Zusammenhänge zwischen physiologischen Mechanismen zur Hitzestressbewältigung und dem phänotypischen Erscheinungsbild des Gehäuses bei thermotoleranten Landgastropoden

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Nur ein Narr macht keine Experimente.

Charles Darwin (1809 – 1882), englischer Naturforscher

Inhaltsverzeichnis

Zusammenfassung

1. Promotionsthema

Zusammenhänge zwischen physiologischen Mechanismen zur Hitzestressbewältigung und dem phänotypischen Erscheinungsbild des Gehäuses bei thermotoleranten Landgastropoden

2. Einleitung

2.1 Grundlagen

Untersuchungen zur Lebensweise xerothermophiler Landschnecken und die damit assoziierten morphologischen, physiologischen und im Verhalten begründeten Anpassungen an die Umwelt besitzen eine lange Tradition. Neben Studien zum Lebensraum, der Verteilung und der Landkolonisierung unterschiedlicher, hitzeresistenter Gastropodenfamilien (Mazek-Fialla 1934, Baur & Raboud 1988, Aubry *et al.* 2005, Aubry *et al.* 2006) wurde untersucht, wie die Tiere bezüglich ihres Metabolismus' an heißes und trockenes Klima angepasst sind. Frühere Studien konnten nachweisen, dass die Aktivitätsphasen (einhergehend mit einem erhöhten Metabolismus) der Tiere zum Zwecke der Nahrungsaufnahme und der Fortbewegung vornehmlich in der kühleren, feuchteren Nacht und in den frühen Morgenstunden liegen (Mazek-Fialla 1934, Machin 1968, Pomeroy 1968). Darüber hinaus betreiben die meisten xerothermophilen Schnecken eine Ästivation (Sommerruhe), welcher eine Senkung der Metabolismusrate zu Grunde liegt, die in einer Einsparung von Wasser und Energiereserven resultiert (Schmidt-Nielsen 1971, Guppy & Withers 1999, Bishop & Brand 2000). Angesichts der Tatsache, dass der Boden in ariden Gegenden durch Sonnen-einstrahlung oftmals sogar für xerothermophile Gastropoden letale Temperaturen erreicht, bevorzugen die Tiere erhöhte Standorte wie beispielsweise Gräser, Büsche, Baumstämme und sogar Zäune, die sie nach Beendigung ihrer aktiven Phasen am Boden aufsuchen und erklettern (Pomeroy 1968, Yom-Tov 1971). Schon wenige Zentimeter über dem Grund fällt

die Lufttemperatur auf ein Niveau, in dem die Schnecken Hitze überdauern können (Köhler *et al.* 2009).

Die Befähigung, große Hitze tolerieren zu können und dafür Austrocknung und Überhitzung in Kauf zu nehmen, bedarf einiger physiologischer Anpassungen. Der Hepatopankreas xerothermophiler Gastropoden ist ein zentrales Stoffwechselorgan, welches nicht nur für die Verdauung und Resorption von Nahrung zuständig ist, sondern auch weitere wichtige Aufgaben im Metabolismus erfüllt (Sumner 1965, Taieb & Vicente 1999). Histologisch sind im Hepatopankreas Resorptionszellen und auch Calciumzellen unterscheidbar, wobei letztere in die Osmoregulation (Taieb & Vicente 1999) und die Regulation des Säure-Base-Haushaltes (Burton 1976) involviert sind. Starke Erwärmung verfügt über das Potential, den Säure-Base-Haushalt, das osmotische Ionengleichgewicht als eine Folge von Wasserverlust und den intrazellulären pH-Wert negativ zu beeinflussen und eine metabolische Azidose hervorzurufen (Barnhart 1986, Heisler 1986, Ryan & Gisolfi 1995). Wiederholt hat sich herausgestellt, dass sich als eine Reaktion auf Hitzestress die Anzahl der Calciumzellen im Hepatopankreas durch Hyperplasie und Hypertrophie erhöht hat (Zaldibar *et al.* 2007, Dittbrenner *et al.* 2009), was die wichtige Aufgabe dieser Zellen bei der Stressbewältigung unterstreicht.

Nicht zuletzt soll als weitere physiologische Anpassung die Induktion von Stressproteinen (Hitzeschockproteine, Hsp) genannt werden. Diese Proteine besitzen essentielle Aufgaben im Zellstoffwechsel. Neben konstitutiv in der Zelle vorliegenden Stressproteinen können einige Hitzeschockproteinfamilien bei Stresszuständen, vor allem bei thermalem Stress, induziert werden (Lindquist & Craig 1988, Feder & Hoffmann 1999, Pörtner & Farrell 2008). Insbesondere Hsp70 wurde in vielen Studien mit Hitzetoleranz in Verbindung gebracht (Köhler *et al.* 1992, Bukau & Horwich 1998, Feder & Hoffmann 1999, Lewis *et al.* 1999, Köhler *et al.* 2000). Die hohe Bedeutung dieser Hsp-Familie besteht in der Fähigkeit, neben der Chaperonfunktion bei der Faltung und Stabilisierung neu synthetisierter Polypeptide, auch teildenaturierte und somit in ihrer Funktion beeinträchtigte Proteine bis zu einem gewissen Grad zu renaturieren, folglich also proteotoxischen Stress zu kompensieren (Gething & Sambrook 1992, Köhler *et al.* 1992, Parsell & Lindquist 1993, Mayer & Bukau 2005). Die Induktion von Hsp70 kann infolgedessen als Indikator für den proteotoxischen Stressstatus eines Organismus‘ gesehen werden (Köhler *et al*. 1992, Triebskorn & Köhler 1996, Feder & Hoffmann 1999, Lewis *et al*. 1999, Köhler *et al*. 2009). In der Literatur existieren einige Studien, die sich mit der Hsp-Expression thermophiler Gastropoden auseinandersetzen.

Mizrahi *et al.* (2009) konnten zeigen, dass vor allem bezüglich Dürrestress auch die Induktion von sHsp's, zur Familie der niedermolekularen Stressproteine gehörende Hsp's, Relevanz besitzt und die Synthese von Hsp70 und Hsp90 insbesondere bei sehr trockenresistenten Pulmonaten der Familie *Sphincterochila* verzögert eintreten kann. Köhler *et al*. (2009) und Scheil *et al*. (2011) konstatierten die wichtige Rolle von Hsp70 bei der Stresskompensation von *Xeropicta derbentina* [Krynicki 1836] und *Theba pisana* [Müller 1774], zwei invasiven, u.a. im mediterranen Raum vorkommenden, xerothermophilen Gastropodenarten und fanden darüber hinaus große intraspezifische Variation bei der Fähigkeit, Hsp70 zu induzieren, was vermutlich oftmals auf Selektionsprozesse in distinkten Mikrohabitaten zurückzuführen ist (Köhler *et al.* 2000). Verschiedene Studien zeigten, dass genetische Unterschiede zu intraspezifischen Variationen in der Hsp-Induktion beitragen können (Sørensen *et al.* 2001, Jensen *et al*. 2009, Bahrndorff *et al*. 2010). Zudem ist die Induktion von Stressproteinen sehr energieaufwendig und somit auch an andere Faktoren, wie beispielsweise an das Energiebudget und an den gesundheitlichen Zustand der Organismen gekoppelt (Sanchez *et al*. 1992, Heckathorn *et al*. 1996, Tomanek & Somero 1999, Köhler *et al*. 2000). Demgemäß folgt die Induktion von Stressproteinen einer Kinetik, die, ausgehend von einem Konstitutivlevel, unter leichtem Stress zu einer Aktivierung der Stressgentranskription und damit zu einem Hsp-Anstieg führt und nach dem Erreichen eines Maximallevels bei moderatem bis stärker werdendem Stress wegen des Verbrauchs von Energiereserven und Zellzerstörung schließlich in die Destruktionsphase mit einer damit einhergehenden Senkung des Hsp-Levels mündet (Eckwert *et al*. 1997). Aufgrund der Tatsache, dass die Synthetisierung von Stressproteinen auf der einen Seite zwar einen hohen Energieaufwand bedeutet, auf der anderen Seite jedoch überlebensnotwenig für die Unversehrtheit der Körperzellen und der Integrität physiologischer Abläufe ist, bildet die Frage nach distinkten Hitzebewältigungsstrategien xerothermophiler Landschnecken einen interessanten Forschungsansatz.

Es existieren wenige Studien, die sich u.a. damit beschäftigen, ob und wie sich die Stressproteininduktion über die Lebensspanne, beziehungsweise über verschiedene Jahreszeiten verändert. Sørensen & Loeschcke (2002) und Mayer & Bukau (2005) geben erste Hinweise, dass jüngere Lebewesen im Vergleich zu älteren Tieren in der Lage sind, höhere Mengen an Stressproteinen zu synthetisieren. Zudem existieren Arbeiten über tageszeitliche und jahreszeitliche Schwankungen in der Hsp-Expression bei Fischen und marinen Mollusken (Köhler *et al*. 2001, Nakano & Iwama 2002, Schill *et al*. 2002, Tomanek & Sanford 2003), jedoch nur wenige zu Pulmonaten (Mizrahi *et al*. 2011, Scheil *et al*. 2011). Bezüglich

circadianer Schwankungen in der Hsp70-Expression in Verbindung mit xerothermophilen Schnecken besteht also noch Forschungsbedarf.

Hitzeliebende Landschnecken sind also physiologisch optimal an deren bevorzugte, aride Gebiete angepasst. Es wurde gezeigt, dass sich Umweltparameter wie beispielsweise die Umgebungstemperatur, die Sonneneinstrahlung, Windgeschwindigkeiten, die Art der Vegetation und das Bodensubstrat, auf das Verhalten, die Physiologie und die Entwicklung ektothermer Tiere auswirken (Stevenson 1985). Darüber hinaus sind diese Umweltvariablen eng in komplexen Interaktionen miteinander verflochten und erfordern von den Pulmonaten nicht nur Überlebens-, sondern auch ausgeprägte Adaptionsmechanismen (Yom-Tov 1971, Staikou 1999, Dittbrenner *et al.* 2009, Mizrahi *et al.* 2009, Scheil *et al.* 2011). Begleitend hierzu unterliegen die Gastropoden aufgrund von Schwankungen der Umweltbedingungen in unterschiedlichen Mikrohabitaten und wegen individueller physiologischer und morphologischer Charakteristika auch Selektionsdrücken (Cain & Sheppard 1954, Cook & O'Donald 1971, Cowie 1985, Johnson 2011, Johnson 2012). Im Interesse vieler Malacologen und deshalb Gegenstand zahlreicher Studien ist die hohe intraspezifische Variation der Gehäusefärbung vieler Schneckenfamilien und der damit assoziierten Farbmuster. Untersuchungen belegen den hohen Anteil der genetischen Ausstattung an der Ausprägung der Gehäusefärbung (Jones 1973, Jones *et al.* 1977, Cain 1984, Cowie 1984). Für die Schalenfärbung von *Cepaea nemoralis* [Linnaeus 1758] beispielsweise wurde gezeigt, dass vorrangig fünf miteinander gekoppelte Genloci u.a. die Schalenfärbung, die Anwesenheit bzw. das Fehlen einer Bänderung und deren Intensität und Kontinuität regulieren (Jones 1980). Auch die dunkle Bänderung mit ihrer variablen Ausprägung auf dem weißen Gehäuse von *T. pisana* wird über ausgewählte, miteinander gekoppelten Allele determiniert, kann jedoch durch Allele auf anderen Loci noch beeinflusst werden (Cain 1984, Cowie 1984). Dies erlaubt eine Vielzahl sehr diverser Morphausprägungen bei bestimmten Gastropodenfamilien (beispielsweise *T. pisana, X. derbentina, Cernuella virgata* [Da Costa 1778]), während andere Gattungen in der Anzahl unterschiedlicher Morphen eher restriktiv sind (*C. nemoralis, Cepaea hortensis* [Müller 1774]).

Divergente Gehäusefärbungen von Landschnecken mit populationscharakteristischen Unterschieden in Morphfrequenzen werden in der Literatur häufig mit Selektionsprozessen assoziiert. Es wurde gezeigt, dass sonnenexponierte Mikrohabitate eine Häufung von Individuen mit heller Schale ohne Bänderung aufweisen, wohingegen gebänderte Pulmonaten vorzugsweise in schattigem Unterholz abundant sind (Heller 1981, Cain 1983, Cowie 1985,

Cook 1998, Slotow & Ward 1997, Johnson 2011). Dies sei vor allen Dingen darauf zurückzuführen, dass helle Individuen eine hohe Albedo aufweisen und somit viel Sonnenenergie abstrahlen, also folglich in der Sonne nicht so schnell aufheizen. Dunkle, gebänderte Organismen hingegen profitieren in buschigen und waldigen Gebieten von einem erhöhten Tarnvermögen und sind weniger sichtbar für Fraßfeinde wie beispielsweise Vögel oder Kleinsäuger (Cain & Sheppard 1954, Heller 1981, Heller & Gadot 1984, Cowie & Jones 1985, Cowie 1990, Slotow *et al.* 1993, Rosin *et al.* 2011, Adrian Surmacki mit Arbeitsgruppe, Posen, unpubliziert). Dem gegenüber existieren auch Studien, die keine bzw. nur marginale Effekte der Gehäusebänderung auf die Erwärmung der Gastropoden offenlegen (Knights 1979, Scheil *et al.* 2012), sondern vielmehr eine Assoziation der Gehäuseerwärmung mit zunehmender Größe als selektives Agens postulieren (Cook & O'Donald 1971, Knights 1979, Cowie 1992, Slotow *et al.* 1993). Heath (1975) veranschaulichte an *C. nemoralis*, dass große Individuen wegen eines kleineren Oberflächen-Volumen-Verhältnisses nach Aufheizung nur langsam abkühlen und sich deshalb bevorzugt in kühleren Gegenden aufhalten. Es bleibt jedoch die Frage, warum eine unterschiedliche regionale Verteilung der Schalenmorphen vorliegt, wenn die Selektion durch Sonneneinstrahlung und damit einhergehender Aufheizung offensichtlich nicht von allen Studien als einziges und ausschließlich wirksames Agens für die Gehäusediversität bestätigt werden konnte. Außerdem gibt es zuweilen unter gegebenen Bedingungen an einem Standort sowohl helle als auch dunkle Individuen.

Eine zusätzliche Aufgabe der Chaperone, primär für Hsp90, zunehmend aber auch für Hsp70 konstatiert, ist epigenetischer Natur. Diese Moleküle sind in der Lage, hinsichtlich mancher Merkmale die Entwicklung eines Organismus' zugunsten eines bestimmten Phänotyps zu kanalisieren und damit eine genetisch determinierte, aber kryptisch bleibende Variation phänotypisch zu unterdrücken (Rutherford & Lindquist 1998, Roberts & Feder 1999, Queitsch *et al*. 2002, Rutherford 2003). Studien zeigen, dass beispielsweise Stress (Hoffmann & Parsons 1997, Imasheva *et al*. 1997, Kristensen *et al*. 2003) oder die pharmakologische Inhibition von Hsp90 bzw. Mutationen in für Hsp90 codierenden Genen (Rutherford & Lindquist 1998, Queitsch *et al*. 2002) einen Anstieg in der phänotypischen Variation verschiedener Populationen von *Drosophila* oder *Arabidopsis* begünstigt. Dieses Phänomen des genetischen „Abpufferns" von Variabilität und damit von epigenetischer Wirksamkeit wurde auch für Hsp70 postuliert (Roberts & Feder 1999, Bergmann & Siegal 2003, Suzuki & Nijhout 2006). Die Ausbildung der phänotypischen Variation, also die Ausprägung verschiedener Morphen, wird demzufolge vor allem unter stressvollen Bedingungen initiiert, wenn Stressproteine für Reparaturarbeiten an teildenaturierten Proteinen akut benötigt werden

und nicht mehr für Kanalisierungsprozesse zur Verfügung stehen (de Visser *et al*. 2003, Tomala & Korona 2008). Der evolutionsbiologische Vorteil einer im Rahmen der phänotypischen Plastizität auftretenden hohen phänotypischen Variation liegt klar auf der Hand: Die Möglichkeit einer Population, als Antwort auf sich ändernde Umweltbedingungen verschiedene Phänotypen auszubilden, verleiht ihr eine hohe Adaptivität und somit erhöhte Überlebenschancen (Dusheck 2002, Price et al. 2003, Sultan 2007, Bolker 2012).

Zahlreiche Studien beschäftigten sich bisher mit polymorphen Schneckenpopulationen und der Frage, inwieweit genetische Eigenschaften und Umweltbedingungen zur Variation des phänotypischen Erscheinungsbildes beitragen (Jones et al. 1982, Baur 1988, Baur & Raboud 1988, Cowie 1990, Johnson 2011, Johnson 2012). Ungeachtet dessen konnte jedoch immer noch nicht abschließend geklärt werden, welche Bedeutung den verschiedenen Faktoren, die bei der phänotypischen Komposition einer Population mitwirken, zukommt. Eine Reihe von Untersuchungen zeigen, dass Hitzestress die phänotypische Variation verschiedener Organismengruppen erhöhen kann (Child *et al*. 1940, Royer *et al*. 2009, Sisodia & Singh 2009, Hansen *et al*. 2011, Crichigno *et al*. 2012). Auch bei *T. pisana* konnte neben der genetischen Konstellation der Individuen die Umgebungstemperatur als selektiv beziehungsweise epigenetisch wirksam herausgestellt werden (Johnson 2011, Johnson 2012). Da Stressproteine insbesondere bei hohen Temperaturen induziert werden und neben intrazellulären „Reparaturarbeiten" kanalisierende Funktion besitzen, sind sie plausible Anwärter zur Erklärung der unter Temperaturstress erzeugten phänotypischen Variation. Bislang existierte erst eine Feldstudie an xerothermophilen Pulmonaten, die eine hohe Hsp70-Induzierbarkeit mit der Unterdrückung phänotypischer Variation in Verbindung bringt. Köhler *et al*. (2009) konnten zeigen, dass Schneckenpopulationen, welche eine hohe Hsp70-Induzierbarkeit aufweisen, über eine geringe phänotypische Variation verfügen und setzten diesen Umstand mit einer hohen epigenetischen Kanalisierung von Hsp70 in Verbindung. Allerdings fehlen in dieser Studie Angaben zur genetischen Struktur der untersuchten Populationen, was eine Gegenüberstellung der phänotypischen Variabilität mit der genetischen Diversität unmöglich macht. Als Gegenstand weiterer Forschungsarbeiten bietet sich somit die Fragestellung, inwieweit sich die Diversität der Gehäusefärbungen innerhalb einer Schneckenpopulation in der jeweiligen genetischen Struktur spiegelt, an.

Ausgewählte Studien thematisierten bisher den Umstand, dass zumindest einige Gastropodenfamilien nachweislich in der Lage sind, ihre Schalenfärbung im Laufe ihres Lebens zu verändern. Cowie (1984a & 1992) und Cain (1984) beschrieben in ihren

Publikationen die Beobachtung, dass manche Individuen von *T. pisana* mit einer ungebänderten, rein weißen Schale im Laufe ihres Lebens beginnen, frisches Schalenmaterial mit ausgeprägter Bänderung zu synthetisieren. Zudem beleuchtete Cowie (1984a) den Umstand, dass bei der Art *T. pisana* Nachkommen bei Kreuzungsversuchen auch oftmals entgegen aller Erwartungen vom genetisch determinierten Phänotyp abweichen, also beispielsweise rezessive Allele zum Durchbruch kommen, obwohl ein Individuum heterozygot ist. Hier könnte die Vermutung, dass der Phänotyp einer Schneckenschale auch noch postembryonal epigenetisch modifiziert und an Umweltbedingungen angepasst werden kann, ihre Bestätigung finden. Auch hier besteht jedoch noch Forschungsbedarf hinsichtlich der Determinierung der diesen Vorgang triggernden ökologischen und physiologischen Bedingungen. An dieser Stelle verbleibt noch zu bemerken, dass zusätzliche Studien vonnöten sind, die zeigen, ob das Phänomen der wechselnden Gehäusebänderung auch anders herum besteht, also eine gebänderte Schale in eine ungebänderte übergehen kann.

Ebenfalls in diesem Zusammenhang von Interesse ist der Umstand, dass Hsp70 die Melaninsynthese beeinflussen kann. Es wurde gezeigt, dass ein hoher Hsp70-Spiegel in Melanomzellen von Mäusen die Melaninsynthese unterdrückt (Hoshino *et al.* 2010). Dies wird in der Publikation von Hoshino *et al*. (2010) vor allem mit einer Beeinflussung des intrazellulären Melanosomentransportes und einer Hemmung der Genexpression und Aktivität der Tyrosinase, einem Enzym zur Melaninsynthese, in Verbindung gebracht. Unter der Annahme, dass Hsp70-Aktivität auch bei Schnecken einen Einfluss auf die Melaninsynthese besitzt - der Syntheseweg für Melanin ist bei allen Metazoen im Wesentlichen gleich (Swan 1974, Prota 1992) - wäre dies eine Möglichkeit für Hsp70, epigenetisch zu agieren, nicht zuletzt auch deshalb, weil die Melaninsynthese viele verschiedene Syntheseschritte vereint, die sich gegenseitig pleiotrop beeinflussen können. Demzufolge müssten Schnecken, die unter längerfristig wirkendem Umweltstress nur wenig Hsp70 induzieren, mit der Zeit eine gebänderte Schale bekommen (und *vice versa*), da die intrazellulär vorhandenen Stressproteine vor allem zur Regeneration der degenerierten Proteine herangezogen würden, was zu einer Dekanalisierung des Phänotyps mittels fehlender Chaperone und damit zur Melaninsynthese führen würde. Die Hemmung der Melaninsynthese durch erhöhte Hsp70-Level wäre also ein mechanistischer Ansatz in der weiträumigen Vorstellung des Capacitoring, welcher sich bei den Gastropoden in Veränderungen im Phänotyp des Gehäuses manifestieren könnte. In der Tat konnte von Köhler *et al*. (2009) gezeigt werden, dass Individuen von *T. pisana*, eben diese Schnecken, die von Cowie (1984a & 1992) und Cain (1984) als morphwechselnde Gastropodenfamilie identifiziert wurden,

unter thermalem Stress vergleichsweise niedrige Hsp70-Level induzieren. Dies könnte darauf hinweisen, dass speziell diese Gastropodenart bei Temperaturstress mit einem Anstieg der Gehäusevariabilität bei niedriger Stressproteinkonzentration reagiert.

Eine interessante Frage, die es in diesem Zusammenhang noch zu untersuchen gilt, ist, ob sich das Hsp70-Induktionsmuster auch auf den Phänotyp anderer xerothermophiler Schneckenarten wie beispielsweise *X. derbentina* auswirkt, insbesondere auch deshalb, weil für *X. derbentina* eine hohe Induktionskapazität für Hsp70 postuliert wurde (Köhler *et al.* 2009).

2.2 Fragestellungen

Im Rahmen dieser wissenschaftlichen Arbeit wurde untersucht, welche morphologischen und ökologischen Variablen, einschließlich ihrer Interaktionen, zu einer physiologischen Stressbelastung bei xerothermophilen Gastropoden führen. Zu diesem Zweck wurden Proben von *Xeropicta derbentina* nach Aufzeichnung ausgewählter Parameter aus dem Feld entnommen. Als Proxy für den durch die Schnecken erfahrenen Stress sollte der Hsp70-Level ermittelt werden. Das Endresultat soll eine ökologische Schnappschusssituation darstellen, in der der Stresslevel der Tiere den detektierten Bedingungen aus dem Feld gegenübersteht und diese reflektiert. Folgende Fragen wurden für dieses Projekt formuliert:

- Ist die Position einer Schnecke, die Orientierung zur Sonne und die Aufenthaltshöhe über dem Grund bedeutsam für die Stressbelastung der Tiere?
- Inwieweit spielen die Größe der Tiere und damit einhergehend das Oberflächen-Volumen-Verhältnis eine Rolle?
- Welchen Effekt verursacht die variable Gehäusefärbung der Gastropoden und ist die Pigmentierung in die Thermoregulation involviert?

In einem nächsten Schritt sollte untersucht werden, wie sich verschiedene, räumlich getrennte *X. derbentina* - Populationen in ihrer Kapazität zur Hsp70-Induktion unterscheiden und wie sich der jeweilige Induktionslevel auf die Integrität des Hepatopankreas auswirkt. Auf der Basis dieses Ergebnisses sollte geprüft werden, ob ein hoher Stressproteinspiegel mit einem guten zellulären Zustand des Hepatopankreas einhergeht und ob sich die unterschiedlichen

Populationen bezüglich etwaiger Hitzebewältigungsstrategien unterscheiden. Folgende Fragen sollten in dieser Studie Antwort finden:

- Gibt es innerhalb derselben Art zwischen verschiedenen Populationen unterschiedliche biochemische und zelluläre Hitzebewältigungsstrategien und wenn ja, wie kann man diese unterscheiden?
- Kann die genetische Populationsstruktur für solche Strategieunterschiede eine Erklärung bereitstellen?

In einem nächsten Ansatz sollte geprüft werden, wie sich die Hsp70-Induktion der Schnecken im Feld während des Tagesverlaufs verhält und ob sich die Induzierbarkeit einer ausgewählten *X. derbentina*-Population über den Jahresverlauf, also korrespondierend zum Alter der Tiere, verändert. Diese Fragen wurden bearbeitet:

- Wie verläuft eine Hsp70-Induktion über einen gesamten Tag?
- Gibt es Veränderungen im Induktionsmuster während dem Jahresverlauf, welche mit dem Lebenszyklus der Tiere zusammenhängen könnten?

Weiterführend sollte untersucht werden, inwieweit die Größe und die Schalenfärbung der Pulmonaten mit deren Erhitzung durch Beleuchtung und der Wiederabkühlung nach Beendigung der Bestrahlung und somit einem zu erwartenden Selektionsdruck in Zusammenhang stehen. Aufgrund der Tatsache, dass viele Studien in der Literatur diesbezüglich an *Theba pisana* vorgenommen wurden und zum Teil widersprüchliche Ergebnisse lieferten, sollte auch diese Studie mit dieser Gastropodenart erfolgen, um die Ergebnisse mit jenen aus der Literatur vergleichen zu können. Diese Fragen wurden als Bearbeitungsgrundlage formuliert:

- Inwieweit beeinflusst eine Gehäusebänderung die Erwärmung und Auskühlung von *T. pisana* durch Illumination durch die Sonne?
- Welche Rolle spielt das Oberflächen-Volumen-Verhältnis bei der Erwärmung und Wiederauskühlung der Tiere?
- Welchen Einfluss besitzen andere Parameter wie beispielsweise die Dauer der Beleuchtung oder die Tageszeit?

Ergänzend hierzu sollte überprüft werden, ob Umweltparameter, also etwa Außentemperaturen, Sonnenstunden oder die Menge an Regenfall in verschiedenen Jahreszeiten, das Potential besitzen, die phänotypische Variation der Gehäusemorphen

während der Maturation in Populationen von *Theba pisana* Populationen zu beeinflussen. Hierfür sollte eine bereits publizierte Langzeitstudie von Cowie (1992), die Daten zu juvenilen und ausgewachsenen Individuen enthält, mit den herrschenden Umweltbedingungen in Verbindung gebracht werden. Hierbei wurde folgenden Fragen nachgegangen:

- Gibt es Hinweise, dass zusätzlich zur erblichen Komponente auch Umweltbedingungen einen Einfluss auf die phänotypische Variation verschiedener *T. pisana* - Populationen haben und falls ja, in welchem Ausmaß tragen beide Faktoren zur Diversität bei?
- Welche Klimaparameter sind bei einer potentiellen Beeinflussung des Phänotyps besonders wichtig?
- Gibt es distinkte Lebensphasen, die für Veränderungen im Phänotyp besonders empfänglich sind?

In einem abschließenden Ansatz sollte der durch *T. pisana* gewonnene Erkenntnisgewinn auf *X. derbentina* übertragen und mit dem Hsp70 Stresssystem der Tiere in Verbindung gebracht werden. Um beurteilen zu können, ob Hsp70 in natürlichen Populationen von *X. derbentina* das Potential besitzt, als Capacitor zu agieren und die phänotypische Ausprägung des Gehäuses zu beeinflussen, soll von 10 verschiedenen Populationen die genetische Populationsstruktur anhand von *COI*-Sequenzen, der jeweilige konstitutive beziehungsweise maximal induzierbare Hsp70-Level und die phänotypische Variation der Gehäusefärbung unter Zuhilfenahme des jeweiligen Shannon-Wiener-Index', ermittelt werden. Um feststellen zu können, ob, und wenn ja, welche dieser Variablen sich gegenseitig beeinflussen und ob die jeweilige populationsspezifische Höhe des Hsp70-Levels die phänotypische Diversität der Gehäuse beeinflusst, sollen diese Faktoren zueinander in Verbindung gesetzt werden. Folgende Fragen sollten beantwortet werden:

- Unterscheiden sich verschiedene *X. derbentina*-Populationen in ihren konstitutiven und maximal induzierbaren Hsp70-Leveln und in ihrer Induktionskapazität?
- Korrespondieren die Hsp70-Spiegel mit dem jeweiligen populationsspezifischen Grad der phänotypischen Variation?
- Inwieweit reflektiert die genetische Populationsstruktur Variationen der Gehäusefärbungen?
- Reflektiert die genetische Diversität der verschiedenen Populationen die jeweilige Hsp70-Induktionskapazität?

3. Material & Methoden

3.1 Testorganismen

Im Zuge dieser Arbeit wurden Schnecken der Überfamilie Helicoidea betrachtet. Die Überfamilie der Helicoidea wird gemeinsam mit sechs weiteren Überfamilien (Orthurethra, Arionoidea, Limacoidea, Clausilioidea, Orthalicoidea und Elasmognatha) der nicht-achatinoiden Unterordnung der Landlungenschnecken (Stylommatophora) untergeordnet, welche sich in der Ordnung der Lungenschnecken (Pulmonata) und in der Klasse der Gastropoda befinden (Wade *et al*. 2006).

Xeropicta derbentina [Krynicki 1836] ist unter den Helicoidea ein Vertreter der Laubschnecken (Familie: Hygromiidae). Ursprünglich heimisch im osteuropäischen Raum (Kroatien, Nord-Griechenland, Bulgarien, Rumänien, Türkei, Kaukasus), hat sich diese Art seit den späten 1940er Jahren invasiv im Süden Frankreichs ausgebreitet (Labaune & Magnin 1999, Kiss *et al*. 2005, Aubry *et al*. 2006). Diese xerothermophile Gastropodenart erreicht eine Größe von 10-16 Millimetern und weist einen intraspezifischen Schalenpolymorphismus auf (Abbildung 1).

Abbildung 1: Schalenpolymorphismus bei *X. derbentina*

Theba pisana [Müller 1774] gehört innerhalb der Helicoidea zur Familie der Schnirkelschnecken (Helicidae). Diese Pulmonaten sind im west- und südeuropäischen Raum nativ, jedoch existieren auch invasive Populationen in Südafrika, Kalifornien und Australien (Cowie 1990). *T. pisana* erreicht eine Größe von bis zu 21 Millimetern und verfügt ebenfalls über eine sehr hohe Variation bezüglich unterschiedlicher Gehäusefärbungen (Abbildung 2).

Abbildung 2: Schalenpolymorphismus bei *T. pisana*

3.2 Aufnahme morphologischer & ökologischer Variablen aus dem Feld

Die für das erste Kapitel der vorliegenden Arbeit benötigten Messungen wurden im Mai 2010 im Feld (Département Vaucluse, Provence, Südfrankreich) für 7 unterschiedliche Populationen konsekutiv für jede einzelne Schnecke (*X. derbentina*) getätigt. Für jedes Individuum wurden folgende Parameter ermittelt: Die Aufenthaltshöhe über dem Grund mittels einem Zollstock [in cm], die geografische Schalenorientierung zur Sonne [3 Kategorien; Apex zugewandt, abgewandt oder lateral zur Sonne], die Oberflächentemperatur des Gehäuses und die Innentemperatur des Weichkörpers unter Zuhilfenahme eines Präzisionsthermometers [in °C] (ELLAB Copenhagen, Typ DM 852), der Schalendurchmesser [in mm] und die Gehäusefärbung [Einteilung in 4 Kategorien; 1: weiß; 2: weiß mit einem schwachen Band; 3: gräulich mit mehreren leichten Bändern; 4: viele Bänder, verteilt über die ganze Schale; in Anlehnung an Köhler *et al*. 2009). Zusätzlich wurden im Labor das Frischgewicht [in mg] und der Hsp70-Level ermittelt.

Für die im dritten Abschnitt dieser Arbeit beschriebenen Untersuchungen wurde zur Untersuchung der *X. derbentina*-Verteilung im Feld über eine Dauer von 4 Probennahmen hinweg (April, Juni, August und Oktober 2011) ein zufällig ausgesuchtes Wiesenstück in Modène, Südfrankreich mit einer Fläche von 1*3 Metern untersucht. Hierfür wurde jeweils von insgesamt 250 Schnecken die Schalenfärbung, die Schalengröße und die Aufenthaltshöhe ermittelt. Zusätzlich wurde stündlich die Lufttemperatur in unterschiedlichen Höhen über Grund (1, 2, 3, 5, 10, 15, 20, 25, 30 und 40 cm) gemessen und Schneckenproben zur Hsp70-Analyse entnommen.

