day hours. By the end of the night, the organisms climb up vertical objects, seal their shell aperture with an epiphragm and reduce their metabolism to save water and energy (Mazek-Fialla 1934, Machin 1968). It seems to be generally advisable to climb up vertically, independent of the assumption that climbing eventually modifies their exposures to predators from ground-living beetles to birds. Evolution has selected this behavioural trait in all snail species from the Mediterranean but, apparently, there is no optimal height a snail should reach. Both climbing and stress protein induction is energy-costly and, thus both of these two responses to solar radiation likely trade-off against one another. Besides of the necessity to escape from lethal soil surface temperatures, it seems that the amount of climbing quite often is limited by random factors such as the height of the vegetation or hindering conspecifics. On sunny summer days in Vaucluse, the soil surface reaches temperatures of up to 50°C or higher, which are above the lethal limit for the snails (M.A. Di Lellis and S. Troschinski, unpublished data). Only a few centimeters above the soil, the temperatures decline and conditions hence become more comfortable for the snails (Cowie 1985, Kempster and Charwa 2003). With lower temperatures, the cellular stress is decreasing and the Hsp70 level is expected to adjust at a moderate level (Feder and Hofmann 1999, Mukhopadhyay et al. 2003). However, internal temperature as one of the factors within our model set emerged not to be meaningful, with a probability of 15.8-18.5% only, in regard to explain the Hsp70 level variation. However, Köhler et al. (2009) and Pomeroy (1966) showed the height above ground to be predominantly relevant for the body temperature. Several considerations may explain this lack of coherence: in our analysis we were looking on a temperature variation within a span of only 6°C, an exiguous span in terms of the large temperature fluctuations the snails experience through the whole day and are adapted to during the whole summer. Furthermore, it is known that stress protein levels do not solely reflect immediate conditions but rather integrate temporally over the effects experienced within some time span. Therefore, in the

field, spontaneously fluctuating parameters like wind speed or sun shading may instantaneously influence the temperature of a snail but not its actual Hsp70 level. In addition, intraspecific variation in the heat stress response which can be due to genetic and physiological differences between the individuals might interfere with a possible effect of the internal temperature on the Hsp70 level. Experiments in the laboratory revealed an Hsp70 maximum level in these *X. derbentina* populations at around 40°C after 8 hours of this elevated temperature (M.A. Di Lellis, unpublished). Therefore, the examined snails in this study most probably did not reach their thermal stress limit in the field. Besides this, Mizrahi et al. (2009) showed a delayed induction of Hsp70 and Hsp90 in a desert dwelling snail species (*Sphincterochila zonata*) together with an enhanced synthesis of small Hsp molecules (sHsps), which are also induced under stressful conditions. It has been shown, that highly thermotolerant species adapted to environments with hot temperature regimes induced Hsp70 and other stress proteins at higher thermal limits (Hofmann & Somero 1996, Nakano & Iwama 2002, Evgen'ev et al. 2007). For highly thermotolerant species in particular, this strategy might save fitness costs and high temperature regimes might act as a microevolutionary effective agent (Feder & Hofmann 1999). To adress the entirety of Hsp classes in this context might be advisable for further studies.

Another factor with a high validity turned out to be the size of the shell. With an increase in size, the Hsp70 level decreased. This might be due to several reasons. Small snails are developing in growth and maturity. Constitutive Hsp70 molecules own crucial features like chaperoning proteins during folding processes, intracellular protein trafficking, and assembly of proteins besides acting as inducible proteotoxic defense (Mayer & Bukau 2005). Since we used an α-Hsp70 antibody which was sensitive to all isoforms of Hsp70 and detected both constitutive and inducible stress protein molecules, small snails could have had a higher base level of Hsp70 caused by their development at stage. Another important point to consider is desiccation due to shell size. In general, larger specimens possess a more favourable surface-to-volume ratio than smaller individuals and therefore, water loss is minimized. As suggested by Mizrahi et al. (2009), land snails living under harsh environmental circumstances and suffering from desiccation use Hsp induction as important survival strategy. Hence, high Hsp70 levels in small individuals can be evoked by increased desiccation processes. Furthermore, we found coherence in shell size and the residing height above the ground. On the basis of the present dataset it is impossible to decide which of these two correlating

parameters is aetiologically defining the Hsp70 level in *X. derbentina*. Lab exposure experiments in which individuals of *X. derbentina* were kept at defined temperature, however, did not reveal any influence of body size on Hsp70 induction (Köhler et al. 2009, M.A. Di Lellis, unpublished data).

The shell orientation in relation to the position of the sun as a single factor, turned out to be rather neglectible, since this parameter alone could explain only a minuscule part of the Hsp70 level variation. Nevertheless, in combination with the height above ground, we obtained an interesting effect. When orientated to the South ('x'), the residing height above the ground has rather an increasing effect on the Hsp70 level. When orientated in other directions ('y' or 'z'), elevated heights decreased the Hsp level in the snails. The reason for this effect remains unclear, and its elucidation requires a thermodynamic computer model of the snail's shell and its inner organs which allows to simulate the thermal effects of virtual illumination from different sides of an individual. Currently, such a computer model is envisaged to be built up in our group. Whether the difference in Hsp70 expression in different body tissues which has been reported for mollusks (Lyons et al. 2003, Mizrahi et al. 2009, Arad et al. 2010) plays a role in this context, can therefore be addressed in the future.

In our correlation analyses, the shell colouration pattern turned out to have an effect on the Hsp70 level. Since the colouration pattern correlated with other continuous variables such as the internal temperature and the size of the snail, we had to exclude this parameter from the models. However, correlation analyses revealed the Hsp70 level to rise with increasing shell pigmentation. Due to sunlight reflectance, it was postulated that brighter coloured shells generally heat up slower than darker shell patterns (Yom-Tov 1971, Dittbrenner et al. 2008). However, current results of our group show that there are no differences in the thermal capacity of the different morphs in *Theba pisana*, another Mediterranean helicoid snail species living in the same habitat as *X. derbentina* (Scheil et al. 2012). Since in *X. derbentina* the increase in shell pigmentation is strongly and negatively correlated with size, we assume that shell colouration pattern distribution could depend on the individual's age. Hoshino et al. (2010) showed that melanin production in cultured mouse melanoma cells was suppressed in cells with a high Hsp70 induction. The long and sun-intense summer time in Provence might lead to shell bleaching by suppression of the melanin synthesis via long term Hsp70 action in the growing snail. Since the shell pattern apparently is connected to the trait

'size' in *X. derbentina*, selection cannot work on it independently. But also in other species the intrinsic character 'bright shell' cannot be unequivocally favoured by evolution – otherwise the often-reported high variation in shell colouration in Helicoidea like *Theba pisana*, and Cepaea species, would not be maintained in natural populations (Cowie 1990, Silvertown et al. 2011).

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

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

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Literature

Anderson DR, Burnham KP, Thompson WL (2000) Null hypothesis testing: Problems, prevalence, and an alternative. J Wildl Manage 64:912–923

Arad Z, Mizrahi T, Goldenberg S, Heller J (2010) Natural annual cycle of heat shock protein expression in land snails: desert *versus* Mediterranean species of *Sphincterochila*. J Exp Biol 213:3487-3495

Aubry S, Labaune C, Magnin F, Kiss L (2005) Habitat and integration within indigenous communities of *Xeropicta derbentina* (Gastropoda: Hygromiidae), a recently introduced land snail in South-Eastern France. Diversity and Distributions 11:539–547

Bradford M (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principles of protein-dye binding. Anal Biochem 72:248-254

Burnham KP, Anderson DR (2002) Model selection and multimodel inference: a practical information-theoretical approach, Springer, New York

Burnham KP, Anderson DR (2004) Multimodel inference: understanding AIC and BIC in model selection. Sociol Method Res 33:261–304

Clusella Trullas S, van Wyk JH, Spotila JR (2006) Thermal melanism in ectotherms. J of Therm Biol 32:235-245

Cowie RH (1985) Microhabitat choice and high temperature tolerance in the snail *Theba pisana* (Mollusca:Gastropoda). J. Zool. Lond. (A) 207:201-211

Cowie RH (1990) Climatic selection on body colour in the land snail *Theba pisana* (Pulmonata: Helicidae). Heredity 65:123–126.

Dittbrenner N, Lazzara R, Köhler H-R, Mazzia C, Capowiez Y, Triebskorn R (2009) Heat tolerance in Mediterranean land snails: histopathology after exposure to different temperature regimes. J Mollus Stud 75, 9–18

Evgen'ev MB, Garbuz DG, Shilova VY, Zatsepina OG (2007) Molecular mechanisms underlying thermal adaptation of xeric animals. J Biosci 32:489–499

Feder ME, Hofmann GE (1999) Heat-shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology. Annu. Rev. Physiol. 61:243–282

Helmuth B, Broitman B, Yamane L, Gilman SE, Mach K, Mislan KAS, Denny MW (2010) Organismal climatology: analyzing environmental variability at scales relevant to physiological stress. J. Exp. Biol. 213:995-1003

Hofmann GE, Somero GN (1996) Interspecific variation in thermal denaturation of proteins in the congeneric mussels Mytilus trossulus and M. galloprovincialis: evidence from the heatshock response and protein ubiquitination. Mar Biol 126:65–75

Hoshino T, Matsuda M, Yamashita Y, Takehara M, Fukuya M, Mineda K, Maji D, Ihn H, Adachi H, Sobue G, Funasaka Y, Mizushima T (2010) Suppression of Melanin Production by Expression of HSP70. J Biol Chem 285:13254-13263

infoclimat.fr, retrieved 02 November 2011

Irvine DR, Hibbs DE, Shatford JPA (2009) The relative importance of biotic and abiotic controls on young conifer growth after fire in the Klamath-Siskiyou region. Northwest Sci 83:334-347

Johnson JB, Omland KS (2003) Model selection in ecology and evolution. Trends Ecol. Evol. 19:101–108

Kempster V, Charwa S (2003) Soil surface temperature and mortality in land snails: Implications for successful management. Austral J Agricult 43:1351-1356

Köhler H-R, Triebskorn R, Stocker W, Kloetzel PM, Alberti G (1992) The 70kD heat shock protein (Hsp 70) in soil invertebrates: a possible tool for monitoring environmental toxicants. Arch Environ Contam Toxicol 22:334–38

Köhler H-R, Zanger M, Eckwert H, Einfeldt I (2000) Selection favours low Hsp70 levels in chronically metal-stressed soil arthropods. J Evol Biol 13:569-582

Köhler HR, Lazzara R, Dittbrenner N, Capowiez Y, Mazzia C, et al. (2009) Snail Phenotypic Variation and Stress Proteins: do Different Heat Response Strategies Contribute to Waddington's Widget in Field Populations? J Exp Zool Part B - Mol Develop Evol 312B: 136–147

Lewis S, Handy RD, Cordi B, Billinghurst Z, Depledge MH (1999) Stress proteins (Hsps): methods of detection and their use as an environmental biomarker. Ecotoxicol 8:351-368

Lyons C, Dowling V, Tedengren M, Gardestrom J, Hartl MJ, O'Brien N, Van Pelt F, O'Halloran J, Sheehan D (2003) Variability of heat shock proteins and glutathione S-transferase in gill and digestive gland of blue mussel, *Mytilus edulis*. Mar Environ Res 56:585-597

Machin J (1968) The permeability of the epiphragm of terrestrial snails to water vapor. Biol Bullmar biol Lab, Woods Hole 134:87-95

Mayer MP, Bukau B (2005) Hsp70 chaperones: cellular functions and molecular mechanism. Cell Mol Life Sci 62:670–684

Mazek-Fialla K (1934) Die Lebensweise xerophiler Schnecken Syriens, Griechlands, Dalmatiens und der Türkei und die Beschaffenheit ihrer subepithelialen Drüsen. Z Morph Ökol Tiere, 28:445-468

Mizrahi T, Heller J, Goldenberg S, Arad Z (2009) Heat shock proteins and resistance to desiccation in congeneric land snails. Cell Stress Chaperon 15: 351-363

Mukhopadhyay I, Nazir A, Saxena DK, Chowdhuri DK (2003) Heat shock response: Hsp70 in environmental monitoring. J Biochem Mol Toxicol 17:249–254

Nakano K, Iwama G (2002) The 70-kDa heat shock protein response in two intertidal sculpins, Oligocottus maculosus and O. snyderi: relationship of hsp70 and thermal tolerance. Comp Biochem Physiol A Mol Integr Physiol 133:79–94

Pomeroy DE (1966) The ecology of Helicella virgata and related species of snails in South Australia. PhD thesis, University of Adelaide

Pörtner HO, Farrell AP (2008) Ecology: Physiology and climate change. Science 322:690–92

Scheil AE, Gärtner U, Köhler H-R (2012) Colour polymorphism and thermal capacities in *Theba pisana* (O.F. Müller 1774). J Thermal Biol

Silvertown J, Cook LM, Cameron RAD, Dodd M, McConway K, Worthington J, Skelton P, Anton C, Bossdorf O, Baur B, Schilthuizen M, Fontaine B, Sattmann H, Bertorelle G, Correia M, Oliveira C, Pokryszko B, Ożgo M, Stalažs A, Gill E, Rammul Ü, Sólymos P, Féher Z, Juan X (2011) Citizen science reveals unexpected continental scale evolutionary change in a model organism. PLoS ONE, 6: e18927; doi:10.1371/journal. pone.0018927

Stevenson RD (1985) The relative importance of behavioral and physiological adjustments controlling body temperature in terrestrial ectotherms. The Am Naturalist 126/3:362-386

Symonds MRE, Moussalli A (2010) A brief guide to model selection, multimodel inference and model averaging in behavioural ecology using Akaike's information criterion. Behav Ecol Sociobiol 65:13-21

Triebskorn R, Köhler H-R (1996) The impact of heavy metals on the grey garden slug, *Deroceras reticulatum* (Müller): metal storage, cellular effects and semi-quantitative evaluation of metal toxicity. Environ Pollut 93:327–343

Yom-Tov Y (1971) Body temperature and light reflectance in two desert snails. Proc Malacol Society London 39:319–326

Kapitel 2: Daily and seasonal changes in heat exposure and the Hsp70 level of individuals from a field population of *Xeropicta derbentina* (Krynicki 1836) (Pulmonata, Hygromiidae) in Southern France

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Abstract

The Mediterranean land snail *Xeropicta derbentina* forms huge populations in Southern France. In order to characterize heat exposure and the induction of the 70-kD heat shock protein (Hsp70) response system during the life cycle of this snail, a selected population from the Vaucluse area, Provence, was investigated encompassing the issues of morphological life cycle parameters (shell size and colouration), the daily courses of heat exposure at different heights above the ground, of shell temperature, and that of the individual Hsp70 levels. The study covered all four seasons of the year 2011. Snails were found to be annual, reaching their final size in August. The shell colouration pattern showed high variation in juveniles (spring) with a strong tendency towards becoming uniformly white at old age in autumn. In all seasons, ambient air temperature decreased with increasing distance from the ground surface during daytime while remaining constantly low in the night. Overall, the Hsp70 level of individuals followed the ambient temperature during diurnal and seasonal variations. Correlation analysis revealed a positive association of individual shell temperature and Hsp70 level for the most part of the life cycle of the snails until late summer, whereas a negative correlation was found for aged animals indicating senescence effects on the capacity of the stress response system.

Keywords: Heat shock response - Mediterranean land snail – Stress proteins – Temperature - Life cycle -

Introduction

Climbing vertical structures to avoid lethal ground temperatures is a common and frequently recognized adaptive behaviour of land snails to their environment (Aubry et al. 2006; Kiss et al. 2005; Storey 2002). Besides other behavioural adaptations like burrowing in the soil during the day or hiding beneath fallen leaves, climbing is one of the most obvious responses of snails to adverse conditions in the field during daytime. Measurements of the ground temperature and several centimetres above show a dramatic decrease of the air temperature even a few centimetres above the ground (Köhler et al. 2009). Shifting the activity to the cooler and moister night hours is another common behaviour of land snails in their response to hot environments (Abdel-Rehim 1983; Di Lellis et al. 2012). In the climate of Southern

France with hot and dry summers, ground temperatures frequently reach 50 °C and more. For snails that consist of roughly 75 % water (Reuner et al. 2008), such temperatures are lethal (Dittbrenner et al. 2009).

In Southern France, the land snail *Xeropicta derbentina* (Krynicki 1836) (Gastropoda, Hygromiidae) is an introduced species originating from the Eastern Mediterranean. First records in France date from 1949 (Altena 1960; Kiss et al. 2005; Aubry et al. 2006). Adults of *X. derbentina* reach shell sizes ranging between 10 and 16 mm in diameter, and are generally characterized by a uniformly white shell. Nevertheless, different colour morphs can be found in the field especially in younger stages. Populations of *X. derbentina* may differ in morph composition, and different morphs were also shown to vary slightly in their heat response (Di Lellis et al. 2012). *X. derbentina* is quite often found in areas that are or at least were used for agricultural purposes (Aubry et al. 2005). Especially in open fields with scarce vegetation, at the border of agricultural areas, and along roads *X. derbentina* can be found in large numbers resting at the top of grass-blades or other vegetation - sometimes forming enormous clusters of hundreds of individuals at a single spot. The climbing behaviour protects the snail from potentially lethal temperatures of the soil in summer even though ambient temperatures frequently exceed 40 °C for several hours a day. This climbing behaviour is most likely responsible for the rapid spread of *X. derbentina* in France as snails resting on vehicles disperse rapidly along small roads (Aubry et al. 2006). Apart from the passive means of transport, the movement of these animals is extremely limited during the day. Once they have climbed up vertically, they remain in the sunlight until sunset. Consequently, *X. derbentina* cannot avoid extreme temperatures during hot summer days and,

therefore, has to deal with the experienced high temperature in a different way to avoid overheating and desiccation.

Being confronted with thermal stress, almost all organisms investigated so far are able to produce heat shock proteins (=stress proteins, Hsps) to counteract this and other stresses (Feder and Hofmann 1999; Sørensen et al. 2003; Kiang and Tsokos 1998) with the exception of some Antarctic fish (Hofmann et al. 2000). Hsps are considered part of an intracellular defence machinery that also includes other physiological mechanisms protecting the cells from damage and denaturation of proteins. The best investigated Hsp family is that of Hsp70 (Mayer and Bukau 2005; Daugaard et al. 2007). These structurally highly conserved proteins act as molecular chaperones that assist in folding newly produced proteins. Also increasing amounts of misfolded proteins inside the cell due to heat-induced denaturation or other stresses, induce the production of Hsp70 proteins (Daugaard et al. 2007; Sørensen et al. 2003). Therefore, the increased concentration of Hsp70 proteins can be used as a marker of effect for proteotoxicity. This marker is frequently used in studies examining the tolerance of organisms against heat (Tomanek and Sanford 2003; Nakano and Iwama 2002; Dittbrenner et al. 2009; Di Lellis et al. 2012; Köhler 2009; Köhler et al. 2009). As a marker of effect, rising Hsp70 levels can be interpreted as a response to the effects of heat. With respect to proteotoxic stress, Hsp70 induction follows a distinct reaction curve (Eckwert et al. 1997; Tomanek 2002). Starting with a base level that is expressed under “normal” conditions, the curve rises with increasing stress. When proteotoxic stress reaches a distinct (and population specific) level, no further induction is possible (Arts et al. 2004; Köhler et al. 2009). Exceeding this point of stress leads to a collapse of the Hsp70 protection system revealed in a rapid decrease in the Hsp70 level, followed by the death of the organism or, at least serious damage of its inner structures (Eckwert et al. 1997; Scheil et al. 2011).

As found in helicoid land snails (Dittbrenner et al. 2009; Scheil et al. 2011), Hsp70 clearly increases when the animals heat up. Apart from the intensity of stress affecting the increase of the Hsp70 level in the organism, the exposed life stage also influences the degree of Hsp70 induction. Young or larval stages are especially known to be able to induce Hsp70 to a higher degree than older or senescent organisms (Mayer and Bukau 2005; Köhler 2009). Furthermore, it was shown that Hsp70 levels varied on a seasonal basis, monthly, or even on a daily scale (Nakano and Iwama 2002; Tomanek and Sanford 2003; Schill et al. 2002; Köhler et al.

2001). The induction of Hsp70 was found to vary depending on the environmental conditions the species or a specific population encountered. For example, two closely related Sphincterochila species from two different habitats (Mediterranean vs. desert) expressed different levels of Hsp70 when they were exposed to adverse conditions, reflecting a pre- adaptation to their environment (Arad et al. 2010; Mizrahi et al. 2010, 2012). To date, little is known about the diurnal changes in the Hsp70 level under field conditions in different seasons of a year, particularly for animals living in non-aquatic systems. We investigated a selected population of *X. derbentina* in respect to the daily course of their Hsp70 level in four different months of 2011. Furthermore, we continuously recorded the ambient temperature at different heights over ground during all samplings. According to the known heatinducibility of stress proteins, we expected the Hsp70 level of the snails to correspond to the external temperature profile recorded in the field. Investigations covered different months and, consequently, different life stages of this annual species. Our aim was to provide a solid data basis to estimate the severity of heat stress and the capacities of the Hsp70 system to counteract this stress during the life-cycle of this annual land snail species.

Material and Methods

Test organism

In this study, *X. derbentina* (Krynicki 1836), a hygromiid land snail, was investigated. All samples of *X. derbentina* were collected from a meadow in the vicinity of Modène, department Vaucluse, Southern France (N44°4.034′ E5° 11.041′). Samples were taken randomly from this population. The sampling site was not used agriculturally and no pesticides were applied by the owner. Sampling took place during four different months in 2011 to make sure that different climatic conditions were present during sampling and different life stages of *X. derbentina* could be collected. Samples were taken on April 18, June 13, August 30, and October 17, 2011. All samples were taken on sunny days with none to only little cloudiness. In April, ten snails were collected hourly and individually submerged in liquid nitrogen after recording the following parameters: (a) the heights at which individuals were resting, measured with a yard stick, (b) the temperatures at the surface of their shells, in the middle of the first whorl, that was exposed to the sun, using a medical precision thermometer (ELLAB Copenhagen, type DM 825), (c) the

shell diameter using a digital calliper, and (d) the patterns of shell colouration as introduced by Köhler et al. (2009). For *X. derbentina*, colour category 1 consisted of white shells only, while in category 2 animals with a single small black or brown band near the umbilicus or a brownish shell colour at the umbilicus side of the shell were grouped. Category 3 snails bore two or more bands near the umbilicus or one large intensely pigmented stripe on the umbilicus side of the shell. Snails that were classified into category 4 showed bands all over the shell as well as on its upper part, in the vicinity of the protoconch. It was avoided to touch snails during steps 1 and 2 of the above-mentioned field measurements to prevent artefacts. All snails taken for the Hsp70 analysis were collected from heights ranging between 5 and 20 cm above ground. In June, samples were taken the same way as in April between 4 am and 11 pm, in August, from 4 am to 10 pm, and in October from 5 am till 12 pm. Morphological species determination of samples from this population were carried out by W. Rähle, University of Tübingen, Germany and E. Gittenberger, University of Leiden, the Netherlands. Genetic determination based on COI gene sequencing was performed by S. Sereda and T. Wilke, University of Giessen, Germany.

Hsp70 analysis

For Hsp70 analysis, only individuals which have been resting between 5 and 20 cm above the ground were taken. The individually frozen samples were homogenized with 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 weight including the shells. All homogenization steps were performed on crushed ice to prevent degradation of proteins. The samples were centrifuged for 10 min at 13,722 rpm (=20,000 rcf) using an Eppendorf Centrifuge 5804R at 4 °C. The protein content of the supernatants was determined according to Bradford (1976) using 96-well plates and a plate reader (Bio-Tek Instruments, Winooski, VT, USA). Total protein (10–40 µg/sample, depending on the intensity of resulting Hsp70 bands in preliminary analyses) was analysed using minigel SDS-PAGE (12 % acrylamide, 0.12 % bisacrylamide, 30’ at 80 V plus 90’ at 120 V). The proteins were transferred to nitrocellulose membranes by semi-dry electrotransfer. After incubation in blocking solution (50 % horse serum in TBS) for 2 h, the nitrocellulose membranes were incubated with the first monoclonal α-Hsp70 antibody (mouse anti-human Hsp70, Dianova, Hamburg, Germany, dilution

1:5,000 in 10 % horse serum / TBS) on a lab shaker at room temperature overnight. This antibody cross-reacts with all isoforms of the Hsp70 family. To remove surplus Hsp70 antibodies, the nitrocellulose membranes were rinsed in TBS for five minutes. After 2 h of incubation with the secondary antibody (goat anti-mouse IgG conjugated to peroxidase, Jackson Immunoresearch, West Grove, PA, dilution 1:1,000 in 10 % horse serum/TBS) the nitrocellulose membranes were rinsed again for 5 min in TBS.
Subsequently, the membranes were stained in a solution containing 1 mM 4 chloro(1)naphthol, 0.015 % H2O2, 30 mM Tris pH 8.5 and 6 % methanol. Digitalization of the nitrocellulose membranes was carried out using an Epson Perfection V350 Photo scanner. For each band, the optical volume (= band area × average grey scale value) was calculated with E.A.S.Y. Win 32 (Herolab, Wiesloch, Germany). The optical volumes of the bands were related to a standard sample containing supernatant of full body extract of *Theba pisana* (Müller 1774) snails. In each minigel SDS-PAGE, this standard sample was run in duplicate. All data (means ± standard deviations) were calculated by ten individuals.

Additional sampling for field distribution and colouring

In addition to the samples taken for Hsp70 analysis, 250 individuals were randomly collected from a randomly chosen area of 1×3 m in the same meadow at each sampling event. For each individual, the pattern of shell colouration, the shell diameter, and the position (height above the ground) was recorded. The shell temperature was not recorded here as these additional samples were exclusively used for investigations on the shell growth and colouration patterns.

Recording of temperature at different heights

During the time of each sampling event, the ambient temperatures were recorded in ten different heights simultaneously. For this purpose, Type T thermocouples were placed 1, 2, 3, 5, 10, 15, 20, 25, 30, and 40 cm above the ground using a wooden stand. Each sensor was read out every 15 seconds using a multi-channel data logger (Agilent 34972A). In April, these measurements were carried out by hand using a medical precision thermometer (ELLAB Copenhagen, type DM 825) and a yard stick. In order to condense these data, hourly mean temperatures were calculated for each height.

Statistics

All data were checked for normal distribution using the Shapiro-Wilk W-Test in JMP 9.0.0. (SAS Institute Inc.). Since the data were not normally distributed, nonparametric tests had to be applied. To compare sample sets describing the change of shell diameter during the year, individual Wilcoxon tests were performed between all examined months. Correction for multiple testing was accomplished by adjusting the significance level according to Bonferroni. The resulting α-level was 0.0083. Correlation between the parameters Hsp70 level, shell temperature, shell diameter, and climbing height were performed using SAS JMP 9.0.0. A Spearman's ρ test was performed to check for significance and α-levels were also corrected according to Bonferroni as mentioned above.

