Mechanismen der Thermotoleranz und der Beeinflussung des Schalenpolymorphismus' bei helicoiden Schnecken

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Der aufregendste Satz in der Wissenschaft, derjenige, der neue Entdeckungen ankündigt, ist nicht „Heureka“ (Ich hab's gefunden!), sondern „Das ist aber komisch …!“

(Isaac Asimov)

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Die vergleichsweise hohe Thermotoleranz und der ausgeprägte Polymorphismus der Schalenfärbung bei einigen Schneckenarten der Überfamilie Helicoidea sowie weiterer Gastropodenspezies sind bekannte Phänomene, die auch schon Gegenstand einer Reihe von Studien waren. Neben Arbeiten zur Aestivation und zum thermoregulatorischen Verhalten dieser Tiere (Pomeroy, 1968; McQuaid et al., 1979; Cowie, 1985; Arad et al., 1993) liegen Erkenntnisse zu den genetischen Grundlagen der Schalenfärbungen und der Korrelation der Morphenverteilungen mit Umgebungstemperaturen vor (Jones, 1973a; Heller, 1981; Cowie, 1984a; Cowie, 1990; Johnson, 2011), und auch die thermische Kapazität unterschiedlicher Schalenmorphen wurde bereits in mehreren Studien untersucht (Jones, 1973a; Heath, 1975; Cook & Freeman, 1986). Reviews zu bisherigen Studien zur Entstehung und Erhaltung des Schalenpolymorphismus' bei *Cepaea* (Jones et al., 1977; Cook, 1998; Prädation: Heller, 1981; Heller & Gadot, 1984) sind ebenso zu finden wie Studien zur morphenabhängigen (Mikro-) Habitatswahl (Johnson, 1981; Cowie, 1985; Hazel & Johnson, 1990) und zur Verbreitung und Ausbreitung der verschiedenen Arten (Baker, 2002; Aubry et al., 2006). Die hohe Thermotoleranz von bestimmten Helicoideen wie beispielsweise *Theba pisana* (MÜLLER, 1774), *Cernuella virgata* (DA COSTA, 1778) und *Xeropicta derbentina* (KRYNICKI, 1836) zeigt sich bereits in der natürlichen Verbreitung dieser Schneckenarten, welche im Mittelmeerraum bis z.T. nach Nordafrika häufig sind, oder auch in ihrem Erfolg nach Einführung in mediterrane und andere warme Gebiete wie z.B. Westaustralien (Cowie, 1985; Baker, 2002; Aubry et al., 2006). Trotz dieser Vielzahl an Untersuchungen sind Informationen zu den physiologischen, zellulären und biochemischen Mechanismen, die der Thermotoleranz und der Entstehung und Erhaltung des Schalenpolymorphismus' zugrunde liegen, bislang nur begrenzt vorhanden, da viele der bisherigen Untersuchungen sich *in puncto* mechanistischer Erklärung meist auf Anpassungen auf Verhaltensebene (Kletterverhalten zur Vermeidung hoher Bodentemperaturen, Aufsuchen von Schatten) und

auf physikalische Faktoren (z.B. Erwärmung, also thermische Kapazität) konzentrieren. Bezüglich der Thermotoleranz lieferten bisher Dittbrenner et al. (2009), Köhler et al. (2009), Arad et al. (2010), Mizrahi et al. (2010) und Di Lellis et al. (2012) erste Erkenntnisse über zugrundeliegende zelluläre und biochemische Mechanismen, die Aestivation (Sommerschlaf) ist der einzige weitere physiologische Vorgang, der in diesem Zusammenhang untersucht ist (Pomeroy, 1968; McQuaid et al., 1979; Arad, 2001). Es besteht also im Hinblick darauf weiterer Forschungsbedarf. Die Ergebnisse von Dittbrenner et al. (2009), Köhler et al. (2009), Arad et al. (2010), Mizrahi et al. (2010) und Di Lellis et al. (2012) deuten darauf hin, dass histopathologische Untersuchungen der Mitteldarmdrüse bzw. Hsp70- Analysen von hitzeexponierten Schnecken hierbei wichtige Untersuchungsgegenstände sein können.