Für die Versuche, die im vierten Kapitel dieser Arbeit beschrieben werden und die sich mit der Erwärmung und Auskühlung im Feld beschäftigen, wurden Individuen von *T. pisana* (aus Biville, Normandie, Nordfrankreich) verwendet. Für die Erwärmungsversuche wurden im Juni 2012 polymorphe Tiere [eingeteilt in 2 Kategorien; gebändert und ungebändert] unter geschützten Freilandbedingungen auf einer Holzplatte aus 30 cm Entfernung mit einer 400 Watt-Halogenlampe für entweder eine oder zwei Minuten bestrahlt. Anschließend wurden die äußere Gehäusetemperatur [in °C], die Innentemperatur [in °C] und der Durchmesser der Schale [in mm] gemessen. Für die Versuche zur Auskühlung der Tiere wurden diese zunächst auf einer Schieferplatte für mindestens 15 Minuten durch Sonnenlicht erwärmt. Anschließend wurden die Tiere auf einem Präzisionsthermometer 20cm vor der Austrittsöffnung eines

Luftgebläses (HP4935, Phillips, Eindhoven, Netherlands) fixiert. Alle 10 Sekunden wurde die Senkung der Innentemperatur bis zum Eintritt eines Temperaturequilibriums notiert.

3.3 Berechnung der phänotypischen Variabilität

Als Maß für die phänotypische Variabilität der verschiedenen *X. derbentina*-Populationen im ersten und sechsten Teil dieser Arbeit wurden auf der Basis der 4 Schalenkategorien (siehe 3.2) Shannon-Wiener-Indices (Hs) nach folgender Formel errechnet:

$$Hs = -\sum_{i=1}^{s} pi * \ln pi$$

(p*i*: Quotient der Anzahl von Individuen der Kategorie *i* und der Gesamtanzahl der Tiere einer Population. *s:* Anzahl der Kategorien). Für den fünften Teil dieser Arbeit, der an *T. pisana* durchgeführt wurde, wurde der Hs anhand von 5 verschiedenen Schalenkategorien bei adulten Tieren und 3 Kategorien bei den Juvenilen errechnet. Hierzu wurde der von Cowie (1992) publizierte Datensatz verwendet.

3.4 Temperaturexpositionen

Um Rückschlüsse auf die Hsp70-Induktionskapazität und die pathologische Wirkung verschiedener Temperaturexpositionen auf den Hepatopankreas ziehen zu können, wurden für die im zweiten und sechsten Kapitel beschriebenen Versuche Individuen verschiedener *X. derbentina*-Populationen zunächst im Mai 2010 aus dem Feld entnommen und bei 25°C für zwei Wochen im Labor auf angefeuchteter Terrarienerde (JBL TerraBasis, Neuhofen, Deutschland) akklimatisiert. Die Fütterung erfolgte nach den Empfehlungen von Cowie & Cain (1983) anhand von Babybrei (HIPP Gute Nacht Bio-Milchbrei, Hafer & Apfel, Pfaffenhofen, Deutschland) und Sepiaschulp. Dann erfolgte die Temperaturexposition im Wärmeschrank in Plastikboxen (6.5 * 18 * 13cm), welche mit feuchten Vliespapiertüchern ausgelegt wurden. Jeweils insgesamt 22 Schnecken für Hsp70- und Histopathologieanalysen wurden für 8 Stunden folgenden Temperaturen ausgesetzt: 25, 33, 38, 40, 43, 45, 48, 50 und 52°C. Nachdem die Größe der Tiere notiert wurde, wurden die Schnecken für die Stressproteinanalysen in flüssigem Stickstoff schockgefroren und bis zur Weiterverarbeitung bei -25°C gelagert, sowie diejenigen für die histopathologischen Untersuchungen präpariert und komplett, jedoch ohne Schale in 2%igem Glutardialdehyd-Cacodylatpuffer fixiert.

3.5 Stressprotein – Analyse

Für die Analysen im ersten, zweiten, dritten und sechsten Kapitel dieser Arbeit wurde der individuelle Level an Hsp70 der Gastropoden quantifiziert. Die einzelnen Individuen wurden mechanisch mit jeweils 2µl Extraktionspuffer (80mM Kaliumacetat, 5mM Magnesiumacetat, 20mM Hepes) je 1mg Schneckengewebe homogenisiert und das Homogenat bei 2000*g* zentrifugiert. Ein Teil des Überstandes wurde zur Bestimmung der Gesamtprotein-konzentration nach Bradford (1976) verwendet, der Rest wurde mit SDS-Puffer versetzt und anschließend in einer Menge von 40µg Gesamtprotein je Probe mittels einer SDS-PAGE (SDS-Polyacrylamidgelelektrophorese) aufgetrennt. Anschließend wurden die Proteine mittels Elektrotransfer auf Nitrocellulose gebracht, auf der sie dann zunächst mit Antikörpern (1. AK: mouse anti-human Hsp70, Kreuzreaktion mit konstitutivem und induzierten Stressprotein; 2. AK: goat anti-mouse IgG, Peroxidase-konjugiert) inkubiert und anschließend durch Umsetzung von 1mM 4-Chloro(1)naphthol-Lösung angefärbt wurden. Abschließend wurde das optische Volumen der Hsp70-Banden mittels E.A.S.Y. Win 32 (Herolab, Wiesloch, Deutschland) densitometrisch erfasst. Alle Proben wurden relativ zu einer Standardprobe, die auf jedem Gel zweifach aufgetragen wurde, quantifiziert, um eventuelle filterabhängige Unterschiede in der Färbung zu berücksichtigen.

3.6 Histopathologische Untersuchungen

Für den zweiten Teil dieser Arbeit wurden die Gastropoden nach Hitzeexposition auf histopathologische Auffälligkeiten im Hepatopankreas untersucht. Hierfür wurden die zuvor von der Schale befreiten Tiere zunächst für mindestens eine Woche im Fixans belassen, um eine gute Durchdringung des Gewebes zu gewährleisten. Anschließend wurden die Proben entkalkt und über ansteigende Alkoholstufen entwässert, bevor sie in Epoxidharz (Technovit, Heraeus Kulzer GmbH, Wehrheim, Deutschland) eingebettet wurden. Darauffolgend wurden 7µm dicke Schnittpräparate angefertigt, die mit Hämatoxylin-Eosin-Lösung angefärbt wurden. Der Hepatopankreas wurde anschließend hinsichtlich seines lichtmikroskopischen Erscheinungsbildes qualitativ beschrieben und semiquantitativ bewertet, wofür ein 5-Kategorien-Bewertungssystem zum Einsatz kam. Hierbei stand Kategorie 1 für den Kontrollstatus, Kategorie 3 für den Reaktionsstatus und Kategorie 5 für den Destruktionsstatus, Kategorien 2 und 4 beschrieben Intermediärzustände. Der Zustand der

Verdauungstubuli, der Resorptions- und Calciumzellen wurde jeweils für jedes Individuum einzeln bewertet und die Werte für Parallelproben anschließend gemittelt.

3.7 Untersuchungen zur genetischen Struktur der Schneckenpopulationen

Für das zweite und sechste Kapitel wurden *X. derbentina*-Populationen hinsichtlich ihrer genotypischen Populationsstruktur untersucht. Hierfür wurde tiefgefrorenes Gewebe aus dem Fuß der Heliciden entnommen, aus dem anschließend mit einem DNeasy Blut & Gewebe-Kit (QUIAGEN, Inc. Mississauga, Ontario, USA) genomische DNA extrahiert wurde. Anschließend erfolgte eine PCR-Amplifizierung des mitochondrischen Cytochrom-C-Oxidase Untereinheit 1 (*COI*) Gens mit einer Länge von 700 Basenpaaren (Primer für Amplifizierung und DNA Sequenzierung: LCO1490 [Folmer et al. 1994] und neuer Primer: HeliR2 5‘-CCTAAAATATGWGAAAYAATACCAAA-3‘). Sequenziert wurde in beide Richtungen mittels eines ABI 3730 XL DNA Analyzers.

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

Einige der untersuchten morphologischen und ökologischen Variablen besitzen einen distinkten Einfluss auf den Hsp70-Spiegel von *X. derbentina*. Die Aufenthaltshöhe über dem Grund erwies sich dahingehend als sehr bedeutender Einflussfaktor auf den Hsp70-Level, als dass höher sitzende Artgenossen einen niedrigeren Hsp70-Spiegel aufwiesen. Wenige Zentimeter über dem im Tagesverlauf bis zu über 50°C heiß werdendem Boden sinkt die Temperatur auf ein weit angenehmeres Niveau (Cowie 1985, Kempster & Charwa 2003). Mit niedrigerer Außentemperatur sinkt auch der proteotoxische Stress und infolgedessen auch das Hsp70-Niveau. Zunächst einmal überraschend erschien der Umstand, dass die Innentemperatur der Gastropoden in unseren Ergebnissen nur marginalen Einfluss auf die Höhe des Hsp70-Spiegels zu haben schien. In der Tat unterschied sich die Innentemperatur

der in dieser Feldstudie untersuchten Organismen jedoch auch nur wenig (max. 6°C), zudem muss beachtet werden, dass der Hsp70-Spiegel über gewisse Zeitspannen integriert und nicht sofort auf kleine Temperaturänderungen, wie sie z.B. durch Wind oder kurze Schattenereignisse auftreten können, reagieren kann. Zudem können genetische und physiologische Unterschiede zwischen den Individuen mögliche Effekte auf den Hsp70-Spiegel durch die Innentemperatur überdecken.

Als ebenfalls von hoher Bedeutung für die Höhe des Hsp70-Niveaus stellte sich die Größe des Gehäuses heraus. Je kleiner die Schnecke war, desto stärker wurde Hsp70 induziert. Zum einen kann das am entsprechenden Alter der Individuen liegen. Im juvenilen Stadium werden für Proteinfaltungsprozesse, Proteintransport und –aggregierung im Zuge des Wachstums und Reifung viele konstitutive Chaperone benötigt (Mayer & Bukau 2005). Zum zweiten kann ein ungünstigeres Oberflächen-Volumen-Verhältnis kleiner Schnecken früher zu Austrocknungserscheinungen und damit zu einer erhöhten Stressproteininduktion führen (Mizrahi *et al*. 2009).

Die Gehäuseorientierung zur Sonne offenbarte keine nennenswerten Effekte auf den Stressstatus der Tiere. Dieser Faktor in Interaktion mit der Höhe über dem Boden, jedoch, zeigte eine Auswirkung. Wenn das Gehäuse mit dem Apex zur Sonne orientiert war, erhöhte sich der Hsp70-Spiegel mit zunehmendem Abstand zum Boden. Bei einer Bestrahlung durch die Sonne von hinten oder von lateral, nahm der Hsp70-Level mit mehr Abstand zum Grund ab. Möglicherweise unterscheiden sich die unterschiedlich lokalisierten Körpergewebe in der Schnecke, wie beschrieben für andere Mollusken (Lyons *et al*. 2003, Mizrahi *et al*. 2009, Arad *et al*. 2010), in ihrer Induktionsfähigkeit für Hsp70.

Die Schalenfärbung der Gastropoden erwies sich ebenso als bedeutend für den Hsp70-Pegel. Dunkel pigmentierte Individuen wiesen einen höheren Stressproteinspiegel auf als ihre hellen Artgenossen. Dies könnte einerseits mit einer erhöhten Aufwärmung der Tiere durch eine stärkere Sonnenlichtabsorption, wie postuliert von beispielsweise Yom-Tov (1971) und Dittbrenner *et al*. (2009), zusammenhängen, andererseits aber auch mit dem Alter der Tiere, denn eine zunehmende Gehäusefärbung war stark negativ mit der Größe der Gastropoden korreliert. In diesem Zusammenhang soll erwähnt werden, dass Hoshino *et al*. (2010) eine Reduktion der Melaninsynthese in Mausmelanomzellen durch erhöhte Hsp70-Induktion zeigen konnte. Möglicherweise waren deshalb adulte Tiere, die bereits länger gegenüber Hitze ausgesetzt waren, tendenziell heller gefärbt.

Zusammenfassend kann gesagt werden, dass große Schnecken größtenteils weiß gefärbt sind und sich vergleichsweise hoch über dem Grund niederlassen, während sie einen recht niedrigen Hsp70-Spiegel halten. Kleine Schnecken tendieren zu dunkleren Schalen, halten sich in niedrigerem Abstand zum Boden auf und zeigen einen höheren Hsp70-Level. Aufgrund der Tatsache, dass Klettern genauso wie die Induktion von Stressproteinen sehr energieaufwendig ist, muss angenommen werden, dass beide Faktoren miteinander in einem energetischen Trade-off-Verhältnis zueinander stehen.

4.2 Kapitel 2: *S Troschinski, MA Di Lellis, S Sereda, T Hauffe, T Wilke, R Triebskorn, H-R Köhler* (*eingereicht bei* PLoS ONE*) Intraspecific variation in cellular and biochemical heat response strategies of Mediterranean* Xeropicta derbentina *[Pulmonata, Hygromiidae].*

Diese Studie veranschaulicht, dass der Hepatopankreas xerothermophiler Gastropoden über metabolische Prozesse stark in die Hitzestressbewältigung integriert ist. Nach der Exposition von Vertretern aus sieben verschiedenen *X. derbentina*-Populationen gegenüber hohen Temperaturen konnten Veränderungen am Hepatopankreas beobachtet werden. Typisch hierfür war die Präsenz fragmentierter Zell-Apices bei Resorptionszellen, welche durch freigesetzte lysosomale Enzyme hervorgerufen worden sein könnten (Moeller *et al*. 1976, Poste *et al*. 1971) Außerdem konnte das verstärkte Auftreten dunkler Zellnuklei, einer gestörten Zellkompartimentierung und einer verringerten Dichte in Calciumzellen, welche wichtige osmoregulatorische Eigenschaften besitzen (Burton 1976, Taib & Vicente 1999, Aviworo, 2011) einen Hinweis auf osmotischen Stress und ein gestörtes Säuren-Base-Gleichgewicht geben (Burton 1976, Barnhart 1986, Heisler 1986, Ryan & Gisolfi 1995). Resorptionszellen konnten im Vergleich mit Calciumzellen allgemeinhin als hitzesensitiver identifiziert werden.

Begleitend zu den histologischen Untersuchungen wurde der Hsp70-Level der Schnecken unter verschiedenen Temperaturexpositionen betrachtet. Auch hier konnte die Induktionskinetik einer Optimumskurve mit einem Maximallevel bei 40°C erfasst werden, wobei sich die Populationen bezüglich der Höhe ihrer Maximalinduktion erheblich unterschieden. Aufgrund der populationsspezifischen Unterschiede in der Hepatopankreashistologie und der Stressproteininduktion ist es möglich, verschiedene Hitzebewältigungsstrategien zu identifizieren. Diese wurden wie folgt klassifiziert:

- Strategie 1: moderate Hsp70-Induktion mit damit einhergehender moderater Zellreaktionen im Hepatopankreas (verfolgt von insgesamt 3 Populationen).
- Strategie 2: Investition in hohe Hsp70-Niveaus mit moderatem, zellulären Zustand (verfolgt von ebenfalls 3 Populationen)
- Strategie 3: kein signifikant erhöhter Hsp70-Spiegel mit dem Risiko eines schnellen zellulären Zerfalls bei hohen Temperaturen (eine Population).

Hierbei wurde beobachtet, dass Calciumzellen sich im Schnitt selbst bei hepatopankreatischen Destruktionszuständen in einem intakteren Zustand befanden als Resorptionszellen. Diese Unterschiede zwischen beiden Zelltypen konnte mit den jeweiligen Hsp70-Niveaus assoziiert werden. Je höher der Hsp70-Spiegel, desto intakter war die zelluläre Struktur der Calciumzellen. Möglicherweise hängt dies mit den wichtigen metabolischen Aufgaben der Calciumzellen unter Stress in Verbindung, außerdem kann in diesem Zusammenhang auch die Frage aufgeworfen werden, ob die Hsp70-Synthese im Hepatopankreas vor allem von diesem Zelltyp übernommen wird.

Aufgrund der Tatsache, dass sich eine hohe Hsp70-Induktion als sehr energieaufwendig gestaltet, kann eine übermäßige Synthetisierung die Fitness der Tiere beeinträchtigen (Feder et al. 1992, Krebs & Loeschke 1994). Eher niedrig gehaltene Hsp70-Niveaus können sich also vorteilhaft auf Energiebudgets auswirken und durch Selektionsprozesse favorisiert werden (Sørensen *et al*. 2001, Zatsepina *et al*. 2001, Mizrahi *et al*. 2009). Tatsächlich zeigte sich in Analysen zur genetischen Struktur der sieben Populationen, dass ebendiese, welche sich genetisch wenig von Nachbarpopulationen unterschieden, eher hohe Hsp70-Niveaus mit einer besseren zellulären Kondition unter Temperaturstress hielten, während davon ausgegangen werden kann, dass Populationen mit großen genetischen Unterschieden zu Nachbarpopulationen aufgrund von mikrohabitatabhängigen Unterschieden unter einem hohen Selektionsdruck standen und auf niedrig gehaltene Hsp70-Niveaus mit einer daraus resultierenden Einsparung von Energiereserven selektiert wurden.

4.3 Kapitel 3: *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

In dieser Studie konnte eine positive Korrelation zwischen der Schalentemperatur und dem Hsp70-Spiegel der Gastropoden im April, Juni und August gezeigt werden. Nur im Oktober gab es eine negative Korrelation dieser beiden Parameter, also einen Abfall des Hsp70-Levels unter höheren Temperaturen. Dies kann mehrere Gründe haben. Zum einen wurde gezeigt, dass ältere Organismen vergleichsweise niedrigere Hsp70-Spiegel aufgrund eines energieaufwendigen Reproduktionssystems besitzen (Sørensen & Loeschke 2002, Mayer & Bukau 2005, Köhler 2009, Mizrahi *et al*. 2011). Zum anderen könnte das Hsp70-Stressabwehrsystem über die heißen Sommermonate ausgeschöpft sein und mit einem zellulären, pathologischen Befund von Hsp70-synthetisierenden Gewebestrukturen einhergehen (Dittbrenner *et al*. 2009, Scheil *et al*. 2011). Außerdem kann ein reduzierter Metabolismus mit einer damit möglicherweise einhergehenden Senkung von Hsp70 wegen einer Ästivation, in der sich die Tiere im Oktober befinden, eine Rolle spielen (Riddle 1981, Umezurike & Iheanacho 1983, Storey 2002, Reuner *et al*. 2008).

Neben den saisonalen Änderungen der Hsp70-Induktion konnten auch Veränderungen im Hsp70-Spiegel über den Tagesverlauf nachgewiesen werden. Die Hsp70-Induktion folgte dem Verlauf der Umgebungstemperatur über den Tag. Im April mit einem Temperaturmaximum von 27,3°C verblieb die Hsp70-Induktion auf geringem Niveau, während im Juni und im August bei weitaus heißeren Maximaltemperaturen (32,9 beziehungsweise 33,7°C) ein Anstieg des Hsp70-Spiegels erfolgte. Im Oktober jedoch (maximal 23,0°C) ließ sich kein Stressproteinanstieg erkennen, was auf niedrige Temperaturen und das Alter der Tiere zurückzuführen ist.

Zudem zeigte sich, dass die Tiere mit dem Voranschreiten des Jahres an Größe zunehmen. Im April konnten die meisten Schnecken noch einem Juvenilstadium zugeordnet werden (definiert als < 10mm, Labaune 2001, Kiss *et al*. 2005), während sie im Juni das Adultstadium erreichten. Vereinzelte Tiere, die bereits im April ausgewachsen waren, wurden als Vertreter eines zweijährigen Lebenszyklus identifiziert (Kiss *et al*. 2005). Die Schalenfärbung der Gastropoden veränderte sich während der Maturation im Jahresverlauf dahingehend, dass die Schale aufhellte und eventuelle Bänderungen von neu synthetisiertem, weißem Schalenmaterial überwachsen wurden und damit verschwanden.

Summa summarum konnte in dieser Studie gezeigt werden, dass es tageszeitliche und jahreszeitliche Schwankungen in der Hsp70-Induktion gibt, die in erster Linie mit der Außentemperatur und dem Alter der Organismen einhergehen.

4.4 Kapitel 4: *T Knigge, MA Di Lellis, T Monsinjon, H-R Köhler (*Journal of Zoology, *unter Revision*) *Relevance of body size and shell colouration for thermal absorption and heat loss in White Garden Snails from Northern France.*

Die Ergebnisse dieser Studie weisen darauf hin, dass sowohl die Körpergröße von *T. pisana* als auch die Gehäusepigmentierung für die Erwärmung beziehungsweise Abkühlung des Weichkörpers Relevanz aufweisen. *De facto* kann jedoch keine Aussage darüber getroffen werden, welcher Parameter wichtiger für die Erwärmung / Abkühlung der Schnecken ist. Der Einfluss der Körpergröße ist erklärbar mit einem höheren Oberflächen-Volumen-Verhältnis kleinerer Schnecken, welches dazu führt, dass sich die Pulmonaten aufgrund einer höheren Absorption elektromagnetischer Strahlung schneller aufheizen, jedoch auch schneller wieder auskühlen (Heath 1975). Zusätzlich zeigte sich, dass hellere Tiere tendenziell weniger aufheizten, was an einem erhöhten Reflektionsvermögen heller Schalen liegen kann (Cain & Sheppard 1954, Heller 1981, Cowie & Jones 1985, Cowie 1990, Slotow *et al.* 1993).

Nach Kurzzeiterwärmung der Tiere ließ sich insbesondere morgens bei starker Sonneneinstrahlung ein signifikanter Effekt der Schalenfärbung auf die Außen- und Innentemperatur erkennen, während dieser Effekt nachmittags nicht gegeben war. In der Literatur wurden Studien mit dem Versuch, einen Effekt der Gehäusefärbung mit einer Erwärmung von Schnecken zu assoziieren, kontrovers diskutiert (Cowie 1992, Slotow *et al*. 1993, Scheil *et al*. 2012). Während ein Teil dieser Studien einen Effekt der Gehäusebänderung auf die Körpertemperatur nachweisen konnten (Cain & Sheppard 1954, Jones et al. 1977, Heller 1981, Johnson 1981, Johnson 2011), fanden andere Studien keinen, oder nur marginale Effekte (Cook & O'Donald 1971, Knights 1979, Goodfriend 1986, Scheil *et al*. 2012). Möglicherweise hängt die Erwärmung der Schale sowohl mit der Struktur des Schalenmaterials (Comfort 1951, Hedegaard *et al*. 2006) als auch von optischen Merkmalen und der Intensität der Sonneneinstrahlung ab.

Die Größe der Schnecken zeigte sich insbesondere morgens nach einminütiger und nachmittags nach zweiminütiger Sonnenbestrahlung als relevant für die Körpererwärmung. Nach einer längeren Erhitzungsperiode der Schnecken konnte ein messbarer Effekt der

Gehäusefärbung bei der Auskühlungsdauer beobachtet werden. Dies äußerte sich darin, dass gebänderte Individuen langsamer auskühlten als ungebänderte, was höchstwahrscheinlich darauf zurückzuführen ist, dass gebänderte Individuen parallel zum Kühlprozess über eine längere Zeitdauer etwas mehr Sonnenlicht absorbierten.

Zusammenfassend kann festgehalten werden, dass sich in dieser Studie die Gehäusefärbung als auch die Körpergröße von *T. pisana* als bedeutend für die Körpererwärmung und –auskühlung erwiesen haben. Eine durch Illumination induzierte Selektion muss also für beide Faktoren angenommen werden.

4.5 Kapitel 5: *H-R Köhler, C Schultz, AE Scheil, R Triebskorn, M Seifan, MA Di Lellis (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

Dieser Studie liegt der Datensatz einer Publikation von Cowie (1992) zugrunde, der im Gegensatz zu anderen Studien zur phänotypischen Verbreitung von Schalenmustern (Johnson 2011, Silvertown *et al*. 2011) sowohl Daten zur phänotypischen Diversität juveniler als auch adulter Individuen von *T. pisana* über 13 Jahre hinweg enthält. Die Studie wurde bei Tenby (Pembrokeshire, Wales, UK) unter moderaten Klimabedingungen durchgeführt.

Korrelationsanalysen zwischen der phänotypischen Ausprägung der Schneckengehäuse (formuliert als Hs-Werte der verschiedenen Populationen) juveniler und adulter Individuen von *T. pisana* zeigten eine hohe Assoziation dieser Parameter zueinander, was auf eine wichtige genetische Komponente und damit auf eine populationsspezifische Determinierung des Phänotyps schließen lässt. Zudem zeigten modellselektive AIC_C-Anwendungen, dass die phänotypische Variation adulter Tiere zusätzlich zur erblichen Komponente auch von distinkten Umweltparametern, genauer gesagt, der durchschnittlichen Winter- und Frühlingstemperatur, beeinflusst wird, wohingegen Juvenile diese Abhängigkeit nicht aufwiesen. Dieser Umwelteinfluss ist in den Daten mit ungefähr einem Drittel der durch die Hs-Werte ausgedrückten, erklärbaren Streuung abgedeckt, während die weiteren zwei Drittel durch erbliche Faktoren determiniert werden. Da kein Zusammenhang zwischen Klimaparametern und reinweißen Pulmonaten als stellvertretendem Morph hergestellt werden konnte, ist davon auszugehen, dass es sich bei der Änderung der phänotypischen, intraspezifischen Variabilität nicht um gerichtete Selektion handelt, wie diese beispielsweise

oft durch Sonneneinstrahlung mit einhergehenden hohen Temperaturen und Sichtbarkeit gegenüber Prädatoren postuliert wurde (Cain & Sheppard 1954, Richardson 1974, Jones *et al*. 1977, Heller 1981, Johnson 1981 & 2011, Cowie 1990, Slotow & Ward, 1997).

In der Tat wurden neben den in der Publikation Cowies (1992) beschriebenen Tieren auch am nördlichen Rand des Verbreitungsgebietes von *T. pisana* Individuen gefunden, die einen Wechsel der Gehäusefärbung von weiß nach gebändert aufwiesen. Dieser Morphwechsel gibt einen Hinweis auf ein sensibles Fenster während des Lebenszyklus‘ von *T. pisana*, in dem innerhalb eines genetisch festgelegten „Spielraumes“ der phänotypischen Plastizität eine Modifikation des Phänotyps als Antwort auf klimatische Bedingungen möglich ist. Die Tatsache, dass eine erhöhte Temperatur in den Gastropoden eine Erhöhung der phänotypischen Variation hervorruft, wäre erklärbar durch den Umstand, dass schon leicht erhöhte Temperaturen mit der physiologischen Stressantwort der Organismen interferieren, welches auch für Kanalisationsprozesse eine wichtige Rolle spielt. Werden Chaperone für die Kompensierung von proteotoxischem Stress benötigt, verlieren sie ihre puffernde, „kanalisierende“ Aufgabe und das phänotypische Erscheinungsbild kann sich verändern. Aufgrund der Tatsache, dass *T. pisana* unter Temperaturstress vergleichsweise wenig Hsp70 und Hsp90 induziert (Köhler *et al*. 2009, Adrien Picot, Universität LeHavre, unpubliziert), kann hier eine Dekanalisierung des Phänotyps angenommen werden, was folglich die Entstehung einer Bänderung verursacht. In diesem Kontext ist es Köhler *et al*. (2009) gelungen, einen Zusammenhang zwischen der Hsp70-Induktionsstärke xerothermophiler Pulmonaten und der Ausprägung phänotypischer Diversität zu finden. Populationen von allerdings mediterranen Helicoidea, die in der Lage sind, hohe Mengen Hsp70 zu exprimieren, sind vorzugsweise mit weißer Schale und wenig phänotypischer Variation ausgestattet, während niedrig Hsp70-induzierende Populationen eine hohe phänotypische Variation mit gebänderten Gehäusen aufweisen. Der Umstand, dass Heliciden mit weißem Gehäuse in einer heißen Umgebung in höherer Frequenz vorkommen, wurde in Perth, Australien, unter anderem schon von Johnson (2011 & 2012) nachgewiesen und mit einer Selektion durch heiße Klimabedingungen in Verbindung gebracht. Auch für *X. derbentina*-Populationen wurde im Zuge dieser Arbeit gezeigt, dass heiße Temperaturen in der Tendenz weiße Gehäuse hervorbringen. Mittels dieser Studie konnte allerdings gezeigt werden, dass bezüglich der Gehäusemorph-Komposition neben einer direktionalen Selektion nachweislich auch andere Faktoren in Betracht gezogen werden müssen. Die Gastropoden in dieser Studie stammten aus einer kühleren Umgebung im nördlichsten Verbreitungsgebiet dieser Art (Tenby, Pembrokeshire, UK) und reagieren auf leicht erhöhte, jedoch möglicherweise noch

nicht Hitzestress-relevante Temperaturen mit einer Dekanalisierung im Phänotyp. Gastropoden aus heißen Gegenden reagieren stressbedingt mit einer höheren Hsp70-Induktion, welche eine weiße Schale begünstigt.

4.6 Kapitel 6: *MA Di Lellis, S Sereda, A Morgenroth, 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* (*eingereicht bei* Functional Ecology) *Phenotypic diversity, population structure, and stress protein-based capacitoring in populations of heat-tolerant land snails.*

Diese Studie zeigte, dass sich zehn verschiedene *Xeropicta derbentina*-Populationen hinsichtlich ihrer phänotypischen Variation, ihrer genetischen Populationsstruktur und ihrer Stressproteininduktionskapazität unterschieden. Die Stressproteininduktion aller Populationen mit Ausnahme einer nicht-induzierbaren Population, wies die bereits angesprochene Induktionskinetik (Eckwert *et al*. 1997, Köhler *et al*. 2009) einer Optimumskurve auf, welche zunächst mit einem Anstieg des Hsp70 Levels und nach Erreichen eines Maximums, das im Durchschnitt bei 40°C lag, mit einer Senkung dessen einherging.

Es konnte gezeigt werden, dass die genetische Populationsstruktur der Schnecken insbesondere den maximal induzierbaren Hsp70-Level gut erklärt, was Rückschlüsse auf Selektionsdruck für hoch induzierbare Hsp70-Niveaus in manchen Mikrohabitaten zulässt. Außerdem war eine niedrige phänotypische Variation innerhalb einer Population stark mit einem hohen Hsp70-Konsititutiv- und Maximallevel assoziiert, während in diesem Fall die genetische Diversität nicht direkt mit der korrespondierenden phänotypischen Diversität in Zusammenhang gebracht werden konnte. Dieser Umstand weist abermals darauf hin, dass Hsp70 Stressproteine das Potential besitzen, als umweltvermittelte „Puffermoleküle“ über Außentemperaturen die morphologische Entwicklung zu beeinflussen.

Auch in dieser Untersuchung erschienen Schneckenpopulationen mit hohen Hsp70-Spiegeln in einer niedrigen phänotypischen Diversität, die sich in einer rein weißen Schale ohne Bänderungen äußerte. Es ist bekannt, dass sich die Melaninsynthese, welche unter divergenten Syntheseschritten und pleiotropen Einflüssen stattfindet, durch erhöhte Hsp70-Niveaus negativ beeinflusst werden kann (Hoshino *et al*. 2010, Mikami *et al*. 2013). In der Tat wurden nachträglich Individuen von *X*. *derbentina* gefunden, die ihre Bänderung im Gegensatz zu *T. pisana* aus dem Norden des Verbreitungsgebietes im Laufe ihrer Maturation

zu einem recht späten Zeitpunkt ihres Lebens noch verlieren (Abbildung 3). Dies könnte durch eine hohe Hsp70-Induktionskapazität zu erklären sein, die wegen eines hohen intrazellulären Hsp70-Spiegels zu kanalisierenden Prozessen und damit zu einer Verringerung der phänotypischen Diversität beiträgt.

Abbildung 3: Individuen von *X. derbentina* verlieren während der Maturation ihre Gehäusebänderung. Die Melaninkonzentration wird mit zunehmender Größe geringer und frisch synthetisiertes Schalenmaterial überwächst die Bänderung.

5. Abschließende Betrachtung

Im Zuge dieser Arbeit konnten viele neue Erkenntnisse gewonnen werden. Anhand von Hsp70 als Proxy wurden für die physiologische Stressantwort wichtige Determinatoren ermittelt. So wurden neben der Aufenthaltshöhe über dem Grund auch die Größe der Organismen und die individuelle Gehäusefärbung als Temperaturstress-beeinflussend identifiziert. Grundsätzlich offenbarte sich folgendes Muster: große Vertreter von *Xeropicta derbentina* im mediterranen Raum besitzen eher helle Gehäuse und halten sich während des Tages in relativ großen Höhen über dem Erdboden auf, während sie einen niedrigen Hsp70-Spiegel halten und *vice versa*. Mit zunehmender Aufenthaltshöhe und infolgedessen mehr Abstand zum erhitzten Boden fällt die Temperatur und damit der Temperaturstress der Organismen (Cowie 1985, Kemster & Charwa 2003). Die Gehäuseorientierung zur Sonne erwies sich nur als Interaktion mit der Aufenthaltshöhe über Grund als bedeutend, was auf Unterschiede in der Induzierbarkeit verschiedener Gewebe interpretiert werden kann (Lyons

et al. 2003, Mizrahi *et al*. 2009, Arad *et al*. 2010). Der im Zuge dieser wissenschaftlichen Arbeit untersuchte Hepatopankreas von *X. derbentina* offenbarte sich als stark in die Hitzebewältigung integriertes Stoffwechselorgan. Generell zeigte sich, dass Calciumzellen toleranter gegenüber Hitzestress sind als die sensibleren Resorptionszellen. Aufgrund intraspezifischer Unterschiede in der Hsp70-Induktion, welche in Verbindung mit der Integrität des Hepatopankreas gebracht wurden, konnten drei verschiedene Hitzebewältigungsstrategien determiniert werden.