Results

Shell growth and colouration

During the 4 days of sampling in 2011, a total number of 1996 individuals were examined. In the course of the year, a significant increase in shell diameter was observed between April (4.62±1.08 mm, n=490), June (9.99±1.30 mm, n= 538), and August (13.35±0.98 mm, n=478; all, Wilcoxon, $p<0.0001$). In October (12.90±0.95 mm, n=490), a slight but significant decrease in shell diameter, compared to August, was found (Wilcoxon, $p<0.0001$, Fig. 1).

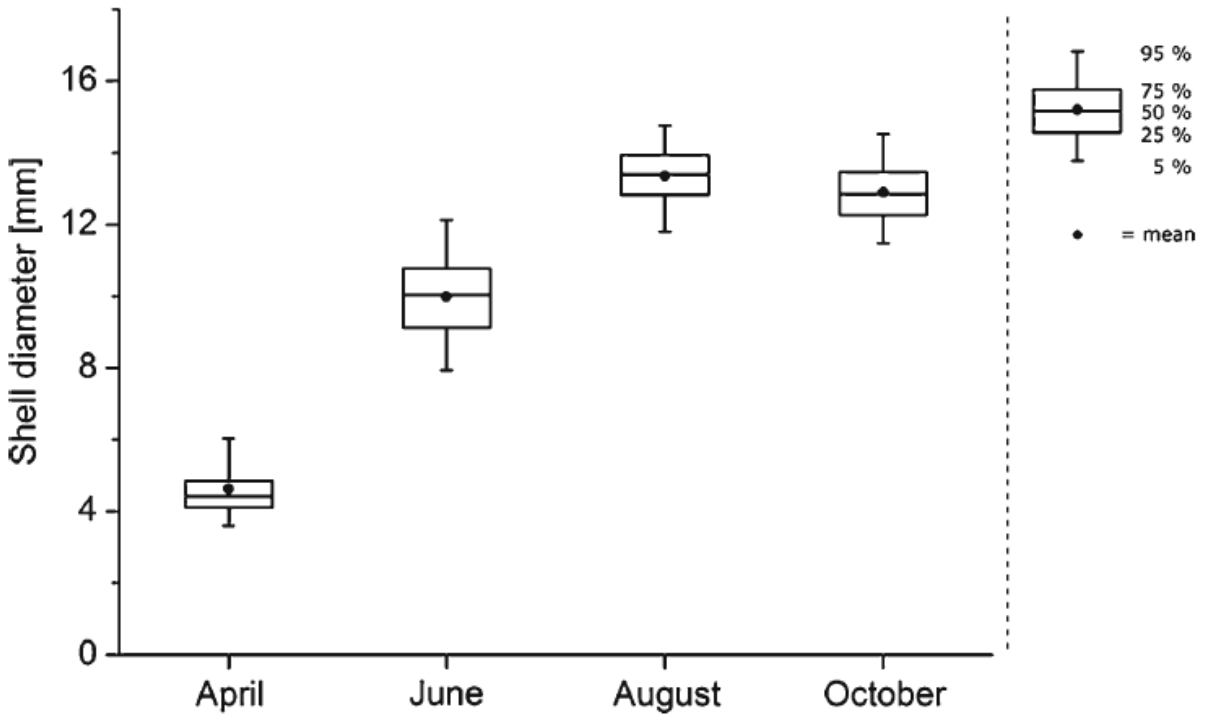

Figure 1. Increase in shell diameter of samples taken in 2011. Boxes indicate 25%, 50%, and 75% percentiles of all samples taken during the corresponding sampling day. Black dots = mean shell diameter, whiskers = 5% and 95% percentiles.

In addition to the observed increase of the shell diameter, snails tended to have paler shell patterns in the course of the year. Although a mixture of the pre-defined categories could be found in April, where category 3 was the predominant colouration (55 % of the total observed snails), almost the entire population displayed a pure white shell in August (96 %) and October (97 %) which was classified as shell pattern category 1. In June, an intermediate situation was present. Compared to the observations from April, a strong increase in the frequency of category 1 snails (78 % category 1 in June vs. 11 % in April) could be found. On the other hand, the number of snails categorized into category 3 decreased from 55 % in April to 5 % in June. The composition of shell patterns in all four samplings is shown in Table 1.

Table 1. Percentage of colour morphs in the selected *X. derbentina* population in four different months in 2011.

Month	**Category 1 [%]**	**Category 2 [%]**	**Category 3 [%]**	**Category 4 [%]**
April (n= 490)	11	30	55	4
June (n= 538)	78	15	5	2
August (n= 478)	96	3	0	1
October (n= 490)	97	3	0	0

Hsp70 induction and ambient parameters

In June, the lowest measured temperature 5 cm above the soil surface was 13.2 °C measured at 4 am. The maximum temperature at the same height was 32.9 °C at 2 pm. In August the temperature 5 cm above ground ranged from 10.4 °C (6 am) to 33.7 °C (3 pm). In these 2 months, the temperature exceeded 30 °C during the day which made conditions different from those in April and October. In April the lowest temperature of all samplings was measured. At a height of 5 cm above the soil surface, it was found to be 4.8 °C (4 am). The maximum temperature at this height in April was 27.3 °C (5 pm). In October, the temperature in 5 cm above ground varied from 7.6 °C (7 am) to 23.0 °C (3 pm). In all months, an increase of air temperature after sunrise was observed as well as a decrease after sunset. By comparing the temperature at different heights, a gradient with decreasing temperatures at increasing heights above the ground was found to be established during the day. At night and during sunrise and sunset, only little temperature differences were recorded at different heights. In April, sunrise was roughly at 6:30 am and sunset roughly at 8:30 pm. In June, sunrise took place around 6 am and sunset around 9:30 pm. In August, sunrise took place at approximately 7 am and sunset at 8:30 pm. In October, sunrise took place at roughly 8 am and sunset at 7 pm. On June 13th, a sudden decrease in ambient temperature was recorded at all heights at 4 pm. At this time, clouds temporarily covered the sky and ambient temperature decreased transiently. Five centimetres above the ground the overall mean temperature of the sampling day in April was calculated to be 14.1 °C, in June 22.3 °C, in August 22.9 °C, and in October 14.7 °C. Temperatures at heights between 1 and 30 cm above ground are presented in Fig. 2 for each sampling.

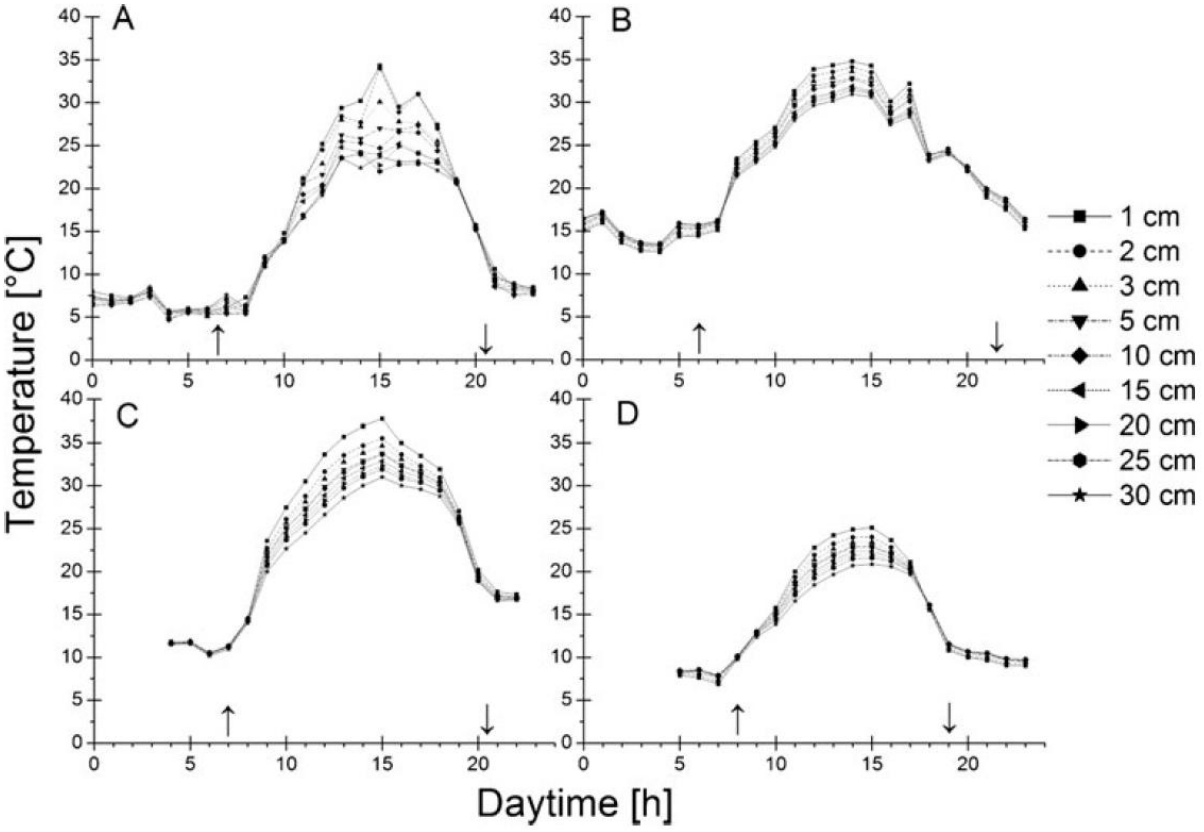

Figure 2. Daily course of the air temperatures at different heights above the ground in Modène, France, during samplings in 2011. a April 18. b June 13. c August 30. d October 17. Sunrise is indicated by an up arrow and sunset by a down arrow.

The daily course of shell temperature largely reflects the course of ambient temperature. A daily increase in shell temperature with progressively increasing time of exposure to solar irradiation was also recorded in all months, as well as a decrease in shell temperature after sunset (Fig. 3). In general, shell temperatures were higher even at night, in June and August compared to the other months.

The analysis of our samples revealed differences between the 4 months of sampling, and even during a single day, changes in Hsp70 induction were found (Fig. 4). Our study showed that, in general, hot months lead to higher Hsp70 levels in *X. derbentina*. In April, a slight increase in the Hsp70 level was revealed from sunrise until noon. The highest relative Hsp70 level in April, however, was just 1.2. In June, the course of the Hsp70 level followed the increase of ambient temperatures in the morning and the decrease of ambient temperatures in the evening (Fig. 5). In addition, a secondary peak of Hsp70 expression was found at night, which

decreased again at around midnight. The highest relative Hsp70 level in June was 2.7. In the samples taken in August, the highest Hsp70 level was 4.4 which was also the maximum recorded for the entire year. Again, an increase of Hsp70 was recorded at sunrise and in the morning when ambient temperatures rose. Except for a relatively low value at 1 pm, a steady increase of Hsp70 levels could be observed till sunset. After sunset, the Hsp70 levels decreased again. In contrast to the other months, samples taken in October did not show any increase in the Hsp70 level during the day. Instead of an increase in the Hsp70 level that follows the ambient temperature, a slight decrease was observed particularly from sunrise until noon. Subsequently, Hsp70 levels rose again at the end of the day until midnight. The highest measured relative Hsp70 level in October was 0.7, even lower than in spring.

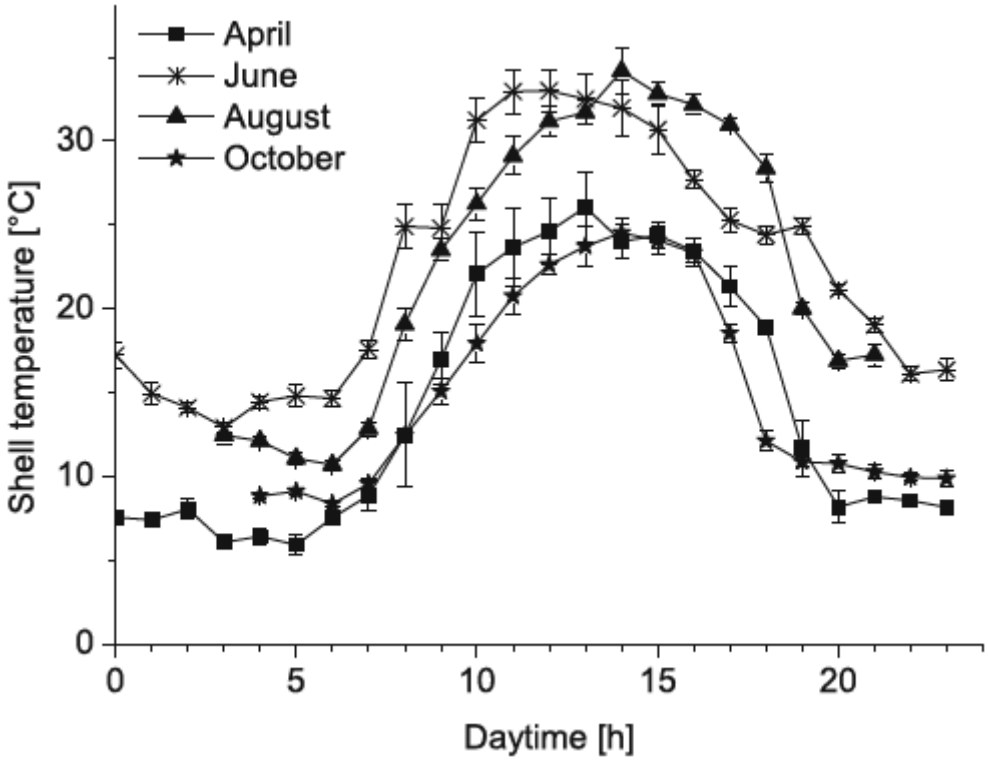

Figure 3. Daily course of the shell temperature in four different months in 2011. Error bars indicate the standard deviation of ten samples taken per hour. Each data point represents the mean value of ten individuals.

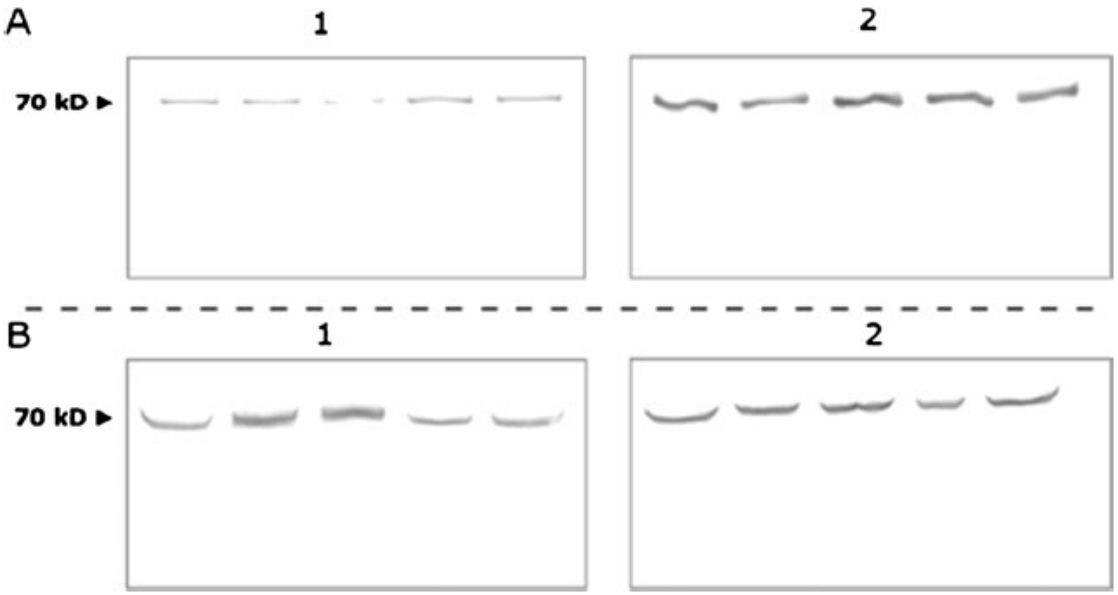

Figure 4. Western blots for two different seasons and two different sampling times. A Samples from June 2011; 1= 0 h, 2=15 h daytime. Ten micrograms of total protein was separated per lane. b Samples from October 2011; 1=0 h, 2= 15 h daytime. Forty micrograms of total protein per lane were separated. Each band represents a single individual.

In April, June, and August, a significant positive correlation between Hsp70 level and shell temperature was found (Spearman's ρ; April, rho=0.3380, n=236, p<0.0001; June, rho=0.5339, n=209, p<0.0001; August, rho=0.3143, n=190, p<0.0001) whereas in October a negative correlation between these two factors was found (Spearman's ρ; rho=−0.3328, n=200, p<0.0001). Compared to the other months of sampling, the majority of the Hsp70 levels measured in October were below those of the other months (Fig.6).

In addition to these results, a negative correlation between the Hsp70 level and the shell diameter was found for snails collected in June (Spearman's ρ; rho=−0.3596, n=209, p<0.0001). For all other samples taken, no significant correlation between these two factors was found. Furthermore, in April a positive correlation between shell diameter and shell temperature was revealed (Spearman's ρ; rho=0.2558, n=236, p<0.0001). No such findings were observed for the samples which were taken in the other months. The factor "climbing height" was recorded for every snail, but no correlation was found between this parameter and any other factor. However, since no general trend was visible for the other months, these occasional

differences must be attributed to stochastic effects and should not lead to further interpretation. Considering that more than 95 % of the population was found to belong to category 1, no statistics were applicable to find correlations between the colouration of the shell and other factors. Only few or no snails were found to contribute to category 3 or 4 in these months.

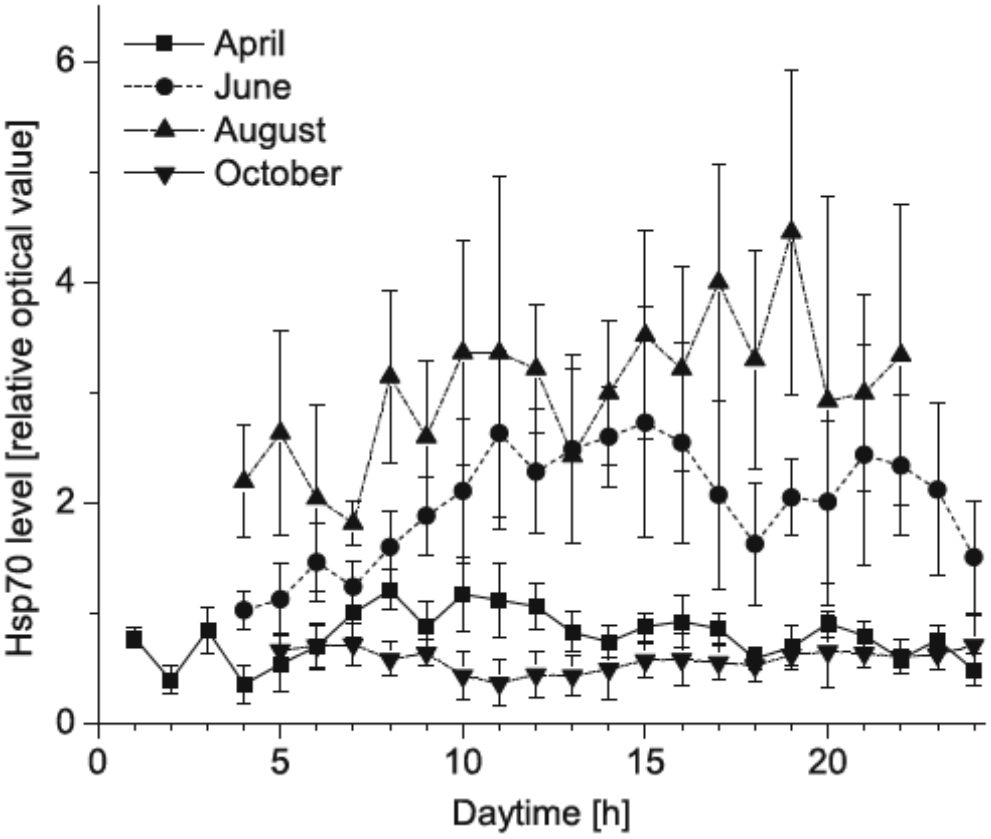

Figure 5. Daily course of mean Hsp70 levels (n=10) obtained from samples taken in 2011. Error bars indicate the standard deviation of ten samples taken per hour.

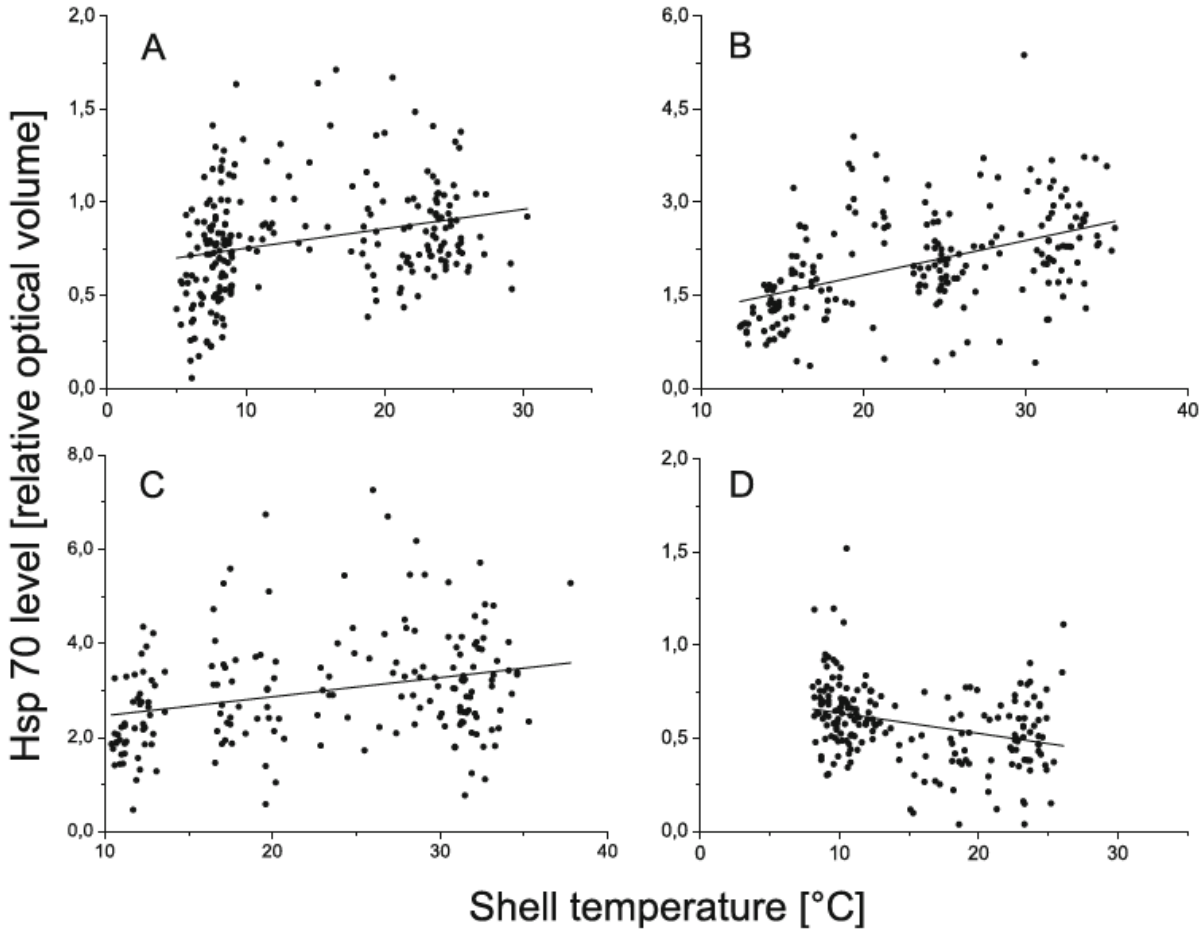

Figure 6. Correlation between Hsp70 level and external shell temperature in the four different months of sampling. a April. B June. c August. d October. In April, June, and August, a significant positive correlation between Hsp70 level and shell temperature was present. In October, a significant negative correlation of these factors was found. For visualization purposes, linear regression lines were added to the figures.

Discussion

In the present study, a field population of *X. derbentina* from Southern France (Modène, Vaucluse) was used to investigate the molecular stress response to ambient temperature. This was accomplished during four different snapshots of a single day, each of these in four different months of one year. In addition to the Hsp70 analysis, we notice the development of colouration and growth in individuals of this population.

Snail growth and colouring

During our samplings in April, June, and August 2011, an increase in shell diameter was found. In April, most of the individuals of this population were around 4.5 mm in diameter; only few were larger than 6 mm. These small snails can most likely be regarded as juveniles that had hatched in spring of 2011. Occasionally found snails of ≥9 mm in size were regarded as survivors from 2010. Similar findings were previously reported for the semelparous annual species *Xeropicta arenosa* (Staikou and Lazaridou-Dimitriadou 1991) in northern Greece as well as by Kiss et al. (2005) for French populations of *X. derbentina* [as long as there is no clarity as to whether *X. derbentina* (Krynicki 1836) and *X. arenosa* (Ziegler) are actually the same species, we treat them as two different ones]. Also for the population in focus of this study, an annual life cycle must be proposed according to the findings of Kiss et al. (2005) and Staikou and Lazaridou-Dimitriadou (1991). In both cases, as well as in our findings, the growth of the snails was continuous from spring until autumn. In our samples the population reached its final mean shell size in August 2011. Even the observed slight decrease in mean shell diameter in October compared to that of August does not support a biennial lifecycle. If hatching of the next generation would have taken place until October, or if snails would have entered aestivation, the mean shell diameter of the sampled population in October would have been much smaller than observed. During the entire sampling in October, no juvenile snails were found in the field.

With respect to the change of the shell colouration pattern of the snails during the course of the year, it was obvious that almost all individuals of the population carried a uniformly white shell when snails have grown to their adult body size in late summer. Particularly morphotypes that fit the pre-defined “category 3” disappeared during the year. Our data suggest that colouration pattern category 3 is typical for at least part of the juvenile snails. The banding may “disappear” when newly produced parts of the shell are forming the next whorl of the shell. Alternatively, the shell pigmentation may fade because of bleaching in ultraviolet light. Our study, however, did not yield information to clarify this question. In other studies (Köhler et al. 2009; Di Lellis et al. 2012) hints on this phenomenon are already given. In their studies, samplings in May revealed partly phenotypic “mixed” populations of *X. derbentina* as well.

Hsp70 induction

Since another study (Di Lellis et al. 2012) has revealed influence of the factor "climbing height" on the Hsp70 level, we have only used snails that were taken from a pre-defined range of height for stress protein analysis. Within this range, no significant effect of the climbing height or correlation between this factor and another parameter was found. This enabled us to relate the stress protein response to the factors "temperature" and "season".