Die Assoziation eines niedrigen Hsp70-Niveaus mit der zunehmenden Größe der Tiere hängt sehr wahrscheinlich mit dem Alter der Tiere zusammen (Entwicklung eines energetisch teuren Reproduktionssystems, über den Sommer überstrapaziertes Stressabwehrsystem, Ästivation; Sørensen & Loeschke 2002, Storey 2002, Mayer & Bukau 2005, Reuner *et al*. 2008, Dittbrenner *et al*. 2009, Scheil *et al*. 2011) und trägt durch ein günstigeres Oberflächen-Volumen-Verhältnis zu verzögerten Austrocknungserscheinungen bei (Mizrahi et al. 2009). In diesem Kontext konnte gezeigt werden, dass es neben tageszeitlicher, stark an die Außentemperatur gebundener Variation der Hsp70-Induktion eine jahreszeitliche Veränderung der Induktionskapazität gibt, die gegen den Spätsommer hin wesentlich schwächer ausfällt. Zudem zeigte eine weitere Untersuchung im Zuge dieser Arbeit, dass sich große Individuen von *T. pisana* bei Exposition gegenüber elektromagnetischer Strahlung langsamer aufheizten und danach auch wieder langsamer auskühlten, was ebenfalls mit einem günstigeren Oberflächen-Volumen-Verhältnis erklärbar war. Die Gehäusefärbung der Tiere zeigte sich in diesem Kontext ebenso sowohl unter Kurzzeit- als auch unter Langzeiterwärmung als relevant, als dass im Vergleich gebänderte Individuen stärker aufheizten und langsamer auskühlten.

Da die zunehmende Größe mit einer heller werdenden Schalenfärbung bei *Xeropicta derbentina* korreliert ist, kann ein altersbedingter Verlust der Schalenbänderung angenommen werden, der möglicherweise durch eine Hemmung der Melaninsynthese durch über den Sommer hoch induzierte Hsp70-Level hervorgerufen wurde (Effekt von Hsp70 in Mausmelanomzellen: Hoshino *et al*. 2010; humane Melanomzellen: Mikami *et al*. 2013). Tatsächlich zeigte sich auch in einer weiteren Studie im Zuge dieser Abhandlung, dass tendenziell keine Bänderung bei stark Hsp70- induzierenden *X. derbentina*-Populationen, deren Individuen unter dem Einfluss erhöhter Hsp70-Niveaus standen, ausgebildet war. Des Weiteren konnte die genetische Populationsstruktur zwar mit der Variation der Hsp70-

Konstitutiv- und Maximallevel, jedoch nicht mit der intraspezifischen phänotypischen Variation assoziiert werden.

Als Vertreter einer unter Stress relativ niedrigen Hsp70-induzierenden Gastropodenart aus dem nördlichen Rand des Verbreitungsgebietes (Wales) (Köhler *et al*. 2009, Adrien Picot, Universität LeHavre, unpubliziert) zeigte sich in diesem Zusammenhang jedoch, dass *Theba pisana* – Populationen unter leichtem Temperaturstress während eines sensiblen Fensters im Zuge der Maturation zu einer Zunahme der phänotypischen Diversität mit einer damit einhergehender Bildung einer unspezifischen, nichtdirektionalen Gehäusebänderung neigen. Als determinierend für den adulten Phänotyp von *T. pisana* konnten in einer Meta-Studie neben einer genetisch erblichen Komponente die herrschenden Umweltbedingungen, genauer: erhöhte Umgebungstemperaturen im Winter und im Frühjahr, festgestellt werden.

Der Vergleich zwischen diesen unterschiedlich stark Hsp70-induzierenden Gastropodenarten und -populationen macht es möglich, für Hsp70 eine umweltvermittelte „Puffer"-Funktion zwischen genotypischer und phänotypischer Variation annehmen zu können. Diese Funktion besitzt das Potential, durch Hsp70 einen ungebänderten und reinweißen Phänotyp über die Hemmung der Melaninsynthese zu kanalisieren, während bei einer niedrigen Hsp70-Induktionskapazität und einer damit einhergehenden niedrigeren Stressproteinkonzentration eine Dekanalisierung des Phänotyps erfolgt (Abbildung 4a+b).

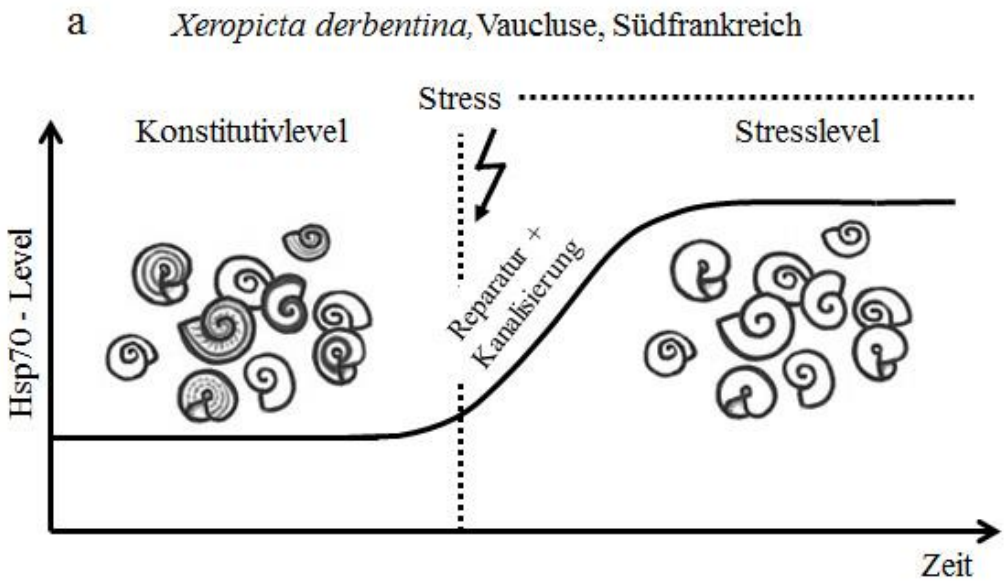

Abbildung 4. a: Illustration der in Kapitel 4.6 beschriebenen Interpretation der Ergebnisse: Phänotypisch diverse *X. derbentina* – Populationen bekommen durch Capacitoring nach längerfristigem Stress aufgrund einer hohen Hsp70-Induktion *via* Melaninsynthesehemmung ein weißes Gehäuse und werden somit uniformer. Die induzierte Stressproteinmenge reicht hier neben den notwendigen Reparaturen auch zur Kanalisierung eines weißen Phänotyps.

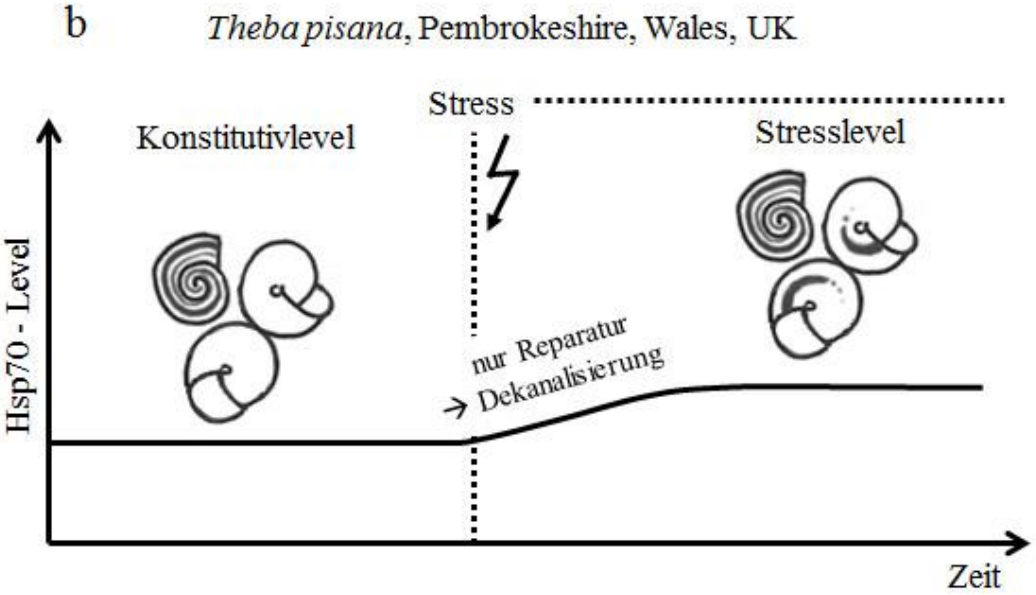

Abbildung 4. b: Illustration der in Kapitel 4.6 beschriebenen Interpretation der Ergebnisse: Die niedrig Hsp70-induzierenden *T. pisana* – Populationen aus dem nördlichen, kühlen Verbreitungsgebiet reagieren unter leichtem Stress während einer sensiblen Lebensphase mit der Entstehung einer unspezifischen Gehäusebänderung. Aufgrund der vermutlich niedrigen Stressproteinkonzentration wird der Phänotyp dekanalisiert, die Populationen werden diverser.

6. Literatur

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Eigenanteil an den durchgeführten Arbeiten in den zur Dissertation eingereichten 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

Die Probennahme im Feld erfolgte gemeinsam mit S. Troschinski, Prof. Dr. H.-R. Köhler und Prof. Dr. R. Triebskorn (Universität Tübingen) nach Hinweisen von Dr. C. Mazzia und Dr. Y. Capowiez (Université d'Avignon et des Pays de Vaucluse). Gesamter Eigenanteil an der Probenaufbereitung im Labor und an der statistischen Analyse, welche unter Einweisung von Dr. M. Seifan (Universität Tübingen) durchgeführt wurde. Eigenhändige Verfassung des Manuskriptes für die Publikation der Daten mit anschließender Korrektur durch Prof. Dr. H.-R. Köhler und Dr. M. Seifan. Fachliche Betreuung durch Prof. Dr. H.-R. Köhler.

Kapitel 2: *S Troschinski, MA Di Lellis, S Sereda, T Hauffe, T Wilke, R Triebskorn, H-R Köhler* (eingereicht bei PLoS ONE*) Intraspecific variation in cellular and biochemical heat response strategies of Mediterranean* Xeropicta derbentina *[Pulmonata, Hygromiidae].*

Eigenanteil an der Hsp70-Probengewinnung und –aufbereitung. Die histologische Bearbeitung der Proben, die statistische Auswertung und die Erstellung des Manuskriptes erfolgte durch S. Troschinski. Genetische Analysen mit statistischer Teilauswertung durch S. Sereda, T. Hauffe und Prof. Dr. T. Wilke (Universität Giesen). Diskussion der Ergebnisse mit S. Troschinski. Fachliche Betreuung durch Prof. Dr. H.-R. Köhler (Universität Tübingen) und Prof. Dr. R. Triebskorn (Universität Tübingen).

Kapitel 3: *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

Beitrag bei der über das Jahr verteilten Probengewinnung und Messungen im Feld. Die Bearbeitung der Proben, die statistische Auswertung als auch das Erstellen des Manuskriptes erfolgte durch A. Dieterich. Inhaltlicher Beitrag durch U. Fischbach, M. Ludwig und Prof. Dr. U. Gärtner im Rahmen des *Twinning-Projects* (Hochschule Esslingen). Fachliche Betreuung durch Prof. Dr. H.-R. Köhler (Universität Tübingen) und Prof. Dr. R. Triebskorn (Universität Tübingen).

Kapitel 4: *T Knigge, MA Di Lellis, S Schwarz, T Monsinjon, H-R Köhler (unter Revision für das* Journal of Zoology) *Relevance of body size and shell colouration for thermal absorption and heat loss in White Garden Snails from Northern France.*

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Kapitel 5: *H-R Köhler, C Schultz, AE Scheil, R Triebskorn, M Seifan, MA Di Lellis (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

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Kapitel 6: *MA Di Lellis, S Sereda, A Morgenroth, 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* (eingereicht bei Functional Ecology) *Phenotypic diversity, population structure, and stress protein-based capacitoring in populations of heat-tolerant land snails.*

Gesamtanteil an der Probengewinnung und -aufbereitung, an der statistischen Analyse (mit Ausnahme der genetischen Analysen, welche von S. Sereda, T. Hauffe und Prof. Dr. T. Wilke (Universität Giessen) duchgeführt wurden) und Erstellung des Manuskriptes zur Publikation. Beitrag zur Entwicklung der Hypothese: A. Morgenroth, P. Arnold, S. Lang, S. Troschinski, A. Dieterich und S. Krais (Universität Tübingen), Dr. Y. Capowiez, Dr. C. Mazzia (Université d'Avignon et des Pays de Vaucluse) und A. Picot, Dr. T. Knigge, Dr. T. Monsinjon (Université LeHavre). Die Korrektur des Manuskriptes vor Einreichung erfolgte durch Prof. Dr. H.-R. Köhler, Prof. Dr. T. Wilke und Prof. Dr. R. Triebskorn (Universität Tübingen). Fachliche Betreuung durch Prof. Dr. H.-R. Köhler.

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}**	**$HS_{shell\ p}$**
1	Modène Mazzia	12	4,47	23	3,3	139	0,87	1,2
2	Modène wine yard	12	2,02	25	3,5	70	0,89	1,24
3	Modène West	12	4,2	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

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).

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.

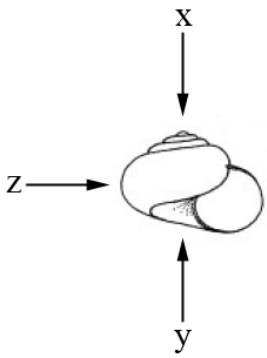

Fig. 1 Code for the factor 'orientation towards the sun'. [x] Apex directed to the sun; [y] umbilicus directed to the sun; [z] shell laterally directed to the sun. The respective characters symbolize the position of the sun

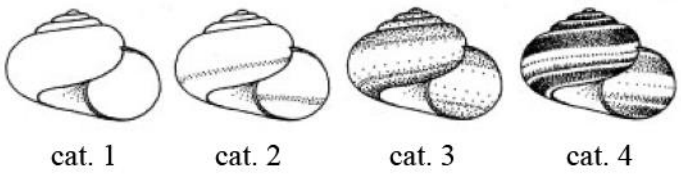

Fig. 2 Code for the factor 'shell colouration pattern', according to Köhler et al. (2009) modified. Cat. 1 = white; cat. 2 = white with a single pale band; cat.3 = greyish with several light bands; cat. 4 = dark with lots of intense bands

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.

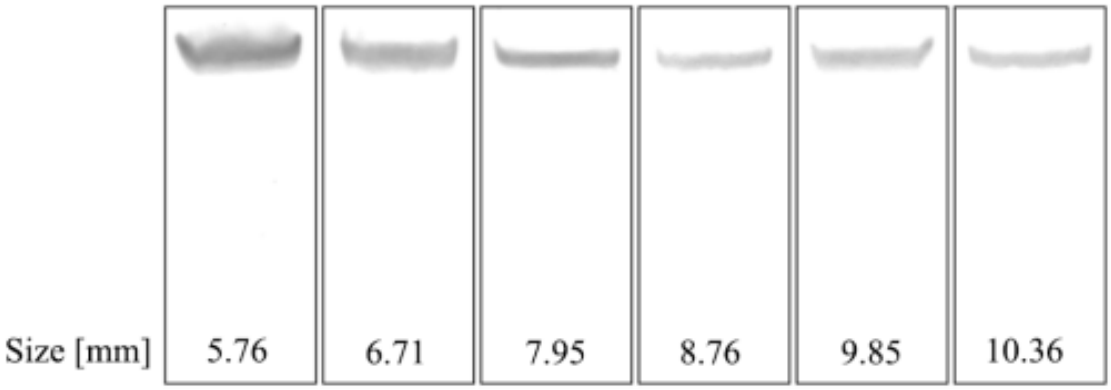

Fig. 3 Representative Western blot bands for Hsp70 in snails featuring different size. The α-Hsp70 antibody was sensitive to all isoforms of Hsp70 and detected both constitutive and inducible stress protein molecules

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 $\alpha=0.05$ 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.

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 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 $\alpha=0.05$

* 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

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. 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

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			

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. 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

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			

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

		β
	S	-0.0562
	T	-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
	S*T	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
	H	-0.0013
	T	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
	H*T	0.0000
Temperature* Orientation	T*O(x)	-0.0001
	T*O(y)	-0.0001
	T*O(z)	0.0002

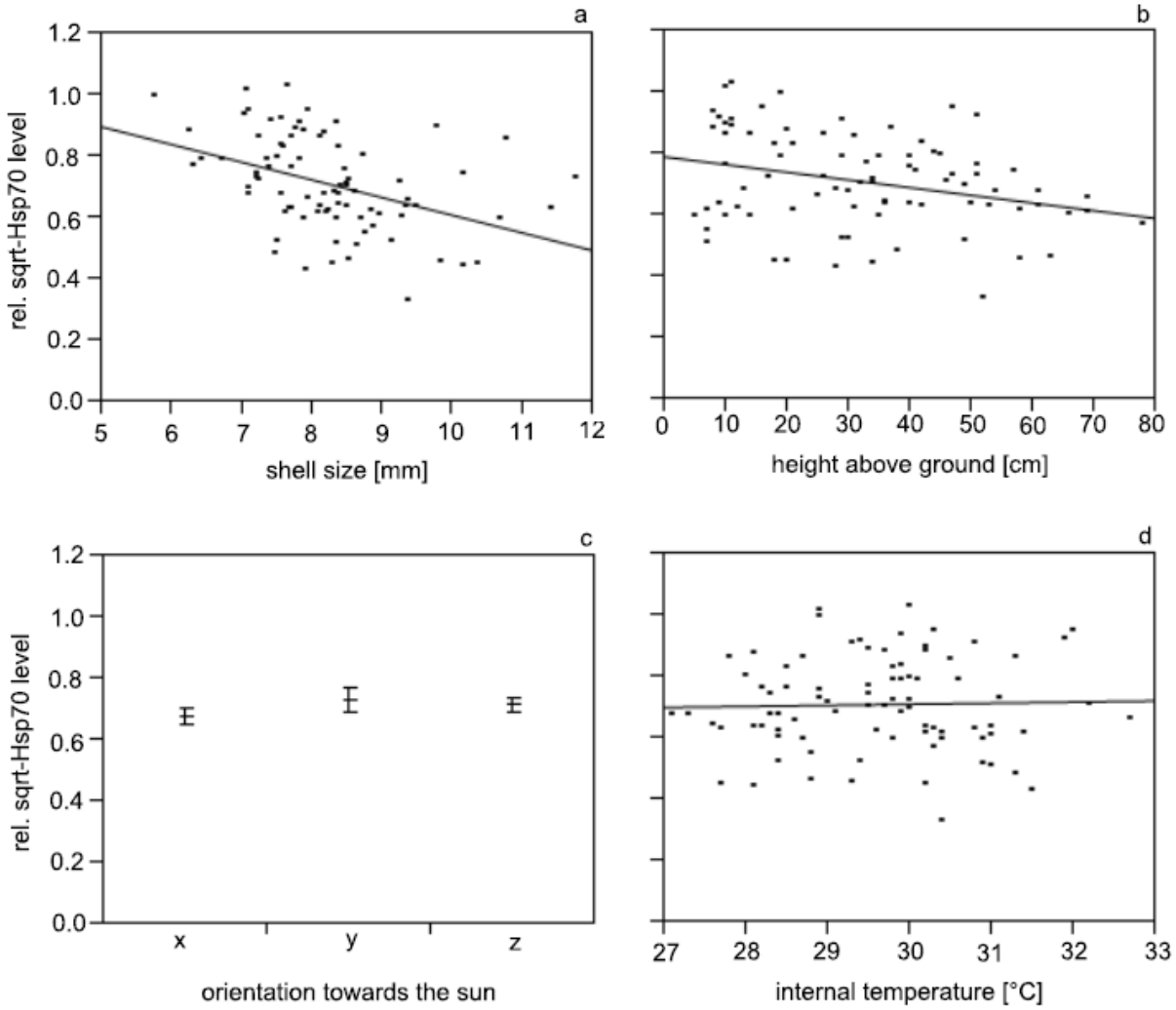

Fig. 4 All single factors used for model selection analysis plotted against sqrt Hsp70 level. Because the applied model selection approach does not determine significance levels but likelihood estimates, these graphs lack confidence intervals and p-levels. These figures serve as completion for the correlation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

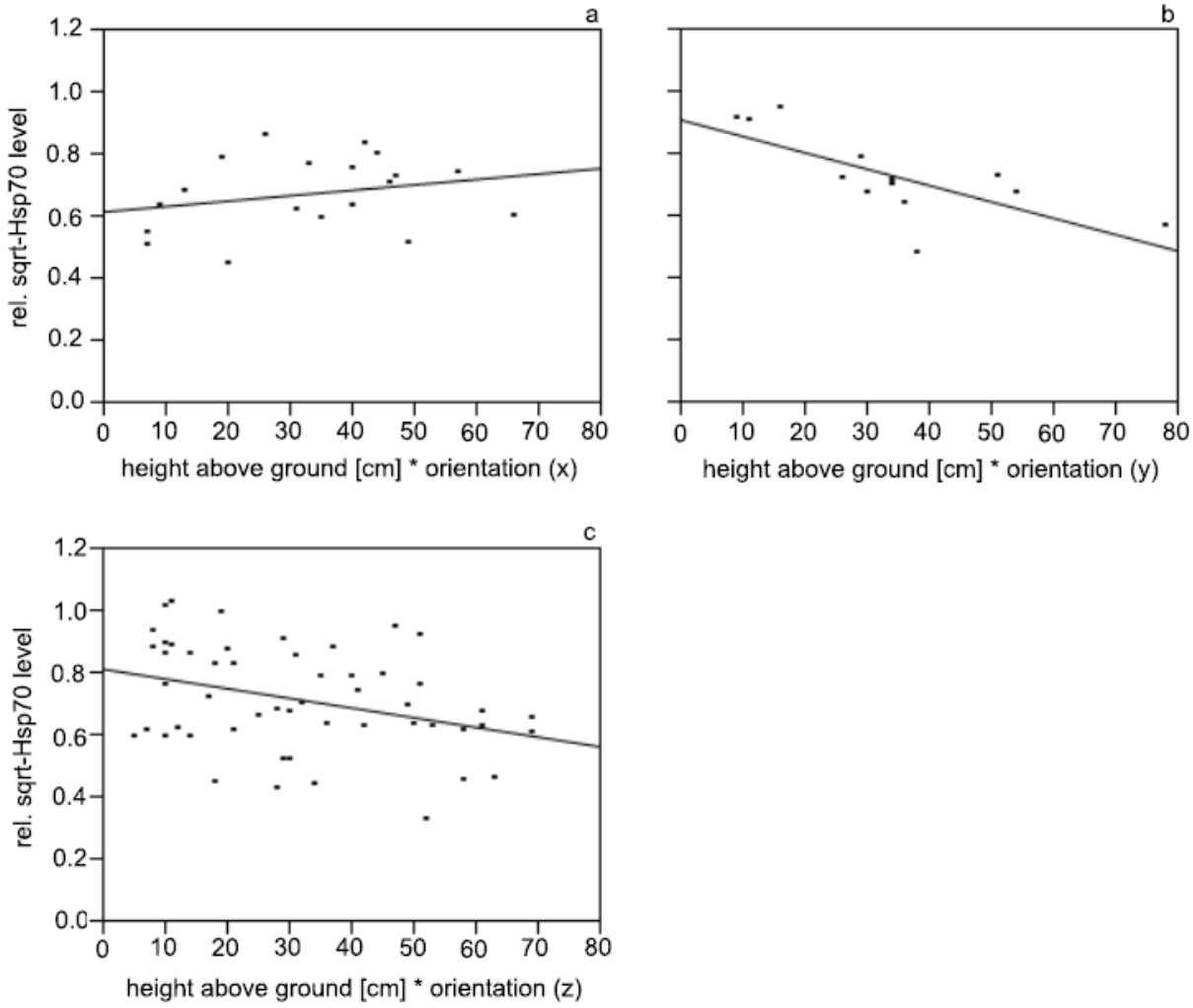

Fig. 5 Interaction 'height above ground * orientation towards the sun', each category is plotted against sqrt Hsp70 level. Because the applied model selection approach does not determine significance levels but likelihood estimates, these graphs lack confidence intervals and p-levels. These figures serve 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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Kapitel 2: 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. 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. Generally, calcium cells of the hepatopancreas were more heat resistant than digestive cells - this phenomenon was associated with elevated Hsp70 levels at 40°C. 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 almost identical habitats within a close range, populations of the same species have developed different stress response strategies which 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, 1999). 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, 1999) and the acid-base balance (Burton, 1976), both of which can be affected by high temperature.

Stress proteins 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 may contribute to Hsp70 expression levels (Bahrndorff *et al.* 2010, Jensen *et al.* 2009, Sørensen *et al.* 2001), 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 animals. 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 (e.g. 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*., 2012), as well as on the genetic structure in the Helicidae (e.g., 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 microallopatric populations.

Specifically, we studied whether different populations of the same species collected within a range of a few kilometers in almost identical habitats have developed 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 the pulmonate land snail species *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 from each population were exposed as a group in one plastic container, respectively, 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, they 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.

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. Shell sizes of all individuals were determined by a sliding caliper.

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 rotation microtome (Reichert Jung 2050 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 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 · Nucleus oval in shape · Clear cellular compartmentation (vacuolisation: apical small, basal large vacuoles)	· Irregular cell shape · Irregular nucleus shape · Irregular cellular compartmentation (irregular vacuolisation)	· Cells damaged · Necrosis
Calcium cells	· Cone-shaped cells (broad basis, slim apex) · Large round-shaped nucleus · Dense and consistent cytoplasm	· Irregular cell shape · Irregular nucleus shape · Irregular cytoplasm	· Cells damaged · Necrosis
Tubule	· Smooth apices · Smooth basic · Tight lumina	· Irregular apices · Large lumina	· Tubule damaged

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 20000*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 between all samples.

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 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.

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 proved 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.).

Network analysis

Cryptic species may coexist within the range of morphologically undistinguishable heliciid snails (Dépraz et al., 2009). 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 (R Development Core Team 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, Dixon, 2003).

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). 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).

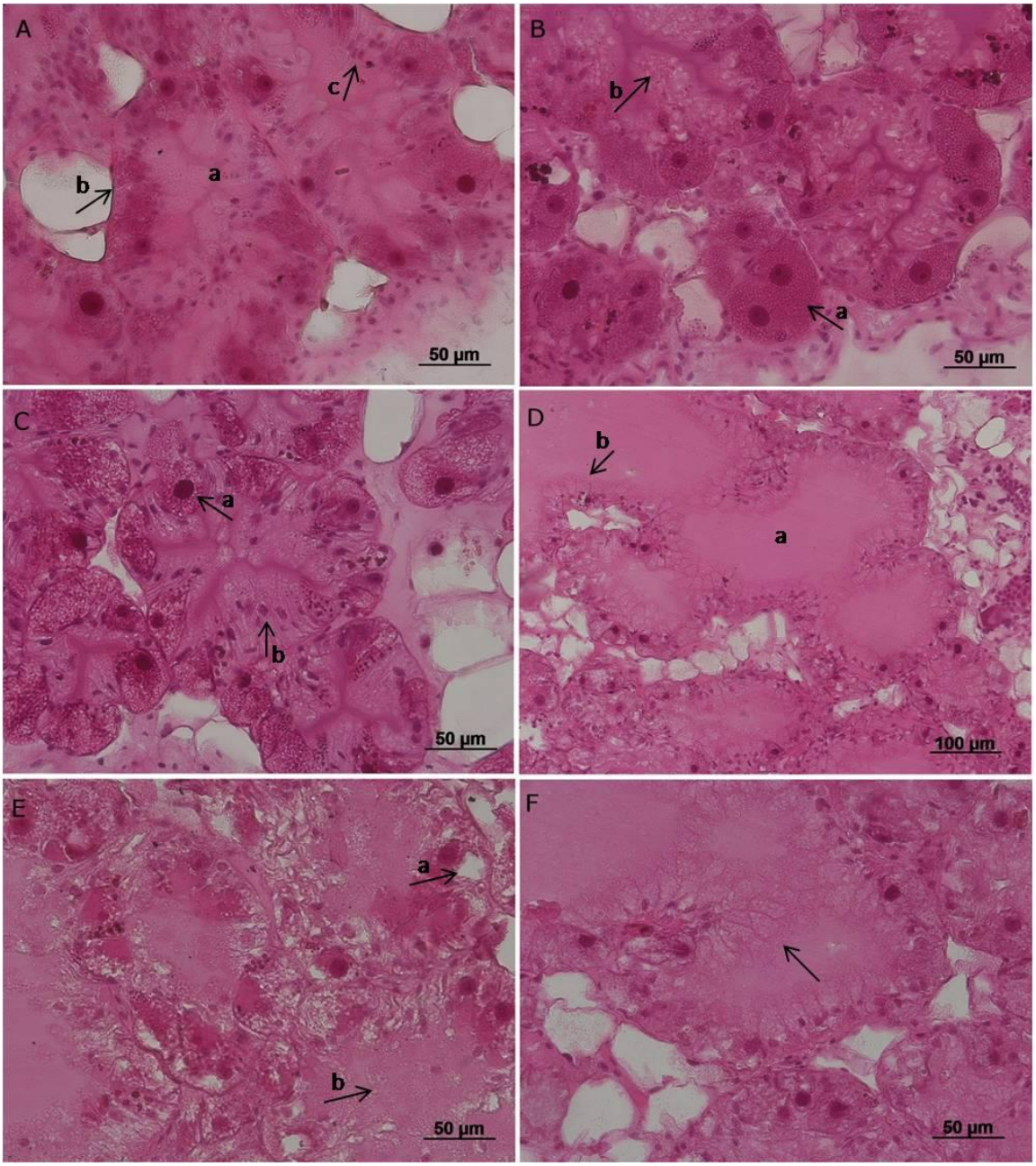

Figure 1. Digestive gland of *Xeropicta derbentina* in different reaction states. A. Digestive gland of a control animal. a. indicates tight lumina, b. a smooth base of the tubule and c. shows an oval-shaped nucleus and regular vacuolization of the digestive cells. **B.** Digestive gland of a control 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.