In Southern France, *X. derbentina* snails that consist of 78 % water [including shell, measured as a mean of 15 fully hydrated snails dried to the nearest 0.01 g body weight, measurements performed by A.D. and U.F. in July 2011; similar results were found in *Cantareus apterus* (Born 1778) by Reuner et al. (2008)], have to face comparatively hot conditions during the day. Due to their inactivity during the day, they are not able to take up water from food or from their environment to cool down or to prevent desiccation. During all samplings, activity of snails was found to take place in the cooler night until the early hours of the morning when the sun has not yet heated up the ground. No activity was observed during the day, thus escaping higher temperatures by moving into shaded regions is not an option for *X. derbentina*. Rising ambient temperature results in higher temperature on the surface of the shell and, consequently, also in higher temperature inside the body (Di Lellis et al. 2012). To prevent misfolding of proteins and to counteract consequences of heat stress and desiccation, Hsp70 is usually up-regulated (Sørensen et al. 2003; Mayer and Bukau 2005; Köhler 2009; Kiang and Tsokos 1998; Feder and Hofmann 1999). In our study, a positive correlation between the Hsp70 level and the temperature at the shell surface could be observed for April, June, and August only. In the samples taken in October, a negative correlation for these two variables was found. When comparing the temperature– stress response relationships from April and October, it became obvious that snails lost their ability to react properly to heat stress in October, even though ambient temperature in these 2 months was almost the same. These findings may have occurred for the following reasons.

It is known that older, senescent individuals have reduced Hsp70 levels compared to younger ones (Sørensen and Loeschcke 2002; Mayer and Bukau 2005; Köhler 2009). This may be due to an energetic trade-off between the maintenance of the stress response system and reproduction (Mizrahi et al. 2011). Furthermore, continuously repeated exposure to high temperature during summer, accompanied

by a shortage in energy supply may have reduced the ability of the snails' cells to fully express the energy-costly Hsp system. Moreover, the overwhelming of this stress response machinery in turn could have resulted in cellular pathology as shown by Dittbrenner et al. (2009), Scheil et al. (2011), and S. Troschinski, University of Tübingen (unpublished data) for Mediterranean land snails. The limitation of the stress protein system by environmental parameters resulting in a reduced capacity of organisms to overcome environmental stressors has already been postulated by Nakano & Iwama (2002) and Tomanek (2002). In cases of "overwhelmed" stress physiology, additional stressor action will not result in an induction but rather in a decrease of Hsp70 levels (Eckwert et al. 1997; Tomanek 2002). It is likely that the present results obtained for the October snails should be seen as a consequence stemming from an exhaustion of the stress response system as it was shown before by Scheil et al. (2011) for *X. derbentina*.

Another assumption that could explain the absence of Hsp70 induction in the October snails is, as reported in many studies, that snails, especially in the Mediterranean area, are often able to enter an aestivating phase when conditions turn unfavourable. During this phase metabolism is reduced and the internal milieu of the snails changes (Herreid II 1977; Riddle 1981; Umezurike and Iheanacho 1983; Storey 2002). In our French field population snails did not enter the aestivation phase in April, June, and August as they were foraging on the ground in the night hours during sampling. In October, snails were almost exclusively found resting on the vegetation and only very few snails were active during the night. If snails had entered a temporal aestivation phase due to physiological exhaustion, the low level and limited induction of Hsp70 in snails collected in October could be explained according to the findings of Reuner et al. (2008) who found dormant snails not to express much Hsp70 compared to heat-shocked active ones. Kiss et al. (2005) have shown that populations of *X. derbentina* may be able to change their survival strategy and shift from an annual to a biennial life-cycle, and some of these populations were found to aestivate. In our population, it is more likely that snails entered a short-term aestivationlike phase to temporarily cope with a prolonged phase of dry conditions during autumn 2011. Equivalent to the findings in 2011, predominately small snails were found in spring 2012 on the same sampling ground (personal communication C. Mazzia, University of Avignon). Therefore, it is highly unlikely that large parts of the population had entered a prolonged aestivation phase. In this case, snails with intermediate shell sizes would have been found in spring 2012. As no aestivation was observed, apart from some

periods in autumn, we conclude that aestivation is not part of the survival strategy of the investigated population.

Our results reveal not only a seasonal change of Hsp70 level as reported in several other studies (Nakano and Iwama 2002; Tomanek 2002; Tomanek and Sanford 2003; Arad et al. 2010), but, for one of the few times (Ulmasov et al. 1999), also a daily change in Hsp70 expression in the field. Regarding this daily course it is obvious that Hsp70 levels follow the increase in ambient temperature. In April, where temperatures were lower than in June or August, only a slight increase in the Hsp70 level could be shown during the day. This slight increase indicates that ambient temperatures at that time seemed to generally be below the threshold temperature at which *X. derbentina* starts to up-regulate Hsp70 for their survival. According to Köhler et al. (2009) this threshold temperature should be estimated to be around 30 °C. In experiments where *X. derbentina* was exposed to different temperatures, 24–25 °C was used as a control (Dittbrenner et al. 2009; Köhler et al. 2009; Scheil et al. 2011). On the day of data collection in April, temperatures >25 °C occurred for 5 h only with a measured maximum of 27.3 °C. In June, the Hsp70 levels followed the rise of ambient temperatures till early afternoon and decreased again with sunset in the evening. Additionally at night, a slight elevation of the Hsp70 level was found. This additional Hsp70 peak most likely corresponds to the activity period of the snails that typically starts a few hours after sunset, when temperatures had decreased. During this period, snails were often found on the ground, eating, moving around, and probably being in a phase where the snails have to deal with balancing their internal milieu and producing new proteins (Herreid II 1977; Riddle 1981; Umezurike and Iheanacho 1983; Storey 2002). This happens at a time of the year when snails have not yet reached their final body size, as shown by our results on shell size. Hence, the induction of Hsp70 during the night could be seen as a consequence of the need to chaperone newly synthesized proteins necessary for the animals' growth (Köhler 2009; Mayer and Bukau 2005). In August, the daily course of Hsp70 was shown to remain at a high level but with high standard deviations. A possible reason for this effect could be the interaction of high temperature at that time of the year and the energycostly proceeding maturation of reproductive organs. This may have led to a beginning collapse of the Hsp70 protection system in some individuals. Particularly those snails that are still growing and have not entered maturation seemed to produce high levels of Hsp70 to counteract heat stress; others, which have grown to their final size, may have started with egg production which poses an additional stress on them, overcharging

the capacity of the molecular stress response and resulting in a sub-optimal Hsp70 level. In October temperatures did not reach 25 °C. The recorded maximum in October was 23.0 °C. Given the fact that such a temperature is not high enough to induce Hsp70, only a "base level" of constitutional Hsp70 should remain which was the case in our investigation.

The negative correlation of Hsp70 level and temperature with the rather small range of this "base level" supports the above mentioned assumption of a "physiological exhaustion" of most *X. derbentina* individuals by the long-term heat exposure plus reproduction effects that they have experienced in late summer and particularly autumn.

Our study showed growth and stress response of *X. derbentina* to be in accordance with the requirements posed on an annual population of invertebrates. In spring and early summer, the Hsp70 response remains adequate to counteract possible heat effects, as the strong positive association of ambient temperature and Hsp70 level indicates. This situation seems to continue for a number of individuals also until the late summer, while others already show symptoms of exhaustion of the stress response system. In autumn, the limited capacity to induce Hsp70 suggests senescence. Most individuals die at the end of the year.

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References

Abdel-Rehim AH (1983) The effects of temperature and humidity on the nocturnal activity of different shell colour morphs of the land snail Arianta arbustorum. Biol J Linn Soc 20(4):385–395. doi:10.1111/j.1095-8312.1983.tb01599.x

Altena COR (1960) On the occurrence of a species of Xeropicta in France. Basteria 24:21–25

Arad Z, Mizrahi T, Goldenberg S, Heller J (2010) Natural annual cycle of heat shock protein expression in land snails: desert versus Mediterranean species of Sphincterochila. J Exp Biol 213 (20):3487–3495. doi:10.1242/jeb.047670

Arts M, Schill RO, Knigge T, Eckwert H, Kammenga JE, Köhler HR (2004) Stress proteins (hsp70, hsp60) induced in isopods and nematodes by field exposure to metals in a gradient near Avonmouth, UK. Ecotoxicology 13(8):739–755. doi:10.1007/s10646- 003-4473-5

Aubry S, Labaune C, Magnin F, Kiss L (2005) Habitat and integration within indigenous communities of Xeropicta derbentina (Gastropoda: Hygromiidae) a recently introduced land snail in southeastern France. Divers Distrib 11(6):539–547. doi:10.1111/ j.1366-9516.2005.00189.x

Aubry S, Labaune C, Magnin F, Roche P, Kiss L (2006) Active and passive dispersal of an invading land snail in Mediterranean France. J Anim Ecol 75(3):802–813. doi:10.1111/j.1365- 2656.2006.01100.x

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Anal Biochem 72(1–2):248–254. doi:10.1006/ abio.1976.9999

Daugaard M, Rohde M, Jäättelä M (2007) The heat shock protein 70 family: Highly homologous proteins with overlapping and distinct functions. FEBS Lett 581(19):3702–3710. doi:10.1016/ j.febslet.2007.05.039

Di Lellis M, SeifanM, Troschinski S,Mazzia C, Capowiez Y, Triebskorn R, Köhler H-R (2012) Solar radiation stress in climbing snails: behavioural and intrinsic features define the Hsp70 level in natural populations of Xeropicta derbentina (Pulmonata). Cell Stress and Chaperones:1–11 IN PRESS. doi:10.1007/s12192-012-0344-4

Dittbrenner N, Lazzara R, Köhler H-R, Mazzia C, Capowiez Y, Triebskorn R (2009) Heat tolerance in Mediterranean land snails: histopathology after exposure to different temperature regimes. Journal of Molluscan Studies 75(1):9–18. doi:10.1093/mollus/ eyn033

Eckwert H, Alberti G, Köhler H-R (1997) The induction of stress proteins (hsp) in Oniscus asellus (Isopoda) as a molecular marker of multiple heavy metal exposure: I. Principles and toxicological assessment Ecotoxicology 6(5):249–262. doi:10.1023/ a:1018682928839

Feder ME, Hofmann GE (1999) Heat-shock proteins, molecular chaperones, and the stress response: Evolutionary and ecological physiology. Annual Reviews of Physiology 61:243–282. doi:10.1146/annurev.physiol.61.1.243

Herreid CF II (1977) Metabolism of land snails (Otala lactea) during dormancy, arousal, and activity. Comparative Biochemistry and Physiology Part A: Physiology 56(2):211–215. doi:10.1016/ 0300-9629(77)90187-6

Hofmann GE, Buckley BA, Airaksinen S, Keen JE, Somero GN (2000) Heat-shock protein expression is absent in the Antarctic fish Trematomus bernacchii (family Nototheniidae). J Exp Biol 203(15):2331–2339

Kiang JG, Tsokos GC (1998) Heat Shock Protein 70 kDa: Molecular Biology, Biochemistry, and Physiology. Pharmacol Ther 80 (2):183–201. doi:10.1016/s0163-7258(98)00028-x

Kiss L, Labaune C, Magnin F, Aubry S (2005) Plasticity of the life cycle of Xeropicta derbentina (Krynicki, 1836), a recently introduced snail in mediterranean France. Journal of Molluscan Studies 71(3):221–231. doi:10.1093/mollus/eyi030

Köhler H-R, Bartussek C, Eckwert H, Farian K, Gränzer S, Knigge T, Kunz N (2001) The hepatic stress protein (hsp70) response to interacting abiotic parameters in fish exposed to various levels of pollution. Journal of Aquatic Ecosystem Stress and Recovery (Formerly Journal of Aquatic Ecosystem Health) 8(3):261–279. doi:10.1023/a:1012935931161

Köhler HR (2009) Die Rolle von Stressproteinen bei der Anpassung an Umweltbedingungen: Ökophysiologische, ökotoxikologische und evolutionsbiologische Implikationen. Umweltwissenschaften und Schadstoff-Forschung 21(2):150–159. doi:10.1007/s12302-009-0041-9

Köhler H-R, Lazzara R, Dittbrenner N, Capowiez Y, Mazzia C, Triebskorn R (2009) Snail phenotypic variation and stress proteins: do different heat response strategies contribute to Waddington's widget in field populations? Journal of Experimental Zoology Part B: Molecular and Developmental Evolution 312B(2):136–147. doi:10.1002/jez.b.21253

Mayer M, Bukau B (2005) Hsp70 chaperones: Cellular functions and molecular mechanism. Cellular and Molecular Life Sciences 62 (6):670–684. doi:10.1007/s00018-004-4464-6

Mizrahi T, Heller J, Goldenberg S, Arad Z (2010) Heat shock proteins and resistance to desiccation in congeneric land snails. Cell Stress & Chaperones 15(4):351–363. doi:10.1007/s12192-009-0150-9

Mizrahi T, Heller J, Goldenberg S, Arad Z (2011) Heat shock protein expression in relation to reproductive cycle in land snails: Implications for survival. Comp Biochem Physiol A-Mol Integr Physiol 160(2):149–155. doi:10.1016/j.cbpa.2011.05.031

Mizrahi T, Heller J, Goldenberg S, Arad Z (2012) The heat shock response in congeneric land snails (Sphincterochila) from different habitats. Cell Stress and Chaperones 17(5):639–645. doi:10.1007/s12192-012-0340-8

Nakano K, Iwama GK (2002) The 70-kDa heat shock protein response in two intertidal sculpins, Oligocottus maculosus and O. snyderi: relationship of hsp70 and thermal tolerance. Comparative Biochemistry and Physiology - Part A: Molecular & Integrative Physiology 133 (1):79–94. doi:10.1016/s1095-6433(02)00115-0

Reuner A, Bruemmer F, Schill RO (2008) Heat shock proteins (Hsp70) and water content in the estivating Mediterranean Grunt Snail (Cantareus apertus). Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology 151(1):28–31. doi:10.1016/j.cbpb.2008.05.004

Riddle WA (1981) Hemolymph osmoregulation and urea retention in the woodland snail, Anguispira alternata (Say) (Endodontidae). Comparative Biochemistry and Physiology Part A: Physiology 69 (3):493–498. doi:10.1016/0300-9629(81)93009-7

Scheil AE, Köhler H-R, Triebskorn R (2011) Heat tolerance and recovery in Mediterranean land snails after pre-exposure in the field. Journal of Molluscan Studies 77(2):165–174. doi:10.1093/ mollus/eyr003

Schill RO, Gayle PMH, Kohler HR (2002) Daily stress protein (hsp70) cycle in chitons (Acanthopleura granulata Gmelin, 1791) which inhabit the rocky intertidal shoreline in a tropical ecosystem. Comp Biochem Physiol C-Toxicol Pharmacol 131(3):253–258. doi:10.1016/s1532-0456(02)00012-1

Sørensen JG, Kristensen TN, Loeschcke V (2003) The evolutionary and ecological role of heat shock proteins. Ecol Lett 6(11):1025– 1037. doi:10.1046/j.1461-0248.2003.00528.x

Sørensen JG, Loeschcke V (2002) Decreased heat-shock resistance and down-regulation of Hsp70 expression with increasing age in adult Drosophila melanogaster. Funct Ecol 16(3):379–384. doi:10.1046/j.1365-2435.2002.00639.x

Staikou A, Lazaridou-Dimitriadou M (1991) The life-cycle, population-dynamics, growth and secondary prouction of the snail Xeropicta arenosa Ziegler (Gastropoda, PulmonataI in northern Greece. Zool J Linn Soc 101(2):179–188. doi:10.1111/ j.1096-3642.1991.tb00892.x

Storey KB (2002) Life in the slow lane: molecular mechanisms of estivation. Comparative Biochemistry and Physiology - Part A: Molecular & Integrative Physiology 133(3):733–754. doi:10.1016/s1095-6433(02)00206-4

Tomanek L (2002) The Heat-Shock Response: Its Variation, Regulation and Ecological Importance in Intertidal Gastropods (genus Tegula). Integr Comp Biol 42(4):797–807. doi:10.1093/icb/ 42.4.797

Tomanek L, Sanford E (2003) Heat-Shock Protein 70 (Hsp70) as a Biochemical Stress Indicator: an Experimental Field Test in Two Congeneric Intertidal Gastropods (Genus: Tegula). Biol Bull 205 (3):276–284

Ulmasov K, Zatsepina O, Molodtsov V, Evgen'ev M (1999) Natural body temperature and kinetics of heat-shock protein synthesis in the toad-headed agamid lizard Phrynocephalus interscapularis. Amphibia-Reptilia 20(1):1–9. doi:10.1163/ 156853899X00015

Umezurike GM, Iheanacho EN (1983) Metabolic adaptations in aestivating giant African snail (Achatina achatina). Comparative Biochemistry and Physiology Part B: Comparative Biochemistry 74 (3):493–498. doi:10.1016/0305-0491(83)90216-x

Kapitel 3: Intraspecific variation in cellular and biochemical heat response strategies of Mediterranean *Xeropicta derbentina* [Pulmonata, Hygromiidae]

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Abstract

Dry and hot environments challenge the survival of terrestrial snails. To minimize overheating and desiccation, physiological and biochemical adaptations are of high importance for these animals. In the present study, seven populations of the Mediterranean land snail species *Xeropicta derbentina* were sampled from their natural habitat in order to investigate the intraspecific variation of cellular and biochemical mechanisms, which are assigned to contribute to heat resistance. Furthermore, we tested whether genetic parameters are correlated with these physiological heat stress response patterns. Specimens of each population were individually exposed to elevated temperatures (25 to 52°C) for 8h in the laboratory. After exposure, the health condition of the snails' hepatopancreas was examined by means of qualitative description and semi-quantitative assessment of histopathological effects. In addition, the heat-shock protein 70 level (Hsp70) was determined. Generally, calcium cells of the hepatopancreas were more heat resistant than digestive cells - this phenomenon was associated with elevated Hsp70 levels at 40°C.We observed considerable variation in the snails' heat response strategy: Individuals from three populations invested much energy in producing a highly elevated Hsp70 level, whereas three other populations invested energy in moderate stress protein levels - both strategies were in association with cellular functionality. Furthermore, one population kept cellular condition stable despite a low Hsp70 level until 40°C exposure, whereas prominent cellular reactions were observed above this thermal limit. Genetic diversity (mitochondrial cytochrome c oxidase subunit I gene) within populations was low. Nevertheless, when using

genetic indices as explanatory variables in a multivariate regression tree (MRT) analysis, population structure explained mean differences in cellular and biochemical heat stress responses, especially in the group exposed to 40°C. Our study showed that, even in similar habitats within a close range, populations of the same species use different stress response strategies that all rendered survival possible.

Introduction

Animals which live in dry and hot habitats have to cope with desiccation and overheating. Land snails are particularly affected by these adverse conditions due to their water-permeable skin (Machin 1964) and, therefore, developed a range of behavioral, physiological, and morphological adaptations to ensure survival in arid habitats. Climbing on vegetation to escape from hot ground temperature and restriction of activity phases to favorable time periods can be regarded as behavioral adaptations (Pomeroy 1968, Yom-Tov 1971). As a physiological mechanism of adaptation, aestivation accompanied by a decrease of the metabolic rate (Guppy & Withers 1999, Bishop & Brand 2000) during dry and hot seasons allows land snails even to survive in extreme arid climates of deserts (Schmidt-Nielsen et al. 1971). Morphological adaptations are reflected in variations in shell structure, shell aperture, and size, as well as in body and shell color, or the thickness of the epiphragm (Yom-Tov 1971, Riddle 1983, Goodfriend 1986).

The hepatopancreas plays a major role in the metabolism of mollusks (Sumner 1965, Taieb & Vicente 1998). Alterations and cellular damage caused by different stressors, including heat stress, rapidly occur in the hepatopancreas, which makes this organ suitable to monitor and study cellular responses (Kammenga et al. 2000, Triebskorn 2005). It is also known that calcium cells, representing one cell type of the hepatopancreas, play an important role in osmoregulation (Taieb & Vicente 1998) and acid-base balance (Burton 1976), both of which can be affected by high temperature.

Stress or heat shock proteins (Hsp) can be induced by heat and many other proteotoxic stressors in several organisms (Lindquist & Craig 1988, Feder & Hofmann 1999). The 70kD stress protein family, Hsp70, is a main component of the cellular heat stress response system and protects the cell against the proteotoxic action of elevated temperature and numerous other stressors (Lindquist 1986,

Parsell & Lindquist 1993, 1994). Though isoforms of this protein class are constitutively present already under homeostatic conditions in the cell - e.g. acting as chaperones during protein folding processes, stabilizing proteins in intracellular trafficking, and playing an essential role in assembly, degradation and intracellular localization of proteins (Hendrick & Hartl 1993, Fink 1999, Mayer & Bukau 2005) - the expression of some isoforms of Hsp70 is up-regulated under the influence of a proteotoxic stressor and, therefore, can be used as a marker for proteotoxic stress. Moreover, several studies indicate that genetic differences among populations may be associated with Hsp70 expression levels (Sørensen et al. 2001, Jensen et al. 2009, Bahrndorff et al. 2010), though the effect of population structure on heat response differences in invertebrates is, in general, not well understood.

A land snail species that seems to be particularly well adapted to heat stress is the pulmonate *Xeropicta derbentina* (Krynicki, 1836). These snails are highly abundant in the Mediterranean region and can build up large populations with hundreds of thousands of individuals. During daytime, they remain inactive on vegetation, fully exposed to sunlight. Although several studies exist on thermotolerance and adaptations to heat stress in land snails in general (Yom-Tov 1971, Staikou 1999, Mizrahi et al., 2010) and in Mediterranean species in particular (Dittbrenner et al. 2009, Köhler et al. 2009, Scheil et al. 2011, Di Lellis et al. 2012, Dieterich et al. 2013), as well as on the genetic structure in the Helicidae (Pfenninger & Magnin 2001, Pfenninger & Posada 2002, Pfenninger et al. 2003), comprehensive investigations combining cellular and biochemical reactions to heat stress with population structure information are lacking. We here used *X. derbentina* as a model organism and investigated variations in heat response mechanisms after exposure to elevated temperatures in seven populations.

Specifically, we studied whether different populations of the same species collected within a range of a few kilometers in similar habitats utilize different biochemical and cellular strategies to deal with heat stress, and tested whether physiological heat stress response data can be explained with population structure information. For this purpose, genetically characterized specimens were individually exposed to elevated temperatures (25 to 52°C) under laboratory conditions. Then, effects of heat stress were assessed by histopathological (cellular) and stress protein level (biochemical) biomarkers, and the correlation between genetic structure and heat stress response data tested using multivariate statistics.

Material and Methods

Sampling sites

Individuals of seven populations of *Xeropicta derbentina* were collected in the last week of May 2010 in the Vaucluse area, Provence, Southern France (Table 1). All sampling sites were dry, open, and sun-exposed habitats and similar in structure and vegetation.

For each sampling site, approximately 200 snails were collected and kept separately in plastic containers (20.5 × 30 × 19.5 cm). For genetic analyses, 20 snails per sampling site were collected and stored in liquid nitrogen.

Table 1. Coordinates and locality of the different sampling sites.

Population	Locality	Coordinates
1	Modène 1	N 44° 6.055' E 5° 7.937'
2	Modène 2	N 44° 6.157' E 5° 7.733'
3	Modène 3	N 44° 6.391' E 5° 7.032'
4	St. Pièrre	N 44° 6.053' E 5° 8.311'
5	Mazan 1	N 44° 1.511' E 5° 6.446'
6	Mazan, Bon Reméde	N 44° 2.653' E 5° 8.213'
7	Mazan 2	N 44° 3.974' E 5° 8.084'

Experimental setup

In the laboratory, the snails were acclimatized to 25°C for 2 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. Twenty-two individuals per population were exposed as a

group in individual plastic containers to temperatures of 25, 33, 38, 40, 43, 45, 48, 50 and 52°C for 8h. As the two highest temperature regimes (50 and 52°C) were lethal for the snails, these groups were excluded from both histopathological and stress protein analyses. Due to constraints in lab capacity, snails exposed to 38 and 45°C were not investigated histologically. 25°C was used as control temperature.

Even though tub effects cannot be totally excluded in such an experimental design, we adjusted the conditions in the different plastic boxes equal, to the best of our possibilities, in order to minimize them.

After eight hours of exposure, eight randomly selected individuals from each experimental group were used for the histological studies. For the stress-protein analyses, ten individuals per group were individually shock-frozen in liquid nitrogen and stored at -25°C until further analysis. In order to avoid a potential bias introduced by body size, we conducted a pre-test correlating Hsp70 values (both base level and levels recorded after temperature exposure) and body size (sliding caliper measured shell diameter).

Histopathological analyses

First, the shells of the snails were cracked between two glass slides and removed. Immediately after cracking, the snails were fixed in 2% glutardialdehyde (25% glutardialdehyde dissolved in 0.01M cacodylate buffer, pH 7.4) and stored for at least one week at 4°C. After overnight de-calcification in a 1:2 mixture of formic acid and ethanol (70%) to remove leftover shell fragments, the samples were dehydrated in a graded series of ethanol and embedded in epoxy resin (Technovit, Heraeus Kulzer GmbH, Wehrheim, Germany). Tissue sections with a thickness of 7 µm were prepared using a Reichert Jung 2050 rotation microtome, stained with haematoxylin-eosin, and analyzed by light microscopy.

For each individual, the condition of the hepatopancreas cells (digestive and calcium cells only; excretory cells were excluded from the analysis) and the structural appearance of the tubules were qualitatively described and semi-quantitatively assessed according to the method described by Dittbrenner et al. (2009). For the semi-quantitative assessment, five categories at a scale from 1 to 5 reflecting the histopathological damage were defined: category 1, control status; category 3, status of reaction; category 5, status of destruction; categories 2 and 4

are chosen as intermediate stages between 1 and 3 or 3 and 5, respectively. Table 2 shows the criteria for each cell type and the tubule tissue for the classification into the three main categories.

The condition of the tubules and the two cell types were individually assessed for each snail and, finally, the individual assessments were averaged to get a mean assessment value (MAV) for each population at a given temperature. In addition, the average percentage of calcium cells in the hepatopancreas was determined according to the method by Dittbrenner et al. (2009).

Table 2. Criteria for histopathological effects in the tubule and cell types in the hepatopancreas for classification in the three main categories of the semi-quantitative assessment.

	Category 1: control status	Category 3: status of reaction	Category 5: status of destruction
Digestive cells	· Columnar in shape	· Irregular cell shape	· Cells damaged
	· Nucleus oval in shape	· Irregular nucleus shape	· Necrosis
	· Clear cellular compartmentation (vacuolisation: apical small, basal large vacuoles)	· Irregular cellular compartmentation (irregular vacuolisation)	
Calcium cells	· Cone-shaped cells	· Irregular cell shape	· Cells damaged
	(broad basis, slim apex)		
	· Large round-shaped nucleus	· Irregular nucleus shape	· Necrosis
	· Dense and consistent cytoplasm	· Irregular cytoplasm	
Tubule	· Smooth apices	· Irregular apices	· Tubule damaged
	· Smooth basic	· Large lumina	
	· Tight lumina		

Stress protein analyses (HSP70)

Deep-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 (2 µL 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 method of Bradford (1976) via protein-dye binding assay 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 Western blotting. The membranes were blocked in a 1:2 mixture of horse serum and TBS (50mM Tris, pH 5.7, 150mM NaCl) for 2 hours. Subsequently, the membranes were incubated in the first antibody solution containing monoclonal α-Hsp70 antibody (mouse anti-human Hsp70, Dianova, Hamburg, Germany, dilution 1:5000 in 10% horse serum in TBS) on the 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.