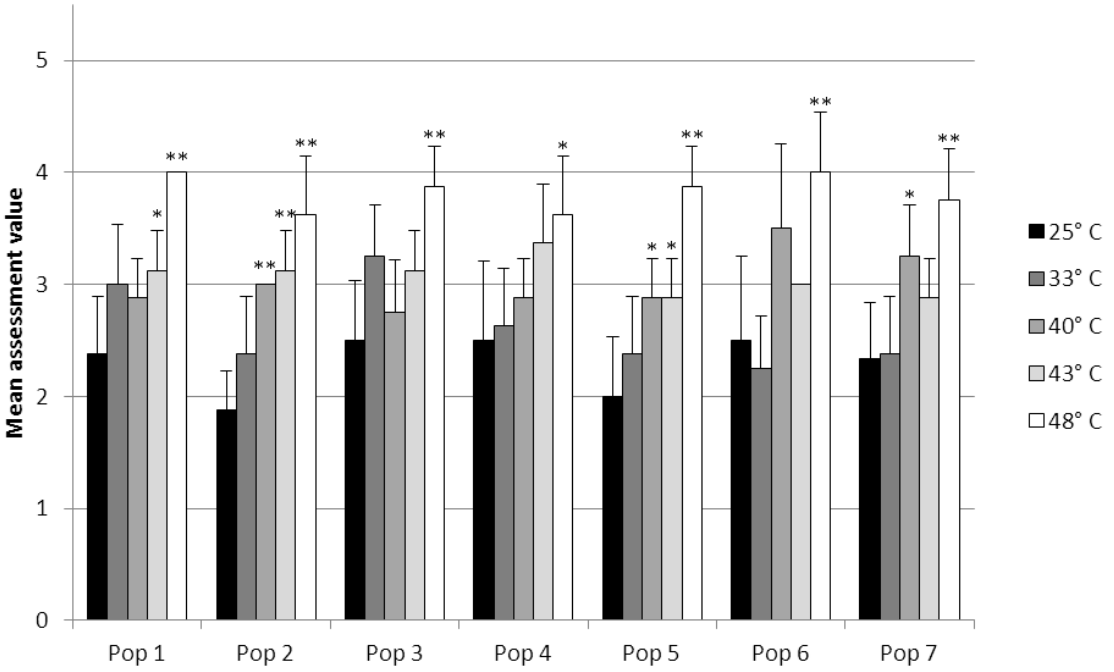

Figure 2. The structural condition of the hepatopancreatic tubules. Mean assessment values for each population at elevated temperature. Shown are means and SD; *n*=8. Asterisks show significant differences of the respective exposure groups compared to the control at 25°C after Bonferroni correction: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

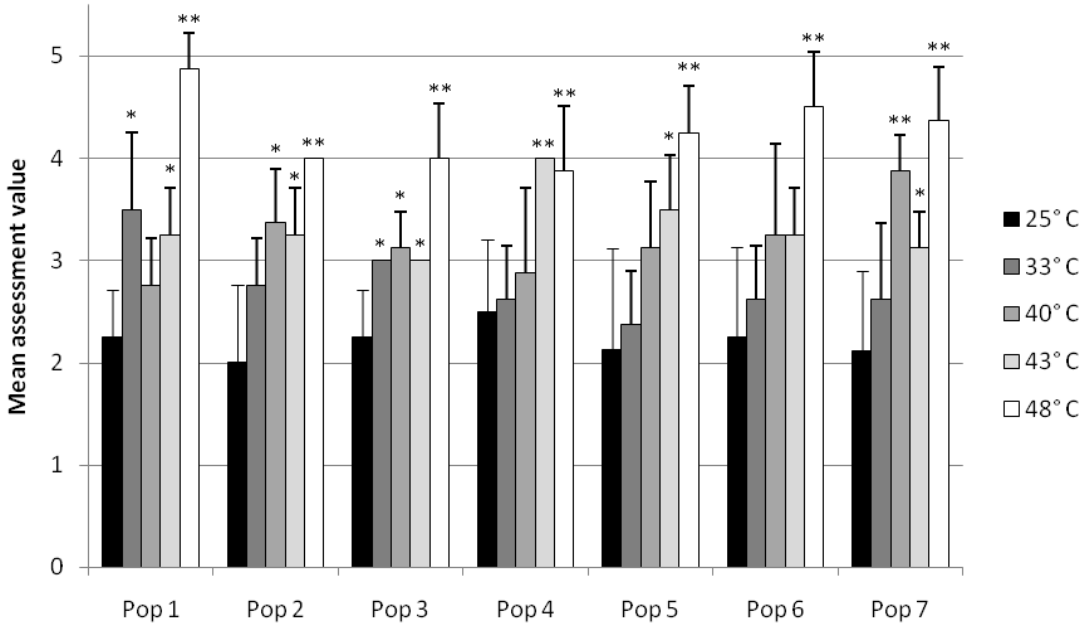

Figure 3. The condition of the digestive cells of the hepatopancreas. Mean assessment values for each population at elevated temperature. Shown are means and SD; *n*=8. Asterisks show significant differences of the respective exposure groups compared to the control at 25°C after Bonferroni correction: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

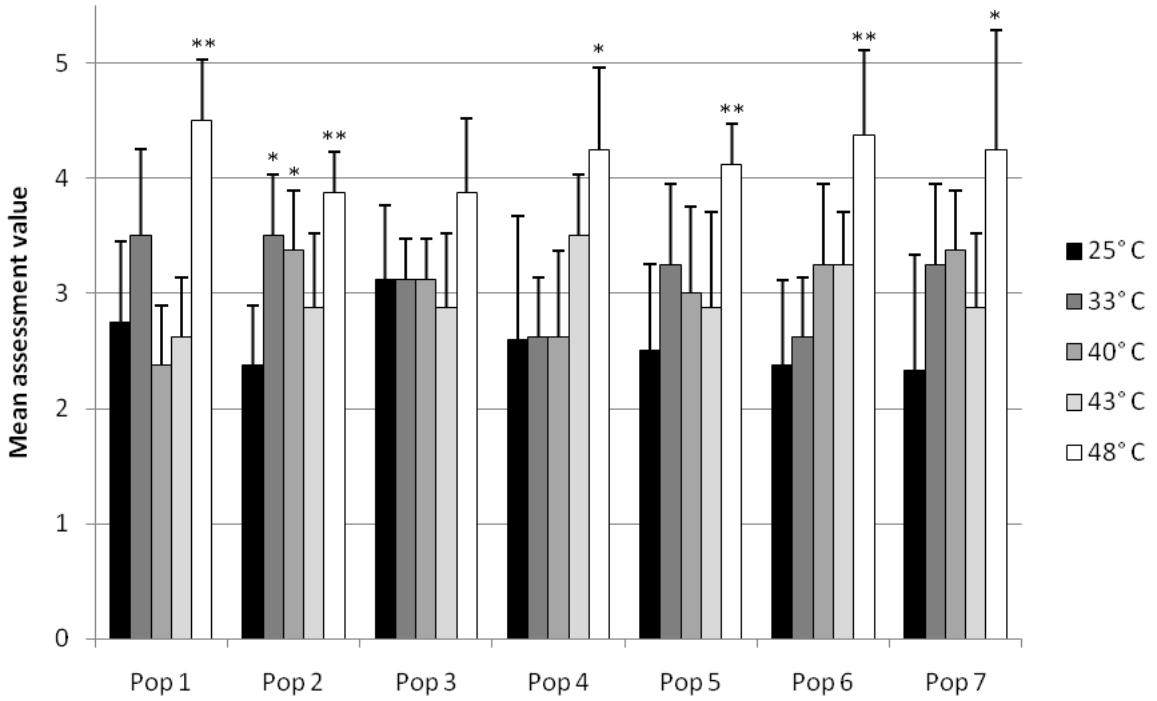

Figure 4. The condition of the calcium cells of the hepatopancreas. Mean assessment values for each population at elevated temperature. Shown are means and SD; *n*=8. Asterisks show significant differences of the respective exposure groups compared to the control at 25°C after Bonferroni correction: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

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*. (2009) 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, 2, 3, 4, 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.

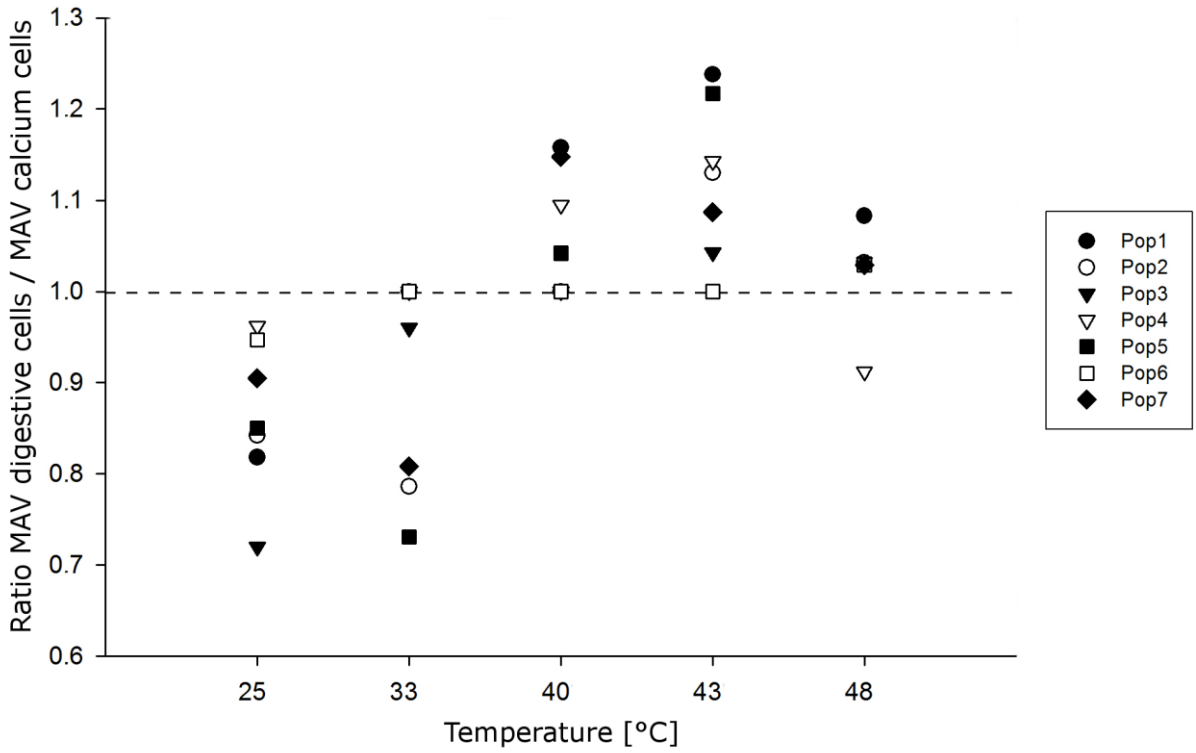

Figure 5. Ratio of the mean assessment values obtained for digestive cells and calcium cells. The ratio for each population at different temperature regimes is illustrated. Data below the dashed line indicate a better condition of digestive cells, compared to calcium cells. Data above the line indicate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

Stress protein analyses

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 (shell diameter). Average shell sized varied from 7.66 ± 0.71 mm (population 3) to 10.86 ± 0.79 mm (population 1). However, the correlation analysis did not show 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 whereas populations 2 and 4 were not able to raise its Hsp70 level remarkably.

To test for significant differences, exposure groups were compared to 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) from that at 25°C. 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).

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).

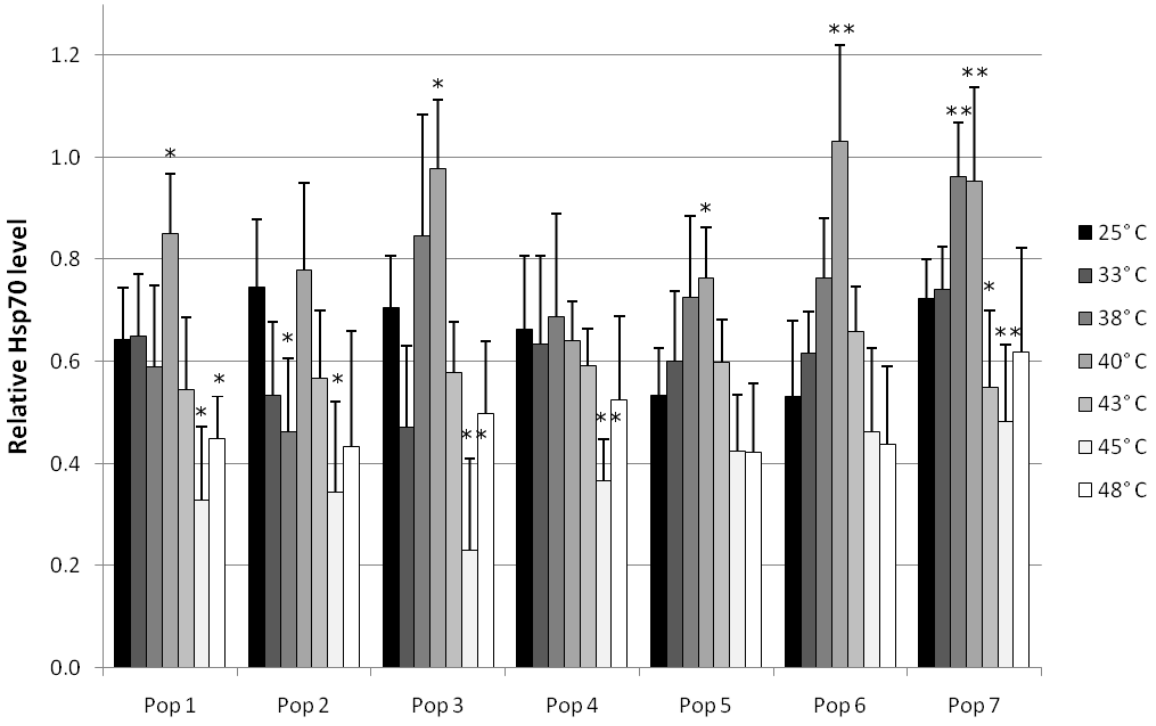

Figure 6. Relative Hsp70 level of different populations after exposure to elevated temperature for 8h. Shown are means and SD; $n = 10$. Asterisks show significant differences of the respective exposure groups compared to the control at 25°C after Bonferroni correction: $0.0017 < P \leq 0.0083$: (*)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

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. 7). Generally, the Hsp70 level increased up to its maximum at 40°C along with increasing cellular responses (assessment values 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 shall be further described in the following.

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) quite constant (mean assessment value, 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 cells 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.

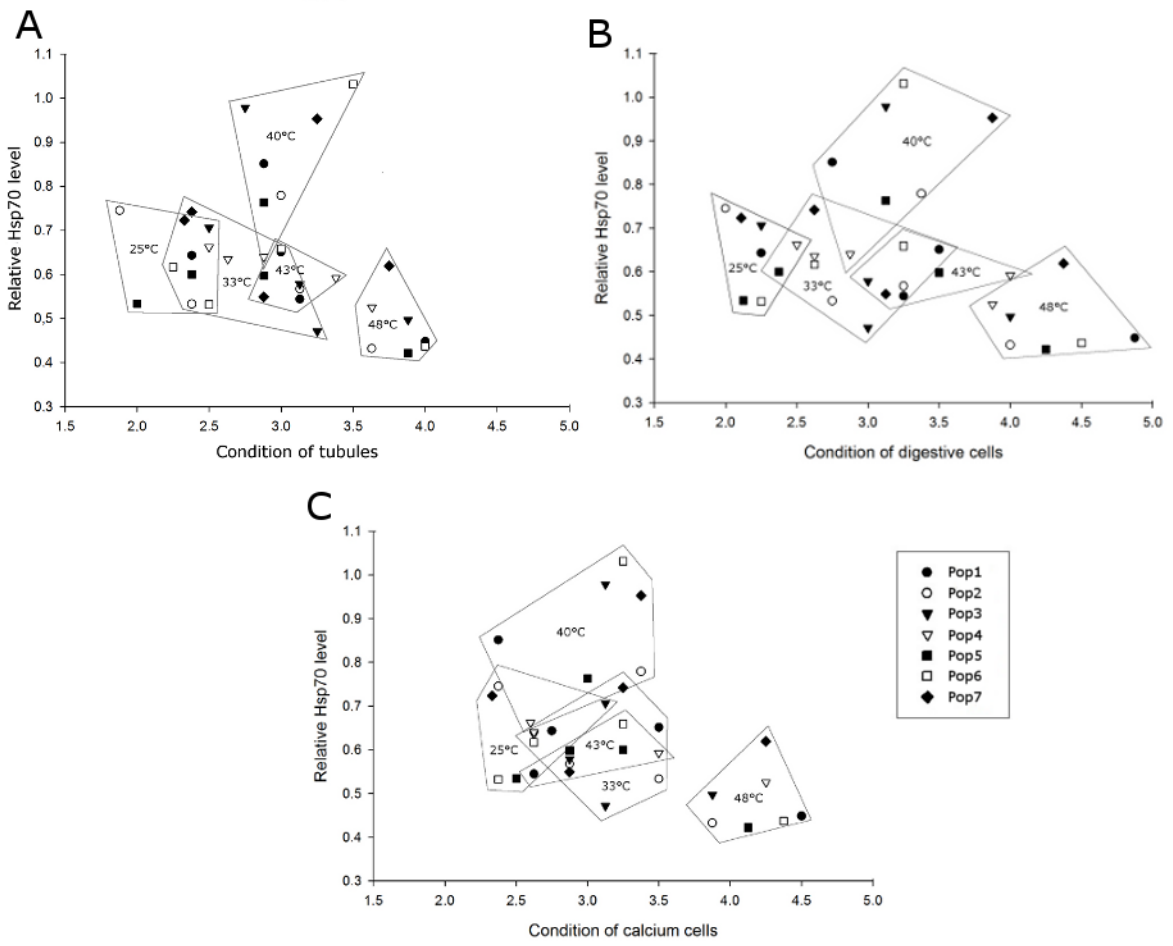

Figure 7. Correlation of relative Hsp70 level vs. histopathological mean assessment values. Data obtained for the populations of the respective exposure groups (25, 33, 40, 43 and 48°C) are framed, respectively. **A.** Relative Hsp70 level vs. condition of the tubules. **B.** Relative Hsp70 level vs. condition of the digestive cells. **C.** Relative Hsp70 level vs. condition of the calcium cells.

Genetic analyses

Network analysis

The statistical parsimony analysis of 138 individuals tested 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 < 3.0). The highest diversity was found in populations P4 and P1; population P7 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 P4, which showed relatively high values for both F_{ST} and H_{MH} and thus is most dissimilar to all other *X. derbentina* populations studied.

Table 4. Within- and between-site genetic differentiation calculated for *Xeropicta derbentina* populations (P1-P7) from Southern France based on the COI gene.

	P1	P2	P3	P4	P5	P6	P7
P1	2.244±1.29	0.05	0.13	0.75	0.01	0.10	0.21
P2	0.0304	1.673±1.024	0.03	0.77	0.09	0.01	0.07
P3	0.0733	0.0210	1.635±1.007	0.79	0.21	0.03	0.06
P4	0.2870	0.3379	0.3149	2.497±1.405	0.75	0.77	0.80
P5	0.0050	0.0557	0.1148	0.3161	2.096±1.221	0.14	0.27
P6	0.0684	0.0095	0.03276	0.4067	0.1001	1.129±0.767	0.03
P7	0.2353	0.1173	0.1077	0.5507	0.2903	0.0877	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}).

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 (actual trees not shown here). 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 explained 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}, respectively).

Interestingly, 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) in the 25°C, 33°C, and 48°C groups, 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°C, 40°C, 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 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	Explanatory variables for primary grouping	Correlation of explanatory and dependent variables for primary grouping
25°C	50% (1.31)	(P1,P3,P4),(P2,P5,P6,P7)	$H_{MH}3$	Hsp70 (+), histology (d)
33°C	58% (1.25)	(P2,P4,P5,P6,P7),(P1,P3)	$H_{MH}3$	Hsp70 (+), histology (i)
40°C	78% (1.03)	((P1,P4),(P3,P5)),(P2,P6,P7)	$F_{ST}1$	Hsp70 (-), histology (i)
48°C	50% (2.95)	(P1,P5,P6),(P2,P3,P4,P7)	$F_{ST}2$	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, 1999). Besides other factors, the metabolic rate can increase due to high temperature (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 found to be 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 causes 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 a 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 (Taib & Vicente, 1999) 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 even after a time of 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 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*., 2012), 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, Cancio & Marigomez, 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, Alberti & Köhler (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 falls down, 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, 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 association of relative Hsp70 levels with the cellular mean assessment values in our study (Fig. 7), the investigated populations follow different strategies to arrange with thermal stress:

(1) strategy 1: investment in medium Hsp70 levels and keeping cellular condition on the level of moderate response (populations 1, 2, and 5), (2) strategy 2: spending energy in high Hsp70 levels associated with cellular condition on a moderate level (populations 3, 6, and 7), and (3) 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.8 which illustrates the association of Hsp70 level and histopathological condition for each population.

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 & Loeschke, 1994). Consequently, lower levels of Hsp70 might save fitness costs. Despite high levels of Hsp70 can improve thermotolerance, too high levels can act contradictorily (Krebs & Feder, 1998). 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*) which 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 which have been selected for thermotolerance, one must conclude that populations 6 and 7 of our study did not evolve 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 (e.g., Sørensen *et al*. 2001; Jensen *et al*. 2009; Bahrndorff *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 (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 a strong selection pressure that is particular acting at 40°C, that is, an ecologically realistic, yet the highest non-lethal temperature. Moreover we also see spatial effects resulting in regional adaptations to heat stress challenges under this condition. In contrast, under the other conditions tested (i.e., 25°C, 33°C, and 48°C), local adaptations 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) exposed another population of *X. derbentina* for 8h at 45°C, and took samples for stress protein analyses and histological investigations at defined time points. The results of the study showed that Hsp70 was up-regulated already after 0.5h of exposure time, reaching a significant peak after 4h and than 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 was 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 (Taib & Vicente, 1999) and acid-base balance (Burton, 1976). This observation can also be linked to the function of calcium cells in protein synthesis (Sumner, 1965; Taib & Vicente, 1999). 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, in consideration of fitness costs. We observed populations who 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, indicating selection of appropriate stress-responses to particularly this high, but environmentally relevant temperature. Despite this presumable uniformity in the requirements for survival at high temperature, our study nevertheless showed the molecular and cellular components of survival strategies of *X. derbentina* to be very variable but – in concert with one another – apparently equally efficient in different populations.

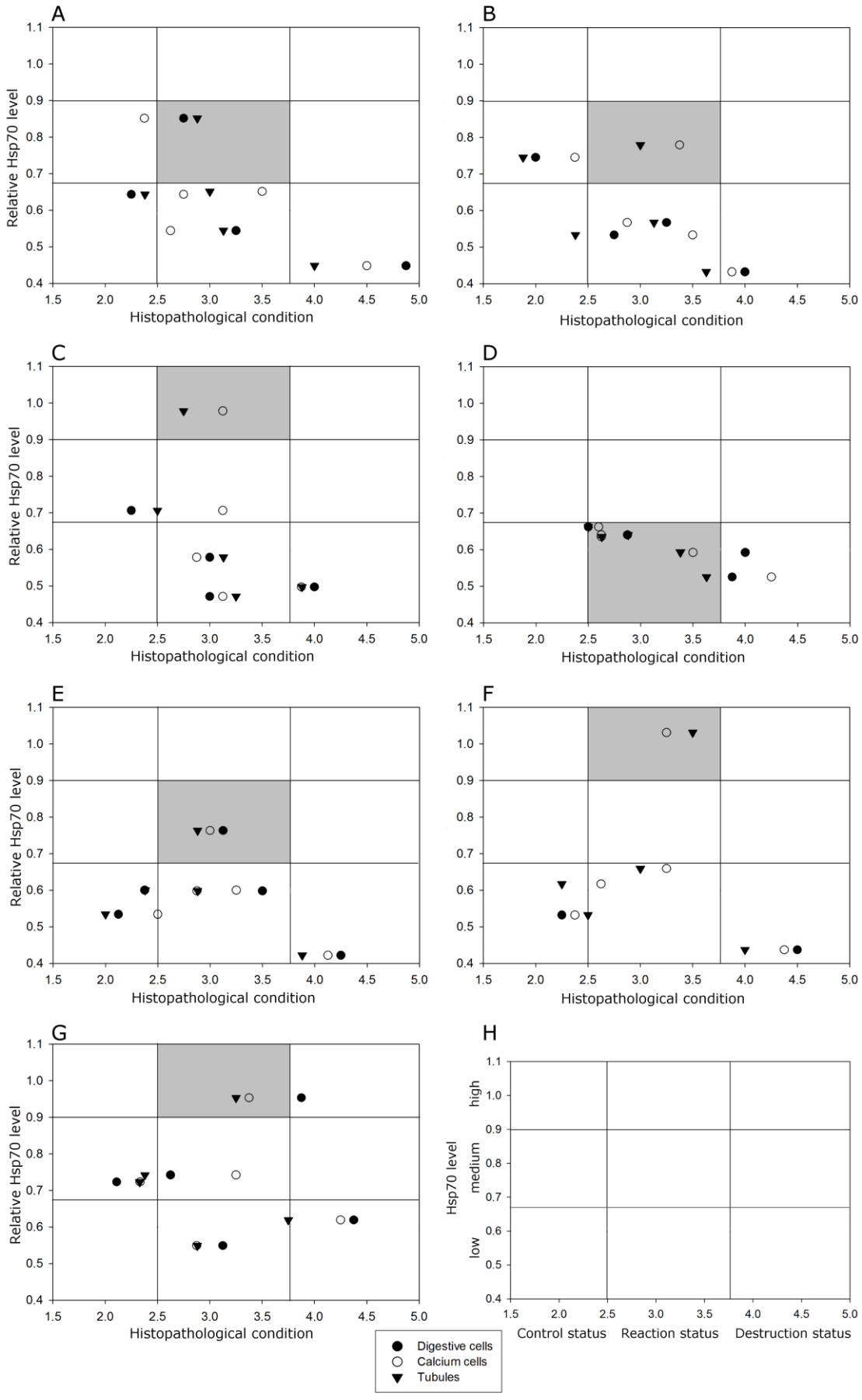
A
B
C
D
E
F
G
H
Relative Hsp70 level
Histopathological condition
Hsp70 level
high
medium
low
Control status
Reaction status
Destruction status
Digestive cells
Calcium cells
Tubules

Figure 8. 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.

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Kapitel 3: 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.

Materials 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 % H_2O_2, 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).

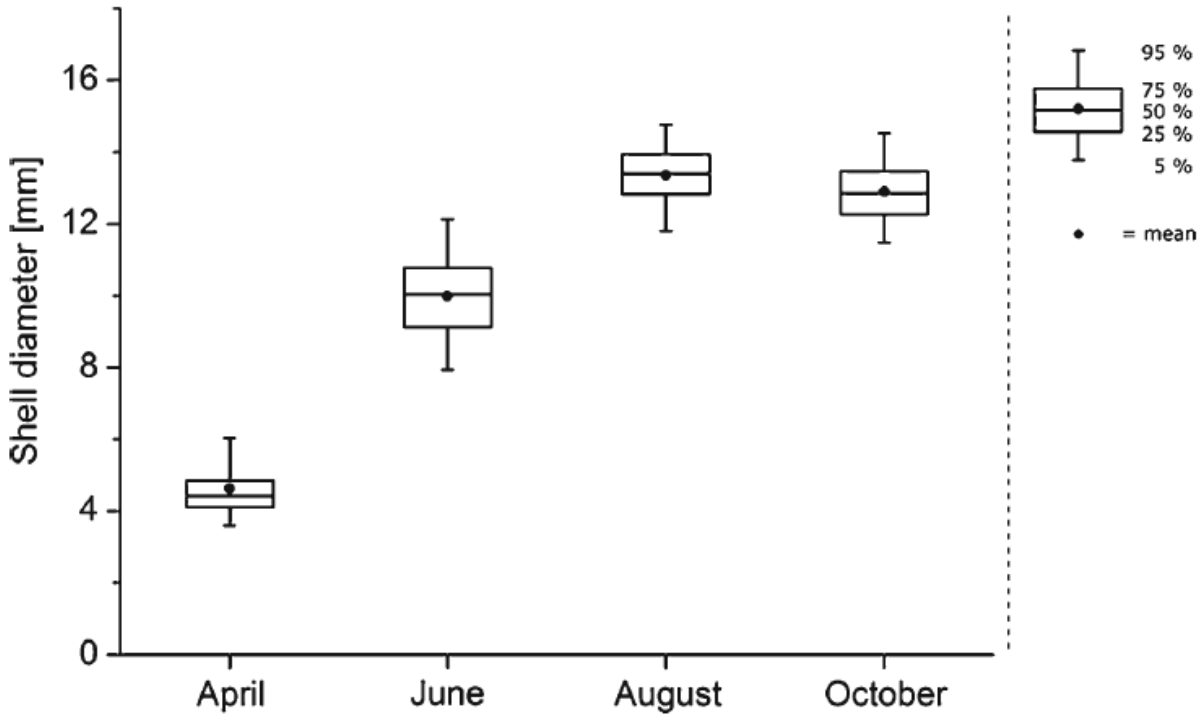

Fig. 1 Increase in shell diameter of samples taken in 2011. Boxes indicate 25%, 50%, and 75% percentiles of all samples taken during the corresponding sampling day. Black dots = mean shell diameter, whiskers = 5% and 95% percentiles

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.

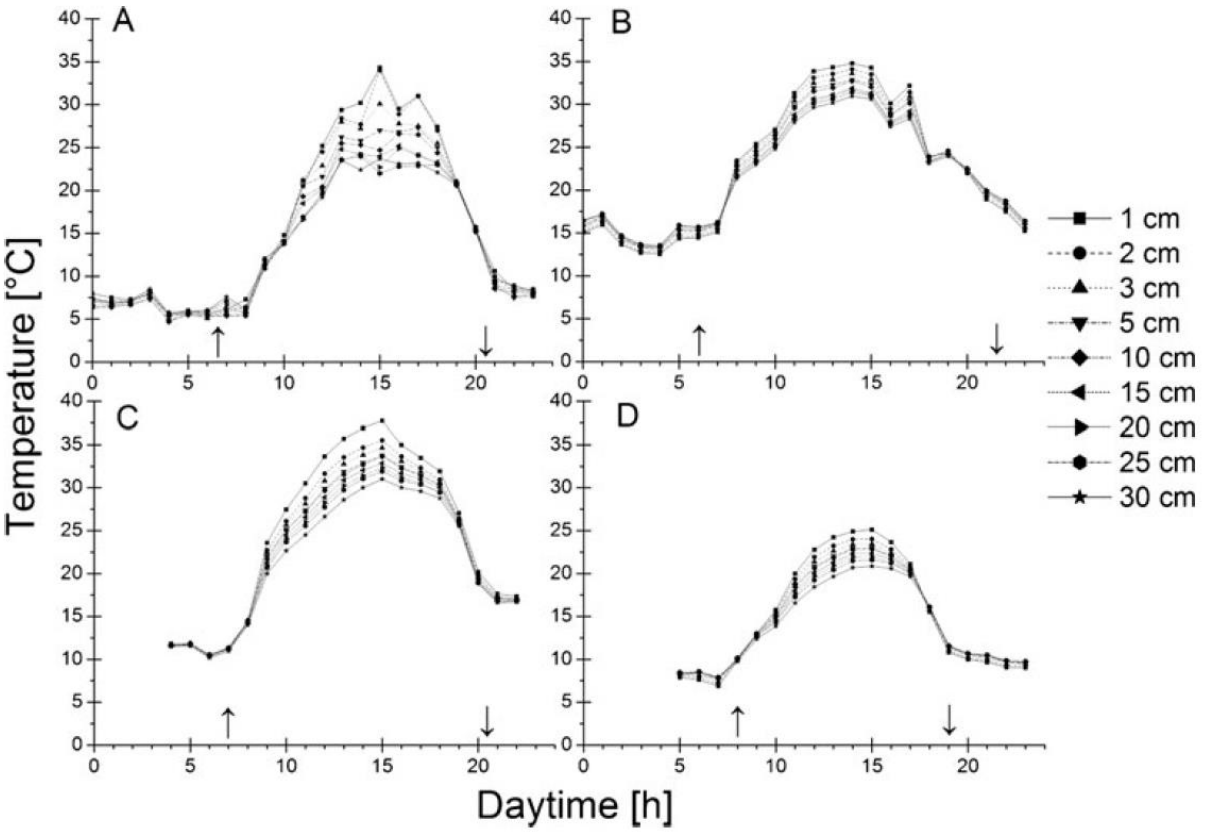

Fig. 2 Daily course of the air temperatures at different heights above the ground in Modène, France, during samplings in 2011. a April 18. b June 13. c August 30. D October 17. Sunrise is indicated by an up arrow and sunset by a down arrow

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.

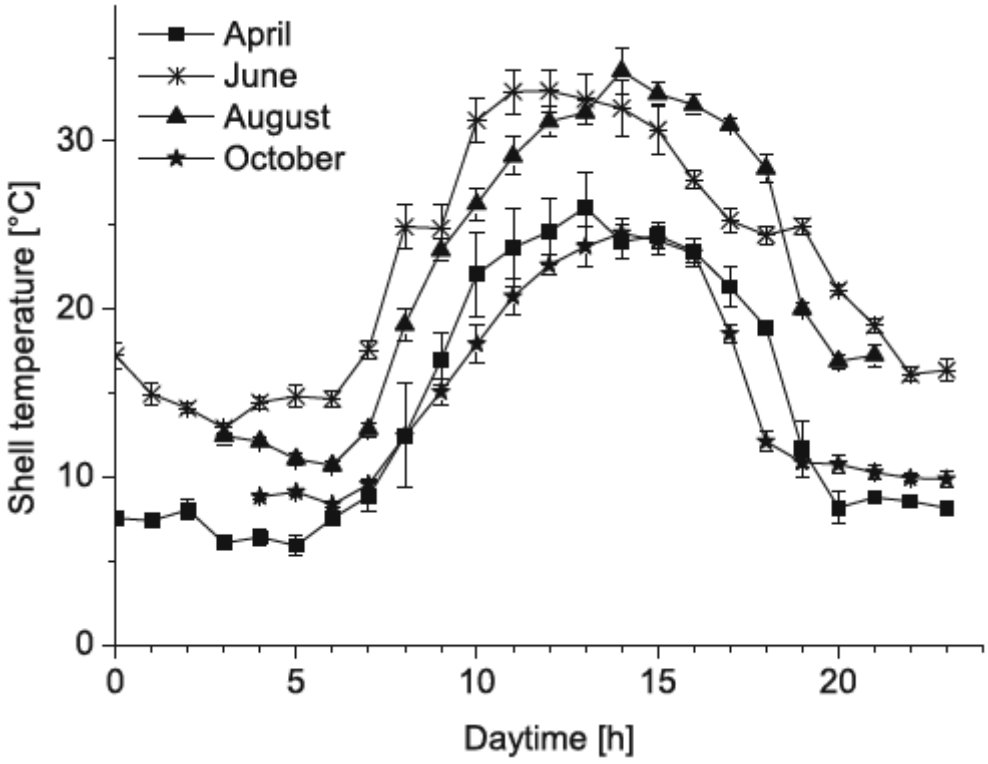

Fig. 3 Daily course of the shell temperature in four different months in 2011. Error bars indicate the standard deviation of ten samples taken per hour. Each data point represents the mean value of ten individuals

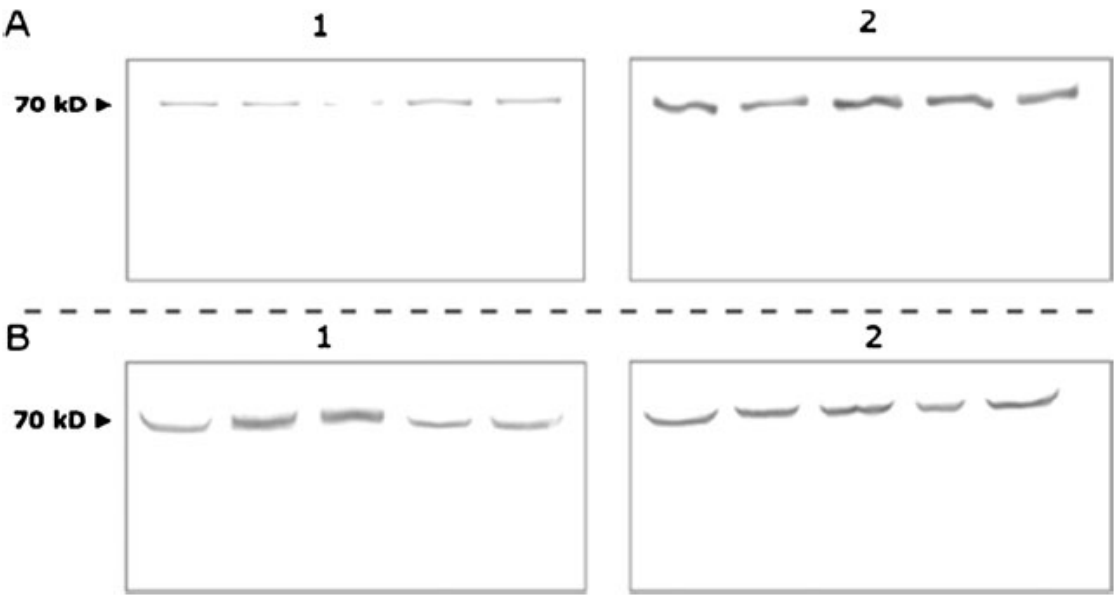

Fig. 4 Western blots for two different seasons and two different sampling times. A Samples from June 2011; 1= 0 h, 2=15 h daytime. Ten micrograms of total protein was separated per lane. b Samples from October 2011; 1=0 h, 2= 15 h daytime. Forty micrograms of total protein per lane were separated. Each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

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.

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.

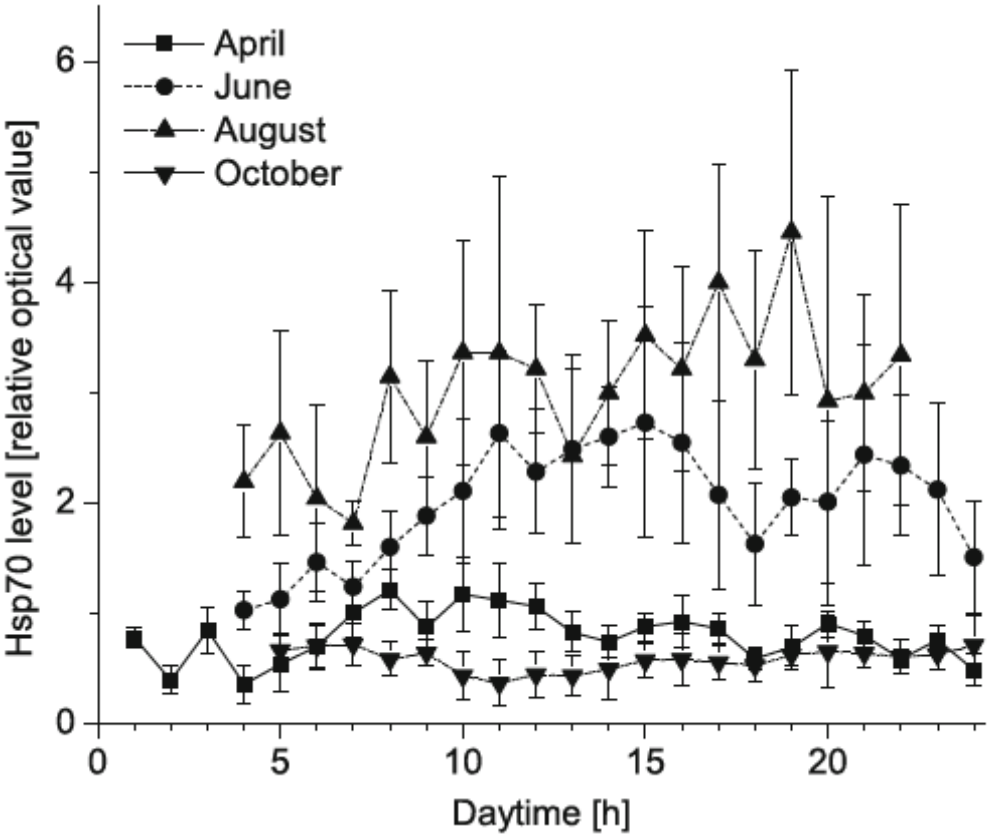

Fig. 5 Daily course of mean Hsp70 levels (n=10) obtained from samples taken in 2011. Error bars indicate the standard deviation of ten samples taken per hour

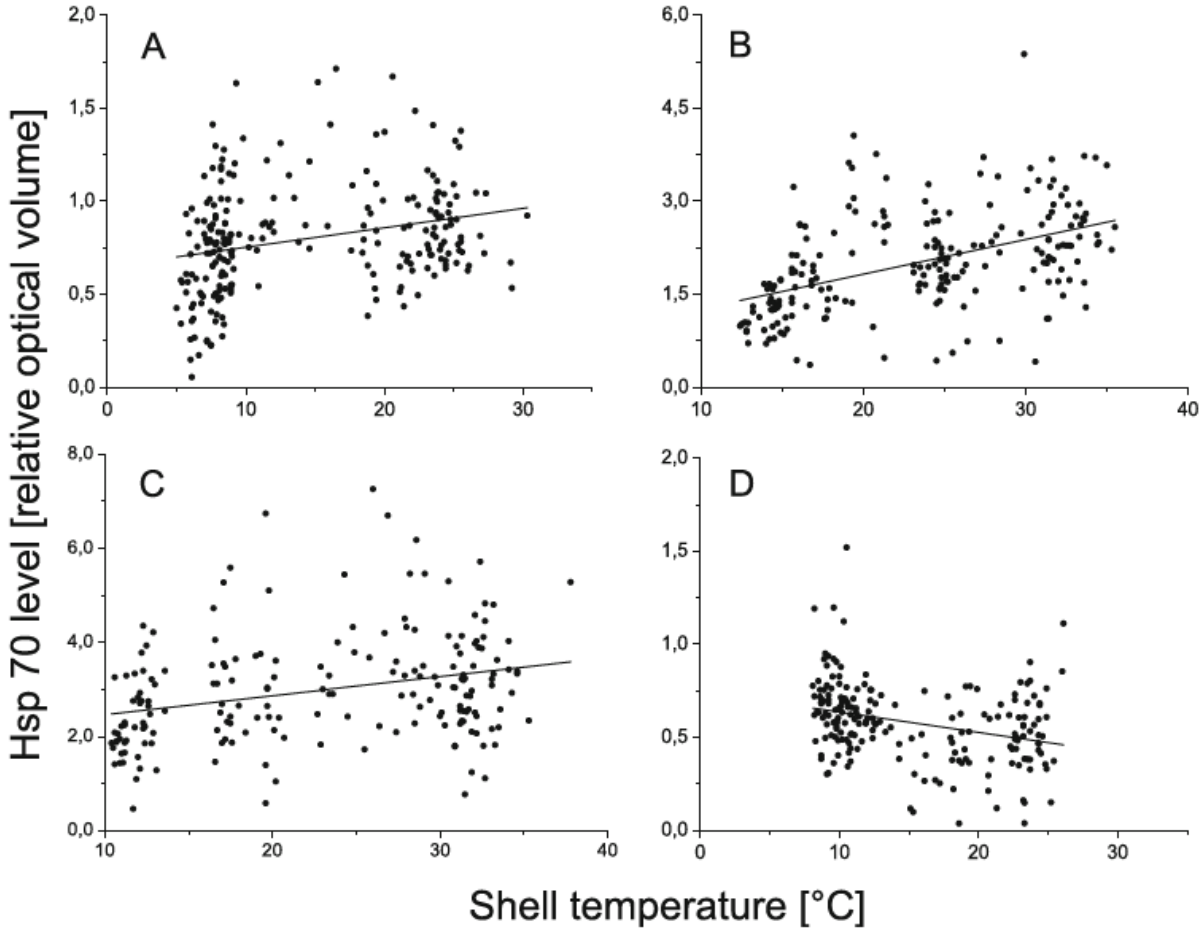

Fig. 6 Correlation between Hsp70 level and external shell temperature in the four different months of sampling. a April. B June. c August. d October. In April, June, and August, a significant positive correlation between Hsp70 level and shell temperature was present. In October, a significant negative 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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Kapitel 4: Relevance of body size and shell colouration for thermal absorption and heat loss in White Garden Snails from Northern France

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Abstract

The internal temperature of land snails depends on environmental factors such as exposure to electromagnetic radiation and airflow. In controlled field experiments, we quantified heating by thermal absorption of light and airflow-induced heat loss in individuals of the White Garden Snail, *Theba pisana*, from Normandy, France. Heating experiments revealed a significant positive correlation of the internal body temperature with the time of constant illumination, the shell temperature, and the air temperature at different daytime. The size of the snails was negatively correlated for both of the given illumination times: smaller animals heated up stronger than larger ones. The temperature at the surface of the shell depended significantly on the time of illumination and the daytime. AIC-based quality assessment of multiple regression modelling additionally showed that, for explaining both shell surface and internal temperature of the soft body, exposure time, daytime, shell size and colouration contributed to the respectively best models. The heat loss of the soft body after and during exposure of the snails to sunlight by a constant airflow depended on the initial body temperature, shell size, colouration, and on the ambient temperature of the air. Modelling also revealed the importance of shell size and colouration: large and banded animals cooled down slower than small and unbanded ones.

Keywords: environmental stress, illumination, land snails, polymorphism, *Theba pisana*, warming

Introduction

Several terrestrial pulmonate snail species of the superfamily Helicoidea exhibit large intraspecific variation in the colour and pattern of their shells. Even though origin, modulation in frequency, and the mechanisms of maintenance of the shell polymorphism in helicoid snails is not completely understood, many studies have addressed the genetic background and the association of morphs with particular microhabitats (Jones, 1973; Jones, Leith & Rawlings, 1977; Heller, 1981; Cowie, 1984; Cowie, 1990; Cook, 1998; Johnson, 2011). Some helicoids are highly thermotolerant and, thus, adaptive responses to high temperature during aestivation for instance, and behavioural aspects of thermoregulation have been investigated in these snails for a long time (Pomeroy, 1968; McQuaid, Branch & Frost, 1979; Cowie, 1985; Arad *et al.*, 1993). In this context, there have been also attempts to quantify the thermal capacity of different shell morphs (Jones, 1973; Heath, 1975; Cook & Freeman, 1986; Scheil, Gärtner & Köhler 2012). Nowadays, temperature and/or solar radiation, and visual appearance to predators are assumed to act as predominant selective factors on shell and body colour variation in helicoid snails (Cain & Sheppard, 1954; Richardson, 1974; Jones *et al.*, 1977; Heller, 1981; Johnson, 1981; Cain, 1983; Heller & Gadot, 1984; Cowie & Jones, 1985; Cowie, 1990; Slotow & Ward, 1997; Johnson, 2011). This is based on the plausible assumption that uncoloured (*i.e.* unbanded or effectively unbanded) shells have a higher albedo and, therefore, should heat up less and more slowly when exposed to light in open habitats. On the contrary, individuals with more intense shell pigmentation (banded) are considered to be less visible to potential predators and therefore are favoured in woodlands, assuming that in such habitats the trade-off with thermal aspects is less relevant.

While the (human perception-based) assumption that banded snail shells are less conspicuous in front of a heterogeneous background than unbanded conspecifics has been supported recently by a study on the avian perception of polymorphic snails (Adrian Surmacki and co-workers, Poznań, unpublished), there is still a controversial debate on the ecological and microevolutionary relevance of differences in thermal capacities which might exist between banded and pale shells in different species. Jones (1973), Heath (1975), and Cook & Freeman (1986) have suggested a higher thermal capacity of darker shell morphs but have focused on species which either exhibit numerous dark stripes, dashes, and dots which cover almost the entire surface of the shell in the pigmented morph (*Littoraria pallescens*) or develop sharp, intensely stained bands that can cover half of an individual´s shell surface (*Cepaea* species). On the contrary, Knights (1979) did not find any colouration effect on the mortality of

Cepaea hortensis morphs in a field exposure experiment but rather unshaded conditions to selectively favour smaller individuals and shade to favour larger ones. In a lab study on shell size and selection, also Cook & O`Donald (1971) and Goodfriend (1986) showed larger shell sizes in helicoid snails to be favoured by cooler and moister conditions. In respect to shell colouration, Scheil *et al.* (2012) at most found a marginal difference between the thermal capacities of unbanded and banded shells of *Theba pisana*, a species which displays multiform variation in the extension and intensity of bands but always has a white background colour. Since the study of Scheil *et al.* (2012) used empty shells to avoid measurement disturbances by the snail´s soft body, it is therefore hard to judge on the ecological relevance of shell colouration pattern in living *T. pisana* individuals. Also the much more intense pigmentation of coloured *Cepaea* and *Littoraria* shells does not allow extrapolation of existing findings to the rather 'fuzzily' pigmented morphs of *Theba*.

Numerous helicid and hygromiid snails, including *T. pisana*, but also *Cernuella*, *Xeropicta*, or *Helicella* species exhibit a white background colour and a variable banding of moderate intensity. To get an idea of the relevance of illumination as a potential selective factor which may act in varying intensity on differently coloured morphs and differently sized individuals in the natural environment, we used *T. pisana* as a representative for other species with shells with a constantly white background and similar banding shape and intensity. By exposing individuals of different size and morphotype (banded *vs.* unbanded) in a field plot with defined illumination and wind speed we aimed to unravel the relevance of body size and shell colouration for thermal absorption and heat loss, in relation to the impact of environmental parameters like duration of illumination or time of the day.

Material and methods

Test animals

To minimize interactions of defined artificial illumination with natural sunlight, we avoided experimental conditions dominated by strong solar irradiation and, therefore, chose an area with moderate climate in the Northern part of the distribution range of the White Garden Snail, *Theba pisana* (Müller, 1774), for our investigations. Specimens of *T. pisana* were collected in mid-June 2012 at the dune system of Biville in Normandy, France (49°36´17´´N, 01°49´13´´W). The snails were separated into banded (b) and effectively unbanded (u)

morphotypes (Fig. 1) according to the classification of Cowie (1992). Intermediate forms that could not be clearly attributed to either category were omitted from the study. Until use in the experiments, the animals were kept in plastic containers at ambient temperature and natural light regime on vegetation taken from the sampling site. Humidity was regulated by adding moist filter paper. The snails were fed organic grown lettuce and carrots *ad libitum* and the boxes were cleaned every second day from the faeces, also eliminating any dead snails that may have occurred. To ensure calcium supply for shell formation, a piece of cuttlebone was added to each of the containers.

Fig. 1: Representative individuals of *T. pisana* from the Biville dunes. Experiments were conducted with effectively unbanded individuals (left) and banded conspecifics (right).

Heating experiments

Heating experiments were carried out at two time points of the day: morning (m) and afternoon (a). The ambient air temperatures corresponding to these time points were 15°C ± 1°C (m) and 17°C ± 1°C (a), respectively. 128 banded and 125 unbanded snails ranging from 8.7 to 18.8 mm (average shell diameter 13.74 ± 1.89 mm) were placed on a wooden board and heated by a 400 W halogen spot light at 30 cm distance from the wooden surface providing 42 klx at this surface. Direct exposure to sun light and wind was prevented by placing the experimental setting in a shaded, wind-sheltered environment. The animals were exposed to the artificial light source for either one or two minutes, both being time spans during which the snails usually remained inside the shell after displacement from their plastic containers. Temperature at the outer surface of the shell (at a point directed towards the illumination source) and internal temperature of the soft body were measured by a medical precision thermometer equipped with a needle sensor (DM 852, Ellab, Copenhagen, Denmark). Care was taken not to cause artificial heat loss during the measurements and, therefore, the snails were wrapped into insulating tissue whilst measuring the temperature. Subsequently, body size was recorded by measuring the individual shell diameter with a digital calliper rule as described by Köhler *et al.* (2009).

Cooling experiments

For the cooling experiments, the decrease of the internal temperature of the soft body was recorded in 30 banded and 32 unbanded snails of 9.7 to 18.9 mm size (average shell diameter 14.29 ± 2.93 mm). The animals were heated up prior to these measurements by exposing them to direct, full sunlight (80-100 klx) on a plate of slate for at least 15´, after which a thermal steady state without further increase of body temperature could be assumed. Two behavioural situations were encountered (*a*) snails that immobilised on the ground resulting in internal temperatures of 30°C or more and (*b*) snails that climbed a vertical object but stayed exposed to sunlight and which reached temperatures of less than 30°C. Subsequently, the snails were individually mounted on a needle thermometer (DM 852, Ellab) in such a manner that they remained vertically fixed at 27 cm above ground in a distance of 20 cm from the exhaust port of an air blower (HP4935, Philips, Eindhoven, Netherlands) which provided a constant air flow of 1.78 ± 0.11 m/s at ambient temperature. The position was arranged such that the animals remained exposed to full sunlight during the measurements, and cooling was

essentially due to removal from the ground and/or the artificial wind source. Changes in internal soft body temperature were recorded continuously every 10 seconds until the body temperature had stabilised and no further temperature decrease was apparent (= equilibrium temperature). Equilibrium was defined as the state whenever soft body temperature did not vary to a larger extent than 0.1°C within 30´´. For each individual, we recorded initial (internal) temperature (T_0), equilibrium temperature (T_{equ}), the difference between them (ΔT), and the time until equilibrium (t_{equ}). Furthermore, individual body size was recorded as described above.

Statistical analysis

As a measure of dependence of two variables we used linear regression analysis. Significance was checked with ANOVA at $P \leq .05$. Independent datasets were checked pairwise for significant difference using Student´s t-test at $P \leq .05$. Datasets were checked for normality using the Shapiro-Wilk test prior to analysis.

Furthermore, an information-theory model selection approach based on Akaike's Information Criterion (AIC) was used to evaluate the quality of multiple regression models in a series of competing models. This approach allows to rank a number of different models according to their data fit and to estimate the significance of all single factors and interactions contributing to the models. This analysis aimed at uncovering the impact of different factors on the thermal absorption and the heat loss of a snail, respectively. We conducted this analysis for both approaches separately. To model thermal absorption, we included the independent factors ˈsizeˈ, ˈshell patternˈ, the interaction between ˈsizeˈ and ˈshell patternˈ, the time of the day, and the exposure time. As dependent variable, the external temperature of the shell and the internal temperature of the soft body were tested in separate analyses, since both factors were correlated to each other and therefore could not be combined in a single run. For the cooling experiment, we also conducted two analyses because of correlations between coefficients with ˈtime until equilibriumˈ as dependent variable. For the first analysis, we chose ˈshell size, ˈshell patternˈ and the interaction between both coefficients as factors, for the second one the internal temperature, the shell pattern as well as their interaction.

In a first step, competing models were constructed from the predictors. After checking for normal distribution of the dependent variables (internal/external temperature, time until

equilibrium) to fulfil the univariate requirements (Shapiro-Wilk test, P>.05), we fitted the data to the models by the use of univariate statistical methods (multiple regression modelling, ANOVA). In the next step, the AIC_C, a modified variant of the AIC being adequate for small sample sizes (Symonds and Moussalli 2011), was calculated for each model according to the following equation:

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

where *n* is the sample size, *k* is the number of parameters used in each respective model and *MSS* is the mean sum of squares.

Subsequently, the models were ranked according to their AIC_C value: the lower the AIC_C, the better is the explanatory power of the model. To assess the relative strength of each respective model compared with the best one, we calculated Δ_i, the difference of the AIC_C of each model to the best ranked one. According to Symonds & Moussalli (2011), models with Δ_i values smaller than 2 can be considered to be comparably meaningful in explaining the variation within the data. Furthermore, we 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 measure gives an impression on the chance of a model to be the best ranked one on a repeated data collection, and can easily be transformed into percentage values, because all ω_i within the whole set of models sum up to one. As last step, we estimated parameter coefficients through the whole set of models by calculating a weighted average, to avoid model selection bias.

For all statistical data analysis, we used JMP, version 9 (SAS Institute Inc., Cary, NC). All data on measurements in this paper are given as means ± SD.

Results

Thermal absorption

When exposed to artificial illumination, the shell and also the soft body of *T. pisana* individuals heated up rather quickly. Illumination for 2´ resulted in significantly higher shell and soft body temperatures than only 1´ illumination (t-test, for both parameters P<.0001). When the dataset was split according to the time of constant illumination, the data for 1´

illumination revealed the same correlations of temperatures with shell size and colouration as the data for 2´ illumination. In both data subsets the internal temperature of the soft body showed a significant, negative correlation with the shell size (ANOVA, P=.0005 for 1´; P=.0012 for 2´) and a significant, positive correlation with the temperature of the shell at its surface (ANOVA, P<.0001 for both 1´ and 2´) (Fig.2). However, the shell surface temperature did not significantly depend on the size of the individuals (ANOVA, P=.3489 for 1´; P=.5254 for 2´).

When testing the parameters ˈtime of dayˈ and ˈshell colourationˈ in each of the two data subsets, results also resembled one another. In the afternoon, at a higher ambient temperature, also the shell surface temperature of the snails was significantly higher after illumination than in the morning (t-test, P<.0001 for both 1´ and 2´). Concomitantly, this held true for the internal temperature of the snail´s soft body as well (t-test, P<.0001 for both 1´ and 2´) (Fig.3). In contrast, the shell colouration pattern did only show an insignificant trend to lower temperatures in effectively unbanded individuals in comparison to banded ones. This was found for the temperature at the shell surface (t-test, P=.0527 for 1´; P=.2072 for 2´) and the temperature inside the snail (t-test, P=.1356 for 1´; P=.1726 for 2´). However, further statistical analyses (ANCOVA) showed for the shell colouration pattern to contribute significantly to lower internal and surface temperatures in unbanded individuals in the morning after one and two minutes of illumination (Tab. 1).

Tab 1: Results of analysis of covariance (ANCOVA). Given is the test statistic (F), the probability level (p-level) and part. Eta².

Exp.		Temperature	Shell size			Shell colouration pattern		
[min]	Time of day	[°C]	F	p-Level	part. Eta²	F	p-Level	part. Eta²
1	morning	internal	50,34	**<0,001**	0,46	72,48	**<0,001**	0,55
1	morning	shell surface	17,95	**<0,001**	0,23	33,01	**<0,001**	0,36
1	afternoon	internal	1,47	0,231	0,02	0,21	0,652	0,00
2	morning	shell surface	2,40	0,126	0,04	4,50	**0,038**	0,07
2	afternoon	internal	4,18	**0,045**	0,07	0,02	0,902	<0,001
2	afternoon	shell surface	37,24	**<0,001**	0,39	1,17	0,284	0,02

The AIC_C-based model selection procedure to explain variation in the shell surface temperature revealed that, of a total of 13 potentially relevant models, only a single 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.2). The model with the lowest AIC_C value and, correspondingly, the highest explanatory power for shell temperature variation (81.7%) was built by the factors 'size', 'time of day', 'shell colouration', and 'exposure time'. All these parameters had a very high probability value (≥99.9%), representing the chance to be part of the best model and, therefore, clearly contributed to the variation in shell surface temperature, whereas the interaction between size and shell colouration has shown to be redundant in this analysis and does not own explanatory power in this respect. Another important indication to the robustness of our results is the fact that the null model is ranked relatively low (rank 11), implying that the chosen measurements clearly improved our ability to predict the change in shell surface temperature. The Correlation coefficients (β) for the model selection analysis are given in table 4.

Tab.2: Tested models for explaining the variation in shell surface temperatures in land snails, listed in decreasing succession, the best ranked model at the top. 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, TD: time of day, SP: shell pattern, TE: time of exposure

Rank	S	TD	SP	TE	S*SP	AICc	*w*	Parameter
1	•	•	•	•		137,51	0,817	5
2	•	•	•	•	•	140,51	0,182	6
3		•		•		151,95	0,001	3
4				•		310,67	0,000	2
5			•	•		310,79	0,000	3
6	•		•	•		313,60	0,000	4
7	•			•		313,71	0,000	3
8		•				434,36	0,000	2
9	•					491,76	0,000	2
10	•		•			491,96	0,000	3
11						495,07	0,000	1
12			•			496,94	0,000	2
13					•	497,45	0,000	2
Probability	0,999	1,000	0,999	1,000	0,182			

The best models which aimed at explaining the variation in the internal soft body temperature used the same variables as mentioned above (Tab. 3). Also for this dependent variable, the model with the lowest AIC_C value which explained 68.2% of soft body temperature variation used the factors 'size', 'time of day', 'shell colouration', and 'exposure time' with a

probability of 100% each. Also the second ranked model showed almost equally high support and used, in addition to the above-mentioned variables, the interaction between shell size and colouration, however with a probability of 31.8% only. Robustness of the results again was indicated by the low rank of the null model (rank 11).

Tab.3: Tested models for explaining the variation in internal temperatures in land snails, listed in decreasing succession, the best ranked model at the top. S: shell size, TD: time of day, SP: shell pattern, TE: time of exposure

Rank	S	TD	SP	TE	S*SP	AICc	*w*	Parameter
1	•	•	•	•		143,15	0,682	5
2	•	•	•	•	•	144,67	0,318	6
3		•		•		215,81	0,000	3
4	•		•	•		263,92	0,000	4
5	•			•		268,06	0,000	3
6				•		288,05	0,000	2
7			•	•		288,98	0,000	3
8	•		•			483,97	0,000	3
9	•					485,75	0,000	2
10		•				490,30	0,000	2
11						514,66	0,000	1
12					•	515,89	0,000	2
13			•			517,02	0,000	2
Probability	1,000	1,000	1,000	1,000	0,318			

Tab.4: Correlation coefficients (β) for the model selection analysis "thermal absorption". Right: analysis for shell surface temperature, left: internal temperature.

Parameter	Category	ß-Coefficient
S		-0,441
TD	morning	-1,057
	afternoon	1,057
SP	banded	0,323
	unbanded	-0,323
TE	1'	-1,982
	2'	1,982
S*SP	banded	-0,018
	unbanded	0,018

Parameter	Category	ß-Coefficient
S		-0,187
TD	morning	-1,336
	afternoon	1,336
SP	banded	0,253
	unbanded	-0,253
TE	1'	-1,872
	2'	1,872
S*SP	banded	0,003
	unbanded	-0,003

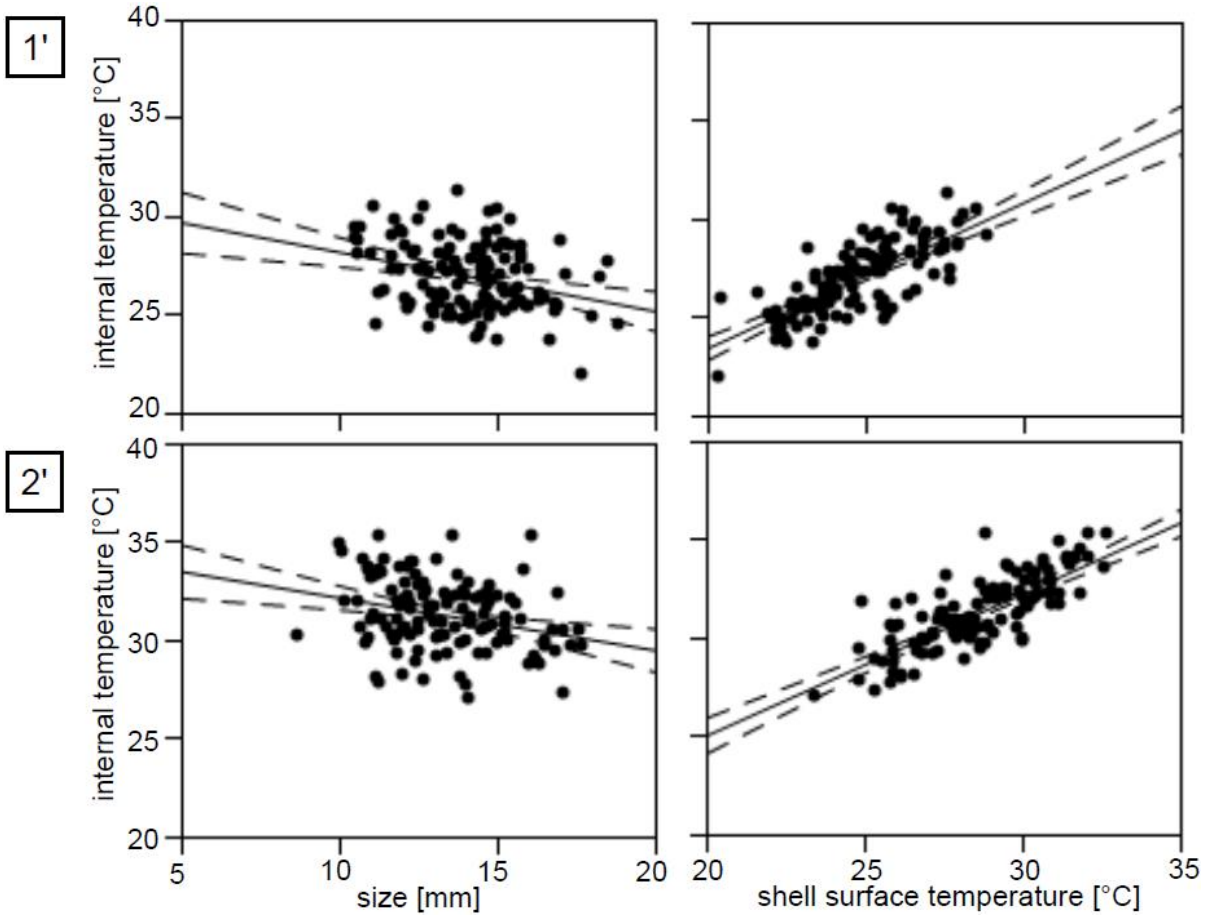

Fig. 2: Internal temperature of the soft body [°C] vs. size [mm] (left) and vs. shell surface temperature [°C] (right) in the heating experiments. Top row: illumination for 1´, bottom row: illumination for 2´. Significances in the text. Regression lines (solid) and 95% confidence interval (dashed) are for visual purpose only.

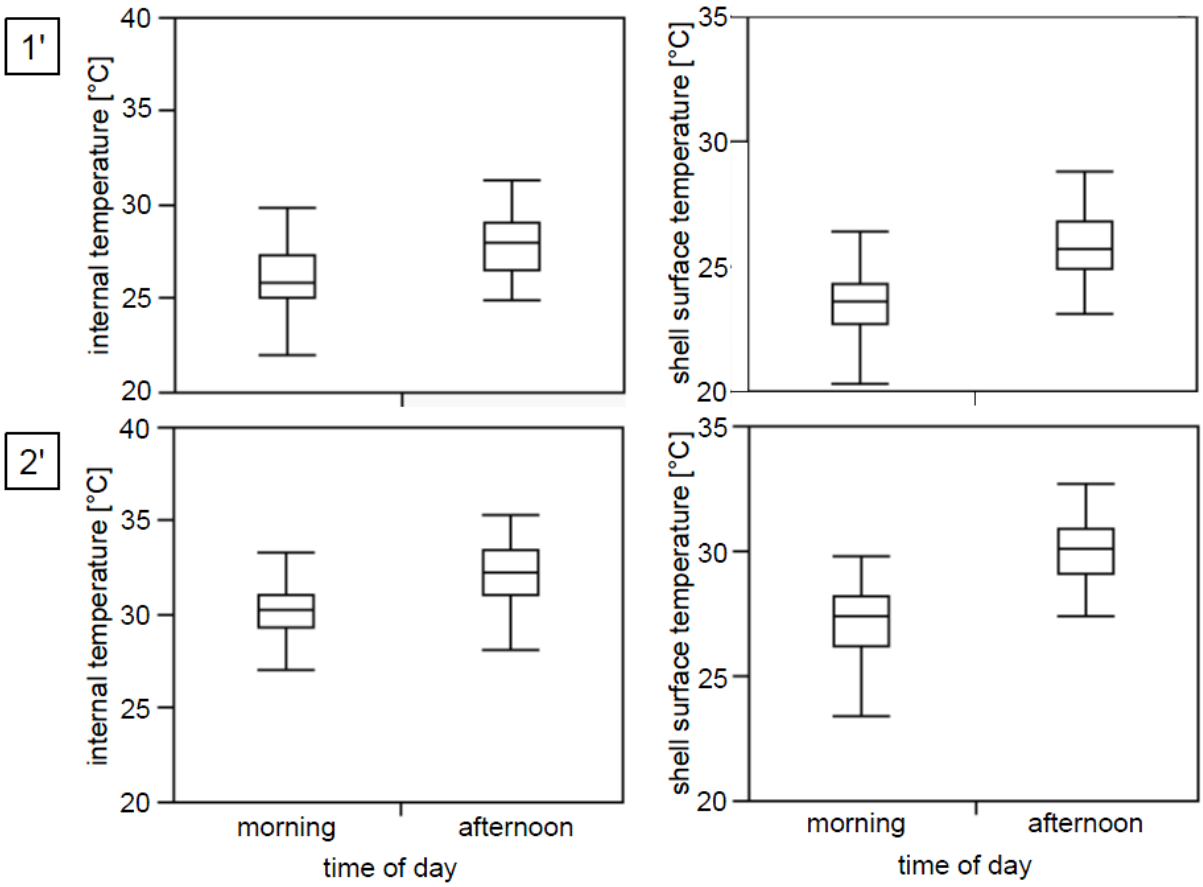

Fig. 3: Internal temperature of the soft body [°C] (left) and shell surface temperature [°C] (right) vs. time of day in the heating experiments. Top row: illumination for 1´, bottom row: illumination for 2´. Significances in the text. Box plots indicate percentiles: bottom whisker (10%), bottom of box (25%), middle line (50%), top of box (75%), and top whisker (90%).