For each population, the maximum percentage of stress protein (Hsp70) induction was determined as the quotient of the Hsp70 level for the respective exposure groups and the Hsp70 level of the control group at 25°C (control = 100%).

DNA isolation, amplification and sequencing

Genomic DNA was extracted from the foot tissue of deep-frozen specimens using the DNeasy Blood & Tissue Kit (QIAGEN, Inc., Mississauga, Ontario, USA). We amplified a fragment of the mitochondrial cytochrome *c* oxidase subunit I (COI) gene with a target length of 700 base pairs (excluding primer sequence). Forward

and reverse primers for PCR amplification and DNA sequencing were LCO1490 (Folmer et al. 1994) and the newly developed primer HeliR2 5'-CCTAAAATATGWGAAAYAATACCAAA-3'. Bidirectional DNA sequencing according to the 'Sanger' chain-termination method was performed by LGC Genomics (Berlin, Germany) using an ABI 3730 XL DNA analyzer. Consensus sequences were generated in BioEdit 7.0.9.0 (Hall 1999) and deposited in GenBank (GenBank accession numbers KF734452- KF734589).

Statistical analyses

Correlation analyses of histopathological and stress protein data

Histopathological and biochemical (Hsp70) data were analyzed using JMP 9 (SAS Institute Inc., Cary, NC). As the Shapiro-Wilk test showed data not to be normally distributed, the nonparametric Wilcoxon U-Test was used to detect significant differences between the control group and each treatment. To counteract the problem of multiple comparisons, a Bonferroni correction was used. For histopathological data, the levels of significance were defined as: $0.0025 < P \leq 0.0125$: * (slightly significant); $0.00025 < P \leq 0.0025$: ** (significant); $P \leq 0.00025$: *** (highly significant). For the data of the stress protein induction (Hsp70 level), the levels of significance were defined as: $0.0017 < P \leq 0.0083$: * (slightly significant); $0.00017 < P \leq 0.0017$: ** (significant); $P \leq 0.00017$: *** (highly significant) after Bonferroni correction.

Correlations of relative Hsp70 levels vs. snail shell sizes were conducted using JMP 9. Correlations of the relative Hsp70 levels vs. the histopathological assessment values recorded for the respective cell types and the tubule condition as well as the illustration of the ratio of digestive cell / calcium cell integrity vs. temperature and the correlation of relative Hsp70 level vs. histopathological condition illustrating the population's heat response strategies were done with SigmaPlot 2000 (SPSS Inc.).

Analysis was conducted with JMP 9. Based on the results for Hsp70 levels which revealed considerable differences in the populations´ maximum of stress protein induction at 40°, we used the Hsp70 response at this temperature to test for statistically significant differences among populations. The separate analysis of the factors was necessary since they showed a significant interaction (2-way ANOVA, $F = 4.411$, $p < 0.0001$). Normality and homogeneity of variance were confirmed with

the Shapiro-Wilk test (W = 0.9791; p = 0.2923) and the Levene´s test ($F_{6,63}$ = 1.8059; p = 0.1122). To detect significant differences among populations in this treatment, we used an ANOVA followed by the Tukey-Kramer HSD post-hoc test. Level of significance was set to 0.05. The box plot illustration was done with SigmaPlot 2000.

Network analysis

Cryptic species may coexist within the range of morphologically undistinguishable heliciid snails (Dépraz et al. 2010). For excluding this possibility for our sample populations of *X. derbentina*, we constructed a statistical parsimony haplotype network from all sequences generated in order to test whether all haplotypes can be connected in a parsimonious fashion. The analysis was done using the program TCS 1.21 (Clement et al. 2000) with the default connection limit of 95%.

Calculation of population indices

For testing whether genetic parameters of the populations studied significantly reflect mean differences in cellular and biochemical heat stress response, three population indices were calculated from the COI dataset. They comprised within-site ('diversity') and between-site ('divergence') parameters.

The first parameter was nucleotide diversity π (average number of nucleotide differences per site within populations based on the K2P model of sequence evolution), estimated in Arlequin 3.5.1.2 (Excoffier et al. 2005).

The two divergence parameters were Nei's (1973) pairwise fixation index (F_{ST}) and haplotype divergence (H_{MH}) based on the Morisita-Horn index (Horn 1966), both calculated in the R 2.15 statistical environment (2011). For the former index, we used the adegenet package (version 1.3-6) (Jombart 2008); for the latter index we treated haplotypes as species (Helmus et al. 2007, Schrader et al. 2013) and estimated the dissimilarity between the haplotype structures of two groups with the vegan package (version 1.17-7) (Oksanen et al. 2013).

Correlation of physiological heat stress response and genetic data

In order to test whether genetic snail parameters, in principle, are correlated with physiological heat stress responses, we performed Multivariate Regression Tree (MRT) analyses (De'ath 2002). This multivariate statistics was specifically designed to assess relationships between species information and environmental data. According to the original author, the method is particularly suited to analyze complex environmental information that includes imbalanced and missing information, and non-linear relationships among variables (De'ath 2002). Using this method, the genetic indices estimated above were assessed for their predictive value to discriminate splits in hierarchical dichotomous clustering of physiological heat stress response data (i.e., Hsp70 and histological data). The clusters and their dependence on the physiological data are graphically represented by a tree. MRT´s are not based on traditional significance testing but on 10-fold cross-validation (CV) in order to determine the number of nodes and the importance of predictor variables (De'ath 2002). Accordingly, the selected genetic indices maximize the homogeneity of measures of stress response within groups of populations and this separation is consistent even if 10% of the populations are omitted.

As it is not possible to use between-population indices (i.e., H_{MH} and F_{ST}) for explaining within-population heat stress responses, we performed classical multidimensional scaling (PCoA) with the coordinates of each population assigned to the ordination axes (De'ath 2002, Legendre & Gallagher 2001). Hsp70 and histological data were assembled according to populations. Then we constructed individual MRTs for each set of temperature specific heat stress response data (i.e., 25, 33, 40, and 48°C) with the mvpart 1.6-0 package (De'ath 2013) in R 2.15 and 1000 CV. Dependent physiological variables were mean Hsp70 levels and mean assessment values for tubules, calcium cells, and digestive cells; genetic explanatory variables consisted of π and PCoA transformed divergence parameters $H_{MH}1$-$H_{MH}3$ (3 axes), and $F_{ST}1$/$F_{ST}2$ (2 axes).

Results

Histopathology

Qualitative assessment

The observed reactions in the hepatopancreas were qualitatively similar in all seven populations. Hence, the results can be summarized as follows.

Tubules: In the control group, the lumina of the tubules were narrow and the cellular bases and apices appeared relatively smooth. After exposure to higher temperatures, tubules of the digestive gland showed enlarged lumina with ruptured apices and also irregular bases primarily caused by hypertrophic calcium cells (Fig. 1D, F). Cell fragments, notably of the digestive cells, could be found in the lumina after 48°C exposure (Fig. 1F).

Digestive cells: The digestive cells showed a regular vacuolisation and compartmentation and an oval-shaped nucleus in the control group (Fig. 1A). Following elevated temperature levels, we found incidence of an irregular cellular compartmentation and vacuolization with enhanced and partially fused vacuoles (Fig. 1B). The cell apices appeared convex, protruded into the lumen of the tubules, and were often ruptured (Fig. 1B, D, F). Deformation and enlargement of the nuclei could be detected. Especially in the groups exposed to higher temperature, the cell apices were ruptured, cell borders were disengaged, and nuclei damaged (Fig. 1D, F). Both cell lysis and necrosis increasingly occurred in the 48°C group (Fig. 1F).

Calcium cells: In the control group, calcium cells showed a dense cytoplasm and spherical nuclei (Fig. 1B). With elevated temperature, reduced density of the cytoplasm with bright spots, disturbed compartmentation, and an increasing vacuolisation could be observed (Fig. 1C, E). Furthermore, the nuclei of the calcium cells were either enlarged or deformed with reduced size and, additionally, appeared dark. Cell shape was altered and, in few cases, hypertrophy of the cells could be detected (Fig. 1C). Necrosis could be observed at 48°C (Fig. 1F).

Semi-quantitative assessment

Within all populations, the structural symptoms observed in the temperature-exposed test groups and categorized as described above were compared to the control group (25°C).

The integrity of the hepatopancreatic tubules became significantly different from the control status after exposure to 40°C (population 2), 43°C (population 2), and 48°C (populations 1, 2, 3, 5, 6 and 7). Slightly significant differences from the control status occurred at 40°C (populations 5 and 7), 43°C (populations 1 and 5), and 48°C (population 4) (Fig. 2).

The digestive cells reacted in a significantly different way from the control group after exposure to 48°C in all populations. Already slightly significant differences were detected after exposure to 33°C (populations 1 and 3), 40°C (populations 2 and 3), and 43°C (populations 1, 2, 3 and 7). Population 7 showed a significant impairment after exposure to 40°C or 48°C, and a slightly significant deterioration of the digestive cells in the 43°C group (Fig. 3).

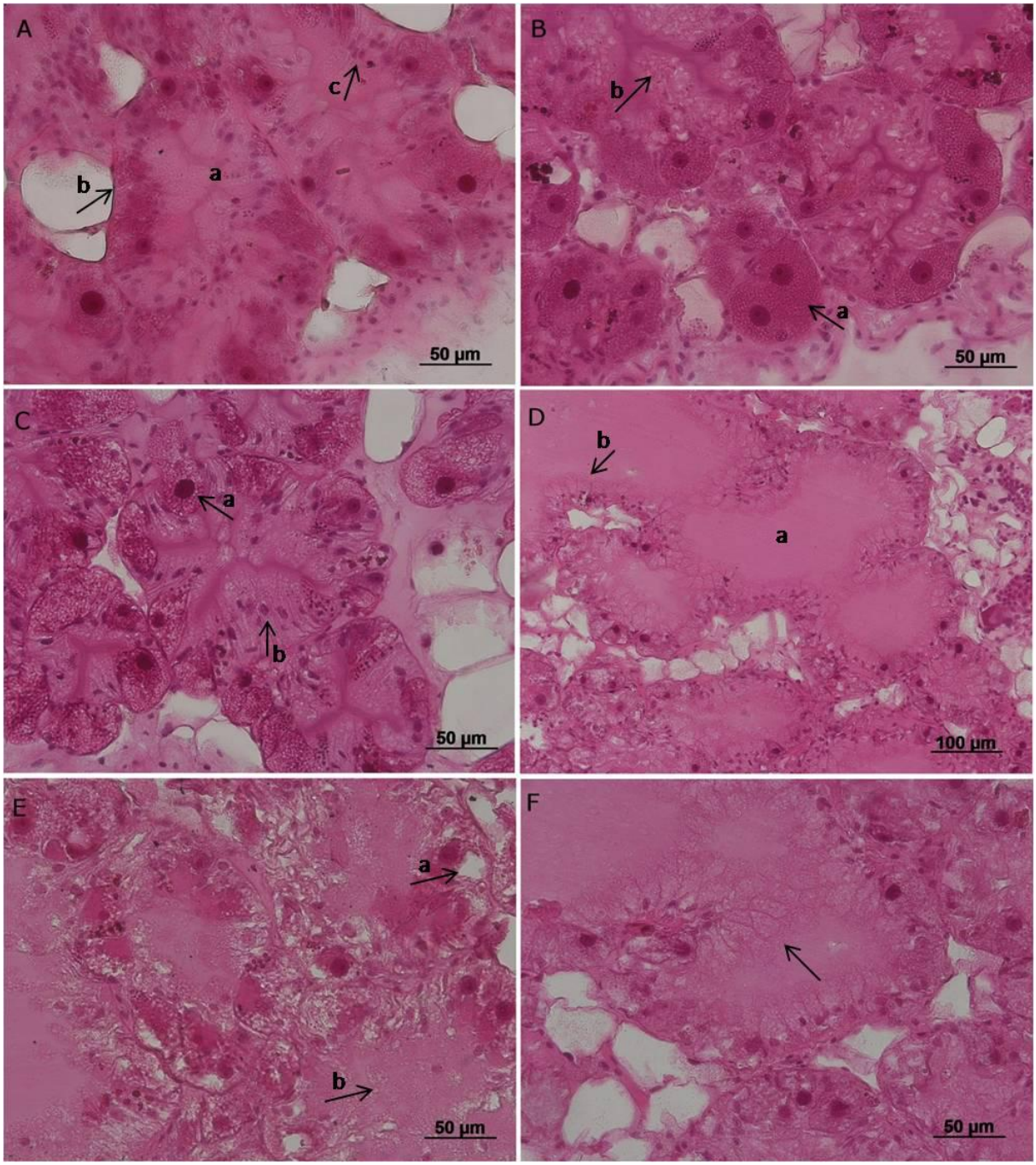


Figure 1. Digestive gland of *Xeropicta derbentina* in different reaction states. A. Digestive gland of a control animal. a. indicates tight lumina, b. a smooth base of the tubule and c. shows an oval-shaped nucleus and regular vacuolization of the digestive cells. **B.** Digestive gland of a control animal. a. shows a calcium cell with dense cytoplasm and round nucleus. b. indicates an irregular vacuolization of the digestive cells with partially fused vacuoles. **C.** Digestive gland in state of reaction. a. indicates dark nuclei and an irregular cytoplasm of the calcium cells. Also hypertrophy of the calcium cells occurs. **D.** Digestive gland in state of reaction. a. shows enlarged lumina of the tubule and b. shows pronounced and ruptured apices of the digestive cells. **E.** Digestive gland in state of destruction. a. indicates a very irregular cytoplasm with bright spots in the calcium cells. b. shows cell fragments in the lumen of the tubule. Cell borders are disengaged. **F.** Digestive gland in state of destruction showing necrosis. The arrow indicates ruptured cell apices. Cell borders are disengaged.

The calcium cells displayed slightly significant reactions after exposure to 48°C (populations 4 and 7). Population 2 exhibited a slightly significant impairment already after 33 and 40°C exposure and a significant difference from the control status after exposure to 48°C. Also populations 1, 5, and 6 showed first significant deterioration of the calcium cells after exposure to 48°C. Population 3 did not show any significant alterations to the control, even under high temperature regimes, but the calcium cells were already in a 'status of reaction' in the control group (Fig. 4).

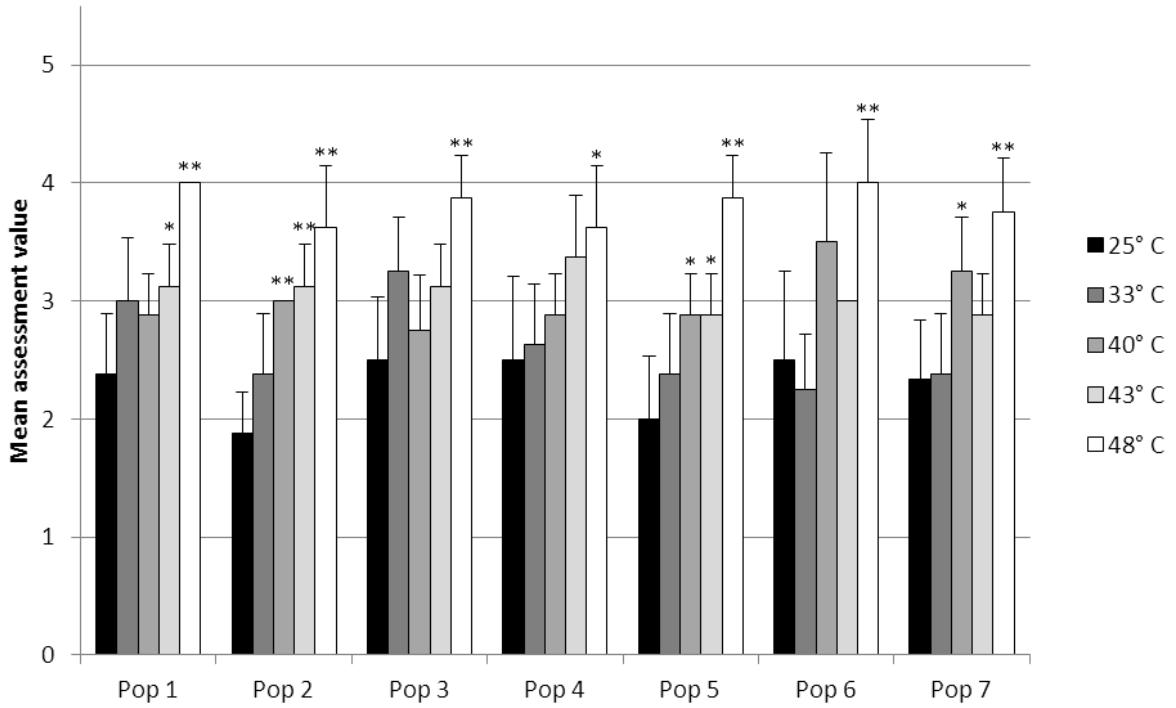


Figure 2. The structural condition of the hepatopancreatic tubules. Mean assessment values for each population at elevated temperature. Shown are means and SD; *n*=8. Asterisks show significant differences of the respective exposure groups compared to the control at 25°C after Bonferroni correction: $0.0025 < P \leq 0.0125$: (*) and $0.00025 < P \leq 0.0025$ (**).

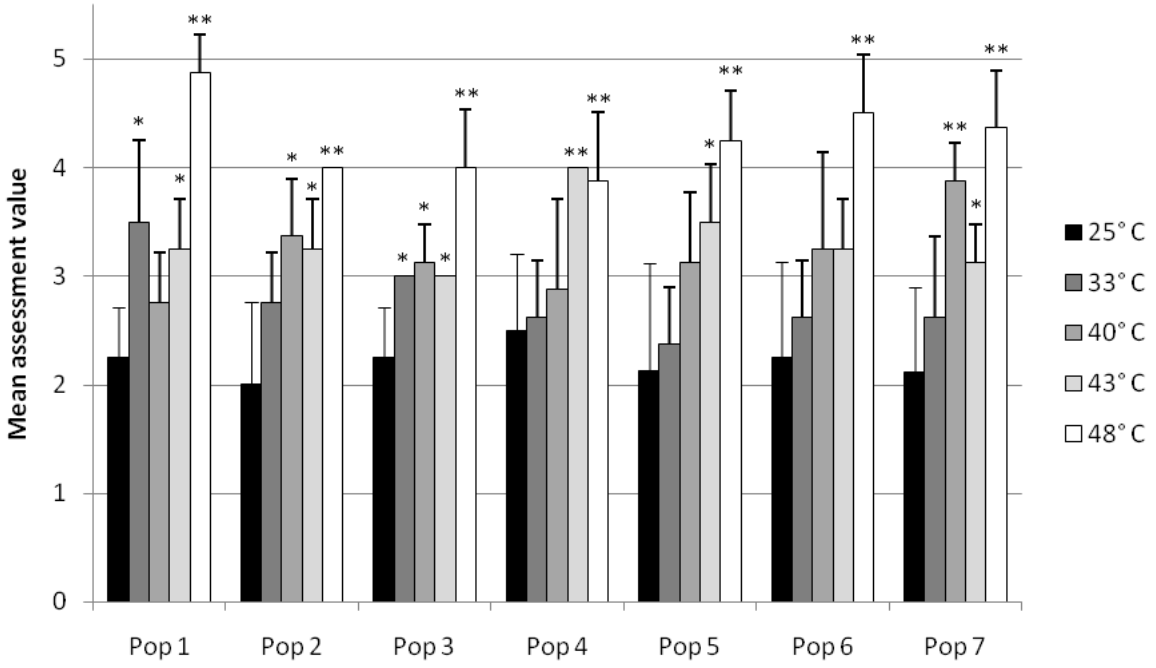


Figure 3. The condition of the digestive cells of the hepatopancreas. Mean assessment values for each population at elevated temperature. Shown are means and SD; *n*=8. Asterisks show significant differences of the respective exposure groups compared to the control at 25°C after Bonferroni correction: 0.0025 < *P* ≤ 0.0125: (*) and 0.00025 < *P* ≤ 0.0025 (**).

Percentage of calcium cells

No significant increase in the ratio of calcium cells in the digestive gland could be detected at elevated temperature levels in any of the studied populations. In comparison to the results of Dittbrenner et al. [27] who observed a gradual increase of calcium cells after exposure to different temperature regimes in two populations of *X. derbentina*, our investigated populations already showed a rather high number of calcium cells (around 40-50% of all digestive gland cells) in the control group (25°C) and were not able to increase this ratio significantly at high temperatures.

Ratio of digestive cell and calcium cell integrity

Digestive cells and calcium cells showed different modes of reaction at elevated temperature in the investigated populations. A ratio of the integrity of the digestive cells and the integrity of the calcium cells was calculated for each population and exposure group, illustrated in Fig. 5. In general, digestive cells remained in a better

health condition than the calcium cells at lower temperatures (25 and 33°C). With elevated temperature, however, the digestive cells became more deteriorated than the calcium cells. Populations 1-5, and 7 showed a continuously increasing deterioration of the digestive cells, compared to the condition of the calcium cells, up to exposure to 43°C (or 40°C, population 7). In population 6, however, the condition of the digestive cells was slightly 'better' than the condition of the calcium cells in the 25°C group, but following elevating temperature, the integrity of these two cell types was rather equal. For the 48°C exposure, the assessment values of both cell types generally became relatively equal caused by degradation of either cell type. In population 4, however, even at this high temperature, the digestive cells were in a slightly 'better' condition than the calcium cells.

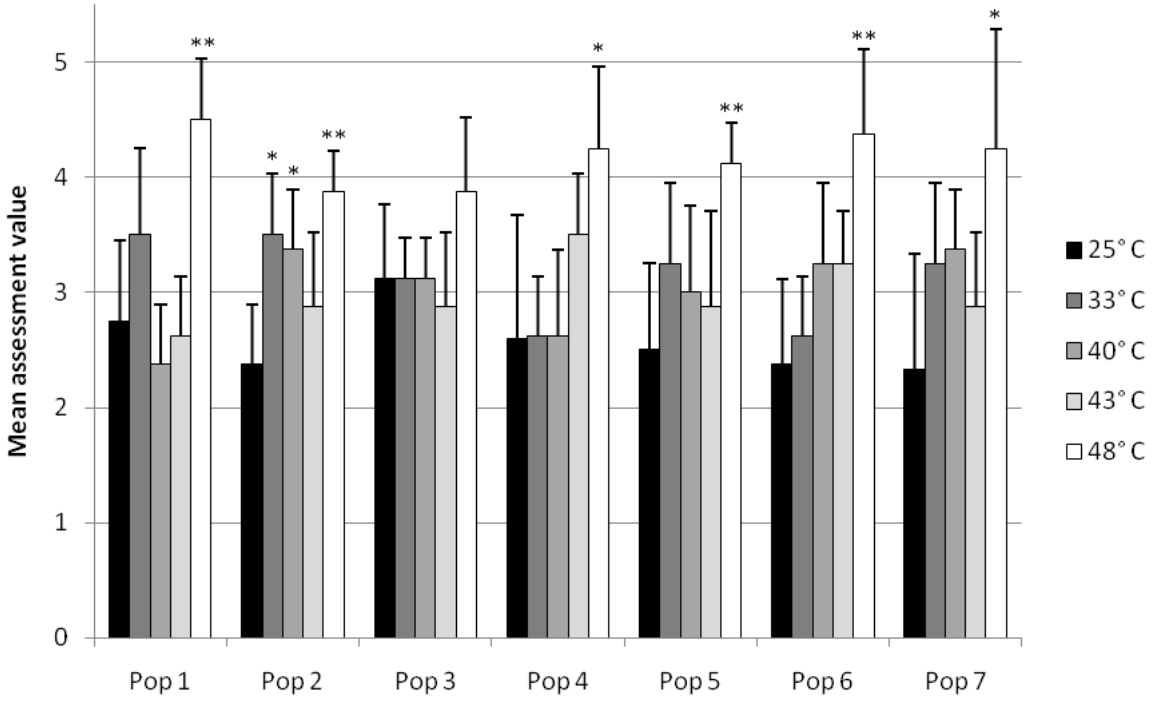


Figure 4. The condition of the calcium cells of the hepatopancreas. Mean assessment values for each population at elevated temperature. Shown are means and SD; *n*=8. Asterisks show significant differences of the respective exposure groups compared to the control at 25°C after Bonferroni correction: $0.0025 < P \leq 0.0125$: (*) and $0.00025 < P \leq 0.0025$ (**).

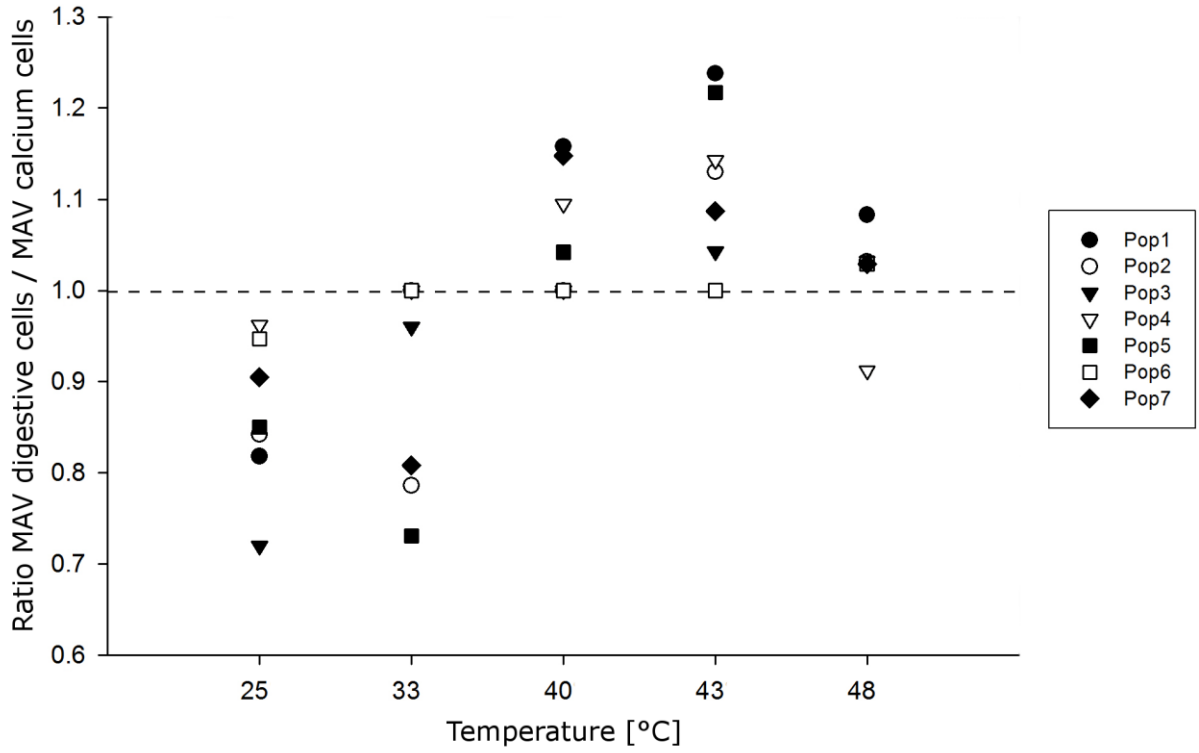


Figure 5. Ratio of the mean assessment values obtained for digestive cells and calcium cells. The ratio for each population at different temperature regimes is illustrated. Data below the dashed line indicate a better condition of digestive cells, compared to calcium cells. Data above the line indicate the opposite.