Heat loss

All snails which had heated up while staying on the ground (internal temperatures of 30°C or more) or at vertical objects (initial temperatures of less than 30°C) cooled down following an exponential temperature decay curve when they were exposed to a constant air flow with ambient temperature, even though they were still exposed to solar radiation during this time (Fig. 4). Although the shape of the heat loss curve did not differ much among the individuals, the ΔT was significantly associated with the internal temperature at the beginning of cooling, T_0 (ANOVA, P<.0001) and the equilibrium temperature of the soft body (ANOVA, P=.0249) the latter of which was inevitably limited by the ambient air temperature (Fig. 5). T_0 was also

found to correlate with the shell size indicating that larger snails exhibited higher internal temperatures after exposure to the sun for ≥15′ than smaller conspecifics, on the average .61°C per mm shell diameter (ANOVA, P=.0376).

As a proxy for 'cooling efficiency', we used the time until an individual has reached thermal equilibrium in its soft body (t_{equ}). This parameter showed a significant positive correlation with the shell size (ANOVA, P=.0124) indicating slower heat loss with increasing size of the animal. In the experimental setup in which individuals have been exposed to an airflow plus natural sunlight, colouration of the shell (which was not associated with size) had also an effect on t_{equ}: it took banded individuals a longer time to reach thermal equilibrium than effectively unbanded ones (t-test, P=.0285) (Fig 6).

The first model selection procedure for the factors 'shell size', 'shell colouration pattern', and their interaction revealed two of eight potentially relevant models to exhibit high and almost identical empirical support to explain t_{equ} variation (Tab. 5). The most prominent factor was the size of the shell which contributed to both of these models with an expectation probability of 79.3% to be part of the best model and explained 36.0% of t_{equ} variation alone. The shell colouration pattern was less important (45.2% probability) but was found to contribute to one of the two best models. The interaction of these two factors was not important. Results did not change much when we replaced the factor 'size' by the correlating variable 'T_0' in the second set of models. Again, two out of eight models showed high and almost equal support (Tab. 6). T_0 clearly contributed to t_{equ} variation with a probability of 99.6% to be part of the best model (which explained a total of 38.1% of t_{equ} variation). Also in this model series the variable 'shell colouration' could not be neglected since it turned out to be part of the best model with a probability of 50%. Again, the interaction of size with pattern did not bear any importance. Robustness of the results was indicated by the low ranking of the null models in both model series (rank 6 out of 8).

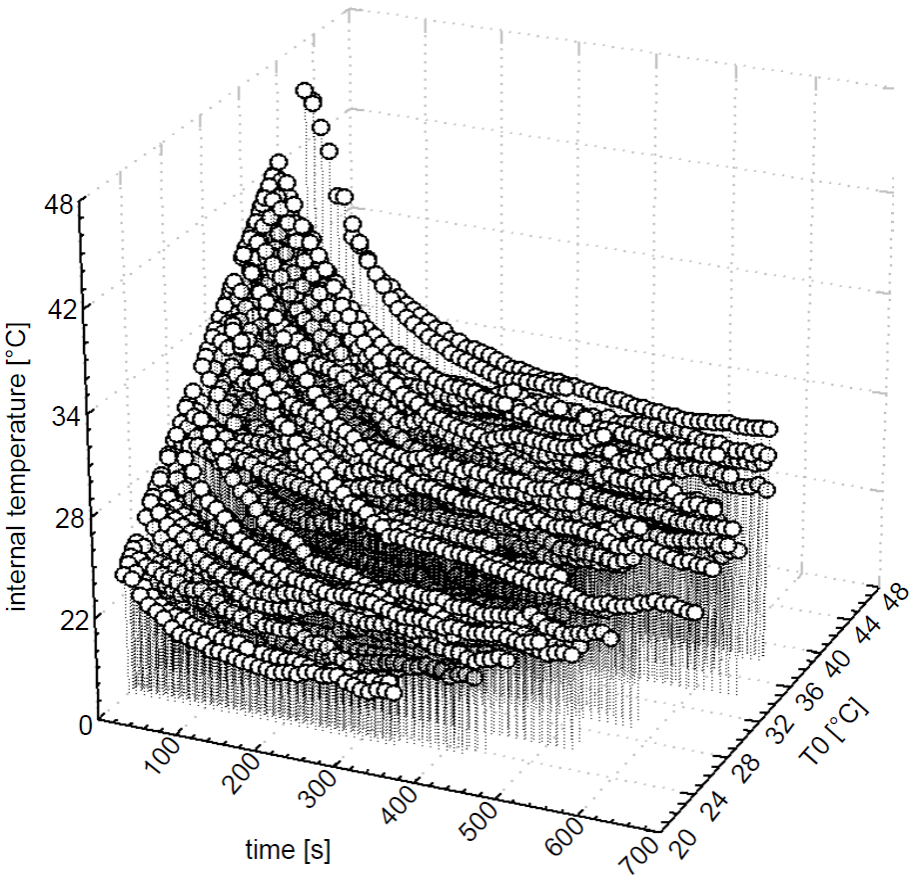

Fig. 4: Internal temperature of the soft body [°C] vs. internal temperature of the soft body at t=0 (T_0) [°C] vs. time [s] in the cooling experiments.

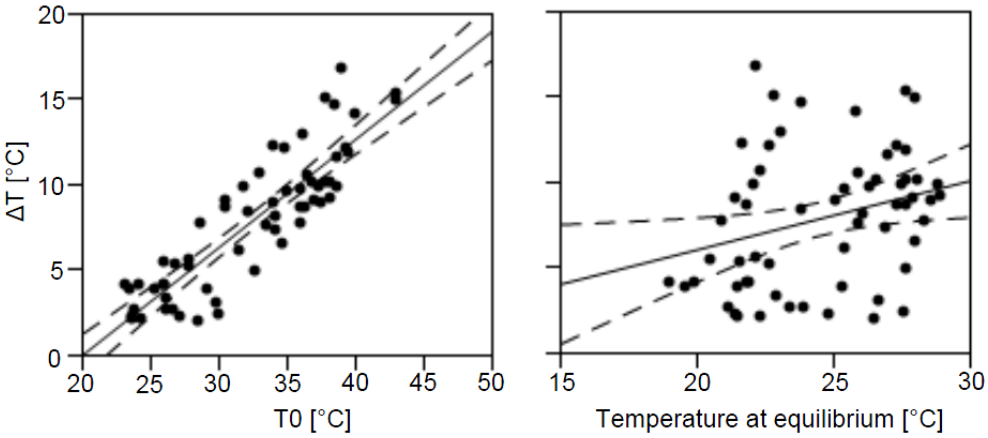

Fig. 5: Heat loss (difference between the internal temperature of an individual´s soft body at t=0 and the temperature at equilibrium [°C]) vs. internal temperature of the soft body at t=0 (T0) [°C] (left) and vs. temperature at equilibrium [°C] (right) in the cooling experiments. Significances in the text. Regression lines (solid) and 95% confidence interval (dashed) are for visual purpose only.

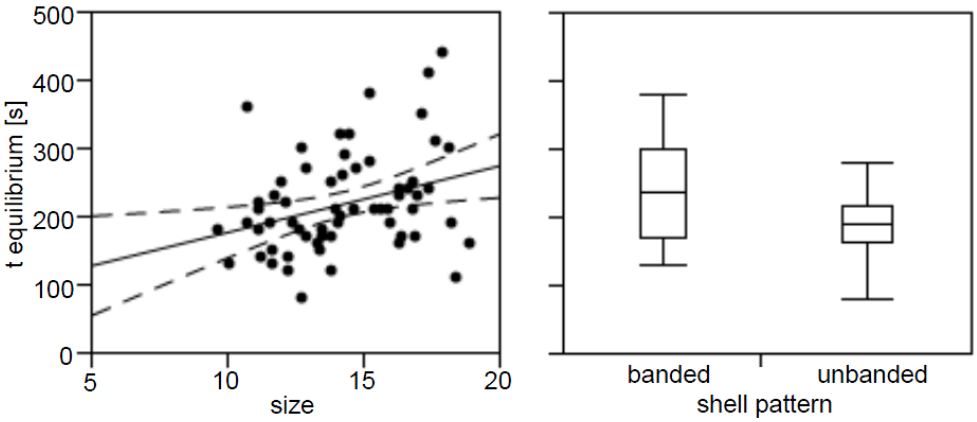

Fig. 6: Time until equilibrium [s] vs. size [mm] (left) and vs. shell colouration pattern (right) in the cooling experiments. Significances in the text. The regression line (solid) and 95% confidence interval (dashed) is for visual purpose only, for the explanation of the box plots see the legend to Fig. 3.

Tab 5: Tested models including the parameter 'size' for explaining t_{equ} variation, listed in decreasing succession, the best ranked model at the top. Models with high empirical support are arranged above the dashed line ($\Delta_i \leq 2$). S: shell size, SP: shell pattern

Rank	S	SP	S*SP	AICc	*w*	Parameter
1	•			532,32	0,360	2
2	•	•		532,96	0,261	3
3	•		•	534,87	0,101	3
4		•		535,02	0,093	2
5	•	•	•	535,55	0,071	4
6				535,66	0,068	1
7		•	•	537,50	0,027	3
8			•	538,12	0,020	2
Probability	0,793	0,452	0,219			

Tab 6: Tested models including the parameter 'T_0' for explaining t_{equ} variation. T_0: initial (internal) temperature, SP: shell pattern

Rank	T0	SP	T0*SP	AICc	*w*	Parameter
1	•	•		524,50	0,381	3
2	•			524,53	0,375	2
3	•		•	526,79	0,121	3
4	•	•	•	526,83	0,119	4
5		•		535,02	0,002	2
6				535,66	0,001	1
7		•	•	537,99	0,000	3
8			•	538,56	0,000	2
Probability	0,996	0,502	0,240			

Tab 7: Correlation coefficients (β) for the model selection analysis "heat loss". Right: analysis with the factor 'size', left: initial internal temperature 'T_0'.

Parameter	Category	ß-Coefficient
S		-0,231
SP	banded	6,913
	unbanded	-6,913
S*SP	banded	-0,704
	unbanded	0,704

Parameter	Category	ß-Coefficient
T_0		-0,245
SP	banded	4,729
	unbanded	-4,729
T_0*SP	banded	-0,478
	unbanded	0,478

Discussion

This study addressed several aspects of thermal exchange between land snails and their environment under semi-natural conditions: short-time heating-up for 1´ and 2´, long-time heating-up for ≥30´ (allowing behavioural thermoregulation), and cooling-down until thermal equilibrium. In this context, we particularly focused on the relevance of the snails´ body size and shell colouration.

In all treatments, the individual body size was found to be just as relevant for the temperature transfer to and from the snails as their shell pigmentation. Correlation analyses conducted on the data deriving from the short-term heating experiments revealed – besides of the obviously relevant parameters ˈtime of illuminationˈ and ˈtime of dayˈ (as a proxy for ambient temperature) – individual size to be associated with the internal temperature while shell colouration was only in the morning. Also modelling internal temperature showed the higher explanatory power of the parameter ˈsizeˈ alone compared to ˈshell patternˈ alone while, of course, most explanatory power had to be assigned to ˈtime of illuminationˈ and ˈtime of dayˈ. However, one should not neglect relevance of the shell pigmentation since this parameter had a very high probability to be part of the best model. Thus, these experiments did not exclude shell pigmentation as a factor to modulate thermal transfer into the snail´s body but clearly showed that the individual size was a bit more relevant in this respect. Because illumination was applied to the snails for a rather short time only, the chances of the animals to physiologically counteract increasing body temperature are very limited and, therefore, the warming up of a snail´s soft body for the very most part depends on thermodynamic principles. So, the reason for the observation that smaller individuals heat up stronger than

larger ones within the first two minutes of strong illumination is most likely up to the higher surface-to-volume ratio of small individuals. The shell surface temperature however, measured at a single point, should solely depend on the absorption of electromagnetic radiation, defined by properties of the material and the character of radiation, independent of the surface-to-volume ratio of the illuminated object. In accordance with this, we did not find a correlation of body size and surface temperature in our experiments. Merely, the parameter ˈsizeˈ contributed, among other parameters, to the model which explained surface temperature variation best, probably due to the inescapable interactions between the internal temperature and the shell surface.

Similarly, the data recorded for long-term (≥15´) warming-up of individuals in preparation for the heat loss experiment revealed importance of the snails´ body size for their internal temperature. Here, behavioural responses to heating were possible, and the rather long exposure time allowed the animals to climb up vertically in order to escape from the hot surface of the stone plate. It has been shown for *Xeropicta derbentina* that this behavior reduces exposure to heat (Dieterich *et al.*, in press) and also reduces the individual levels of the heat-inducible 70 kD stress protein family, Hsp70 (Di Lellis *et al.*, 2012) in positive relation to the climbing height. The impact of size on the internal temperature during the conditioning phase of our heat loss experiment, therefore, may be just a consequence of a possibly higher mobility of small snails in comparison to big conspecifics.

Also the results on the temperature loss of the soft body during exposure to a constant air flow were in accordance with the theoretical predictions. Due to a smaller surface-volume ratio, larger individuals of *T. pisana* cooled down more slowly than smaller ones, corroborating the results obtained for *C. nemoralis* before (Heath 1975). Both correlation analyses and modelling congruently showed this consistently. So, this study overall provides solid evidence that the size of snails is crucial for the temperature exchange of the body with the environment, both for warming-up and for cooling down. We therefore conclude that selection pressure, exerted by illumination, differs throughout the life cycle of *T. pisana*, simply due to proceeding growth.

In contrast, the relevance of shell pigmentation for the body temperature is less clear, at least for *T. pisana*. As mentioned above, any importance of colouration for the internal temperature was hard to be detected and quantified during the first few minutes of illumination, and was suggested by the modelling approach exclusively. Even though Heath (1975) found

differences in the thermal capacities of lighter and darker shells of *Cepaea nemoralis*, there are also reports which failed to detect such difference in other species. Cowie (1992) did not find any difference in the thermal behavior of coloured and plain white morphs of *T. pisana*. Also white and brown morphs of the desert snail *Trochoidea seetzeni* did not differ in body temperature (Slotow, Goodfriend & Ward, 1993). It is likely, that thermal capacity of the snail shells which not only depends on the optical properties of pigments but also on the character and structure of its material (Comfort 1951; Hedegaard, Bardeau & Chateigner, 2006) does not differ much between differently coloured morphs of *T. pisana*. Consequently, possibly resulting differences in the soft body temperature were simply too small to be detected in our study after just two minutes of illumination. Correspondingly, in a methodologically elaborate study Scheil *et al.* (2012) did not detect differences in the average warming of plain white and banded shells of *T. pisana* after a maximum of three minutes of exposure to a full natural light spectrum. Since, for the most part, wavelengths in the visible (VIS) and infrared ranges are responsible for the heating of snail shells, and near-infrared (NIR) contributes about 45% of the total solar radiation energy at the ground (Gautier, O´Hirok & Ricchiazzi, 1999; Savazzi & Sasaki 2013), not only VIS but also NIR reflectivity of snail shells substantially influence their thermal capacity. For a variety of land snail species, including helicids, Savazzi & Sazaki (2013) showed visible shell patterns to be very low in contrast or even undetectable in the NIR and, thus, suggestions drawn solely from visual appearance of shells are likely to overestimate the importance of pigmentation for their thermodynamics.

As revealed by our heat loss experiment, however, visible colouration may nevertheless cause a measurable effect on a snail´s body temperature after a longer time period. When *Theba* was exposed to a constant airflow plus sunlight, banded individuals cooled down more slowly than unbanded ones, probably due to a slightly stronger absorption of sunlight during the cooling process. Here, the time until internal temperature equilibrium was up to 10 minutes, for some individuals even longer. All these findings are in accordance with the results of Di Lellis *et al.* (2012) who have detected higher levels of Hsp70 in small and dark individuals of *X. derbentina* than in large and white snails from the same populations. Because size and colouration were found to co-variate in that study, however, it was not possible to attribute expression of this heat-inducible stress protein to either parameter.

How do we now have to assess the potential of illumination to act as a selective factor for shell colouration? Given the results presented here, we have to accept that other intrinsic features of snails like size are more relevant in this context, at least for the existing

morphotypes of *T. pisana* in a moderate climate. This conclusion is in accordance with the results of Knights (1979) who also found illumination to act selectively on size but not on shell colouration in *C. hortensis* in Somerset, England. Factors other than illumination, such as *e.g.* visibility to predators (Rosin *et al.*, 2011) likely are much more selective on shell colouration in temperate regions. Nevertheless, we did find evidence that the colouration pattern of the shells contributed to thermal absorption and heat loss of the snails, although to some subordinate degree in our experiments. Consequently, even a slight impact of this parameter may be of relevance in Mediterranean or desert populations of this species, such as those which have been investigated by Cowie (1990) or Johnson (2011) in, *e.g.*, Northern Africa, California, and Australia. We, therefore, would like to make a plea for a differential view on this problem. While, in some places on earth, illumination may really be a predominantly selective factor, as commonly reported in the literature, in quite a number of other places it may not. Consequently, the maintenance of shell colour polymorphism in some *Theba* populations can be the result of changes in the direction of selection between different years in hot habitats (Johnson, 2011; Johnson, 2012) or of epigenetic non-directional changes in the temperate zone (Köhler *et al.*, 2009; Köhler *et al.*, in press). The intensity and duration of illumination at which shell colouration becomes relevant for selective processes, however, remains to be investigated.

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Kapitel 5: Historic data analysis reveals ambient temperature as a source of phenotypic variation in populations of the land snail *Theba pisana*

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Abstract

Pulmonate land snails are often polymorphic in their shell coloration pattern. To quantify the contribution of environmental parameters to the nondirectional change in phenotypic variation, we used a historic dataset on *Theba pisana* morph frequencies and climate data for statistical modelling. We found significant correlations of the degree of phenotypic diversity between juveniles and corresponding adult individuals within the same and the subsequent generation. Among climate parameters, the phenotypic diversity of adults correlated significantly and positively with the mean and maximum ambient temperatures in the winter and spring only. There was no correlation between high or low temperatures and the frequency of distinct morphs. Akaike's information criterion-based model selection revealed the particular importance of only parental phenotypic diversity for next generation juvenile phenotypic diversity. By contrast, phenotypic diversity of the juveniles of the preceding year and the mean temperatures in winter and spring were important for the phenotypic diversity of adult snails. Approximately two-thirds of the explicable variation in phenotypic diversity of adults was explained by inheritance and approximately one-third was expained by ambient temperature. The present study shows that genetics and temperature interact to generate nondirectional changes in phenotypic variation within populations, which also can be reflected by changes in the phenotype of individuals.

Additional keywords: helicidae, morphological diversity, plasticity, polymorphism, Pulmonata

Introduction

Phenotypic variation is an essential prerequisite for natural selection and thus evolution. It is generated by the interplay of genetic variation (part of which may stay cryptic), developmental plasticity (part of which may be narrowed by canalization processes), maternal effects, epigenetic variation, phenotypic plasticity, and a plethora of environmental factors (Simpson, 1950; Pigliucci, 2001; Bird, 2007; Bossdorf, Richards & Pigliucci, 2008; Palmer, 2012). The present study aimed to investigate the relative importance of environmental variation in relation to heritable (i.e. genetic and partly epigenetic) factors (Richards, Bossdorf & Pigliucci, 2010) for the maintenance of polymorphisms in natural populations. In this context, we explicitly consider nondirectional changes in variation of plasticity associated with environmental variation and do not exclusively focus on adaptive plasticity or on the explanation of spatial and temporal patterns of frequencies of particular phenotypes that are associated with their habitat.

What could be a mechanism for environmental action to maintain variation? Waddington (1942) proposed an unknown mechanism that conceals phenotypic variation in organisms until they are stressed. Later studies revealed an increase in phenotypic variation of eucaryotes after stress (Hoffmann & Parsons, 1997; Imasheva *et al*., 1997; Kristensen, Dahlgaard & Loeschcke, 2003). Microorganisms, in particular, enhance their chances of survival under stress by generating phenotypic diversity, and this can either have a genetic basis or is revealed by a mechanism termed genetic buffering (Aertsen & Michiels, 2005).

Rutherford & Lindquist (1998) and Queitsch, Sangster & Lindquist (2002) showed that mutations in the gene for the stress protein Hsp90 or its pharmacological inhibition reveal previously dormant phenotypic variation. The term ‘genetic capacitoring’ was coined, which was meant to symbolize the buffering of genetic function by the action of epigenetic protein. Later studies also provided evidence for other molecular chaperones, including Hsp70, being involved in genetic buffering/capacitoring (Roberts & Feder, 1999; Bergman & Siegal, 2003; Suzuki & Nijhout, 2006). It is still a matter of discussion whether and to what extent genetic capacitoring is relevant for the evolvability of variation (Hartman, Garvik & Hartwell, 2001; Wagner, 2005). Nevertheless, the involvement of chaperones such as Hsp90 or Hsp70 in genetic capacitoring would maintain the activity of proteins destabilized by mutations and would thus allow the accumulation of genetic variation. Under conditions of environmental stress, when chaperones are needed for repair and are detracted from capacitoring (de Visser

et al., 2003; Tomala & Korona, 2008), the previously hidden genetic variation will consequently be transduced into the phenotype by the modification of an individual's development (so-called 'decanalization'), and will thus increase phenotypic variation within a group of conspecifics. Although capacitoring favours the 'mainstream' (i.e. the high frequencies of particular morphs), decapacitoring should favour the increase of frequencies of 'rare' morphs. Even though the present study does not explicitly focus on Hsp70 or Hsp90, the aspect of decanalization is mentioned again in the Discussion.

Addressing the interaction of environmental stress factors and nondirectional change in phenotypic variation requires large datasets on phenotypic characters of abundant organisms and on climate parameters. The data should ideally cover different life stages (juveniles and adults) of several generations, which requires, at least for most eucaryotes, research over a long time. Environmental conditions should fluctuate during this period and should not be too extreme because strong selection pressure may create a bottleneck, resulting in the reduction of variation. Accordingly, the best way to address the question is to take advantage of long historical datasets: some of the best, continuous, multi-generational records of phenotypic variation in free-living populations are from snails.

A large number of terrestrial pulmonate snail species in the superfamily Helicoidea exhibit large intraspecific variation in the colour and pattern of their shells. Despite evidence for selection for distinct phenotypes in distinct habitats, there is still uncertainty about the mechanisms responsible for the stability of such morphological variation, which persists in several species in different habitats. Probably as a result of their visual attractiveness, their easy accessibility, and their exceptionally high population density in some habitats, quantitative data on snail morphotype assemblages (populations) have been recorded ever since the first third of the last century. Among them are re-surveys in which previously sampled sites have been revisited decades later and for which data are readily available (Cameron, 1992, 2001; Cook & Pettitt, 1998; Cowie & Jones, 1998; Cook, Cowie & Jones, 1999; Cameron & Pokryszko, 2008; Oz˙go & Kinnison, 2008), as well as other studies with non-accessible raw data (Murray & Clarke, 1978; Wall, Carter & Clarke, 1980; Cain, 1983; Cain, Cook & Currey, 1990). However, there are at least three studies focussing on the shell pattern variation in helicid snails that have generated accessible data from more than 20 000 individuals: one from the UK (Cowie, 1992), one from Australia (Johnson, 2011), and one covering all of Europe (Silvertown *et al*., 2011). However, the datasets of these studies are not equally suitable for addressing the aim of the present study. The continental scale study of

Silvertown *et al*. (2011) on *Cepaea nemoralis* compared data compiled during a citizen science project in 2009 with historic data from the 1960s and 1970s, although no data are available for the years in between. The work of Johnson (2011) on *Theba pisana* offers data recorded continuously over 34 years (1977–2010) but concentrated exclusively on adults (therefore excluding the possibility of investigating adult–juvenile correlations) of presumably a single population living in a habitat with a steep gradient in vegetation density resulting in clear climatic selection pressure. The dataset of Cowie (1992), also on *T. pisana*, exclusively met our requirements, even though 'only' 13 years were covered (1977–1989): the data were almost continuously obtained for six populations in habitats with moderate climate (Wales) and phenotypes of juveniles were also recorded. Consequently, for our modelling of phenotypic variation, we use data and climate data for region and time obtained from the dataset of Cowie (1992). To identify individuals that exhibit signs of modifications in shell colour and pattern during their postembryonic development, we also sampled *T. pisana* from populations living at the other side of the British Channel in a climate similar to Tenby. We addressed the following questions:

• Is there evidence for a contribution of both heritable and environmental variation to phenotypic variation in these snail populations? If so, to what extent do both sources contribute?

• If environmental variation turns out to be relevant, which climate parameters are the most important?

• Are distinct stages in the snails' life cycle particularly susceptible to environmental variation? If environmental factors shape nondirected change of variation independent of selection, a number of individuals must change their phenotypes during their life. This should be reflected by a change in the coloration pattern of the spiral whorls of the individual shells.

In the present study, we use the term 'phenotypic variation' in the sense of the phenotypically realized range of characters, whereas the term 'phenotypic diversity' refers to the amount of phenotypic variation as measured by the Shannon–Wiener diversity index.

Material and Methods

Data mining

The present study concentrated on shell pattern polymorphism in natural populations of the pulmonate land snail *T. pisana* (Müller) from South Wales. All data on the scores of different morphs were taken from a 13-year study conducted by Cowie (1992). Between 1977 and 1989, the shell pattern of 21 277 individuals was recorded from six *T. pisana* populations around Tenby, Pembrokeshire, UK (51.67°N, 4.71°W). According to the sampling protocols of Cowie (1984a), which differentiated between adult and juvenile individuals, snails were always collected between 24 May and 31 August of each year (except 1982). *Theba pisana* is biennial in Pembrokeshire, breeding only once at the end of their second year and subsequently dying (Cowie, 1984b). This means that adults breed in year *x* and their offspring also hatch in year *x*. The F1 generation becomes half grown in year *x* + 1 (1-year-old 'juveniles'; Cowie, 1984a) and reach adulthood in year *x* + 2 when they breed. The offspring of these F1 snails also hatch in year *x* + 2, become half grown ('juvenile') in year *x* + 3 and reach adulthood in year *x* + 4, when they reproduce. Consequently, in each year and every population, 2-year-old individuals (adults) and 1-year-old individuals (juveniles) could be separated easily by size. Cowie (1992) distinguished five shell morphs in adults (unbanded plain, unbanded dotty, banded dark, banded yellow, and banded intermediate) and three morphs in juveniles (unbanded plain, unbanded dotty, and banded).

We used Shannon–Wiener diversity indices (*H*s) calculated from all the morph scores listed in Cowie (1992) for each year and population, and separately for adults and juveniles, as a measure of the degree of morphological variation, according to Köhler *et al*. (2009):

$$Hs = -\sum_{i=1}^{s} pi * \ln pi$$

(where *p*i: is the proportion of morph i individuals out of the total number of individuals investigated for each year, population, and generation; and *s* is the number of morphs)

In *T. pisana*, there is substantial variation within morphs. Cowie (1992) classified all shell phenotypes throughout his study into the above-mentioned set of morphs. *H*s therefore reflects the changes in relative frequencies of the morphs: diversity will increase only if less common morphs increase in frequency.

Because data for the adults of a single population (site 1 of Cowie, 1992) in 1988 displayed an unrealistically low *H*s, possibly as a result of the small number of individuals (10) and a bias towards a single morphotype, this sample was excluded from further analysis.

UK Meteorological Office (Exeter, Devon) climate data for Tenby, Pembrokeshire, included monthly averages of: (1) mean daily temperature (from May 1977); (2) maximum daily temperature (from May 1977); and (3) minimum daily temperature (from May 1977), as well as data on the monthly sum of (4) sun hours (from January 1975) and (5) mm precipitation/rain (from July 1977). From this dataset, each year's seasonal climate data were calculated for these parameters by calculating arithmetic means for winter (December of the preceding year, January, February), spring (March, April, May), summer (June, July, August), and autumn (September, October, November).

Statistical modelling

To assess the effect of heritable variation (genetics and heritable epigenetic tags) on morphology, correlation analyses of the Shannon–Wiener indices (*H*s) for adults versus *H*s for juveniles of the preceding, same, and subsequent year were carried out. To test for climatic impact, correlation analyses were conducted for *H*s (adults) or *H*s (juveniles) versus the above-mentioned climate data for the winter, spring, summer, and autumn of the respective year for *H*s (adults) also of the preceding year (because *T. pisana* at Tenby is biennial). In addition, we tested whether either unbanded (sum of two subdivisions of 'unbanded' for both juveniles and adults), banded (for juveniles; for adults, the sum of three subdivisions of 'banded'), or dark banded morphs (for adults) were favoured by any climate parameter that may explain differences in the *H*s among the years (correlation analysis of the proportion of unbanded, banded or dark banded individuals versus climate data of the same year). Furthermore, we determined which morphotypes of adults and juveniles contribute to the changes in diversity (correlation analysis of the proportion of unbanded, banded or dark banded individuals versus *H*s). *H*s values were checked for normality using Shapiro–Wilk tests before these analyses. Populations were not analyzed separately as a result of the limited amount of available data for each population. Results were visualized by calculating linear regression curves.

To identify the relative importance of the heritable and climatic parameters on the phenotypic variation of adult and juvenile snails, we used an information theory (IT) approach (Burnham & Anderson, 2002, 2004) the goal of which, similar to stepwise multiple regression, is to select the best explanatory variable from a large set of potential factors. In this approach, variable selection takes into account multiple competing hypotheses, and allows inferences through the whole set of potential models, thereby taking into account the fact that no single model (i.e. variable composition) can perfectly reflect nature. The IT-model selection approach that we used is based on Akaike's information criterion (AIC). Using this approach, we created all relevant models based on the combination of the heritable variables ('*H*s juveniles of the year *x* - 1' to model '*H*s adults', '*H*s adults of the year *x* - 1' to model '*H*s juveniles') and of the climate variables, each of which were calculated for winter, spring, summer, and autumn, separately (average of mean daily temperature, average of maximum daily temperature, average of minimum daily temperature, sum of sun hours and sum of precipitation/rain in mm). We then tested which of these factors and factor combinations had an effect on the phenotypic variation in the snail dataset.

The first step in model selection was to formulate a set of relevant competing models according to the aims of the present study. Using univariate regressions, we generated a set of relevant multiple regression models, each with its variance and coefficient estimates. For each of the models we calculated the AIC_C, a modified variation of the AIC that is adequate for small sample sizes, according to the equation (Burnham & Anderson, 2002; Symonds & Moussalli, 2011):

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

where *n* is the sample size (*N* = 40 for *H*s adults, *N* = 38 for *H*s juveniles, representing the sum of cases in which data were recorded for given populations and years); *MSS* is the mean sum of squares of the specific model; and *k* is the number of parameters, including the intercept, which were used in the specific model. For each model, *R*2 was used to measure the proportion of the variation around the dependent variable's mean explained by the model.

The model with the lowest AICC value was considered to have the best explanatory power and the best fit to the data. Using this model selection method, we do not assess the statistical power of single models but, instead, rank their relative importance. In general, models with a high number of parameters are penalized more heavily to avoid over-fitting and are therefore disadvantaged in favour of competing models with fewer parameters.

As a second step in model selection, we assessed the relative strength of the candidate models by calculating the difference between the AICC 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 that were considered worse) compared to the best one, instead of arbitrarily ignoring them. From the Δ_i parameters, we further calculated the Akaike weight (ω_i). This likelihood weight assesses the probability of the specific model being the best one in replicated data collection. The Akaike weights 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 fitting the data. Finally, we estimated the parameter coefficients (i.e. their relative importance and their positive or negative effect) by calculating a weighted average across all models. This process strongly reduced model selection bias and ensured that we took into account as many possible scenarios as the biological logic of the system dictates, instead of restricting ourselves as a result of limited statistical analysis (Johnson & Omland, 2003, Symonds & Moussalli, 2011).

Because of strong correlations between the average mean temperature, the average maximum temperature, and the average minimum temperature in every season, the parameters 'average maximum temperature' for winter, spring, summer, and autumn, as well as 'average minimum temperature' for winter, spring, summer, and autumn, were excluded from the modelling to prevent multicollinearity. Accordingly, the parameters 'sum of sun hours' in spring and autumn, correlating with the respective average mean temperatures in these respective seasons, and 'precipitation rate in summer', correlating with the mean average temperature in summer, were also omitted from the models.

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

Modification of an individual's phenotype

Our hypothesis that environmental variation contributes to changes in phenotypic variation in *T. pisana* implies the assumption that external factors could have the ability to modify an individual snail's phenotype during its development. Even though continuous modifications of the banding pattern of *T. pisana* individuals during their ontogeny have been described by Cain (1984) and, particularly for the Tenby populations, also by Cowie (1984a), we specifically looked for *T. pisana* individuals showing rather abrupt changes in the pattern

expressed on the shell, which, as a record of ontogeny, would be indicative of sudden unveiling of cryptic variation during development. For this investigation, we chose an area with a similar climate to Tenby, Wales: the dunes close to Biville in the region of Cotentin, Normandy, France. In this area, the monthly mean maximum daytime and minimum night-time temperatures throughout the year are almost identical to south-west Wales (http://www.worldclimateguide.co.uk).

Results

Statistical modelling

To check whether the phenotypic variation (as assessed by *H*s) of adults mirrors that of the juveniles of the preceding year (i.e. the juveniles of the same generation), a pairwise linear correlation analysis was conducted. The correlation was significant and positive ($P = 0.0004$; Fig. 1A). *H*s for juveniles and adults of the same year and population (which belong to neither identical, nor subsequent generations) also correlated positively and significantly ($P = 0.0003$; Fig. 1B). Furthermore, the shell pattern diversity of juveniles correlated in a highly significant and positive manner ($P < 0.0001$) with the diversity of the adults of the parental generation (i.e. adults of the preceding year x - 1) (Fig. 1C). These analyses indicate populationspecific determination of phenotypic variation.