Stress protein analyses

Our pre-test on a possible relevance of shell size on stress protein expression did not reveal any significant effect of shell size on Hsp70 expression neither on the Hsp70 base level nor on the Hsp70 levels recorded after temperature exposure.

The actual stress protein analyses indicated that almost all populations showed an up-regulation of their stress protein level until 40°C followed by a decrease of Hsp70 values at higher temperatures. However, populations 2, 3, 4, and 7 already exhibited a high base level of Hsp70 at control temperature, and populations 2 and 4 were not able to raise their Hsp70 levels remarkably.

To test for significant differences, exposure groups were compared to the control group (25°C) within each population. A slightly significant increase of the Hsp70 level could be detected at 40°C (populations 1, 3, 5, and 7). In population 7, we detected a significant increase in the Hsp70 level after exposure to 38 and 40°C. Also, population 6 showed a significant increase of stress proteins at 40°C

exposure. In the high temperature group of 45°C, the decrease of the stress protein level was slightly significant (populations 1 and 2) or significant (populations 3, 4 and 7) compared to the control group. Population 2 already showed a slightly significant decrease at 38°C (Fig. 6). In addition, a slightly significant decrease was observed after exposure to 43°C (population 7) and 48°C (population 1).

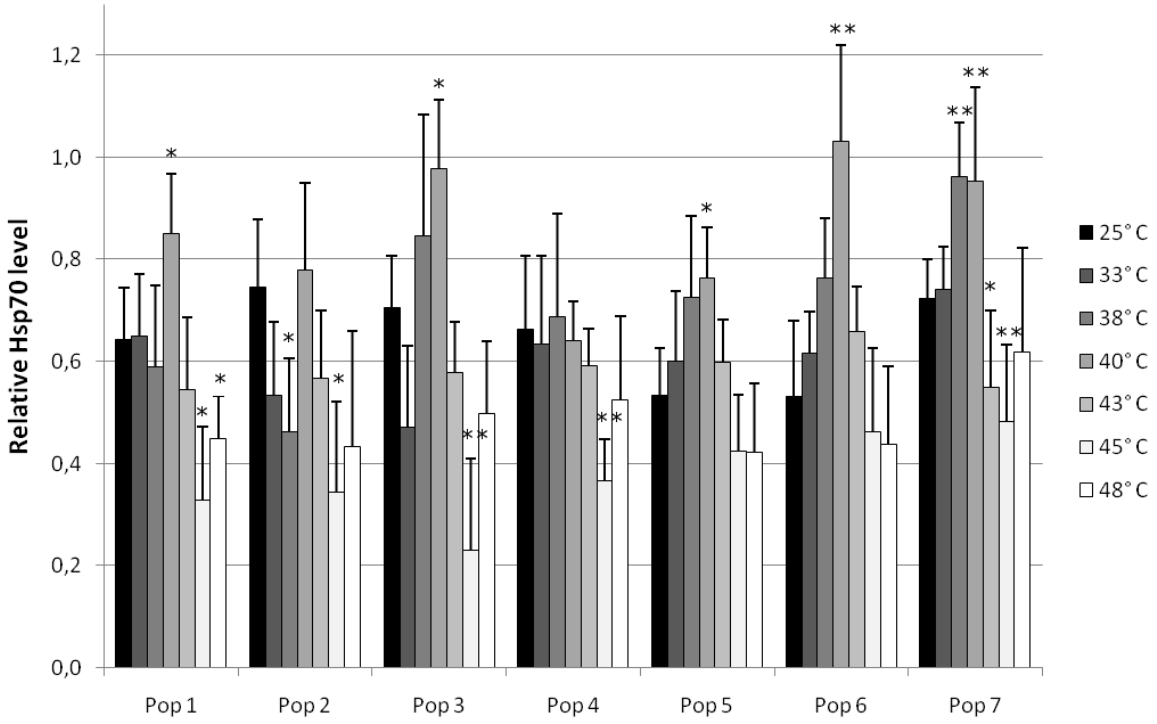


Figure 6. Relative Hsp70 level of different populations after exposure to elevated temperature for 8h. Shown are means and SD; n = 10. Asterisks show significant differences of the respective exposure groups compared to the control at 25°C after Bonferroni correction: $0.0017 < P \leq 0.0083$: (*) and $0.00017 < P \leq 0.0017$ (**).

All populations showed a maximum induction of Hsp70 at 40°C, except for populations 4 and 7, which peaked in their Hsp70 level at 38°C. Population 6 revealed the highest maximum stress protein induction with 193.9 %, whereas population 4 was not able to increase its stress protein level appreciably (104.2 %). Population 3 also showed a relatively high maximum induction followed by population 5 and 7. A rather low maximum stress protein induction was detected in population 1 and 2 (Table 3).

Table 3. Maximum levels of Hsp70 induction in different populations after exposure to elevated temperature regimes.

Population	Temperature	Maximum Hsp70 induction
1	40°C	132.3 %
2	40°C	125.7 %
3	40°C	155.9 %
4	38°C	104.2 %
5	40°C	142.8 %
6	40°C	193.9 %
7	38°C	140.0 %

The maximum induction was calculated as the ratio vs. the Hsp70 induction at the control temperature (25°C).

In general, maximal Hsp70 induction was observed in the 40°C exposure group (38°C not histopathologically analyzed). Therefore, we compared Hsp70 data of this group with those of the other populations. Our analysis of variance showed differences among groups ($F_{6,63}$ = 9.1987; $p < 0.0001$). Population 4 was significantly different from population 1 ($p = 0.0291$), population 3 ($p < 0.0001$), population 6 ($p < 0.0001$), and population 7 ($p = 0.0002$). Population 3 was significantly different from populations 2 ($p = 0.0472$) and 5 ($p = 0.0246$). Population 6, too, showed a significant difference from populations 2 ($p = 0.0043$) and 5 ($p = 0.002$) (Fig. 7). For details see supplementary information (Table S1).

Table S1. Results of the Tukey Kramer HSD post-hoc test for the comparison of Hsp70 levels after 40°C exposure among populations (p-values are shown).

	Population 1	Population 2	Population 3	Population 4	Population 5	Population 6	Population 7
Population 1	*						
Population 2	0,9213	*					
Population 3	0,4538	0,0472	*				
Population 4	0,0291	0,3458	<0,0001	*			
Population 5	0,8224	1,000	0,0246	0,4925	*		
Population 6	0,0958	0,0043	0,9812	<0,0001	0,0020	*	
Population 7	0,7028	0,1198	0,9997	0,0002	0,0679	0,8867	*

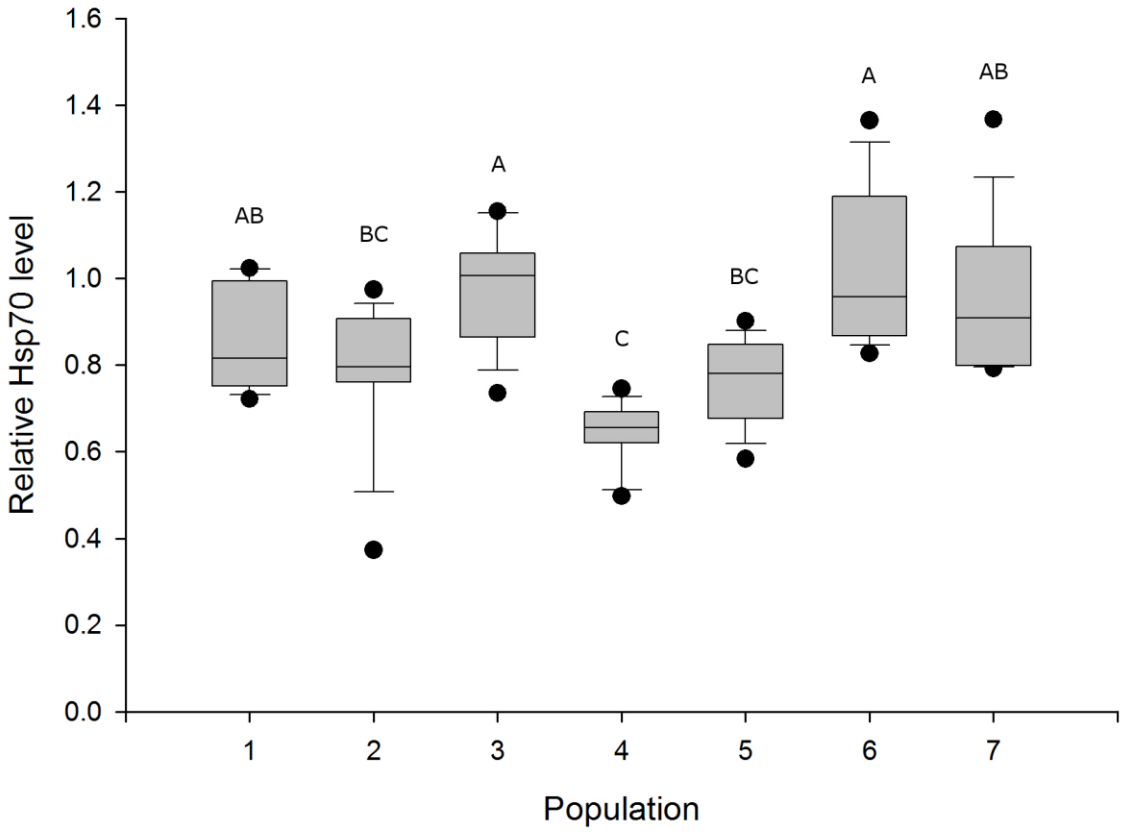


Figure 7. Comparison of Hsp70 levels after exposure to 40°C among populations. Different letters indicate statistically significant differences between groups. Data is plotted as the median, 10th, 25th, 75th, and 90th percentiles. Points show minimum and maximum values.

Correlation of Hsp70 level and histopathology

The relative Hsp70 level was plotted against the histopathological assessment of the tubule structure, the digestive cells, and the calcium cells, respectively (Fig. 8). Generally, the Hsp70 level increased up to its maximum at 40°C along with increasing cellular responses (MAV 2.5-3.5), and decreased in parallel to further increasing cellular injury with rising temperature. However, the respective populations showed different patterns in respect to this correlation: populations 3, 6, and 7 reached a high stress protein level at 40°C, whereas the other populations, especially population 4, kept their stress protein levels relatively low. Despite their high Hsp70 levels, populations 6 and 7 showed stronger cellular alterations than the other ones. Population 4, however, already revealed distinct cell damages at

43°C exposure. A conspicuous improvement of the cellular condition after stress protein level elevation could be observed in population 1.

The differences in temperature stress-response patterns between the studied populations can be further described as follows. Generally, all populations showed a good cellular condition in the control group of 25°C for all assessed parameters (condition of tubule structure, digestive cells and calcium cells) which went along with low (populations 1, 4, 5, and 6) or intermediate Hsp70 levels (populations 2, 3, and 7). Only individuals of population 3 already revealed their calcium cells to be in the 'status of reaction' in response to 25°C.

Population 1 showed, along with an increasing Hsp70 level, distinct improvements in cellular condition. The cells of this population were in the 'status of reaction' already after exposure to 33°C (accompanied by a low stress protein level), but a conspicuous improvement of cellular integrity associated with an increase in Hsp70 level occurred after 40°C heat exposure. Digestive and calcium cells revealed an improved histological picture in which the calcium cells were even in 'better' condition than those of the control group at 25°C.

Despite of high Hsp70 base levels, population 3 was able to increase its stress protein induction to rather high levels at 40°C exposure. In the 33°C group, we detected a low Hsp70 level associated with general cellular reactions. After increasing the Hsp70 level (40°C), the tubule structure improved whereas the condition of digestive and calcium cells stayed in the same category (status of reaction). Contrary to population 1, population 3 did not show any improvement in the structure of digestive and calcium cells in the company of high Hsp70 levels. Though, individuals of this population where, in spite of changes in the Hsp70 level, able to keep the status of digestive cells (up to 43°C) and calcium cells (up to 40°C) relatively constant (MAV around 3.0). Here, the cellular integrity of the hepatopancreas seemed to be rather independent from Hsp70.

In contrast, population 4 kept its stress protein level on a relatively low level in all exposure groups. This was nevertheless associated with a good cellular condition until 40°C. However, only this population showed prominent cellular deterioration after exposure to 43°C - especially the condition of the digestive cells obviously declined - whereas all other populations revealed better cellular condition at this temperature.

Populations 6 and 7 were able to raise their stress protein level significantly after 40°C exposure, but exhibited cellular reactions in all assessment groups (all assessed parameters were in 'status of reaction'). Especially population 7 showed strongly damaged digestive cells.

Populations 2 and 5 revealed a relatively low Hsp70 level at elevated temperatures. In spite of a slightly increase of stress proteins in the 40°C group, histological reactions could be observed particularly in the digestive and the calcium cells of population 2. Population 5 showed increasing deterioration of the cellular status with elevated temperature regimes, whereas, accompanied by an increase in stress proteins, the cellular condition was at a moderate level at 40°C.

Despite of a decrease of Hsp70 in all populations after exposure to 43°C for 8h, we observed, compared to the 40°C exposure, a general structural improvement of cells in population 7. Compared to 40°C, also a 'better' condition in digestive cells (populations 2 and 3) and calcium cells (populations 2, 3, and 5) was detected at 43°C. Population 1 still showed a reasonably 'good' condition of the calcium cells at 43°C (MAV 2.6), which differed only marginally from the cell status at 40°C.

In the 48°C exposure group, the cellular condition declined from the maximum hand in hand with a decrease of the Hsp70 in all populations. Conspicuously, population 7 showed a higher Hsp70 level compared to the other populations, and population 2 revealed totally damaged digestive cells (MAV 4.8).

As mentioned before, digestive and calcium cells showed divergent modes of reaction when subjected to elevated temperature regimes. In general, the digestive cells were in 'better' condition than the calcium cells after exposure to 25 and 33°C, and the stress protein levels stayed on a low or moderate level. After an increase of Hsp70 induction in the 40°C group, the calcium cells revealed a 'better' status compared to the condition of the digestive cells (populations 1, 4, 5, and 7), or both cell types were in rather equal conditions (populations 2, 3, and 6). After exposure to 43°C, the condition of the calcium cells was generally 'better' than the condition of the digestive cells in all populations, accompanied by a decrease in the stress protein level.

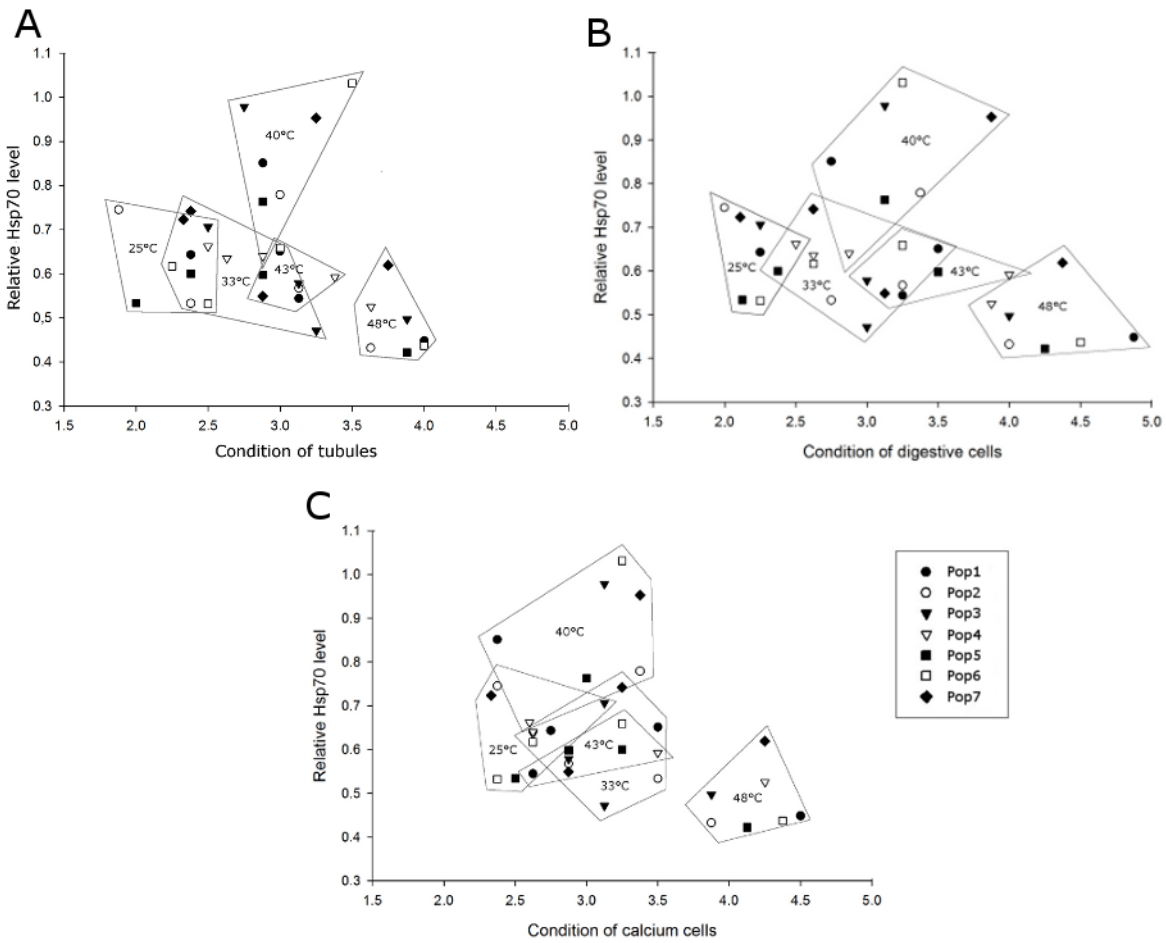
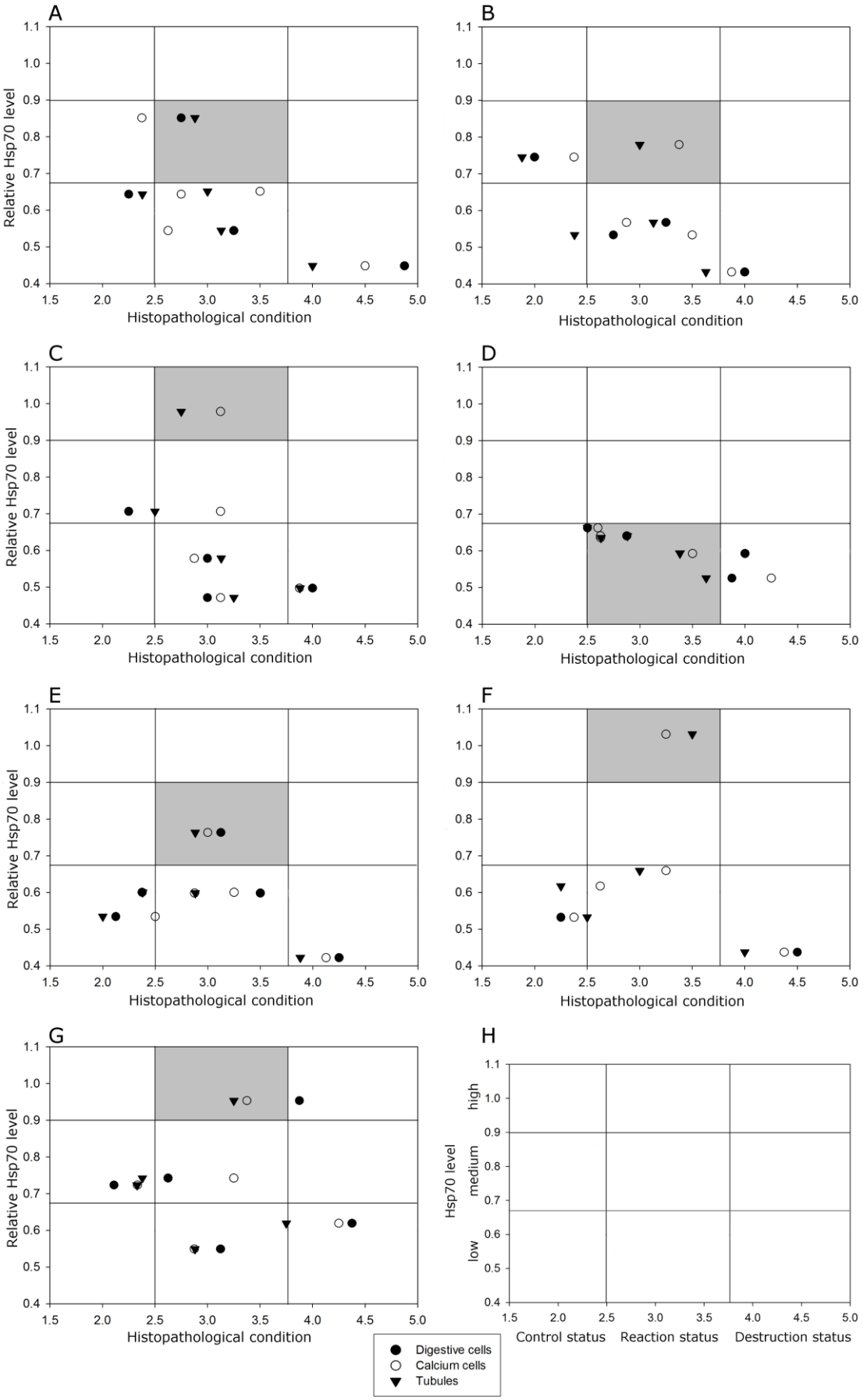


Figure 8. Correlation of relative Hsp70 level vs. histopathological mean assessment values. Data obtained for the populations of the respective exposure groups (25, 33, 40, 43 and 48°C) are framed, respectively. **A.** Relative Hsp70 level vs. condition of the tubules. **B.** Relative Hsp70 level vs. condition of the digestive cells. **C.** Relative Hsp70 level vs. condition of the calcium cells.


A
B
C
D
E
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G
H
Relative Hsp70 level
Histopathological condition
Hsp70 level
high
medium
low
Control status
Reaction status
Destruction status
Digestive cells
Calcium cells
Tubules

Figure 9. Correlation of relative Hsp70 levels vs. mean histopathological assessment values for each population. Shaded squares reflect the respective population's strategy of Hsp70 induction at the histological 'status of reaction' of the hepatopancreas. The position of vertical and horizontal lines is arbitrary and for visual purposes only. **A.** Population 1 **B.** Population 2 **C.** Population 3 **D.** Population 4 **E.** Population 5 **F.** Population 6 **G.** Population 7 **H.** Explanation of subdivisions of Hsp70 levels and histopathological conditions as applied to plots A-G.

Genetic analyses

Network analysis

The statistical parsimony analysis of the 700 base pair long COI fragment of 138 individuals resulted in a single parsimonious network with a total of 6 haplotypes (network not shown here). The majority of the sequences belonged to two haplotypes (66% and 19% of all sequences), which were present in all populations. More than 99% of nucleotide positions were shared among haplotypes, strongly suggesting that all individuals belong to the same species.

Population indices

Genetic diversity within populations (π) was generally low (all < 0.004). The highest diversity was found in populations 4 and 1; population 7 was homogeneous (Table 4). Genetic differences among populations, expressed by F_{ST} and H_{MH}, were also relatively low (Table 4). The only exception was population 4, which showed relatively high values for both F_{ST} and H_{MH} and thus is most dissimilar to all other *X. derbentina* populations studied.

Correlation of physiological heat stress response and genetic data

The results of the individual MRT analyses under different temperature settings are provided in Table 5 (group-specific trees given in Newick format). Under all temperature conditions, physiological data are well explained by genetic variables (particularly divergence parameters) as indicated by R^2 ranging from 50-78% (CV errors 1.03-2.95). The highest R^2 was observed under the 40°C condition (i.e., the condition under which the populations showed the highest Hsp70 activities). There, a divergence parameter (F_{ST}) could explain the physiological parameters that led to the primary grouping (i.e., the first split) of the tree.

At 33°C, the proportion of variance explained by the first split of the tree was 58% with the physiological parameters being explicated by the divergence parameter

H_{MH}. Both at 25°C and 48°C, R^2 for the first split of the trees was 50% with the physiological parameters being explained by divergence indices (H_{MH} and F_{ST}).

Interestingly, in the 25, 33, and 48°C groups divergence parameters are positively correlated with Hsp70 levels (i.e., specimens that show high genetic differentiations to specimens from neighboring populations are characterized by high HSP70 levels), whereas for the 40°C condition, the correlation is inversely. In contrast, high divergence values are associated with adverse histopathological effects under 25°C conditions, whereas under 33, 40, and 48°C conditions, specimens that show high genetic differentiations to specimens from neighboring populations typically show fewer adverse effects.

Cross-validations produced large errors, which can be explained by the relatively small number of populations studied.

Table 4. Within- and between-site genetic differentiation calculated for *Xeropicta derbentina* populations (1-7) from Southern France based on the COI gene.

	1	2	3	4	5	6	7
1	0.0032±0.0018	0.05	0.13	0.75	0.01	0.10	0.21
2	0.030	0.0024±0.0015	0.03	0.77	0.09	0.01	0.07
3	0.073	0.021	0.0023±0.0014	0.79	0.21	0.03	0.06
4	0.287	0.338	0.315	0.0036±0.0020	0.75	0.77	0.80
5	0.005	0.056	0.115	0.316	0.0030±0.0017	0.14	0.27
6	0.068	0.010	0.0328	0.407	0.100	0.0016±0.0011	0.03
7	0.235	0.117	0.108	0.551	0.290	0.088	0

On diagonal line: nucleotide diversity (π); above diagonal: haplotype divergence (H_{MH}) based on the Morisita-Horn index; below diagonal: pairwise fixation index (F_{ST}).

Table 5. Results of the MRT analyses of PCoA transformed physiological heat stress response data (Hsp70 and histology) constrained with population structure information of *Xeropicta derbentina* under four temperature conditions.

Temperature setting	R^2 (CV-error)	Tree topology of populations (Newick format)	Explanatory variables for primary grouping	Correlation of explanatory and dependent variables for primary grouping
25°C	50% (1.31)	(1,3,4),(2,5,6,7)	$H_{MH}3$	Hsp70 (+), histology (d)
33°C	58% (1.25)	(2,4,5,6,7),(1,3)	$H_{MH}3$	Hsp70 (+), histology (i)
40°C	78% (1.03)	((1,4),(3,5)),(2,6,7)	$F_{ST}1$	Hsp70 (-), histology (i)
48°C	50% (2.95)	(1,5,6),(2,3,4,7)	$F_{ST}2$	Hsp70 (+), histology (i)

R^2: cross-validated proportion of variance explained by the primary grouping (i.e., first split of the tree); P1-P7: populations studied; π: nucleotide diversity; $H_{MH}3$: axis 3 of transformed haplotype diversity; $F_{ST}1$, $F_{ST}2$: axes 1 and 2 of transformed pairwise fixation index; (+): positive correlation; (-): negative correlation; improved histopathology (i); deteriorated histopathology (d).

Discussion

Heat stress affects organisms at different physiological levels, including biochemical defense reactions mirrored by the cellular status of central metabolic organs as, e.g., the hepatopancreas in mollusks. The hepatopancreas is strongly involved in metabolic processes even under normal conditions (Sumner 1965, Walker 1970, Taieb & Vicente 1998). Besides other factors, the metabolic rate can increase due to high temperatures (Gillooly et al. 2001) and, thereby, increases the need of nutrient supply by the hepatopancreas. As found in the qualitative assessment of the hepatopancreatic tubule structure, the lumina of the tubules were dilated after exposure to higher temperatures, which could have been the result of an increased metabolic rate associated to a demand in nutrient supply.

An increased amount of ruptured cell apices primarily of the digestive cells occurred preferentially after exposure to higher temperatures, which could be explained by an activated release of lysosomal enzymes. Lysosomal membranes are known to disintegrate under elevated temperatures (Moeller et al. 1976), which cause lysosomal enzymes to be released. Poste et al. (1971) showed that an extracellular release of these enzymes from damaged lysosomes can destroy cell membranes and generally alter cell structures. The combination of high temperature and low pH (also as a result of high temperature) enhance the disruption of lysosomal membranes and also the activity of released enzymes (Moeller et al. 1976).

Predominantly after exposure to high temperature, the calcium cells showed dark nuclei which are indicative of a low pH (Avwioro 2011), disturbed compartmentation, and reduced density of the cytoplasm. Calcium cells play an important role in osmoregulation (Taieb & Vicenet 1998) and the acid-base balance (Burton 1976). It is known that heat can negatively affect the acid-base balance (Heisler 1986) and the ion-balance as a result of water-loss by increased evaporation which leads to osmotic stress and acidosis. High temperatures can also lower the pH (Barnhart 1986), which leads to an accumulation of acidic metabolic products in snail tissue, causing metabolic acidosis (Ryan & Gisolfi 1995). Related to these facts, we assume that the observed heat effects in the calcium cells are associated with osmotic stress and a disturbed acid-base balance. Occasionally, we observed calcium cells exhibiting some heat stress symptoms in the control group (population 3). This might be due to the fact that these snails had already encountered high temperatures in the field and did not fully recover during acclimatization time in the lab prior to the experiments. Scheil et al. (2011) also observed cellular reactions in the control group of a *X. derbentina* population after acclimation and concluded that this might have been caused by pre-exposure in the field.

Our results showed digestive cells to be more heat sensitive than calcium cells. In almost all populations, the condition of the digestive cells stayed 'below' the status of reaction at temperatures of up to 33°C, as illustrated in Fig. 5. With rising temperature, they showed irregular vacuolization and fused vacuoles, both being indicative for an active cellular response, and ruptured cell apices which are likely due to the release of lysosomal enzymes as mentioned above. A main function of the digestive cells is resorption of nutrients and intracellular digestion. Because the two lowest temperatures correspond to natural environmental conditions allowing these

snails to be active and feed, it becomes reasonable that their metabolism ensures a stable function of this cell type at these temperatures. When temperatures rise, e.g. in the morning of hot summer days (Dieterich et al. 2013), the snails remain attached inactive on vegetation. During this period, they are fully exposed to the sun, heat up, and need to cope with heat stress. The above-mentioned functions of the calcium cells become more important under these conditions, so we can assume that under these circumstances, a functional status of these cells is of higher importance than that of the digestive cells.

In several studies, an increase in the percentage of the extension of calcium cells in the digestive gland, caused by hyperplasia, hypertrophy, or loss of digestive cells as an adaptation to heat stress, was observed (Dittbrenner et al. 2009, Zaldibar et al. 2007). In this study, the ratio of calcium cells did not differ among the treatment groups in any of the investigated populations. Only in few cases, hypertrophy of calcium cells could be detected, and also no decrease in the number of digestive cells was observed. However, the populations investigated in this study already revealed a rather high percentage of calcium cells (about 40-50%) in the control group, so we assume that snails were not able to raise this level remarkably in response to heat. Also in a study by Scheil et al. (2011), a high percentage of calcium cells in controls and only a minor increase in their surface ratio were observed when *X. derbentina* was exposed to 45°C.

In order to better understand these histological findings, it is necessary to compare the histopathological results to those obtained for stress proteins.

In response to increasing temperatures, all populations showed an up-regulation of Hsp70 up to a distinct level. This maximum was followed by a decrease in stress protein level as a result of exposure to higher temperatures. These findings are in accordance with the kinetics of stress protein induction described by Eckwert et al. (1997). The induction of Hsp70 is known to be due to proteotoxic effects of stressors in cells and the subsequent initiation of stress gene transcription (compensation phase). After reaching the maximum level of stress protein induction, the stress response decreases, presumably caused in most cases by a pathological impairment of the stress protein machinery (destruction phase). In our study, the histopathological data confirm this interpretation, particularly for temperatures >40°C.

It is known that land snails living under extreme environmental conditions and suffering from heat overload and desiccation use Hsp induction as important survival strategy (Mizrahi et al. 2010, Mizrahi et al. 2012). Furthermore, the expression of Hsp70 proteins is thought to be very energy costly (Sanchez et al. 1992, Heckathorn et al 1996, Köhler et al. 2000). Differences among species and populations in the intensity in which stress proteins are induced could be associated with differences among them in how temperature has affected their energy budgets (Tomanek & Somero 1999). With respect to the relative Hsp70 levels (in association with the cellular mean assessment values) in our study which predominantly differed at 40°C among the populations (Fig. 7 and 8), the investigated populations follow different strategies to arrange with thermal stress:

Strategy 1: investment in medium Hsp70 levels and keeping cellular condition on the level of moderate response (populations 1, 2, and 5), strategy 2: spending energy in high Hsp70 levels associated with cellular condition on a moderate level (populations 3, 6, and 7), and strategy 3: no investment in significantly elevated Hsp70 levels, but nevertheless insurance of cellular functionality until a certain temperature, at the risk of a rapid cellular decay at extreme temperature (population 4). These strategies are reflected in Fig. 9, which illustrates the association of Hsp70 level and histopathological condition for each population. The distribution of Hsp70 maxima and statistical analyses displayed in Fig. 7 support our grouping of the snail populations in these strategies.

What could be the benefits of the strategies involving either high or low levels of Hsp70? Due to the energy consumption associated with the synthesis of Hsps and the expense related to the synthesis of other types of proteins at elevated temperature levels (Tomanek & Somero 1999), over-expression of Hsps can reduce fitness (Feder et al. 1992, Krebs & Loeschcke 1994, Krebs & Feder 1998). Consequently, lower levels of Hsp70 might save fitness costs. Another aspect is that in evolution thermal resistance or tolerance to chemical stressors often is achieved in other ways presumably due to the costs of the production of high Hsp70 levels (Feder & Krebs 1998, Köhler et al. 2000, Arts et al. 2004). Mizrahi et al. (2010) demonstrated that a Mediterranean snail species (*Sphincterochila cariosa*), which is rather sensitive to desiccation, showed a higher level of Hsp72 compared to a related, desert-inhabiting snail species (*Sphincterochila zonata*) that is more desiccation resistant. They also suggested that these results are in line with other studies demonstrating higher levels of Hsp70 in heat sensitive species compared to

heat tolerant ones (Sørensen et al. 2001, Zatsepina et al. 2001). Assuming that low Hsp70 levels are indicative of phenotypes that have been selected for thermotolerance, one could conclude that populations 6 and 7 of our study did not exhibit this tolerance as they showed cellular decay despite high Hsp70 levels.

Because each population has its own demographic history (e.g., bottlenecks, immigration, emigration), it is not surprising, that Hsp70 expression levels are specimen and population specific. In fact, previous population- or line-specific analyses of Hsp70 expression levels in other invertebrates clearly demonstrated within and among population differences in Hsp70 levels (Sørensen et al. 2001, Jensen et al. 2009, Bahnrdorff et al. 2010). However, the specific demographic parameters responsible for the correlation of population structure and Hsp70 levels are still poorly understood. We therefore tested several candidate explanatory genetic parameters in this study in order to infer their effects on physiological heat stress response data. Note, however, that the mitochondrial COI gene used in this study can only reflect the phylogeographical and demographic history of our populations studied; it is very likely not directly involved in Hsp70 expression.

Interestingly, our MRT analyses (Table 5) showed that physiological data are well explained by genetic population characteristics in general and by divergence parameters in particular. This is especially evident in the 40°C exposure group (R^2 = 78%), the condition with the maximal stress protein induction. Here, little genetic differentiation between populations is associated with high Hsp70 levels and improved histopathological conditions. In other words, specimens that share haplotypes with specimens from neighboring populations show, on average, higher Hsp70 values and fewer adverse histopathological effects. The overall pattern inferred could possibly be explained by regional population processes that are particularly acting at 40°C, that is, an ecologically realistic, yet the highest non-lethal temperature. In contrast, under the other temperature regimes tested (i.e., 25, 33, and 48°C), local processes appear to be more important.

Why do some populations not show cellular deterioration even though their Hsp70 level remained low after 8h exposure to thermal stress? Scheil et al. (2011) showed that Hsp70 was up-regulated already after 0.5h of exposure time, reaching a significant peak after 4h and then decreased, reaching the base level again after 8h. In addition, they observed no significant impact on the integrity in cellular condition during exposure time. Thus, it cannot be excluded that short-time

induction of stress proteins, which may have occurred within the first hours of exposure (but remain undetected after 8h), protected cells from pathology.

It is particularly striking that digestive cells and calcium cells showed different modes of reaction in response to the tested gradient of elevated temperature in the investigated populations. This variation in reaction patterns of digestive and calcium cells was found to be associated with the Hsp70 level: with increasing stress protein content, the condition of calcium cells improved or, at least, did not decline in all populations. We conclude that Hsp70 has a protective effect especially on the calcium cells and that our studied populations invested energy to ensure the function of this cell type, above all because of its important role in osmoregulation (Taieb & Vicente 1998) and acid-base balance (Burton 1976). This observation can also be linked to the function of calcium cells in protein synthesis (Sumner 1965, Taieb & Vicente 1998). Up to now, there are no studies about the intensity of Hsp70 synthesis in calcium cells of the hepatopancreas in snails. It is, however, reasonable to assume that Hsp70 synthesis took place in the calcium cells, and that an increase in stress protein synthesis could lead to an instant protection effect in this cell type. This topic might be addressed by further studies in the future.

Our study showed that there is considerable variation in the survival strategies in populations of *X. derbentina*. Results indicate that populations invest either more or less energy in elevated Hsp70 synthesis, according to the presumed trade-off with fitness costs. We observed populations that at least invested energy in moderately elevated (population 1, 2, and 5) or high stress protein levels (population 3, 6, and 7) to keep cellular condition stable. Furthermore, one population (population 4), was able to keep cellular functionality despite a low, at most slightly elevated, Hsp70 level until 40°C exposure, whereas prominent cellular reactions were observed beyond this thermal limit in this population only. Generally, we observed that, with an elevation in Hsp70 levels, especially after exposure to high temperature, calcium cells seemed to be more heat tolerant than digestive cells.

Genetic analyses showed that physiological data are well explained by genetic variables, especially for the 40°C exposure group. This possibly indicates strong selective pressures acting at this high, but environmentally relevant temperature. Despite the presumable uniformity in the requirements for the survival of different *X. derbentina* populations at high temperature, our study nevertheless showed the cellular components of survival strategies of snail populations to be very variable. In

concert with one another, these components are apparently equally efficient in different *X. derbentina* populations and enable survival of them in their natural habitats.

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References

Arts MJSJ, Schill RO, Knigge T, Eckwert H, Kammenga JE, et al. (2004) Stress proteins (hsp70, hsp60) induced in isopods and nematodes by field exposure to metals in a gradient near Avonmouth, UK. Ecotoxicology 13: 739-755.

Avwioro G (2011) Histochemical Uses of Haematoxylin - A Review. JPCS 1: 24-34.

Bahrndorff S, Marien J, Loeschke V, Ellers J (2010) Genetic variation in heat resistance and HSP70 expression in inbred isofemale lines of the springtail *Orchesella cinta*. Climate Res 43: 41-47.

Barnhart MC (1986) Control of acid-base status in active and dormant land snails, Otala lacteal (Pulmonata, Helicidae). J Comp Physiol B 156: 347-354.

Bishop T, Brand MD (2000) Processes contributing to metabolic depression in hepatopancreas cells from the snail *Helix aspersa*. J Exp Biol 203: 3603–3612.

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities utilizing the principle of protein dye binding. Anal Biochem 72: 248-254.

Burton RF (1976) Calcium metabolism and acid-base balance in *Helix pomatia*. In: Davies PS, editor. Perspectives in experimental biology. Oxford: Pergamon Press. pp.7-16.

Clement M, Posada D, Crandall KA (2000) TCS: a computer program to estimate gene genealogies. Mol Ecol 9: 1657–1659.

De'ath G (2002) Multivariate regression tress: A new technique for modeling species environment relationships. Ecology 83: 1105–1117.

De'ath G (2013). mvpart: Multivariate partitioning. http://CRAN.R-project.org/package=mvpart.

Dépraz A, Hausser J, Pfenninger M. (2010) A species delimitation approach in the *Trochulus sericeus/hispidus* complex reveals two cryptic species with a sharp contact zone. BMC Evol Biol 9:171.

Dieterich A, Fischbach U, Ludwig M, Di Lellis M, Troschinski S, et al. (2012) Daily and seasonal changes in heat exposure and the Hsp70 level of individuals from a field population of *Xeropicta derbentina* (Krynicki 1836) (Pulmonata, Hygromiidae) in Southern France. Cell Stress Chaperones 18: 405-414.

Di Lellis M, Seifan M, Troschinski S, Mazzia C, Capowiez Y, et al. (2012) Solar radiation stress in climbing snails: behavioural and intrinsic features define the Hsp70 level in natural populations of *Xeropicta derbentina* (Pulmonata). Cell Stress Chaperones 17: 717-727.

Dittbrenner N, Lazzara R, Köhler HR, Mazzia C, Capowiez Y, et al. (2009) Heat tolerance in Mediterranean land snails: histopathology after exposure to different temperature regimes. J Mollus Stud 75: 9-18.

Eckwert H, Alberti G, Köhler HR (1997) The induction of stress proteins (hsp) in *Oniscus asellus* (isopoda) as a molecular marker of multiple heavy metal exposure: I. Principles and toxicological assessment. Ecotoxocology 6: 249-262.

Excoffier L, Laval G, Schneider S (2005) Arlequin (version 3.0): an integrated software package for population genetics data analysis. Evol Bioinform Online 1: 47–50.

Feder JH, Rossi JM, Solomon J, Solomon N, Lindquist S (1992) The consequences of expressing Hsp70 in *Drosophila* cells at normal temperatures. Gene Develop 6: 1402–1413.

Feder ME, Krebs RA (1998) Natural and genetic engineering of the heat-shock protein hsp70 in *Drosophila melanogaster*: consequences for thermotolerance. Amer Zool 38: 503-517.

Feder ME, Hofmann GE (1999) Heat-shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology. Annu Rev Physiol 61: 243–282.

Fink AL (1999) Chaperone-mediated protein folding. Physiol Rev 79: 425–449.
Mayer MP, Bukau B (2005) Hsp70 chaperones: cellular functions and molecular mechanism. Cell Mol Life Sci 62: 670-684.

Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R (1994) DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Mol Mar Biol Biotechnol 3: 294–299.

Gillooly JF, Brown JH, West GB, Savage VM, Charnoy EL (2001) Effects of size and temperature on metabolic rate. Science, 293: 2248-2252.

Goodfriend GA (1986) Variation in land-snail shell form and size and its causes: a review. Syst Zool 35: 204-223.

Guppy M, Withers P (1999) Metabolic depression in animals: physiological perspectives and biochemical generalizations. Biol Rev 74: 1–40.

Hall T (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp Ser 41: 95–98.

Heckathorn SA, Poeller GJ, Coleman JS, Hallberg R (1996) Nitrogen availability alters the patterns of accumulation of heat stress-induced proteins in plants. Oecologia, 105: 413–418.

Heisler N (1986) Acid-base regulation in animals. Amsterdam: Elsevier Science. 492 p.

Helmus MR, Bland TJ, Williams CK, Ives AR (2007) Phylogenetic measures of biodiversity. Am Nat 169: E68–E83.

Hendrick JP, Hartl FU (1993) Molecular chaperone functions of heat-shock proteins. Annu Rev Biochem 62: 349-384.

Horn HS (1966) Measurement of “overlap” in comparative ecological studies. Am Nat 100: 419–424.

Jensen LT, Cockerell FE, Kristensen TN, Rako L, Loeschke V, et al. (2009) Adult heat tolerance variation in *Drosophila melanogaster* is not related to Hsp70 expression. J Exp Zool 313: 35-44.

Jombart T (2008) adegenet: a R package for the multivariate analysis of genetic markers. Bioinformatics, 24: 1403–1405.

Kammenga JE, Dallinger R, Donker MH, Köhler HR, Simonsen V, et al. (2000) Biomarkers in terrestrial invertebrates for ecotoxicological soil risk assessment. Rev Environ Contam Toxicol 164: 93-147.

Köhler HR, Zanger M, Eckwert H, Einfeldt I (2000) Selection favours low hsp70 levels in chronically metal-stressed soil arthropods. J Evol Biol 13: 569–582.

Köhler HR, Lazzara R, Dittbrenner N, Capowiez Y, Mazzia C, et al. (2009) Snail phenotypic variation and stress proteins: do different heat response strategies contribute to Waddingtons´s widget in filed populations? J Exp Zool B Mol Dev Evol 312B: 136-147.

Krebs RA, Loeschcke V (1994) Costs and benefits of activation of the heat-shock response in *Drosophila melanogaster*. Funct Ecol 8: 730–737.

Krebs RA, Feder ME (1998) Hsp70 and larval thermotolerance in *Drosophila melanogaster*: How much is enough and when is more too much? J Insect Physiol 44: 1091-1101.

Legendre P, Gallagher E (2001) Ecologically meaningful transformations for ordination of species data. Oecologia 129: 271–280. doi:10.1007/s004420100716.

Lindquist S (1986) The heat-shock resonse. Annu Rev Biochem 55:1151-91.

Lindquist S, Craig EA (1988) The heat-shock proteins. Annu Rev Genet 22: 631-77.

Machin J (1964) The evaporation of water from *Helix aspersa* I. The nature of the evaporating surface. J Exp Biol 41: 759-769.

Mizrahi T, Heller J, Goldenberg S, Arad Z (2010) Heat shock proteins and resistance to desiccation in congeneric land snails. Cell Stress Chaperones 15: 351-363.

Mizrahi T, Heller J, Goldenberg S, Arad Z (2012) The heat shock response in congeneric land snails (*Sphincterochila*) from different habitats. Cell Stress Chaperones 17: 639-645.

Moeller PW, Fields PA, Dutson TR, Landmann WA, Carpenter ZL (1976) Effects of high temperature conditioning on subcellular distribution and levels of lysosomal enzymes. J Food Sci 41: 216-217.

Nei M (1973) Analysis of Gene Diversity in Subdivided Populations. Proc Natl Acad Sci U. S. A. 70: 3321–3323.

Oksanen J, Guillaume Blanchet, F, Kindt, R, Legendre P, Minchin PR et al. (2013) vegan: Community Ecology Package. http://CRAN.R-project.org/package=vegan.

Parsell DA, Lindquist S (1993) The function of heat-shock proteins in stress tolerance: Degradation and reactivation of damaged proteins. Annu Rev Genet 27: 437–496.

Parsell DA, Lindquist S (1994) Heat shock proteins and stress tolerance. In: Morimoto RI, Tissieres A, Georgopoulos C, editors. The biology of heat shock proteins and molecular chaperones. New York: Cold Spring Harbor Laboratory Press. pp. 457–494.

Pfenninger M, Magnin F (2001) Phenotypic evolution and hidden speciation in *Candidula unifasciata* ssp. (Helicellinae, Gastropoda) inferred by 16S variation and quantitative shell traits. Mol Ecol 10: 2541-2554.

Pfenninger M, Posada D (2002) Phylogeographic history of the land snail *Candidula unifasciata* (Poiret 1801) (Helicellinae, Stylommatophora): fragmentation, corridor migration and secondary contact. Evolution, 56: 1776-1788.

Pfenninger M, Naumann S, Steinke D, Seidendorf B (2003) Taxonomic recognition approach by morphometric and molecular genetic methods of conchologically similar Helicellinae species (Helicoidea, Gastropoda). Malakologische Abhandlungen Dresden 21: 123-130.

Pomeroy DE (1968) Dormacy in the land snail Helicella virgate (Pulmonata: Helicidae). Aust J Zool 16: 857-869.

Poste G (1971) Sub-lethal autolysis: Modification of cell periphery by lysosomal enzymes. Exp Cell Res 67: 11-16.

R Development Core Team. 2011. R: A Language and Environment for Statistical Computing. Vienna: R foundation for Statistical Computing. 409 p.

Riddle WA (1983) Physiological ecology of land snails and slugs. In: Russell-Hunter WD, editor. The Mollusca: Ecology. New York: Academic Press. pp. 431-461.

Ryan AJ, Gisolfi CV (1995) Responses of rat mesenteric arteries to norepinephrine during exposure to heat stress and acidosis. J Appl Physiol 78: 38–45.

Sanchez Y, Taulien J, Borkovich KA, Lindquist S (1992) Hsp104 is required for tolerance to many forms of stress. EMBO J 11: 2357–2364.

Scheil AE, Köhler HR, Triebskorn R (2011) Heat tolerance and recovery in Mediterranean land snails after pre-exposure in the field. J Mollus Stud 77: 165-174.

Schmidt-Nielsen K, Taylor CR, Shkolnik A (1971) Desert snails: Problems of heat, water and food. J Exp Biol 55: 385-398.

Schrader M, Hauffe T, Zhang Z, Davis GM, Jopp F, et al. (2013) Spatially Explicit Modeling of Schistosomiasis Risk in Eastern China Based on a Synthesis of Epidemiological, Environmental and Intermediate Host Genetic Data. PLoS Negl Trop Dis 7: e2327. doi:10.1371/journal.pntd.0002327.

Sørensen JG, Dahlgaard J, Loeschke V (2001) Genetic variation in thermal tolerance among natural populations of *Drosophila buzzatii*: down regulation of Hsp70 expression and variation in heat stress resistance traits. Funct Ecol 15: 289-296.

Staikou AE (1999) Shell temperature, activity and resistance to desiccation in the polymorphic land snail *Cepaea vindobonensis*. J Mollus Stud 65: 171-184.

Sumner AT (1965) The cytology and histochemistry of the digestive gland cells of *Helix*. Q J Microsc Sci 106: 173-192.

Taieb N, Vicente N (1998) Histochemistry and ultrastructure of the crypt cells in the digestive gland of *Aplysia punctata* (Cuvier, 1803). J Mollus Stud 65: 385-398.

Tomanek L, Somero GN (1999) Evolutionary and acclimation-induced variation in the heat-shock responses of congeneric marine snails (genus Tegula) from different thermal habitats: implications for limits of thermotolerance and biogeography. J Exp Biol 202: 2925–2936.

Triebskorn R (2005) Biomarkers, Bioindicators and the Trondheim Biomonitoring System. In: Lehr JH, Keeley J, editors. Water encyclopedia: water quality and resource development. New York: John Wiley and Sons. pp. 29-33.

Walker G (1970) The cytology, histochemistry and ultrastructure of cell types found in the digestive gland of the slug *Agriolimax reticulatus* (Müller). Protoplasma 71: 91-109.

Yom-Tov Y (1971) Body temperature and light reflectance in two desert snails. Proceedings of the Malacological Society of London 39: 319-326.

Zaldibar B, Cancio I, Marigomez I (2007) Reversible alterations in epithelial cell turnover in digestive gland of winkles (*Littorina littorea*) exposed to cadmium and their implications for biomarker measurements. Aquatic Toxicology 81: 183-196.

Zatsepina OG, Velikodvorskaia VV, Molodtsov VB, Garbuz D, Lerman DN, et al. (2001) A Drosophila melanogaster strain from sub-equatorial Africa has exceptional thermotolerance but decreased Hsp70 expression. J Exp Biol 204: 1869-1881.

Kapitel 4: Hsp70 and lipid peroxide levels following heat stress in *Xeropicta derbentina* (Krynicki 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 peroxideation 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 predefined colour category 3 (with a large black band at the umbilicus side of the shell) showed the most prominent Hsp70 response. Lipid peroxideation 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* (Krynicki 1836), a hygromiid land snail species, occurs in high abundance in Southern France, where it was first recorded in 1949 (Altena 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 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 malfolding. Hsps are categorized according to their molecular weight, and best investigated is the 70kDa 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 (Bahrndorff 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, was 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 (·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 xylenol orange method (FOXassay) (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* (Krynicki 1836) 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 (Figure 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.

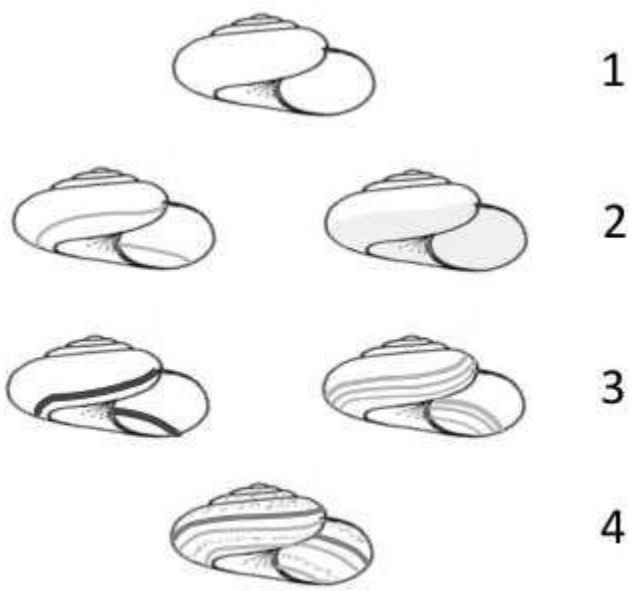

Fig. 1 Illustration of the four different colour morphs of *X. derbentina*, modified version based on Köhler et al. 2009

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 (40µg 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 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 antiboy. 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% H2O2, 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 x 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 Figure 2). All given data were calculated as a mean of ten individuals.