Four cases in which climate data were significantly correlated with *H*s for adults and juveniles were found among all pairwise regression analyses (Fig. 2). Adult phenotypic diversity (*H*s) was positively correlated with the mean daily winter ($P = 0.044$) and spring ($P = 0.010$) temperature and the maximum daily winter ($P = 0.035$) and spring ($P = 0.015$) temperature of the same year. No climatic variable was significantly correlated with juvenile *H*s. Furthermore, no relationship was detected between climate of the previous year on the adult data (i.e. no significant relationship of climate presumably affecting juvenile snails was traced in the adults). This result may be a result of the classification by Cowie (1984a) of juveniles (1-year-old individuals) into three morphs, whereas adults were classified into five morphs, distinguishing three different ‘banded’ morphotypes. This restriction in the number of morphs may have led to a loss of information, and therefore we cannot exclude climatic impact on phenotypic variation in juveniles. However, we definitely could show climatic impact on phenotypic variation in adults.

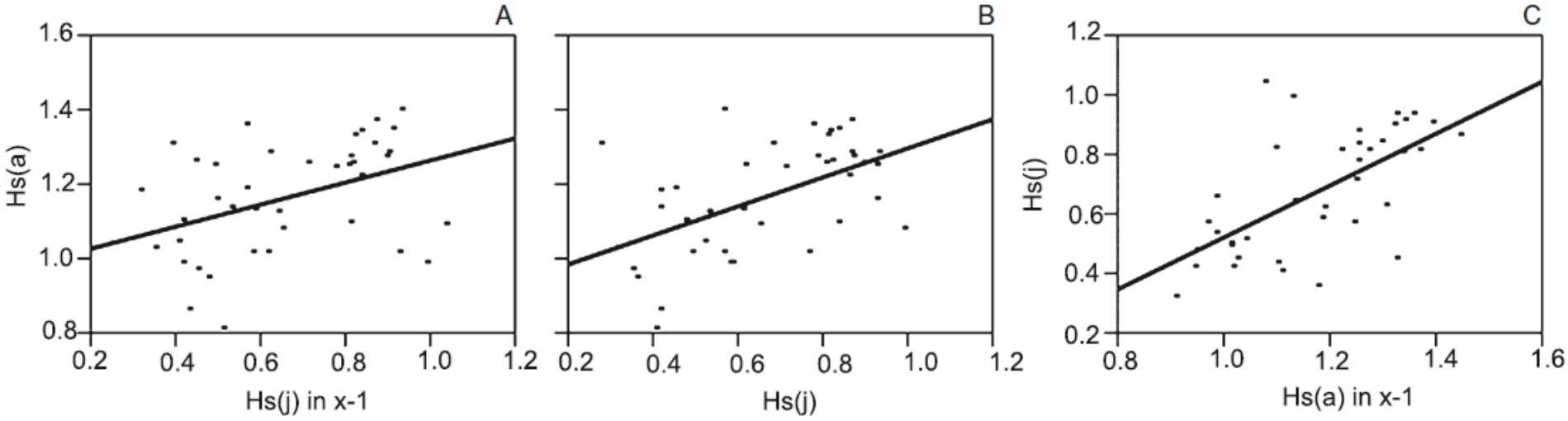

Figure 1. Heritability of phenotypic variation. Single factors plotted against the phenotypic diversity of adult *Theba pisana* [*H*s(a) in plots A ($P = 0.0004$) and B ($P = 0.0003$)] or against the phenotypic diversity of juveniles [*H*s(j) in plot C ($P < 0.0001$)]. Independent variables: Phenotypic variation of juveniles in the preceding year [*H*s(j) in $x - 1$] or in the same year [*H*s(j)] in plots A and B. Phenotypic variation of adults in the preceding year [*H*s(a) in $x - 1$] in plot C. Regression lines are shown for clarity only.

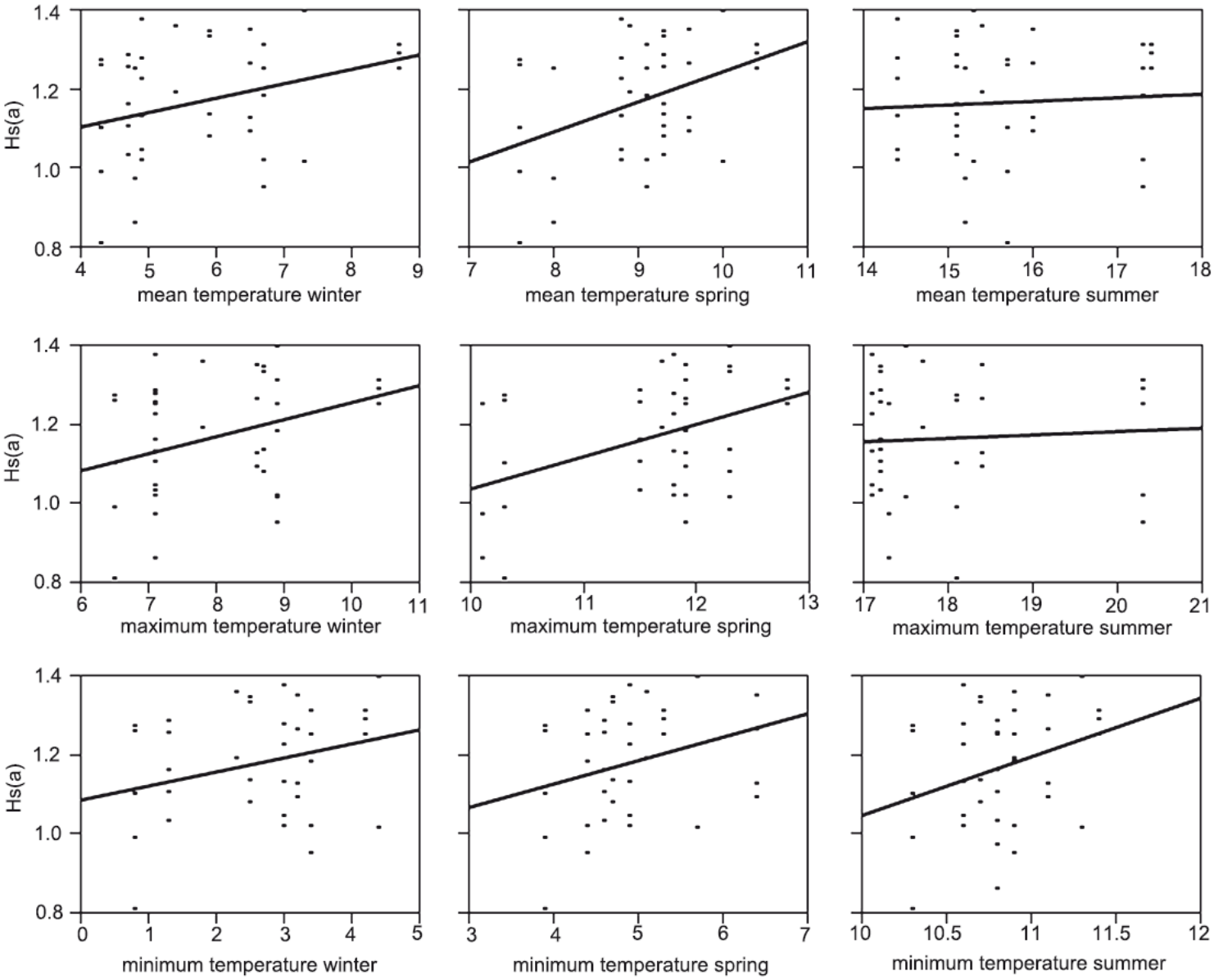

Figure 2. Ambient temperature (°C) versus phenotypic diversity in adult *Theba pisana*. Mean seasonal temperature (top row), maximum seasonal temperature (middle row), and minimum seasonal temperature (bottom row) plotted against the phenotypic diversity of adult snails [*H*s(a)]. Significances: for the average mean temperature in winter (P = 0.044), spring (P = 0.010), and summer (0.504). For the average maximum temperature in winter (P = 0.035), spring (P = 0.015), and summer (0.476). For the average minimum temperature in winter (P = 0.080), spring (P = 0.059), and summer (0.293). Regression lines are shown for clarity only.

The AIC-based model selection procedure revealed that in each of the two analyses (*H*s of adults, with 25 competing models, and *H*s of juveniles, with 22 competing models) only one model had high empirical support. The model with the lowest AICC value and, correspondingly, the highest explanatory power for juvenile phenotypic diversity, was based on the factor '*H*s adults of the preceding year' (which means *H*s of the parental generation) (Table 1). The model that showed by far the highest explanatory power for the phenotypic diversity of adult snails was composed of the independent variables '*H*s juveniles of the preceding year' (in fact, the *H*s of the same generation 1 year previously), 'mean winter temperature of the same year' (i.e. December of year *x* - 1, January, February), and 'mean spring temperature of the same year' (i.e. March, April, May) (Table 2). The next best models in both cases showed Di > 2 (the difference in AIC_C between the next best model and the best ranked one), and therefore cannot be considered as having a comparably good explanatory power (Symonds & Moussalli, 2011).

All the parameters used for the best models (heritable variation in the parental generation for the phenotypic variation of juveniles; juvenile phenotypic diversity, mean winter and spring temperatures for the phenotypic diversity of adults) had high probability values (> 87%), and therefore clearly contributed to explaining phenotypic variation in the six *T. pisana* populations at Tenby (Tables 1, 2). The analysis also revealed that all the other tested variables had negligible contribution to phenotypic variation, both of juveniles and adults. Another important indication of the robustness of the results of the present study is the fact that the respective null models (using the intercept only) were ranked quite low (places 9 and 14, respectively), implying that our chosen variables significantly improved our ability to explain the disequilibrium in phenotypic variation in the snails' shell coloration pattern over 13 years.

The proportion of the variation of *H*s for adult snails that was explained by the best model was 45.45%. The remaining *H*s variation was not explained, and has to be attributed to unknown factors or random processes. The *H*s of juveniles of the preceding year, representing the same generation in the year before, explained only 28.58% of adult *H*s variation. In a model using the independent variables 'mean winter temperature' and 'mean spring temperature', 17.11% of adult *H*s variation was explained, whereas the mean spring temperature alone explained 16.95% and the mean winter temperature alone explained 9.72%. This showed that, among all environmental variables, temperature in spring had the greatest explanatory power, which contributed approximately one-third of all explicable variation of

*H*s in adult snails. Approximately two-thirds were explained by the phenotypic diversity already present in the juveniles. In turn, 40.32% of the *H*s for juveniles was explained by the *H*s of adults of the parental generation in the preceding year (the best model). Even though it was possible to increase the explicable variation of this dependent variable to 42.42% in a suboptimal (according to AICC) model comprising the parameters '*H*s adults of the year *x* - 1', 'mean winter temperature', and 'mean spring temperature', it was obvious that the explicable variation of the *H*s in juveniles was attributed to heritable phenotypic variation and that environmental factors were negligible in this respect. The standardized regression coefficients (b) for all relevant model parameters are presented at the bottom of Tables 1, 2.

By definition, *H*s should strongly depend on the relative frequency of the rare and the common morphs. This was shown for the more common, banded and dark banded morphotypes whose increase lowered *H*s ($P < 0.0001$), whereas higher frequencies of the less common, unbanded morphs resulted in higher *H*s values. ($P < 0.0001$). Despite the abovementioned correlations between *H*s and winter/spring temperatures, we did not find any correlation between the percentage of unbanded individuals and any climate parameter in any season, either for adults (P _ 0.396) or juveniles (P _ 0.170). The same was true for the frequency of banded individuals (adults: P _ 0.417; juveniles: P _ 0.173) and for the 'dark banded' morph among adults (P _ 0.347). This indicates that the above-mentioned correlations of *H*s with temperature cannot be attributed to directional selection that has constantly favoured a single morphotype throughout the years but, instead, to fluctuations in the ratio between common and rare morphs.

Modification of an individual's phenotype

In their natural habitat, individuals of *T. pisana* with abrupt changes in the coloration of their shells were found repeatedly, providing evidence that modifications of the shell pattern occur during the development of this species (Fig. 3). Modifications can be sufficiently drastic to result in changes in morphotype classification on the way from juvenile to adult. In the investigated population from the Biville dunes, most changes occurred in the direction from unbanded to banded (Fig. 3A–C, E). Nevertheless, the opposite was also possible, and a change from dark pigmentation to white (and, later, banded; Fig. 3D) was found in some cases.

Table 1. Tested models for explaining the phenotypic diversity of juvenile *Theba pisana* at Tenby, listed in decreasing succession, the best ranked model at the top. The rank of a model, the model constitution, the Akaike information criterion (AICC)), the Akaike weight (w), and the number of parameters involved are listed. The probability reveals the importance of each parameter to be part of a model. Last row: correlation coefficients (b). Parameters: phenotypic diversity of adults in the preceding year [*H*s(a) in *x* - 1], mean temperature in winter (*T*w), spring (*T*sp), summer (*T*su) and in the autumn of the preceding year (*T*a in *x* - 1), sun hours in spring (*S*sp) and in the autumn of the preceding year (*S*a in *x* - 1), precipitation/rain in winter (*R*w), spring (*R*sp) and the autumn of the preceding year (*R*a in *x* - 1).

Rank	H_s(a) in $x-1$	T_w	T_{sp}	T_{su}	T_a in $x-1$	S_{sp}	S_a in $x-1$	R_w	R_{sp}	R_a in $x-1$	AIC_c	ω	Parameters
1	•										−134.903526	0.7921	2
2	•							•	•		−130.042679	0.0697	4
3	•					•	•				−129.860836	0.0636	4
4	•	•	•								−129.220771	0.0462	4
5	•							•	•	•	−126.805713	0.0138	5
6	•	•	•	•							−126.499133	0.0119	5
7	•	•	•			•	•				−122.503752	0.0016	6
8	•	•	•			•		•	•		−119.285926	0.0003	7
9											−118.559269	0.0002	1
10										•	−116.325166	0.0001	2
11		•									−116.101511	0.0001	2
12							•				−115.818847	0.0001	2
13						•					−115.790326	0.0001	2
14									•		−115.616551	0.0001	2
15								•			−115.581223	0.0001	2
16				•							−115.451636	0.0000	2
17			•								−115.320164	0.0000	2
18									•		−115.307565	0.0000	2
19		•	•								−113.468864	0.0000	3
20						•	•				−113.142501	0.0000	3
21		•	•	•							−110.900936	0.0000	4
22		•	•	•	•						−107.577610	0.0000	5
Probability	0.999	0.060	0.060	0.012	0.000	0.066	0.065	0.084	0.084	0.014			
β	0.8711	0.0030	−0.0037	−0.0006	0.0000	0.0000	0.0001	0.0000	0.0000	0.0000			

Table 2. Tested models for explaining the phenotypic diversity of adult *Theba pisana* at Tenby, listed in decreasing succession, the best ranked model at the top. The rank of a model, the model constitution, the Akaike information criterion (AICC)), the Akaike weight (w), and the number of parameters involved are listed. The probability reveals the importance of each parameter (or interactions of two parameters) to be part of a model. Last row: correlation coefficients (b). Parameters: phenotypic diversity of adults in the preceding year [*H*s(j) in *x* - 1], mean temperature in winter (*T*w), spring (*T*sp), summer (*T*su) and in autumn (*T*a), sun hours in spring (*S*sp) and autumn (*S*a), precipitation/rain in winter (*R*w), spring (*R*sp) and autumn (*R*a).

Rank	H_s(j) in $x-1$	T_w	T_{sp}	T_{su}	T_a	H_s(j) $x-1 * T_w$	H_s(j) $x-1 * T_{sp}$	S_{sp}	S_a	R_w	R_{sp}	R_a	AIC_c	ω	Parameters
1	•	•	•										−164.378813	0.6919	4
2	•	•	•	•									−161.121601	0.1357	5
3	•												−160.5850	0.1038	2
4	•	•	•					•	•				−158.720151	0.0409	6
5	•									•	•		−155.394068	0.0077	4
6	•							•	•				−154.66733	0.0054	4
7			•										−154.548448	0.0051	2
8	•	•	•					•		•	•		−152.861237	0.0022	7
9							•						−151.8210	0.0013	2
10	•									•	•	•	−151.669349	0.0012	5
11		•	•										−151.2181	0.0010	3
12		•											−151.207171	0.0010	2
13						•							−150.687721	0.0007	2
14													−150.377265	0.0006	1
15		•	•			•	•						−148.611947	0.0003	5
16									•				−147.945625	0.0002	2
17		•	•	•									−147.848707	0.0002	4
18										•			−147.8070	0.0002	2
19					•								−147.48654	0.0001	2
20								•					−147.369704	0.0001	2
21				•									−147.259394	0.0001	2
22											•		−147.241837	0.0001	2
23												•	−147.123152	0.0001	2
24								•	•				−144.663422	0.0000	3
25		•	•	•	•								−144.5310	0.0000	5
Probability	0.989	0.873	0.877	0.136	0.000	0.001	0.002	0.049	0.046	0.011	0.011	0.001			
β	0.38804	0.00843	0.05743	−0.00301	0.00001	−0.00012	−0.00044	0.00001	0.00005	0.00000	0.00000	0.00000			

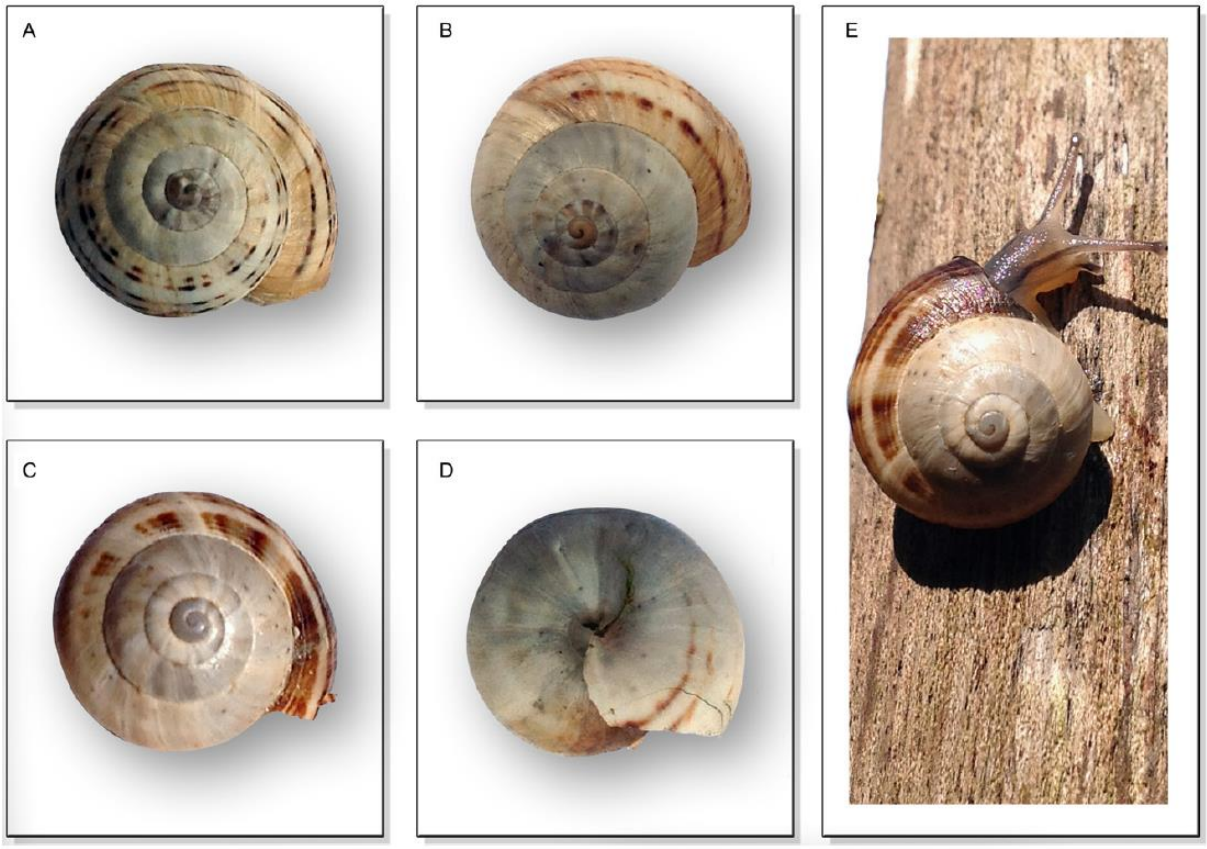

Figure 3. Representative adult individuals of *Theba pisana* that display abrupt changes in their shell coloration pattern. Specimens are from the dunes of Biville, Cotentin, France. Among those that have changed shell coloration during their life, most individuals change from unbanded to one of the banded categories (A, B, C, E). Changes from dark to white also occur (D); furthermore, this individual has developed two faint bands on the umbilicus' side of the shell.

Discussion

Shell colouration pattern: genetics and environmental variation

Shell colour and banding patterns of *T. pisana* are primarily determined by alleles of at least, three loci (Cain, 1984; Cowie, 1984a). In *T. pisana* from Tenby, epistasis among the alleles results predominantly in five morphotypes, which were the basis for the definition by Cowie (1992) of morph categories, to which individuals have been allotted in his study. Cain (1984) investigated additional variation in Mediterranean populations of *T. pisana*. By contrast to other helicid snails such as *Cepaea*, which display distinct coloration and banding patterns,

the polymorphism of *T. pisana* is multiform, with lots of variation within morphs, especially of the intensity of coloration of the bands. The interplay of genetic variation and presumed phenotypic plasticity leads to almost every individual expressing an apparently unique banding pattern as documented by Cowie (1984a) and Cain (1984), and as recently quantified by Johnson (2012) for the intensity of banding. Even though Cowie (1984a, 1992) scored all morphs on the 'juvenile' part of their shells, he pointed out that 'virtually all shells', also those scored as 'unbanded', 'acquire some banding on later whirls. (. . .) There is much variation in the timing of the start of these bands'. Shells with 'the faintest hint of dots' were scored as 'dotty'. We do not question the accuracy of this study but would like to point out the difficulty to define scores in a continuum of varying starts of banding, particularly if banding starts at any time in the transitional period between 'youth' and adulthood. Also Cain (1984) frequently found individuals of *T. pisana* with recently synthesized shell material that differed in its pattern from the rest of the shell, as observed by us in northern France. Very faint dots on the shell may bleach out with increasing age. All this can cause inevitable variation in scoring. Furthermore, Cowie (1984a) also reported on unexpected phenotypes in the offspring from mating experiments such as 'intermediate forms', forms with 'extremely faint' characters, forms that may result from non-expression of an allele despite being homozygous for it, and forms that express a recessive allele even though heterozygous. Such individuals show that even a distinct genotype may vary substantially in its expression of shell colour/pattern. Consequently, the suspicion mentioned in the Introduction that phenotypic variation can result from modifications of genetic variation by varying nonheritable factors during a snail's development cannot be refuted with respect to the shell coloration pattern of this snail species.

Even though the variation of shell coloration in land snails has been examined in many cases, we highlight three innovative aspects of the present study that have not been addressed in this context previously.

1. By contrast to other studies, we focus on the degree of phenotypic variation independent of the frequency of any particular morphs that may have been subject to selection in distinct habitat and climate. Choosing a dataset for populations living in temperate climate at moderate temperatures virtually allowed us to a priori exclude hot temperature or extreme solar radiation as a strong selection factor for a particular white morph. Indeed, we did not find evidence for the selection of any distinct morph.

2. Temperature was shown to be the most influential environmental factor for phenotypic variation. Furthermore, we achieved at least an approximate quantification of temperature-associated influence on morphological variation within the heritable range of phenotypic plasticity.
3. We managed to find evidence for a sensitive 'window' in the life cycle of *T. pisana*, in which the snails' phenotypic variation is allowed to be subject to modifications by climate factors. Such modification is visualized by changes in the coloration of the inner and outer spiral turns of an individual shell.

These statements imply that the association between temperature and phenotypic variation reflects causality. To establish evidence for a causal relationship between these two correlating parameters, we applied Hill's criteria of causation. The following criteria strengthened our interpretation.

• Strength of association: in four cases, temperature and phenotypic diversity were significantly correlated at P as low as 0.01.

• Coherence: temperature and phenotypic diversity did not only show significance in the correlation analyses. High probability values revealed also winter and spring temperatures to contribute clearly to the best model explaining adult phenotypic variation.

• Consistency: our findings are consistent with other studies reporting on increased phenotypic variation at higher temperatures; for example, in three *Drosophila* species (Child, Blanc & Plough, 1940; Imasheva *et al*., 1997; Sisodia & Singh, 2009), Patagonian pejerrey, a Neotropical fish (Crichigno, Battini & Cussac, 2012), diatoms (Hansen *et al*., 2011), and red maple (Royer *et al*., 2009).

• Specificity: all significances are related to temperature parameters in the time from December to May. There was no significance for temperature at any other time of the year or any other climate parameter.

• Temporality: correlation of winter and spring temperatures with phenotypic variation was only demonstrated for adults (i.e. the period of life that starts right during/after the putative elicitor for variation increase).

• Plausibility: a causal relationship of temperature and phenotypic variation would be in accordance with the capacitoring concept, involving temperature-sensitive chaperones as

capacitors of morphological variation (Rutherford & Lindquist, 1998; Queitsch *et al*., 2002). This is explained in detail below.

The role of temperature in nondirectional change in variation and selection

Interpretation of selection in land snails largely concerns the associations of particular morph frequencies with habitat and climate, regardless of questions about the maintenance of polymorphism. Unanimously, climate parameters, particularly temperature and/or solar radiation, and visual appearance to predators are assumed to act as predominant selective factors on shell and body colour variation in helicoid snails (Cain & Sheppard, 1954; Richardson, 1974; Jones, Leith & Rawlings, 1977; Heller, 1981; Johnson, 1981, 2011; Cain, 1983; Heller & Gadot, 1984; Cowie & Jones, 1985; Cowie, 1990; Slotow & Ward, 1997). However, the basis for phenotypic variation may differ among these snails, and the results for a single species may not be generalized. In most studies, the changes in morph frequencies from different years were always attributed to be the result of selection, based on the plausible assumption that (effectively) unbanded shells have a higher albedo and, therefore, should heat up less and more slowly when exposed to light in open habitats. On the other hand, banded shells or individuals with a darker shell colour are suspected to be less visible to potential predators and therefore are favoured in shaded (woodland) habitats, in which the trade-off with thermal aspects is less relevant. It has also been reported for a number of species that oscillation of the direction of selection can account for the maintenance of variation (Reimchen & Nosil, 2002; Cook, 2005; Bell, 2010; Johnson, 2011). However, the lack of correlation of any environmental parameter with a particular morph in the present study does not support the concept of directional selection. Furthermore, the rather fuzzy character of pigment dots and bands in *T. pisana* may prevent exposure to light to act as a strong selective force at Tenby: using sunlightequivalent experimental illumination and infrared camera measurements, Scheil, Gärtner & Köhler (2012) found the radiation absorption of unbanded and banded *T. pisana* shells with white base colour differed at most marginally from one another. This, as well as the presence of individuals with a ‘changing’ phenotype, supports the idea that the environmentally triggered part of the observed change in variation in the Tenby populations of *T. pisana* at the northern edge of the species’ distribution range is nondirectional.

The main result of the present study was the association of high temperature with an increase of rare phenotypes, resulting in an increase in diversity with high winter and spring temperatures. Rare phenotypes are favoured by both apostatic (negative frequency-dependent) selection and decanalization that results in a nondirectional increase in variation, and thus it is hard to attribute one of these two fundamentally different mechanisms to our findings with any certainty. Accordingly, the interpretation of our results can rely only on plausibility because direct evidence for the action of one or the other mechanisms is missing. In *Cepaea hortensis*, apostatic selection has been experimentally established (Allen, Raymond & Geburtig, 1988) but requires, as usual for this kind of selection, biotic components of selection pressure, such as predator–prey interactions. It is hardly conceivable that high temperature should favour rare phenotypes only because they are rare (and does not favour a particular morph) without proposing some indirect interaction with a hypothetical, temperature-dependent and selectively-acting additional biotic component. In this context, capacitoring directly involves a temperature-dependent biochemical regulatory system, and it is known that heat stress can result in decanalization and, consequently, enhanced phenotypic variation. Even though elevated temperature was not found to act as a selective agent on distinct phenotypes in the present study, this parameter nevertheless showed correlation with increased phenotypic diversity. All this is not incongruous: temperature can be selective only, if it affects different phenotypes differently (for which we do not have evidence for the Tenby snails) but it can interfere with the molecular stress response system even after moderate increase. Following the capacitor concept, chaperones like Hsp90 and Hsp70 are key components of both maintenance of cellular homeostasis after proteotoxic stress and canalization processes and, if needed in the molecular stress response (after temperature stress), are drawn off from canalization and from buffering variation. Consequently, this will lead to an increase in variation if the level of chaperones itself is not increased by the ambient temperature. Supporting this idea, our own research has revealed a rather limited potential of *T. pisana* to increase the individual hsp70 and hsp90 levels, even after experimental exposure to a series of very high temperatures (Köhler *et al*., 2009; A. Picot, unpubl. data).

Chaperone-generated capacitoring as a possible contributior to variation

Environmentally produced variation is one of the consequences of the concept of phenotypic plasticity. In this context, ‘plasticity usually refers to environmentally influenced variability in a particular life stage, or (. . .) to variation in the (. . .) form (. . .) produced at a particular stage

of growth' (West-Eberhard, 1989). If we consider temperature to act epigenetically on phenotypic variation in accordance with the phenotypic plasticity concept, this assumption would explain a number of findings deriving from the present study. In such a scenario, ambient temperature would interact with phenotypic plasticity (whose extent is defined on the basis of the inherited range) to enhance phenotypic diversity, even in the absence of a strong selection factor. Furthermore, this theoretical concept would explain the change in morphological diversity from year to year in combination with the long-term maintenance of phenotypic variation (which has been reported for a number of snail species) without proposing high frequency oscillation in the direction of selection: because selection, favouring, for example, individuals with shells with a lower-than-average albedo, acts always on the phenotype, and because, for example, pigmented phenotypes could result from different varieties of genotypes [Cowie (1984a) explicitly noted that some progeny of breeding experiments deviate from Mendelian genetics], selection would neither drastically alter genotypic variation in the population, nor consequently in the offspring (Fig. 4). The juveniles of the next generation then will display considerable phenotypic diversity which, according to our results for *T. pisana*, relies for the most part on the inherited features (inherited range of plasticity) of the parental generation. In a later phase of the life cycle, the ambient temperature may then modify the inherited variation within the limits of phenotypic plasticity again, according to our results in an approximate ratio of one-third (environmental) to two-thirds (inheritance). Again, this resulting phenotypic variation will form the basis that selective factors may act upon. The system will stay stable, even if the direction of selection stays the same in each subsequent year. Exceptional changes in the habitat, of course, may disrupt this proposed homeostasis of genetic and phenotypic variation, although such a process can be excluded for the location and time of interest in the present study.

We admit that we do not have direct evidence for our proposal for an environmental stress response-triggered mechanism, even though it appears reasonable. Because data on chaperone induction are lacking for the *T. pisana* populations from Tenby, Wales, we cannot judge whether genetic capacitoring is involved in the temperature-dependent increase in phenotypic diversity reported in the present study. However, temperature elevation is known to be one of the most important factors for stress protein regulation. The induction of the chaperones Hsp90 and Hsp70 by elevated temperature has already been characterized in *T. pisana* (Köhler *et al*., 2009; Scheil, Köhler & Triebskorn, 2011) and in other polymorphic Helicoidea (Brooks & Storey, 1995; Reuner, Brümmer & Schill, 2008; Arad *et al*., 2010; Mizrahi *et al*., 2010). In response to temperature increase and other environmental stressors, individuals

from the same species but from different populations sometimes show remarkable differences in the inducibility of these stress proteins (Bettencourt, Feder & Cavicchi, 1999; Köhler *et al*., 1999, 2000; Sørensen *et al*., 1999; Sørensen, Dahlgaard & Loeschcke, 2001; Zatsepina *et al*., 2001; Arts *et al*., 2004; Haap & Köhler, 2009). In this context, populations of Mediterranean helicoid land snails (including *T. pisana*) that only moderately up-regulated their Hsp70 response to heat stress were found to display a rather diverse assemblage of shell coloration morphotypes, whereas those with a strong Hsp70 induction displayed little phenotypic variation (Köhler *et al*., 2009). This correlation of gradually modified capabilities in chaperone induction with phenotypic diversity comprises the first evidence for the possible contribution of stress proteins to genetic buffering in field populations of terrestrial snails and strengthens the theory that ambient temperature may act on phenotypic variation via chaperone-mediated capacitoring of developmental processes.