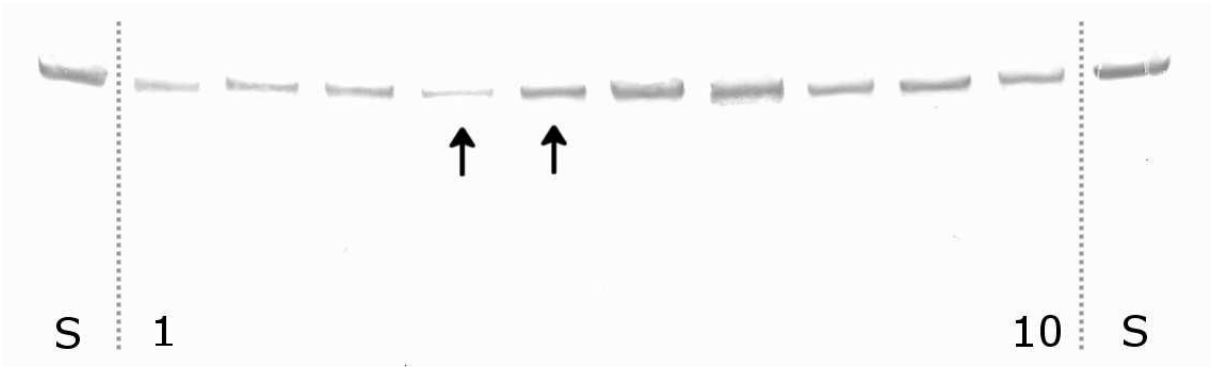

Fig. 2 Representative picture of a stained nitrocellulose membrane. The standard (S) was run in duplicate. Ten randomly chosen samples are shown. The amount of total protein used for analysis was 40 µg for each sample. Arrows indicate two representative samples with a high (lane 5) and a low (lane 4)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

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) 50µl of each reagent was added following this order: 0.25 mM FeSO4, 25 mM H2SO4 and 0.1 mM 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 (3 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 read at 580 nm (A580nm). Subsequently, 1 µl of 1 mM cumenehydroperoxide (Chp) solution was added to the samples, incubated for 30 min at room temperature and again read at 580 nm (A580nm+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 = (A580nm/A580nm+CHP) * CHP1nmol * V/V1 * DF

where V= total sample volume (200 µl), V1=added sample supernatant volume (15 µl) 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).

Results

Hsp70 Analyses

Generally, snails showed a distinct response in their Hsp70 levels after exposure to different temperatures. As shown in figure 3, the overall Hsp70 level (ignoring the shell colouration) 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 (Figure 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 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 (Figure 4).

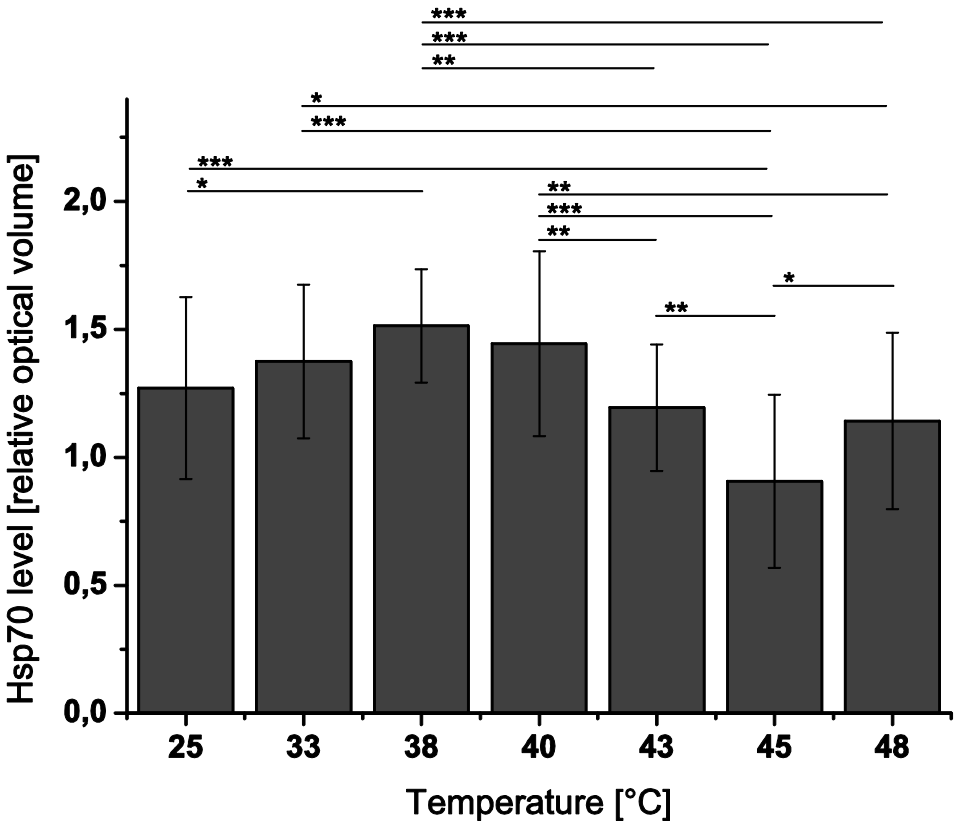

Fig. 3 Hsp70 levels of *X. derbentina* after exposure to different temperatures for 8 hours, irrespective of shell colouration (means ± SD; n = 40). Asterisks show significant differences between the different exposure temperatures: $0.01 < p \leq 0.05$ (*), $0.001 < p \leq 0.01$ (**); $p \leq 0.001$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

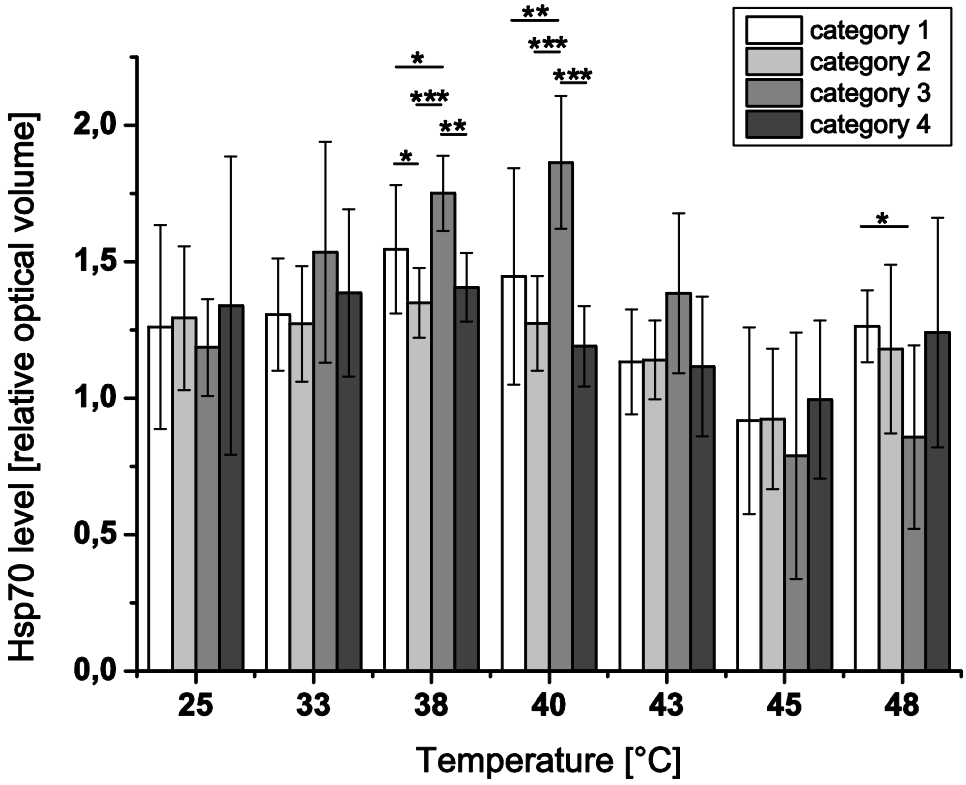

Fig. 4 Hsp70 levels of each morph category after exposure to elevated temperatures (means ± SD; n = 10). Asterisks show significant differences between the categories within an exposure group: $0.01 < p \leq 0.05$ (*), $0.001 < p \leq 0.01$ (**); $p \leq 0.001$ (***).

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 Figure 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 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 (Figure 5).

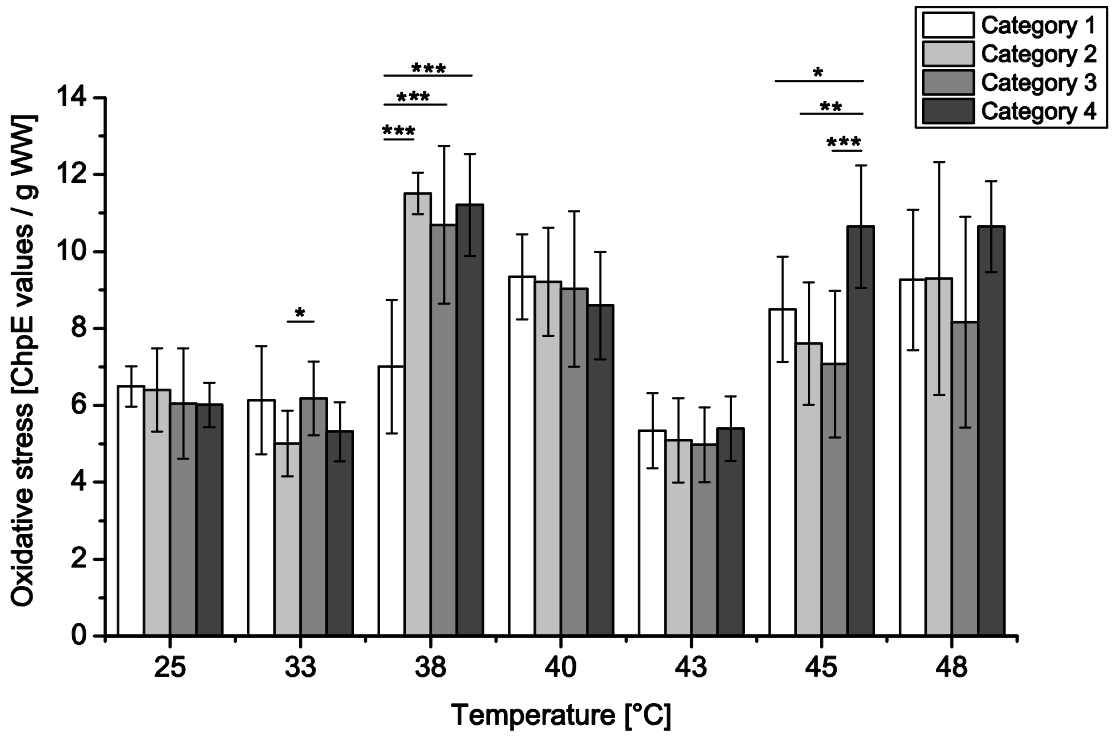

Fig. 5 Lipid peroxide levels (ChpE per gram wet weight) of each colour category after exposure to elevated temperatures (means ± SD; n = 10). Asterisks show significant differences between the colour categories within an exposure group: $0.01 < p \leq 0.05$ (*), $0.001 < p \leq 0.01$ (**); $p \leq 0.001$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

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 know 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 intesity, 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 '8h 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. (2012) exposed snails of *Theba pisana* for 8h at 43°C, took samples at four time points (0, 2, 4 and 8h), and observed a significant increase of lipid peroxides after 4h 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 8h, 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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Literature

Abele D, Burlando B, Viarengo A, Pörtner H-O (1998) Exposure to elevated temperatures and hydrogen peroxide elicits oxidative stress and antioxidant response in the Antarctic intertidal limpet *Nacella concinna.* Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology 120:425-435.

Aebi H (1984) Catalase in vitro Methods. Enzymol 105:121-126.

Altena COvR (1960) On the occurrence of a species of *Xeropicta* in France. Basteria 24:21-25.

Arad Z, Goldenberg S, Avivi T, Heller J (1993) Intraspecific variation in resistance to desiccation in the land snail *Theba pisana.* Int J Biometeorol 37:183-189.

Aubry S, Labaune C, Magnin F, Roche P, Kiss L (2006) Active and passive dispersal of an invading land snail in Mediterranean France. Journal of Animal Ecology 75:802-813.

Bahrndorff S, Holmstrup M, Petersen H, Loeschcke V (2006) Geographic variation for climatic stress resistance traits in the springtail *Orchesella cincta.* Journal of Insect Physiology 52:951-959.

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Analytical Biochemistry 72:248-254.

Cain A (1977) The uniqueness of the polymorphism of *Cepaea* (Pulmonata: Helicidae) in western Europe. Journal of Conchology 29:129.

Chang H-W (1991) Activity and weight loss in relation to solar radiation in the polymorphic land snail *Cepaea nemoralis*. Journal of Zoology 225:213-225.

Comuzzi C, Polese P, Melchior A, Portanova R, Tolazzi M (2003) SOLVERSTAT: a new utility for multipurpose analysis. An application to the investigation of dioxygenated Co(II) complex formation in dimethylsulfoxide solution. Talanta 59:67 80.

Cowie RH (1984) Ecogenetics of *Theba pisana* (Pulmonata: Helicidae) at the northern edge of its range. Malacologia 25:361-380.

Cowie RH (1985) Microhabitat choice and high temperature tolerance in the land snail *Theba pisana* (Mollusca: Gastropoda). Journal of Zoology 207:201-211.

Cowie RH (1992) Shell pattern polymorphism in a 13-year study of the land snail *Theba pisana* (Müller) (Pulmonata : Helicidae) vol 34. vol 1-2. Institute of Malacology, Ann Arbor, MI, ETATS-UNIS.

Daugaard M, Rohde M, Jäättelä M (2007) The heat shock protein 70 family: Highly homologous proteins with overlapping and distinct functions. FEBS Letters 581:3702-3710.

Di Lellis M, Seifan M, Troschinski S, Mazzia C, Capowiez Y, Triebskorn R, Köhler H-R (2012) Solar radiation stress in climbing snails: behavioural and intrinsic features define the Hsp70 level in natural populations of *Xeropicta derbentina* (Pulmonata). Cell Stress and Chaperones 17:717-727.

Di Lellis MA et al. (2014) Phenotypic diversity, population structure, and stress proteinbased capacitoring in populations of *Xeropicta derbentina*, a heat-tolerant land snail species. Cell Stress and Chaperones doi:10.1007/s12192-014-0503-x

Dieterich A et al. (2012) Daily and seasonal changes in heat exposure and the Hsp70 level of individuals from a field population of *Xeropicta derbentina* (Krynicki 1836) (Pulmonata, Hygromiidae) in Southern France. Cell Stress and Chaperones: 1-10. doi:10.1007/s12192-012-0393-8

Dittbrenner N, Lazzara R, Köhler H-R, Mazzia C, Capowiez Y, Triebskorn R (2009) Heat tolerance in Mediterranean land snails: histopathology after exposure to different temperature regimes. Journal of Molluscan Studies 75:9-18.

Feder ME, Hofmann GE (1999) Heat-shock proteins, molecular chaperones, and the stress response: Evolutionary and ecological physiology. Annu Rev Physiol 61:243-282.

Fink AL (1999) Chaperone-Mediated Protein Folding. Physiological Reviews 79:425-449.

Goodhart CB (1987) Why are some snails visibly polymorphic, and others not? Biological Journal of the Linnean Society 31:35-58.

Gutteridge J (1995) Lipid peroxidation and antioxidants as biomarkers of tissue damage. Clinical chemistry 41:1819-1828.

Gutteridge JMC, Halliwell B (1990) The measurement and mechanism of lipid peroxidation in biological systems. Trends in Biochemical Sciences 15:129-135.

Halliwell B, Gutteridge JMC. (1989). Free Radicals in Biology and Medicine, 2nd edn. Clarendon Press, Oxford, UK.

Hazel WN, Johnson MS (1990) Microhabitat choice and polymorphism in the land snail *Theba pisana* (Muller). Heredity 65:449-454.

Heath DJ (1975) Colour, sunlight and internal temperatures in the land-snail *Cepaea nemoralis* (L.). Oecologia 19:29-38.

Hendrick JP, Hartl F (1993) Molecular Chaperone Functions of Heat-Shock Proteins. Annual Review of Biochemistry 62:349-384.

Hermes-Lima M, Willmore WG, Storey KB (1995) Quantification of lipid peroxidation in tissue extracts based on Fe(III)xylenol orange complex formation. Free Radical Biology and Medicine 19:271-280.

Jäättelä M (1999) Heat shock proteins as cellular lifeguards. Annals of Medicine 31:261-271

Jena KB, Verlecar XN, Chainy GBN (2009) Application of oxidative stress indices in natural populations of *Perna viridis* as biomarker of environmental pollution. Marine Pollution Bulletin 58:107-113.

Johnson MS (2011) Thirty-four years of climatic selection in the land snail *Theba pisana.* Heredity 106:741-748.

Kiss L, Labaune C, Magnin F, Aubry S (2005) Plasticity of the life cycle of *Xeropicta derbentina* (Krynicki, 1836), a recently introduced snail in mediterranean France. Journal of Molluscan Studies 71:221-231.

Köhler H-R, Bartussek C, Eckwert H, Farian K, Gränzer S, Knigge T, Kunz N (2001) The hepatic stress protein (hsp70) response to interacting abiotic parameters in fish exposed to various levels of pollution. Journal of Aquatic Ecosystem Stress and Recovery 8:261-279.

Köhler H-R, Lazzara R, Dittbrenner N, Capowiez Y, Mazzia C, Triebskorn R (2009) Snail phenotypic variation and stress proteins: do different heat response strategies contribute to Waddington's widget in field populations? Journal of Experimental Zoology Part B: Molecular and Developmental Evolution 312B:136-147.

Köhler H-R, Schultz C, Scheil AE, Triebskorn R, Seifan M, Di Lellis MA (2013) Historic data analysis reveals ambient temperature as a source of phenotypic variation in populations of the land snail *Theba pisana.* Biological Journal of the Linnean Society 109:241-256.

Krebs RA, Feder ME (1997) Natural variation in the expression of the heat-shock protein Hsp70 in a population of *Drosophila melanogaster* and its correlation with tolerance of ecologically relevant thermal stress. Evolution:173-179.

Kregel KC (2002) Heat shock proteins: modifying factors in physiological stress responses and acquired thermotolerance. Journal of Applied Physiology 92:2177-2186.

Mayer M, Bukau B (2005) Hsp70 chaperones: Cellular functions and molecular mechanism. Cellular and Molecular Life Sciences 62:670-684.

McQuaid CD, Branch GM, Frost PGH (1979) Aestivation behaviour and thermal relations of the pulmonate *Theba pisana* in a semi-arid environment. Journal of Thermal Biology 4:47-55.

Meister A (1988) Glutathione metabolism and its selective modification. J biol Chem 263:17205-17208

Monserrat JM, Geracitano LA, Pinho GLL, Vinagre TM, Faleiros M, Alciati JC, Bianchini A (2003) Determination of Lipid Peroxides in Invertebrates Tissues Using the Fe(III) Xylenol Orange Complex Formation. Arch Environ Contam Toxicol 45:177-183.

Moreno-Rueda G (2008) The colour white diminishes weight loss during aestivation in the arid-dwelling land snail *Sphincterochila* (Albea) *candidissima.* Iberus 26:47-51.

Ozgo M, Schilthuizen M (2012) Evolutionary change in *Cepaea nemoralis* shell colour over 43 years Glob Change Biol 18:74-81.

Pannunzio TM, Storey KB (1998) Antioxidant defenses and lipid peroxidation during anoxia stress and aerobic recovery in the marine gastropod *Littorina littorea.* Journal of Experimental Marine Biology and Ecology 221:277-292.

Pomeroy D (1968) Dormancy in the land snail, *Helicella virgata* (Pulmonata : Helicidae). Australian Journal of Zoology 16:857-869.

Radwan MA, El-Gendy KS, Gad AF (2010) Biomarkers of oxidative stress in the land snail, *Theba pisana* for assessing ecotoxicological effects of urban metal pollution. Chemosphere 79:40-46.

Reuner A, Bruemmer F, Schill RO (2008) Heat shock proteins (Hsp70) and water content in the estivating Mediterranean Grunt Snail (*Cantareus apertus*). Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology 151:28-31.

Richardson AMM (1974) Differential Climatic Selection in Natural Population of Land Snail *Cepaea nemoralis*. Nature 247:572-573.

Richardson AMM (1979) Morph frequencies of empty intact shells from *Cepaea nemoralis* (L.) colonies on snad dunes in South West England. Journal of Molluscan Studies 45:98-107.

Scheil AE, Gärtner U, Köhler H-R (2012a) Colour polymorphism and thermal capacities in *Theba pisana* (O.F. Müller 1774). Journal of Thermal Biology 37:462-467.

Scheil AE, Köhler H-R, Triebskorn R (2011) Heat tolerance and recovery in Mediterranean land snails after pre-exposure in the field. Journal of Molluscan Studies 77:165-174.

Scheil AE, Scheil V, Triebskorn R, Capowiez Y, Mazzia C, Kohler HR (2012b) Shell colouration and antioxidant defence capacity in *Theba pisana* (OF Muller, 1774). Molluscan Res 32:132-136.

Sies H (1997) Oxidative stress: oxidants and antioxidants. Experimental Physiology 82:291-295.

Silvertown J et al. (2011) Citizen Science Reveals Unexpected Continental-Scale Evolutionary Change in a Model Organism. PLoS ONE 6:e18927.

Sørensen JG, Dahlgaard J, Loeschcke V (2001) Genetic variation in thermal tolerance among natural populations of *Drosophila buzzatii*: down regulation of Hsp70 expression and variation in heat stress resistance traits. Functional Ecology 15:289-296.

Sørensen JG, Michalak P, Justesen J, Loeschcke V (1999) Expression of the Heat-Shock Protein HSP70 in *Drosophila buzzatii* Lines Selected for Thermal Resistance. Hereditas 131:155-164.

Staikou AE (1999) Shell Temperature, Activity and Resistance to Desiccation in the Polymorphic Land Snail *Cepaea vindobonensis*. Journal of Molluscan Studies 65:171- 184.

Storey KB (1996) Oxidative stress: animal adaptations in nature. Brazilian journal of medical and biological research = Revista brasileira de pesquisas medicas e biologicas / Sociedade Brasileira de Biofisica [et al] 29:1715-1733.

Troschinski S, Di Lellis MA, Sereda S, Hauffe T, Wilke T, Triebskorn R, Köhler H-R (2014) Intraspecific Variation in Cellular and Biochemical Heat Response Strategies of Mediterranean *Xeropicta derbentina* [Pulmonata, Hygromiidae]. PLoS ONE 9:e86613.

Zhou J, Wang L, Xin Y, Wang W-N, He W-Y, Wang A-L, Liu Y (2010) Effect of temperature on antioxidant enzyme gene expression and stress protein response in white shrimp, *Litopenaeus vannamei*. Journal of Thermal Biology 35:284-289.

Kapitel 5: 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 as a marker for the degree of oxidative stress. 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 increased activities at different temperatures: 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 the pulmonate land snail species *X. derbentina* possesses a set of efficient mechanisms to cope with damaging effects by heat - each with specific temperature-dependent maxima in activity/induction. 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.

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 1996, 1993; Pannunzio and Storey 1998; Lushchak et al. 2001), freezing (Hermes-Lima and Storey 1993; Joanisse and Storey 1996), and also heat stress (Lushchak and Bagnyukova 2006b; Heise et al. 2006; 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 (Daugaard et al. 2007; Sørensen et al. 2001; Feder and Hofmann 1999). 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. unpublished), 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.

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. After scarification, the shell of the snails was 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).

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.

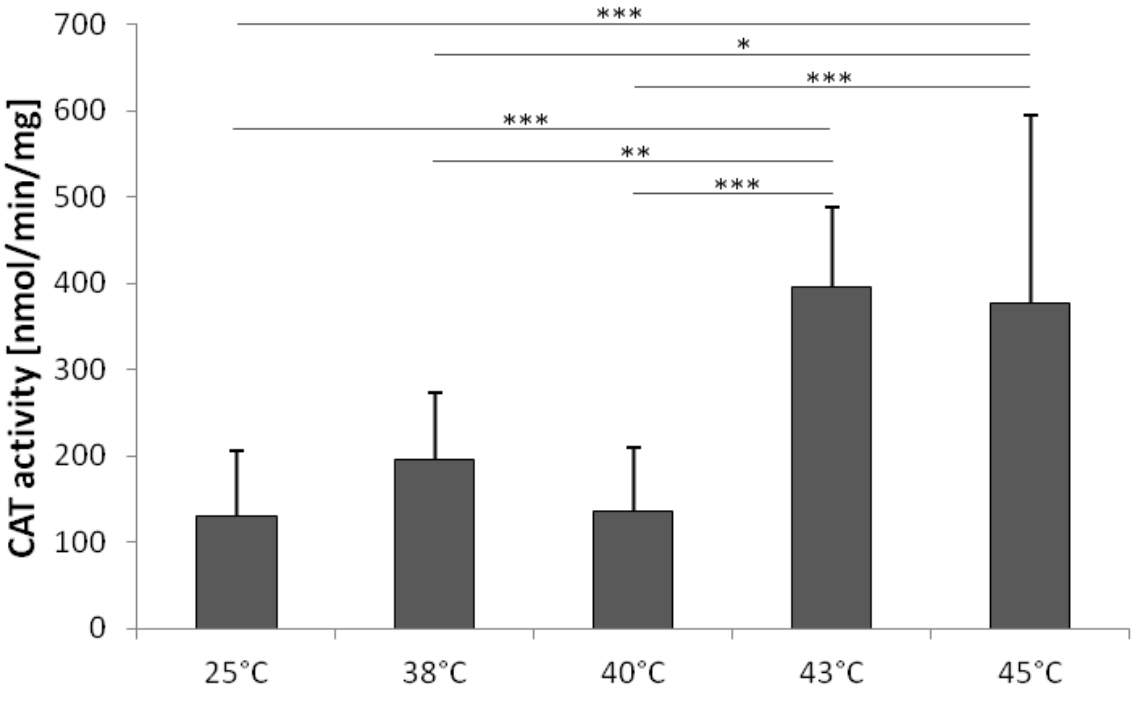

Figure 1. Catalase activity in *X. derbentina* after different temperature treatments (means + SD, n=10). Asterisks indicate significant differences between the groups: $0.01 < p \leq 0.05$ (*), $0.001 < p \leq 0.01$ (**); $p \leq 0.001$ (***).

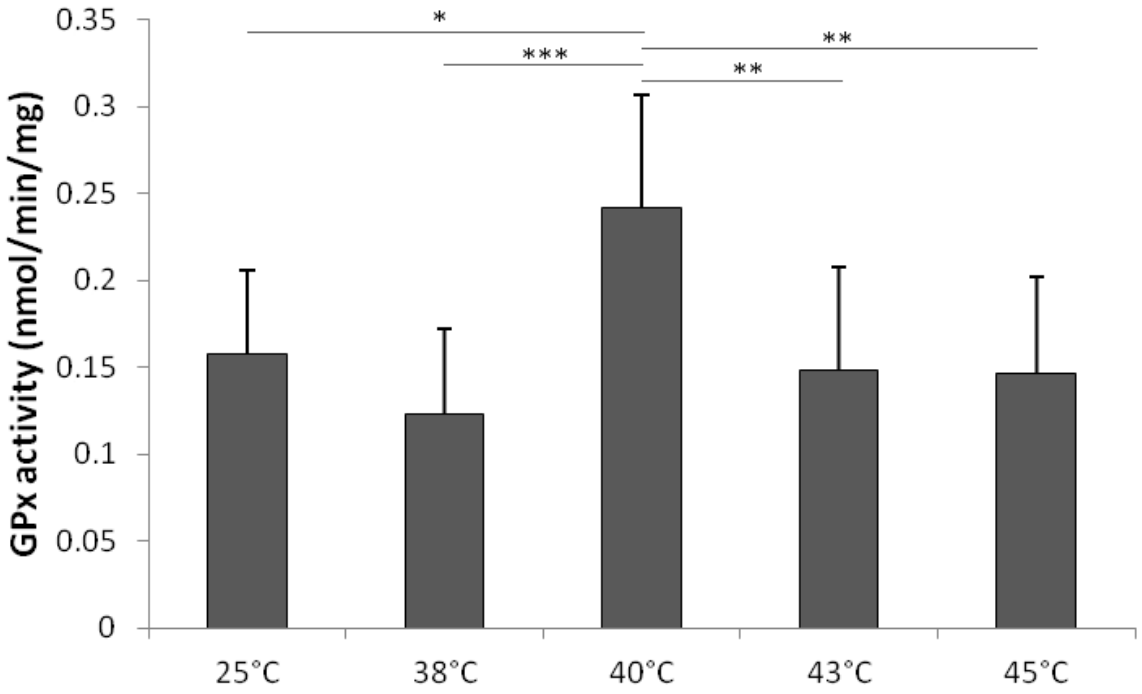

Figure 2. Glutathione peroxidase activity in *X. derbentina* after different temperature treatments (means + SD, n=10). Asterisks indicate significant differences between the groups: $0.01 < p \leq 0.05$ (*), $0.001 < p \leq 0.01$ (**); $p \leq 0.001$ (***).

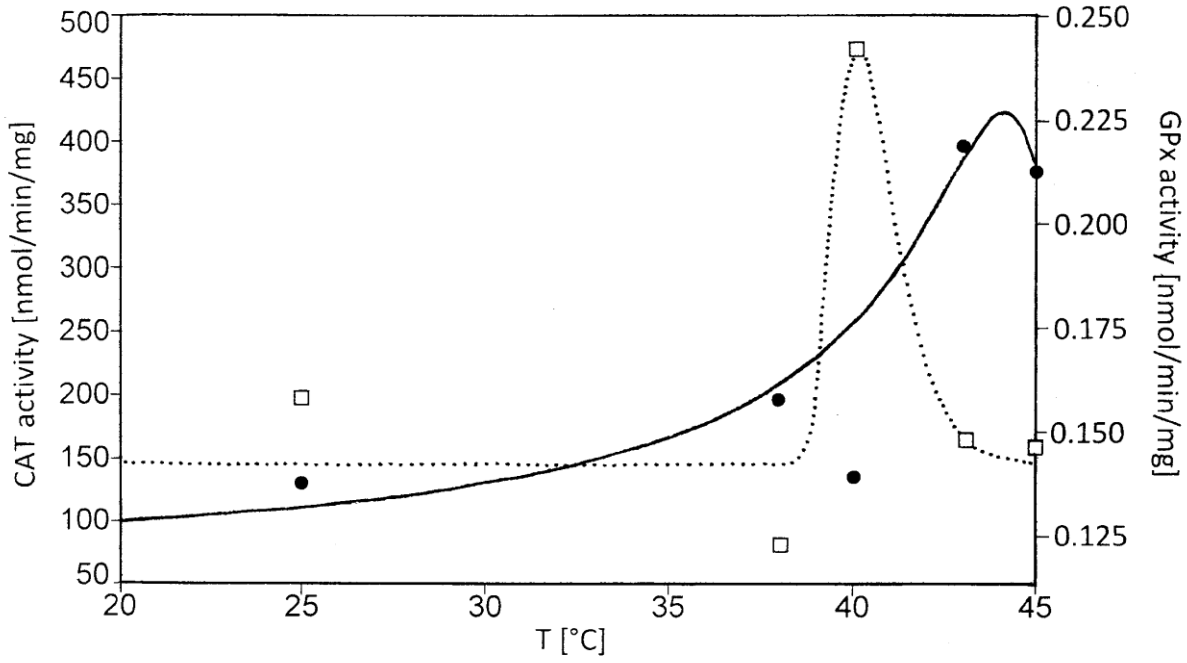

Figure 3. Non-linear regression analysis of catalase (black dots, solid line, left scale, CAT) and glutathione peroxidase (squares, dotted line, right scale, GPx) activities *vs.* temperature (T). $(CAT)^{-1} = 0.011 - (7.369 \cdot 10^{-7})\ T^{2.5} + 3.757\ e^{T}$ with $r^2 = 0.773$

GPx = 0.142 exp [(T / 31.370) + 1.274 – (31.371 exp ((T / 40.063) + 0.242) / 31.370)] with $r^2 = 0.924$

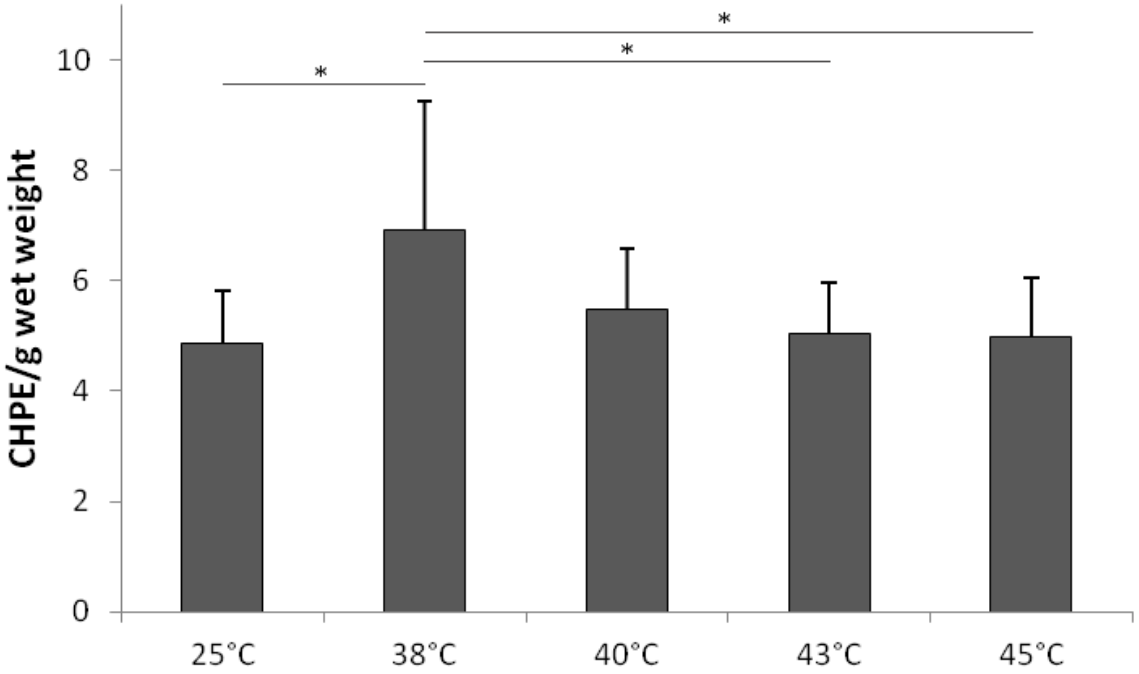

Figure 4. Levels of lipid peroxides (expressed as CHPE per gram wet weight) in *X. derbentina* after different temperature treatments (means + SD, n=10). Asterisks indicate significant differences between the groups: $0.01 < p \leq 0.05$ (*).

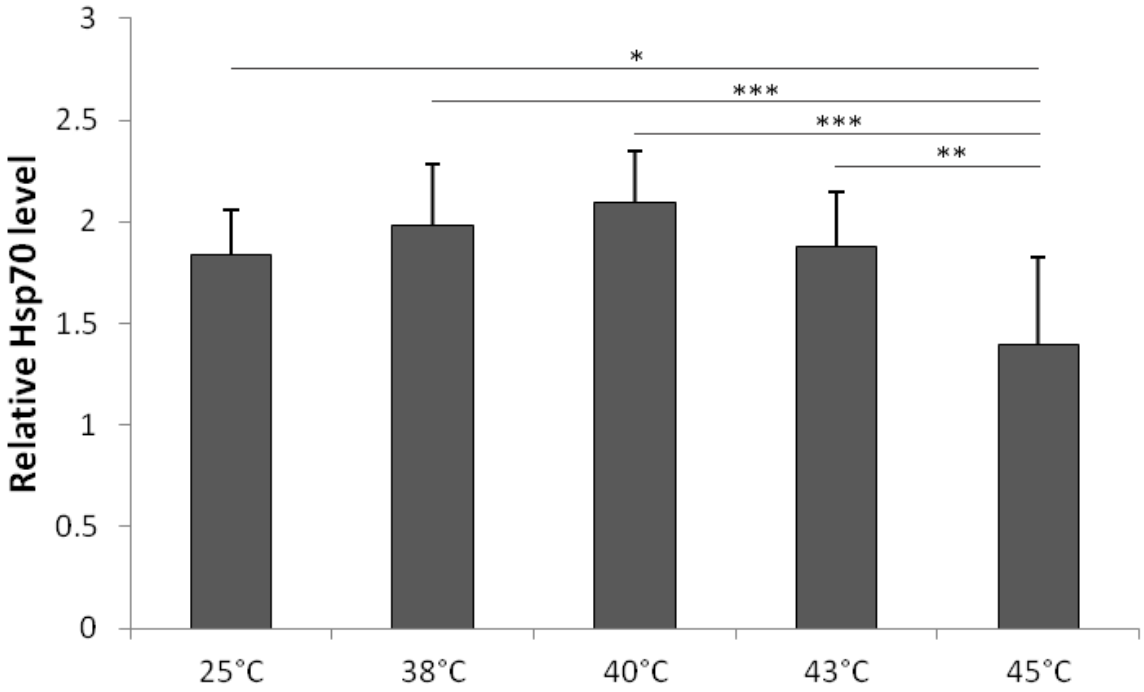

Figure 5. Relative Hsp70 levels in *X. derbentina* after different temperature treatments (means + SD, n=10). Asterisks indicate significant differences between the groups: $0.01 < p \leq 0.05$ (*), $0.001 < p \leq 0.01$ (**); $p \leq 0.001$ (***).

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; Dieterich et al. 2013; Scheil et al. 2011; Di Lellis et al. 2012; 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. unpublished). 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 mechanismsis 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, partucularly 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; Bagnyukova et al. 2007a; Lushchak and Bagnyukova 2006a, b; Verlecar et al. 2007; Bocchetti et al. 2008) and was associated with an increase in antioxidants (Lushchak and Bagnyukova 2006b; Bagnyukova et al. 2006; Bagnyukova et al. 2007b). 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; Troschinski et al. 2014; Di Lellis 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.

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References

Aebi H (1984) Catalase in vitro. Methods Enzymol 105:121-126

Arad Z, Mizrahi T, Goldenberg S, Heller J (2010) Natural annual cycle of heat shock protein expression in land snails: desert versus Mediterranean species of Sphincterochila. The Journal of experimental biology 213 (20):3487-3495

Bagnyukova TV, Chahrak OI, Lushchak VI (2006) Coordinated response of goldfish antioxidant defenses to environmental stress. Aquatic toxicology 78 (4):325-331

Bagnyukova TV, Danyliv SI, Zin'ko OS, Lushchak VI (2007a) Heat shock induces oxidative stress in rotan Perccottus glenii tissues. Journal of Thermal Biology 32 (5):255-260

Bagnyukova TV, Lushchak OV, Storey KB, Lushchak VI (2007b) Oxidative stress and antioxidant defense responses by goldfish tissues to acute change of temperature from 3 to 23 C. Journal of Thermal Biology 32 (4):227-234

Bishop T, Brand M (2000) Processes contributing to metabolic depression in hepatopancreas cells from the snail Helix aspersa. Journal of Experimental Biology 203 (23):3603-3612

Bocchetti R, Lamberti CV, Pisanelli B, Razzetti EM, Maggi C, Catalano B, Sesta G, Martuccio G, Gabellini M, Regoli F (2008) Seasonal variations of exposure biomarkers, oxidative stress responses and cell damage in the clams, Tapes philippinarum, and mussels, Mytilus galloprovincialis, from Adriatic sea. Marine environmental research 66 (1):24-26

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical biochemistry 72 (1):248-254

Daugaard M, Rohde M, Jäättelä M (2007) The heat shock protein 70 family: Highly homologous proteins with overlapping and distinct functions. FEBS letters 581 (19):3702-3710

De Oliveira UO, da Rosa Araújo AS, Belló-Klein A, da Silva RS, Kucharski LC (2005) Effects of environmental anoxia and different periods of reoxygenation on oxidative balance in gills of the estuarine crab Chasmagnathus granulata. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology 140 (1):51-57

Di Lellis MA, Seifan M, Troschinski S, Mazzia C, Capowiez Y, Triebskorn R, Köhler H-R (2012) Solar radiation stress in climbing snails: behavioural and intrinsic features define the Hsp70 level in natural populations of Xeropicta derbentina (Pulmonata). Cell Stress and Chaperones 17 (6):717-727

Di Lellis MA, Sereda S, Geissler A, Picot A, Arnold P, Lang S, Troschinski S, Dieterich A, Hauffe T, Capowiez Y, Mazzia C, Knigge T, Monsinjon T, Krais S, Wilke T, Triebskorn R, Kohler HR (2014) Phenotypic diversity, population structure and stress protein-based capacitoring in populations of Xeropicta derbentina, a heat-tolerant land snail species. Cell stress & chaperones. doi:10.1007/s12192-014-0503-x

Dieterich A, Fischbach U, Ludwig M, Di Lellis M, Troschinski S, Gärtner U, Triebskorn R, Köhler H-R (2013) Daily and seasonal changes in heat exposure and the Hsp70 level of individuals from a field population of Xeropicta derbentina (Krynicki 1836)(Pulmonata, Hygromiidae) in Southern France. Cell Stress and Chaperones 18 (4):405-414

Dittbrenner N, Lazzara R, Köhler H-R, Mazzia C, Capowiez Y, Triebskorn R (2009) Heat tolerance in Mediterranean land snails: histopathology after exposure to different temperature regimes. Journal of Molluscan Studies 75 (1):9-18

Eckwert H, Alberti G, Kohler H-R (1997) The induction of stress proteins (hsp) in Oniscus asellus (Isopoda) as a molecular marker of multiple heavy metal exposure: I. Principles and toxicological assessment. Ecotoxicology 6 (5):249-262

Feder ME, Hofmann GE (1999) Heat-shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology. Annual review of physiology 61 (1):243-282

Fink AL (1999) Chaperone-mediated protein folding. Physiological reviews 79 (2):425-449

Giraud-Billoud M, Vega IA, Tosi ME, Abud MA, Calderon ML, Castro-Vazquez A (2013) Antioxidant and molecular chaperone defences during estivation and arousal in the South American apple snail Pomacea canaliculata. The Journal of experimental biology 216 (Pt 4):614-622. doi:10.1242/jeb.075655

Gorman AM, Heavey B, Creagh E, Cotter TG, Samali A (1999) Antioxidant-mediated inhibition of the heat shock response leads to apoptosis. FEBS letters 445 (1):98-102

Guppy M, Withers P (1999) Metabolic depression in animals: physiological perspectives and biochemical generalizations. Biological Reviews of the Cambridge Philosophical Society 74 (01):1-40

Gutteridge J (1995) Lipid peroxidation and antioxidants as biomarkers of tissue damage. Clinical chemistry 41 (12):1819-1828

Gutteridge JMC, Halliwell B (1990) The measurement and mechanism of lipid peroxidation in biological systems. Trends in Biochemical Sciences 15 (4):129-135. doi:http://dx.doi.org/10.1016/0968-0004(90)90206-Q

Halliwell B (2006) Reactive species and antioxidants. Redox biology is a fundamental theme of aerobic life. Plant physiology 141 (2):312-322

Halliwell B, Gutteridge J (1989) Free radicals in biology and medicine. 2nd edn. Clarendon Press, Oxford, UK,

Heckathorn SA, Poeller GJ, Coleman JS, Hallberg RL (1996) Nitrogen availability alters patterns of accumulation of heat stress-induced proteins in plants. Oecologia 105 (3):413-418

Heise K, Puntarulo S, Nikinmaa M, Abele D, Pörtner H-O (2006) Oxidative stress during stressful heat exposure and recovery in the North Sea eelpout Zoarces viviparus L. Journal of experimental biology 209 (2):353-363

Hendrick JP, Hartl F (1993) Molecular chaperone functions of heat-shock proteins. Annual review of biochemistry 62 (1):349-384

Hermes-Lima M, Storey JM, Storey KB (1998) Antioxidant defenses and metabolic depression. The hypothesis of preparation for oxidative stress in land snails.

Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology 120 (3):437-448

Hermes-Lima M, Storey KB (1993) Antioxidant defenses in the tolerance of freezing and anoxia by garter snakes. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology 265 (3):R646-R652

Hermes-Lima M, Storey KB (1995) Antioxidant defenses and metabolic depression in a pulmonate land snail. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology 268 (6):R1386-R1393

Hermes-Lima M, Storey KB (1996) Relationship between anoxia exposure and antioxidant status in the frog Rana pipiens. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology 271 (4):R918-R925

Hermes-Lima M, Willmore WG, Storey KB (1995) Quantification of lipid peroxidation in tissue extracts based on Fe(III)xylenol orange complex formation. Free Radical Biology and Medicine 19 (3):271-280. doi:http://dx.doi.org/10.1016/0891-5849(95)00020-X

Jena KB, Verlecar XN, Chainy GBN (2009) Application of oxidative stress indices in natural populations of Perna viridis as biomarker of environmental pollution. Marine Pollution Bulletin 58 (1):107-113. doi:http://dx.doi.org/10.1016/j.marpolbul.2008.08.018

Joanisse DR, Storey KB (1996) Oxidative damage and antioxidants in Rana syvatica, the freeze-tolerant wood frog. Am J Physiol 271:R545-R553

Köhler, Zanger, Eckwert, Einfeldt (2000) Selection favours low hsp70 levels in chronically metal-stressed soil arthropods. Journal of Evolutionary Biology 13 (4):569-582. doi:10.1046/j.1420-9101.2000.00210.x

Köhler HR, Lazzara R, Dittbrenner N, Capowiez Y, Mazzia C, Triebskorn R (2009) Snail phenotypic variation and stress proteins: do different heat response strategies contribute to Waddington's widget in field populations? Journal of Experimental Zoology Part B: Molecular and Developmental Evolution 312 (2):136-147

Kregel KC (2002) Invited review: heat shock proteins: modifying factors in physiological stress responses and acquired thermotolerance. Journal of Applied Physiology 92 (5):2177-2186

Lindquist S (1986) The heat-shock response. Annual review of biochemistry 55 (1):1151-1191

Lindquist S, Craig E (1988) The heat-shock proteins. Annual review of genetics 22 (1):631-677

Luna-Acosta A, Bustamante P, Godefroy J, Fruitier-Arnaudin I, Thomas-Guyon H (2010) Seasonal variation of pollution biomarkers to assess the impact on the health status of juvenile Pacific oysters Crassostrea gigas exposed in situ. Environmental science and pollution research international 17 (4):999-1008. doi:10.1007/s11356-009-0287-1

Lushchak V (2011) Environmentally induced oxidative stress in aquatic animals. Aquatic toxicology 101 (1):13-30. doi:10.1016/j.aquatox.2010.10.006

Lushchak VI, Bagnyukova TV (2006a) Temperature increase results in oxidative stress in goldfish tissues. 1. Indices of oxidative stress. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology 143 (1):30-35

Lushchak VI, Bagnyukova TV (2006b) Temperature increase results in oxidative stress in goldfish tissues. 2. Antioxidant and associated enzymes. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology 143 (1):36-41

Lushchak VI, Lushchak LP, Mota AA, Hermes-Lima M (2001) Oxidative stress and antioxidant defenses in goldfish Carassius auratus during anoxia and reoxygenation. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology 280 (1):R100-R107

Machin J (1964) The Evaporation of Water from Helix Aspersa III. The Application of Evaporation Formulae. Journal of Experimental Biology 41 (4):783-792

Mayer M, Bukau B (2005) Hsp70 chaperones: cellular functions and molecular mechanism. CMLS, Cell Mol Life Sci 62 (6):670-684

Meister A (1988) Glutathione metabolism and its selective modification. J biol Chem 263 (33):17205-17208

Mizrahi T, Heller J, Goldenberg S, Arad Z (2010) Heat shock proteins and resistance to desiccation in congeneric land snails. Cell Stress and Chaperones 15 (4):351-363

Mizrahi T, Heller J, Goldenberg S, Arad Z (2012) Heat shock proteins and survival strategies in congeneric land snails (Sphincterochila) from different habitats. Cell Stress and Chaperones 17 (5):523-527

Monserrat JM, Geracitano LA, Pinho GLL, Vinagre TM, Faleiros M, Alciati JC, Bianchini A (2003) Determination of Lipid Peroxides in Invertebrates Tissues Using the Fe(III) Xylenol Orange Complex Formation. Arch Environ Contam Toxicol 45 (2):177-183. doi:10.1007/s00244-003-0073-x

Morimoto RI (1998) Regulation of the heat shock transcriptional response: cross talk between a family of heat shock factors, molecular chaperones, and negative regulators. Genes & development 12 (24):3788-3796

Nowakowska A, Caputa M, Rogalska J (2010) Natural aestivation and antioxidant defence in Helix pomatia: effect of acclimation to various external conditions. Journal of Molluscan Studies 76 (4):354-359

Nowakowska A, Caputa M, Rogalska J (2011) Defence against oxidative stress in two species of land snails (Helix pomatia and Helix aspersa) subjected to estivation. Journal of experimental zoology Part A, Ecological genetics and physiology 315 (10):593-601. doi:10.1002/jez.713

Nowakowska A, Świderska-Kołacz G, Rogalska J, Caputa M (2009) Antioxidants and oxidative stress in Helix pomatia snails during estivation. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology 150 (4):481-486

Pannunzio TM, Storey KB (1998) Antioxidant defenses and lipid peroxidation during anoxia stress and aerobic recovery in the marine gastropod Littorina littorea. Journal of Experimental Marine Biology and Ecology 221 (2):277-292. doi:http://dx.doi.org/10.1016/S0022-0981(97)00132-9

Parsell D, Lindquist S (1993) The function of heat-shock proteins in stress tolerance: degradation and reactivation of damaged proteins. Annual review of genetics 27 (1):437-496

Pomeroy D (1968) Dormancy in the land snail, Helicella virgata (Pulminata: Helicidae). Australian Journal of Zoology 16 (5):857-869

Radwan MA, El-Gendy KS, Gad AF (2010) Biomarkers of oxidative stress in the land snail, Theba pisana for assessing ecotoxicological effects of urban metal pollution. Chemosphere 79 (1):40-46. doi:10.1016/j.chemosphere.2010.01.056

Ramos-Vasconcelos GR, Hermes-Lima M (2003) Hypometabolism, antioxidant defenses and free radical metabolism in the pulmonate land snail Helix aspersa. Journal of experimental biology 206 (4):675-685

Sanchez Y, Taulien J, Borkovich K, Lindquist S (1992) Hsp104 is required for tolerance to many forms of stress. The EMBO journal 11 (6):2357

Scheil AE, Köhler H-R, Triebskorn R (2011) Heat tolerance and recovery in Mediterranean land snails after pre-exposure in the field. Journal of Molluscan Studies 77 (2):165-174

Sies H (1994) Strategies of antioxidant defense. In: EJB Reviews 1993, vol 1993. EJB Reviews. Springer Berlin Heidelberg, pp 101-107. doi:10.1007/978-3-642-78757-7_9

Sies H (1997) Oxidative stress: oxidants and antioxidants. Experimental Physiology 82 (2):291-295

Sørensen J, Dahlgaard J, Loeschcke V (2001) Genetic variation in thermal tolerance among natural populations of Drosophila buzzatii: down regulation of Hsp70 expression and variation in heat stress resistance traits. Functional Ecology 15 (3):289-296

Storey KB (1996) Oxidative stress: animal adaptations in nature. Brazilian journal of medical and biological research = Revista brasileira de pesquisas medicas e biologicas / Sociedade Brasileira de Biofisica [et al] 29 (12):1715-1733

Storey KB (2002) Life in the slow lane: molecular mechanisms of estivation. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology 133 (3):733-754

Storey KB, Storey J (2011) Heat shock proteins and hypometabolism: adaptive strategy for proteome preservation. Res Rep Biol 2:57-68

Tomanek L, Somero GN (2002) Interspecific-and acclimation-induced variation in levels of heat-shock proteins 70 (hsp70) and 90 (hsp90) and heat-shock transcription factor-1 (HSF1) in congeneric marine snails (genus Tegula): implications for regulation of hsp gene expression. Journal of Experimental Biology 205 (5):677-685

Troschinski S, Di Lellis MA, Sereda S, Hauffe T, Wilke T, Triebskorn R, Köhler H-R (2014) Intraspecific Variation in Cellular and Biochemical Heat Response Strategies of Mediterranean Xeropicta derbentina [Pulmonata, Hygromiidae]. PloS one 9 (1):e86613

Verlecar X, Jena K, Chainy G (2007) Biochemical markers of oxidative stress in Perna viridis exposed to mercury and temperature. Chemico-biological interactions 167 (3):219-226

Yom-Tov Y (1971) Body temperature and light reflectance in two desert snails. Journal of Molluscan Studies, vol 39.

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MA Di Lellis, M Seifan, S Troschinski, C Mazzia, Y Capowiez, R Triebskorn, H-R Köhler (2012) Solar radiation stress in climbing snails: behavioural and intrinsic features define the Hsp70 level in natural populations of *Xeropicta derbentina* (Pulmonata). Cell Stress and Chaperones, 17(6): 717-727

A Dieterich, U Fischbach, M Ludwig, MA Di Lellis, S Troschinski, U Gärtner, R Triebskorn, H-R Köhler (2012) Daily and seasonal changes in heat exposure and the Hsp70 level of individuals from a field population of *Xeropicta derbentina* (Krynicki 1836) (Pulmonata, Hygromiidae) in Southern France. Cell Stress and Chaperones, 18(4): 405-414

S Troschinski, MA Di Lellis, S Sereda, T Hauffe, T Wilke, R Triebskorn, H-R Köhler (2014): Intraspecific variation in cellular and biochemical heat response strategies of Mediterranean *Xeropicta derbentina* [Pulmonata, Hygromiidae]. PloS one 9 (1):e86613

A Dieterich*, S Troschinski*, S Schwarz, MA Di Lellis, A Henneberg, U Fischbach, M Ludwig, U Gärtner, R Triebskorn, H-R Köhler (2014): Hsp70 and lipid peroxide levels following heat stress in *Xeropicta derbentina* (Krynicki 1836) (Gastropoda, Pulmonata) with regard to different colour morphs. Cell Stress and Chaperones, DOI 10.1007/s12192-014-0534-3.

MA Di Lellis, S Sereda, A Geißler, A Picot, P Arnold, S Lang, S Troschinski, A Dieterich, T Hauffe, Y Capowiez, C Mazzia, T Knigge, T Monsinjon, S Krais, T Wilke, R Triebskorn, H-R Köhler (2014): Phenotypic diversity, population structure and stress protein-based capacitoring in populations of *Xeropicta derbentina*, a heat-tolerant land snail species. Cell Stress and Chaperones, DOI 10.1007/s12192-014-0503-x

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