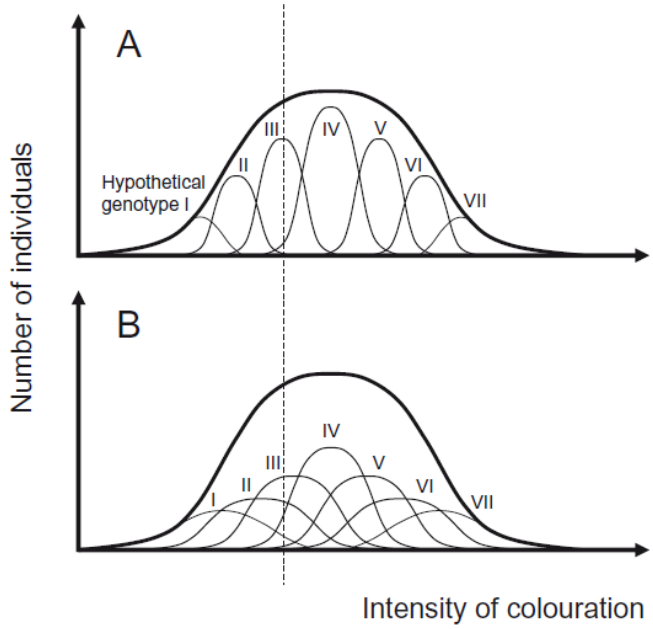

Figure 4. Theoretical concept to visualize the consequences of heritable narrow versus broad ranges of phenotypic plasticity. In this hypothetical example, seven genotypes (I–VII) contribute to morphological variation (intensity of shell coloration) in a given population. A, narrow ranges of phenotypic plasticity in each genotype. B, broad ranges. Directed selection towards higher intensities of coloration (the portion of the population right of the dashed vertical line) will erase genotypes I and II from the population in case A (narrow plasticity range), which will then result in a decrease in phenotypic variation for this trait in the subsequent generation. In case B (broad plasticity range), the same selective impact will allow all genotypes to persist, which will restore the full spectrum of phenotypic variation also in the filial generation.

Temperature-susceptible life stages

The number of studies statistically linking morph frequencies of *T. pisana* to environmental parameters is limited, although it is conspicuous that the most elaborate studies (Johnson, 2011, 2012) and the present study consistently find moderately elevated temperature acting on

snails in the middle of their lifetime to be a key element for the constitution of morphotypes in natural populations. The studies of Johnson (2011, 2012) used *T. pisana* from an area south-west of Perth, Australia, which has a Mediterranean climate with temperatures approximately 9–10 °C higher than in Pembrokeshire, Wales. In this climate, *T. pisana* is annual for the most part (Johnson, 2011), as it also is in Southern France where the study of Köhler *et al*. (2009) was conducted. In both regions, hot conditions during summer enhance the frequency of unbanded phenotypes. This effect was attributed to climate selection in *T. pisana* from Perth (Johnson, 2011, 2012). In Southern France, this effect is also striking in *Xeropicta derbentina* (Dieterich *et al*., 2012) but it remains to be investigated whether it derives from selection or from shell bleaching as a result of Hsp70-induced inhibition of melanin synthesis, as has been reported for mouse cells (Hoshino *et al*., 2010).

Concluding remarks

The present study, performed on a historic dataset, reveals ambient temperature as a source of phenotypic variation in snail populations in the absence of selection for or against a distinct phenotype. Considering the presence of individuals with abrupt changes in the coloration of their shell in natural populations of *T. pisana*, we propose that phenotypic variation can be affected during postembryonic development. From our perspective, the amalgamation of the two concepts on phenotypic plasticity and genetic capacitoring may serve as an elegant explanation for the generation and long-term continuance of temperature-sensitive phenotypic variation in these snails, which in turn provides the substrate for microhabitat-specific selection processes. However, long-term experiments applying artificially elevated temperature in greenhouses or climate chambers are needed to provide direct evidence for our hypotheses.

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Kapitel 6: Phenotypic diversity, population structure, and stress protein-based capacitoring in populations of heat-tolerant land snails

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Abstract

1. The shell colouration of many pulmonate land snail species is known to be highly diverse. Besides a genetic basis, environmentally triggered epigenetic mechanisms including stress proteins as evolutionary capacitors are thought to influence such phenotypic diversity.

2. In this study, we investigated the relationship of stress protein (Hsp70) levels with temperature stress tolerance, population structure, and phenotypic diversity within and between different populations of a xerophilic Mediterranean snail species (*Xeropicta derbentina*).

3. We found Hsp70 levels to vary considerably between populations, and to be significantly associated with the diversity of shell colouration: populations exhibiting low diversity in colouration were shown to comprise individuals expressing higher Hsp70 levels both constitutively and under heat stress than those of phenotypically diverse populations.

4. In contrast, population structure (cytochrome *c* oxidase subunit I gene) did not correlate with phenotypic diversity. However, genetic parameters (both within and between population differences) were able to explain variation in Hsp70 induction at elevated but non-pathologic temperatures.

5. Our observation that (1) population structure had a high explanatory potential for Hsp70 induction and that (2) Hsp70 levels, in turn, correlated with phenotypic diversity while (3) population structure and phenotypic diversity failed to correlate provides empirical evidence for Hsp70 to act as a mediator between genotypic variation and phenotype and thus for chaperone-driven evolutionary capacitance in natural populations.

Keywords

COI, Eco-devo, evolutionary capacitance, Hsp70, *Xeropicta derbentina*

Introduction

Moderate stress is essential for healthy growth and development of organisms (Müller 2003) and, under stressful conditions, organisms tend to increase their phenotypic variation (Waddington 1941, Jablonka et al. 1995, Badyaev 2005). In contrast to the traditional point of view, that only gene-based differences in organisms determine their fitness, there is now increasing evidence that organisms are able to adaptively modify their developmental program according to the environment (ecological and evolutionary developmental biology, eco-evo-devo or, in short, eco-devo) (Dusheck 2002, Sultan 2007, Bolker 2012). Stressful environments are known to influence ontogenetic pathways and therefore may initiate the induction of particular phenotypes. Organisms with a high phenotypic plasticity, that is the capacity to change their phenotype in response to environmental changes (Price, Qvarnström & Irwin 2003), benefit from being more adaptive.

Various authors have investigated polymorphic snail populations in respect to how they are influenced in their phenotypic diversity by different factors such as genetic traits and environmental conditions (Jones, Selander & Schnell 1982, Baur 1988, Baur & Raboud 1988, Cowie 1990, Johnson 2011, Di Lellis et al. 2012, Köhler et al. 2013). The shell colouration pattern is a highly variable feature in pulmonate snail species such as *Xeropicta derbentina* (Krynicki, 1836) or *Theba pisana* (Müller, 1774) (Johnson 1981, Cowie 1990, Köhler et al. 2009). They are known for their great range of morphs, the frequency of which can be associated with different microgeographical specifics (Mazek-Fialla 1934). Yet it is still not fully understood, which combination of factors determines the diversity in shell pattern, and to what extend they are relevant. It is known that several gene loci primarily control shell colour, presence or absence of shell banding, intensity of pigmentation, et cetera (Jones et al. 1982, Cowie 1984). Moreover, habitat structure (such as shaded/exposed) and ambient temperature conditions act upon the composition of phenotypes in snail populations (Johnson 2011, Köhler et al. 2013). These conditions, in turn, might also be reflected in their population structure (which can be inferred with information on their 'genotypic diversity'). Thus genes that echo a population's phylogeographical and demographic history could be informative, even though they are not directly coding for shell colouration. This might be particularly true for populations that are potentially subjected to strong bottle necks caused by, for example, high ambient temperatures. In fact, it has been shown that high temperatures emerged as a manipulative agent, either as selective force or as an epigenetically acting component of the

environment (Johnson 2011, Johnson 2012, Köhler et al. 2013), and organisms possess various mechanisms to deal with high temperatures.

The expression of heat shock proteins (Hsps), for example, is part of the cellular heat response in organisms and has been shown to be directly associated with thermal tolerance (Feder & Hofmann 1999, Pörtner & Farrell 2008). Particularly Hsp70 chaperones own abilities that make them indispensable in the physiological stress response (Lindquist & Craig 1988, Bukau & Horwich 1998). In addition to their function in the process of re-folding damaged proteins, compensating for proteotoxic effects (Gething & Sambrook 1992, Köhler et al. 1992, Parsell & Lindquist 1993, Feder & Hofmann 1999, Lewis et al. 1999, Köhler et al. 2000), they assist in folding and stabilizing nascent protein chains. The structure of Hsp70 is phylogenetically highly conserved, even though the capacities for its induction may vary considerably among populations of the same species (Köhler et al. 2000). Differences in Hsp70 levels can directly account for stress tolerance in organisms (Köhler et al. 1992, Dahlgaard et al. 1998, Feder & Hoffmann 1999, Köhler et al. 2000), which often is influenced by microgeographical selection processes. Since the production of stress proteins, however, is energy consuming, it is reasonable to assume that Hsp induction trades off against other fitness parameters due to constraints in energy allocation (Krebs & Loeschcke 1994, Silbermann & Tatar 2000, Kristensen et al. 2008). On the other hand, a high capacity to transiently elevate chaperoning activity may guarantee survival at occasions of extreme heat pulses. In this context, a combination of a low constitutive (basic) level and a high Hsp induction capacity would allow to benefit from both, a small energy expenditure and a high short-term stress tolerance. Several studies revealed highly thermo- or metal-tolerant species or populations to be selected for reduced constitutive Hsp expression (Krebs & Feder 1997, Bettencourt, Feder & Cavicchi 1999, Köhler et al. 1999, 2000; Sørensen et al. 1999, Sørensen, Dahlgaard & Loeschcke 2001; Zatsepina et al. 2001; Arts et al. 2004, Haap & Köhler 2009). Mizrahi et al. (2009) showed also a delayed induction of Hsp70 and Hsp90 in a highly thermotolerant desert-dwelling snail species, which enhanced small Hsp molecules (sHsps) under thermal stress that are more efficient in production due to their molecular size.

Heat stress has been shown to influence phenotypic variation (Child, Blanc & Plough 1940, Imasheva et al. 1997, Royer et al. 2009, Sisodia & Singh 2009, Hansen et al. 2011, Crichigno, Battini & Cussac 2012) and chaperones were suggested to canalize phenotypic development and, therefore, to act as capacitors of phenotypic variation (Rutherford & Lindquist 1998, Roberts & Feder 1999, Queitsch, Sangster & Lindquist 2002, Rutherford 2003). Thus, it

seems reasonable to hypothesize that they also participate in the regulation of phenotypic variation in natural populations of polymorphic land snails. Specifically, Hsp70 is able to suppress effects of gene-based mutations in proteins in order to preserve the function of them and, therefore, is thought to own capacitor function (Roberts & Feder 1999, Mayer & Bukau 2005). Capacitoring, however, has so far almost exclusively been studied in model organisms, and its role in ecology and evolution of natural populations is still unclear. First indirect evidence for an association of Hsps with capacitoring of phenotypic variation in natural populations was given by Manitasevic et al. (2007) for Hsp90 and Köhler et al. (2009) for Hsp70.

However, the hypothesis that high stress protein levels confer high capacitoring potential and, therefore, low phenotypic variation has not been tested before in a larger number of natural populations. To address this hypothesis, we investigated the correlation of shell colouration diversity, population structure, and the capacity to induce Hsp70 in ten populations of the Mediterranean hygromiid snail *Xeropicta derbentina*. More specifically, the following questions were sought to be answered: Do snail populations differ in their constitutive Hsp70 levels under stress-free conditions, in their maximum induction levels and in their capacity to induce Hsp70? Do Hsp70 levels correspond to phenotypic variation within and among the investigated snail populations, and does population structure reflect the variation in shell colouration? To which extent does genotypic diversity correspond to the capacity to induce Hsp70?

Material & Methods

Test organisms

The hygromiid snail *Xeropicta derbentina* is a xerophilic species and widespread in the circum-Mediterranean region. On hot days, individuals climb up vertical objects to escape from the heated ground, seal their shell aperture, and stay inactive (Mazek-Fialla 1934, Machin 1968, Aubry et al. 2005). During the night when temperatures and air humidity are more moderate, they are agile and feed. *X. derbentina* is an abundant species in southern France that originates in the eastern Mediterranean region and in the Middle East (Aubry et al. 2005, Kiss et al. 2005). The shell of adult individuals reaches a size of 10-16mm in diameter.

Overall, 10 populations were sampled in the Vaucluse department, Provence, Southern France (Table 1) in May 2010 and June 2012. For all populations, species determination was conducted on the basis of morphological traits and a fragment of the mitochondrial cytochrome *c* oxidase subunit I (*COI*) gene. Since *X. derbentina* is annual in Southern France and only few individuals (which are easily identified by their size) reach a longevity of two years (Dieterich et al. 2013), all tested individuals were from an early spring´s hatch and, therefore, of about the same age.

Snails were brought to the laboratory and kept in plastic boxes (20.5 x 30 x 19.5cm) on moist terrarium ground-covering material (JBL TerraBasis, Neuhofen, Germany) under a light regime of 12h/12h and a constant room temperature of 25°C. As recommended by Cowie & Cain (1983), they were fed baby porridge (HIPP *Gute Nacht Bio-Milchbrei, Hafer & Apfel,* Pfaffenhofen, Germany) and cuttlebone.

Heat exposure

After two weeks of laboratory acclimation, snails from each of the 10 populations were exposed to heat stress. The experiments were carried out in heating cabinets using plastic boxes (6.5 x 18 x 13cm) with moist tissue paper as ground cover, each box containing 10 individuals for Hsp70 analysis. Snails were exposed to 25°C (control), 33°C, 38°C, 40°C, 43°C, 45°C, 48°C, and, in populations 1-7, additionally 50°C and 52°C for a duration of 8 hours to measure the basic (at 25°C) and maximum level of stress protein induction. Finally, the gastropods were individually shock frozen in liquid nitrogen and stored in a freezer (-25°C) until Hsp70 and genetic analyses were done.

Because immobility of the snails indicating severe pathological impact was observed at temperatures ≥ 50°C, we omitted data obtained for these temperatures from statistical analysis.

Table 1 Investigated *X. derbentina* populations (Pop. no.), respective sampling sites in Provence, France, and recorded data on phenotypic diversity (Hs), constitutive (Hsp70 const.) and maximum (Hsp70 max) stress protein levels and population structure (π, F_{ST} and H_{MH}). Note that the latter two parameters were obtained by estimating the average pairwise distances between the population of concern and all other populations in the dataset.

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

Hsp70 stress protein analysis

Individual snails were mechanically homogenized, centrifuged, and the total protein concentration in the supernatant determined according to Bradford (1976). For each sample, 40µg of total protein were analyzed by minigel SDS-PAGE and semi-dry Western blotting according to Köhler et al. (2005) using a monoclonal α-Hsp70 antibody (Dianova, Hamburg, Germany) which cross-reacted with both constitutive and inducible stress protein isoforms. The optical volumes of the individual Hsp70 bands were quantified with the E.A.S.Y. Win 32 densitometric image analysis program (Herolab, Wiesloch, Germany). All samples were quantified in relation to a standard prepared from full body homogenate of *Theba pisana* in our laboratory, which was run twice on each gel, to ensure comparability between all samples. This assay was shown to display a methodological variance of ± 2.7% from the mean only (Köhler et al. 2005).

Phenotypic diversity

For each snail population, the shell pigmentation pattern was categorized according to the classification system of Köhler et al. (2009) (for the number of replicates see Table 1): 1 = white; 2 = white with a single pale band; 3 = grayish with several light bands; 4 = dark with lots of intense bands. To determinate the phenotypic variation within each population, we calculated Shannon-Wiener-Indices (Hs) from the shell colouration categories of all available individuals of each locality ($96 \leq n \leq 736$).

Genotyping

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

Statistical analyses

For testing whether genetic parameters of the populations studied significantly reflect mean differences in cellular and biochemical heat stress response, three population indices were calculated 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, Laval & Schneider 2005). The two divergence parameters were Nei's (1973) pairwise fixation index (F_{ST}) and haplotype divergence (H_{MH}) based on the Morisita-Horn index (Horn 1966), both calculated in the R 2.15 statistical environment (R Development Core Team 2011). For the former index, we used the adegenet package (version 1.3-6, Jombart 2008); for the latter 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 2.0-5, Oksanen et al. 2012).

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

We then used generalized additive models (GAM) in order to examine the relationship between Hsp70 level, temperature and population structure. GAMs are often used in case of non-linear relationships between the dependent and independent variables by fitting a polynomial function. Moreover, being an extension of generalized linear models, they allow the use of a gamma error distribution, which in turn prevents the occurrence of any modelled Hsp response below 0. All regression analyses were conducted using the R 2.15 and the mgcv 1.7.22 package (Wood, 2011). Hsp70 data consisted of expression measurements from 10 individuals for each of seven temperatures (25, 33, 38, 40, 43, 45, and 48°C). Preliminary analyses indicated k = 6 (basis dimensions) to be optimal for temperature. In order to facilitate simplicity of interpretation, three basis dimensions were chosen for the other independent variables.

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

Results

Hsp70 induction profiles

With the exception of population 4, all populations showed stress protein induction kinetics to follow an optimum curve in response to elevated temperature regimes. At moderately elevated temperatures, Hsp70 was induced to prevent proteotoxic effects in the cells (compensation phase), followed by a decline of the Hsp70 level at high temperatures, when the maximum response level had been surpassed. Most of the *X. derbentina* populations (Figs 1a-c, e-j) showed a clear stress protein induction with a maximum induction capacity of 120-194% compared to the control level at 25°C. Highest Hsp70 induction was always visible in a temperature range between 38-40°C, except for population 4 (Fig 1d), whose Hsp70 level reached a maximum of just 107% of the constitutive level, at 33°C.

Both constitutive and maximum induction Hsp70 levels were found to differ significantly among the investigated *X. derbentina* populations (Welch-ANOVA; $P < 0.0001$, $F_{1,9}$: 13.3387 and $P < 0.0001$, $F_{1,9}$: 24.5448, respectively) but correlated with one another in a positive way ($P = 0.0009$; $R^2 = 0.7650$) (Fig. 1).

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

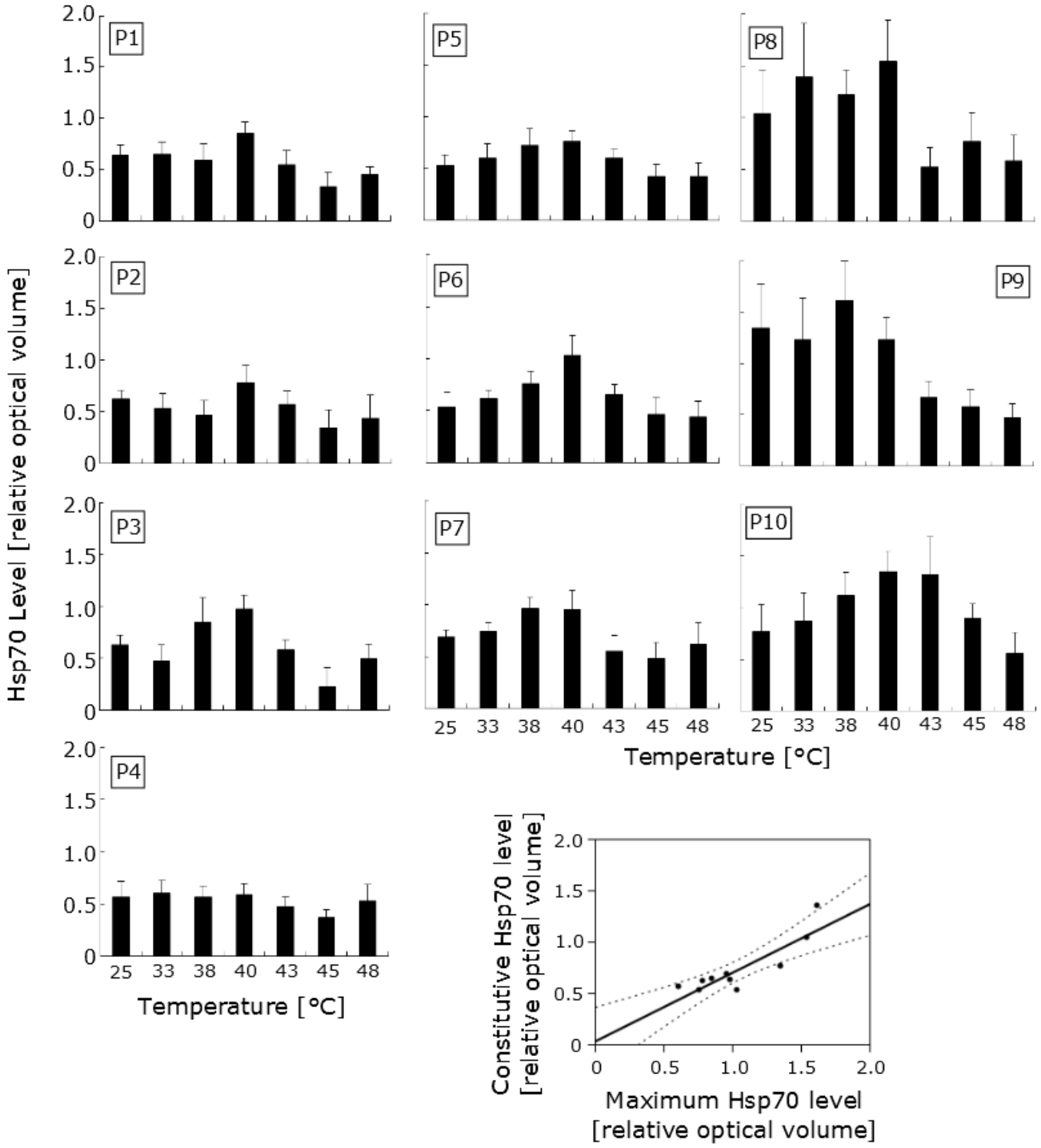

Fig. 1 Hsp70 responses in *X. derbentina* to elevated temperatures. Means and SD. P1 to P10 refer to the respective population numbers. Bottom right: Correlation analyses for *X. derbentina* populations. Constitutive Hsp70 level vs. maximum Hsp70 level. Dotted lines indicate 95% confidence interval of the linear regression curve.

Phenotypic diversity and population structure

Snail populations were found to differ remarkably in respect to their phenotypic diversity. While population 9 was uniformly white, populations 2 and 5 showed rather high Shannon-Wiener indices of about 1.2 (Table 1, Fig 2).

Genetic diversity within populations (π) was generally low (all < 3.0). The highest diversity was found in populations 4 and 1; population 7 was homogeneous (Table 1). Genetic divergences among populations, expressed by F_{ST} and H_{MH}, were also relatively low. The only exception was population 4, resident close to a containerization site, which showed relatively high values for both F_{ST} and H_{MH} and thus is most dissimilar to all other *X. derbentina* populations studied (Table 1).

Correlation of Hsp70 levels and phenotypic diversity

Regression analyses revealed a significant, negative correlation between the constitutive Hsp70 levels and phenotypic diversity (Hs) (P = 0.0139, R^2: 0.5515): the lower the constitutive Hsp70 level of a population, the higher was the corresponding phenotypic diversity of a given population (Fig 2a). As well, we found a significant association between the average maximum induction level and the corresponding Hs-values. Both factors were also negatively correlated to each other (Fig 2b, P = 0.0324, R^2: 0.4552).

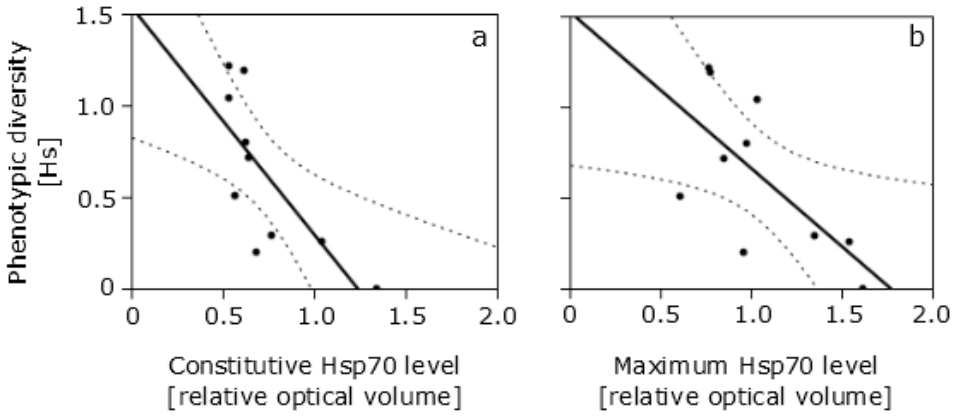

Fig. 2 Correlation analyses for *X. derbentina* populations. a: Constitutive Hsp70 level vs. phenotypic diversity. b: Maximum Hsp70 level vs. phenotypic diversity (Hs). Dotted lines indicate 95% confidence interval of the linear regression curve

Correlation of population structure and phenotypic diversity

Both the diversity parameter π (P = 0.25, R^2 = 0.16) and the two divergence parameters F_{ST} and H_{MH} (P = 0.59, R^2 = 0.004 and P = 0.86, R^2 = 0.004, respectively) missed to correlate with the corresponding Hs values.

Correlation of Hsp70 levels and population structure

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

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

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

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

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

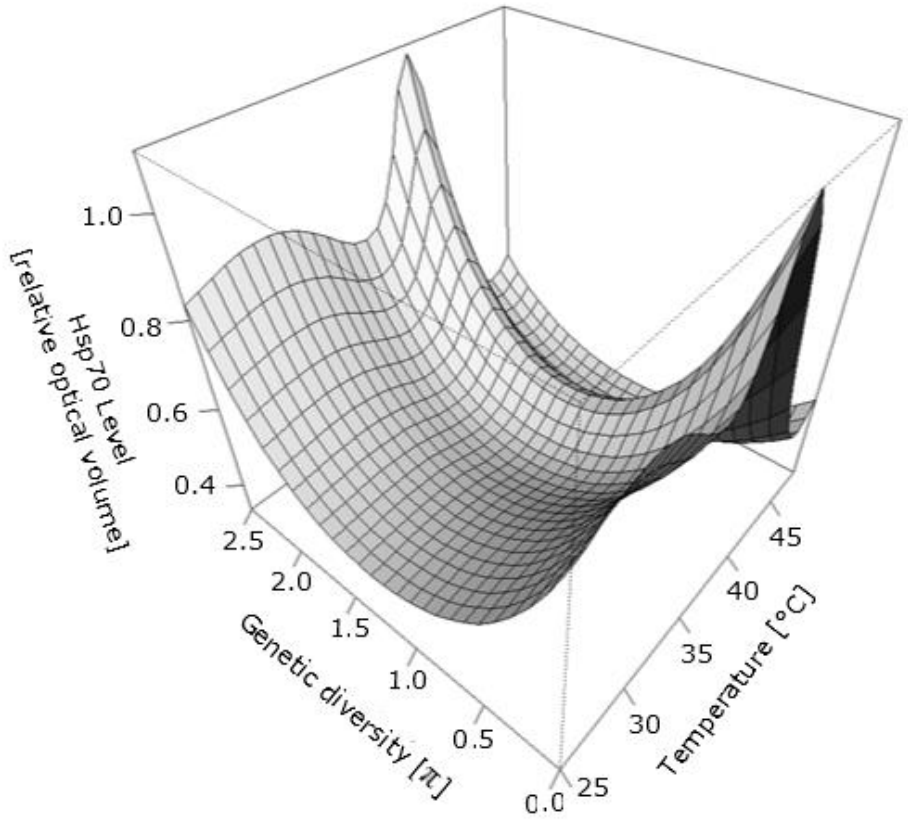

Fig. 3 GAM based prediction for the relationship between Hsp70 expression levels, genetic diversity (π), and ambient temperature.

Structural-equation modelling

Our model, utilized to simultaneously test the causal assumptions that Hsp70 levels depend on ambient temperature and population structure, and that phenotypic diversity is driven by Hsp70 levels, is not rejected ($\chi 2$ = 4.7, df = 3, P = 0.20). This indicates a reasonable approximation of the causal pathways (Fig. 4).

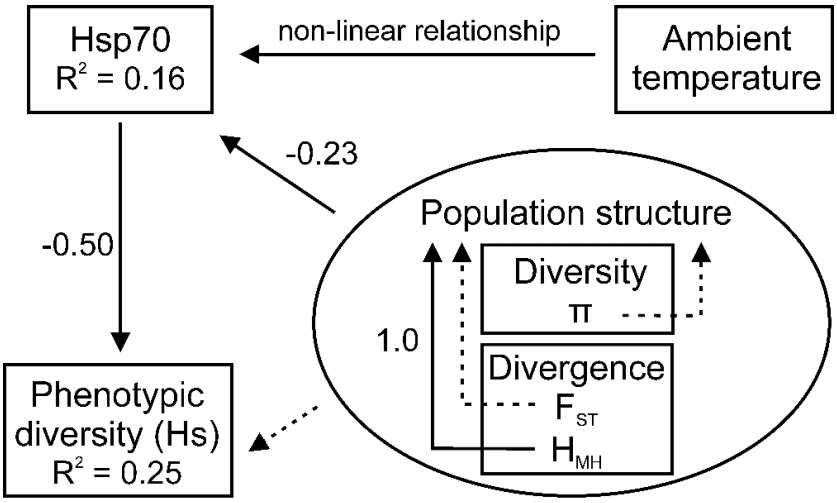

Fig. 4 Structural equation model for causal assumptions of Hsp70 levels, ambient temperature, population structure, and phenotypic diversity. For each path the significant ($\alpha \leq 0.05$) standardized regression coefficients are given. Population structure is a latent variable (shown as ellipse) because it cannot be measured directly and is estimated by its indicators population divergence and diversity. Measured variables are displayed in rectangles including the coefficient of determination for the dependent variables. Dashed lines indicate non-significant relationships.

Discussion

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

work of Manitasevic et al. (2007) and Köhler et al. (2009), our present study provides a much larger dataset and, therefore, stronger evidence for a contribution of stress proteins to the capacitoring of phenotypic variation under natural conditions (Fig. 4).

While Manitasevic et al. (2007) and the present study have focused on actual levels of stress proteins, Köhler et al. (2009) used the 'Hsp70 induction capacity' (ratio between the constitutive Hsp70 level at 25°C and the highest measured Hsp70 level at elevated temperature) as a proxy for potential capacitoring and provided data for 4 snail populations from different species. Supplementing the dataset of Köhler et al. (2009) by data on 'induction capacities' calculated from the present 10 *X. derbentina* populations plus another 6 French helicoid snail populations (A.P., S.L., P.A., unpublished) revealed also negative but just weakly significant correlation between the phenotypic diversity of snail populations and the 'Hsp70 induction capacity' ($P = 0.0474$, $df = 18$, $R^2 = 0.2010$) irrespective of species and habitat. In this context, the positive association between constitutive and maximum stress protein levels as revealed in the present study, however, results in a loss of information when data on constitutive and maximum stress protein levels are transformed into 'induction capacities'. On the basis of the results of the present study we therefore give preference to actual constitutive and maximum Hsp70 levels as measures of capacitoring capability.

In contrast to what should be expected for the hypothetical, "ideal" strategy of adaptation (a low constitutive Hsp70 level, but a high potential to induce Hsp70), we found, as mentioned, a strong positive correlation of the constitutive and the maximum Hsp70 levels in *X. derbentina*. Presumably based on mechanistic constraints, an independent selection for low constitutive Hsp70 levels on one hand and on high Hsp70 induction capacities on the other hand thus does not seem to be possible here and, consequently, selection for low constitutive Hsp levels seems to confer also a low potential of Hsp induction. Consequently, *X. derbentina* populations either follow the strategy to express high constitutive and maximum Hsp70 levels at high energy expenditure and the consequence of a low phenotypic variation or, alternatively, reduce constitutive and maximum Hsp70 levels for the benefit of low energy requirements and at the consequence of a high phenotypic variation. While the first of these two strategies requires a continuous and sufficient energy supply (probably making environmental situations not be perceived as "stressy" by the snails), the latter strategy arranges itself with energetic limitations in "stressy" situations and increases evolvability by displaying previously hidden phenotypic diversity, which is particularly evolutionarily sensible for populations under stress.

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

As mentioned, our investigated *X. derbentina* populations also differed in their shell colouration diversity. We found the phenotypic variation of the shell in different populations to be correlated with the constitutive Hsp70 level as well as with the maximum Hsp70 induction level. In contrast, the population structure of these snails as represented by diversity and divergence parameters did not reveal significant correlations with the phenotypic diversity. Since p-distances (π -values) of *COI* have been shown to be a reliable and well-interpretable proxy for genetic diversity (Kartavtsev & Lee 2006), we have to assume that the potential of *COI* genotypic diversity in view to explain phenotypic variation in the shell pattern of *X. derbentina* is rather limited (Fig. 4). Consequently, epigenetic mechanisms which are able to modify the genotype within the range of phenotypic plasticity have to be proposed for these snails.

Interestingly, populations of *X. derbentina* in the Vaucluse area with low phenotypic (but not necessarily low genotypic) variation are dominated by plain white (category 1) morphs. In this context, the question arises how high Hsp70 levels may be able to favour the emergence

of non-melanized individuals. Since melanin synthesis, the ultimate cause of dark shell colouration, involves multi-step pathways with pleiotropic impact (Rebeiz et al. 2009), Hsp70 might operate in various ways. It has been shown, that increased Hsp70 levels led to suppression in melanin production in mouse melanoma cells *in vitro* as well as *in vivo* (Hoshino et al. 2010). The authors of this publication suggested, that this phenomenon may be based on several reasons: firstly, Hsp70 might affect intracellular trafficking of melanosomes, since it is consent that Hsp70 affects intracellular traffic of vesicles (Bukau & Horwich 1998). Secondly, it influences the expression and activity of tyrosinase, a rate-limiting enzyme in melanin synthesis, by binding to microphthalmia-associated transcription factors (MITF) with subsequent inhibition of the promoter of the tyrosinase gene in the nucleus (Hoshino et al. 2010). Even though mechanisms of gene activation have not been investigated in *X. derbentina*, it cannot be excluded, that the snail populations with predominantly white shells and, therefore, comparably low phenotypic variation are influenced by similar Hsp70 action on melanin synthesis as mammals.

Conclusion

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

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Danksagung

Ein Wort, das von Herzen kommt, macht dich drei Winter warm.

Chinesische Weisheit

Publikationsliste

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