Obwohl einige Studien zu thermischen Kapazitäten unterschiedlicher Schalenmorphen vorliegen, muß aus heutiger Sicht eine kritische Betrachtung der applizierten Methodik vorgenommen werden: Cook & Freeman (1986) benutzten eine Mikroskoplampe für ihre Versuche, somit kam kein natürliches Lichtspektrum zum Einsatz, Heath (1975) führte die Versuche im Freiland ohne Beachtung von Luftströmen (Wind) über einen recht breit gefassten Zeitraum (Mai - August) durch, obwohl bekannt ist, dass Qualität, Intensität und Dauer der Sonneneinstrahlung über die Jahreszeiten hinweg sehr variabel sein können (Burkholder, 1936). Für Jones' Experimente (1973a) sind keine Angaben zur Platzierung und Ausrichtung der Schalen, zu Windverhältnissen und Jahreszeit vorhanden. Für alle diese Experimente gilt, dass die Temperaturmessungen nicht berührungsfrei durchgeführt wurden. Dadurch können unerwünschte Temperaturbeeinflussungen durch den Messvorgang selbst aufgrund von Rückwirkungen nicht ausgeschlossen werden (Edler, 2000). Dennoch wurden und werden die Ergebnisse dieser und ähnlicher Studien, welche u.a. eine stärkere Erwärmung von dunklen Schalenmorphen festgestellt haben, zur Erklärung von unterschiedlichen Morphenverteilungen im Feld herangezogen (Jones et al., 1977; Heller, 1981; Johnson, 1981; Heller and Gadot, 1984; Goodhart, 1987; Cowie, 1990; Johnson, 2011). Diese Studien gehen davon aus, dass die Morphenverteilungen im Feld u.a. dadurch beeinflusst werden, dass dunkle Morphen aufgrund ihrer postulierten höheren thermischen Kapazität Nachteile in eher warmen (Mikro-)Klimaten haben. Jedoch wurden beispielsweise bei *Theba pisana* keine Unterschiede im thermoregulatorischen Verhalten bei den verschiedenen Morphen gefunden (Cowie, 1985), was zusammen mit den oben erläuterten methodischen Schwächen bei bisherigen Studien zur thermischen Kapazität unterschiedlicher Schalenmorphen Zweifel an der Hypothese einer biologisch relevanten höheren Erwärmung dunkler Morphen weckt. Eine kritische Überprüfung der o.g. Studienergebnisse zu thermischen Kapazitäten von

verschiedenen Schalenmorphen mittels eines berührungsfreien Meßverfahrens auf dem heutigen Stand der Technik ist somit vonnöten.

Als eine weitere Erklärungsmöglichkeit für die Morphenverteilung im Feld wurden sowohl für *T. pisana* als auch verschiedene *Cepaea* (HELD, 1838) – Arten Prädationseffekte identifiziert (Cain & Sheppard, 1954; Goodhart, 1956; Heller & Gadot, 1984; Cook, 1986; Goodhart, 1987; Tucker, 1991; Cook, 1998). Diese basieren vornehmlich auf apostatischer Selektion visueller Merkmale, bei welcher der seltenere Morph begünstigt wird, da Räuber, insbesondere Vögel, vor allem den häufigeren Morph fressen (Clarke, 1962a; Clarke, 1962b; Goodhart, 1987), bis dieser zum selteneren Morph wird und die Beutepräferenz des Räubers sich dadurch wiederum verschiebt (Goodhart, 1987). Diese apostatische Selektion trägt zur Erhaltung von verschiedenen Morphen bei, auch wenn sie wahrscheinlich nicht der einzige Faktor ist, auf den sich das Phänomen des sichtbaren Schalenpolymorphismus gründet (Goodhart, 1987). Disruptive und gerichtete Selektion hingegen, für welche eine Einnischung erforderlich ist (die Morphen halten sich vornehmlich auf dem Hintergrund auf, auf dem sie am wenigsten auffällig sind), können zumindest für *Cepaea hortensis in puncto* visueller Merkmale in vielen Fällen ausgeschlossen werden, da die verschiedenen Farb- und Bänderungsmorphen häufig im selben Habitat vorkommen und somit kaum Einnischung beobachtet werden konnte (Goodhart, 1987). Für *Cepaea nemoralis* jedoch wurde in einer aktuellen, großen Studie, die auf Daten, welche über ein großes „citizen science“ Projekt erhoben worden sind, eine morphenabhängige Einnischung beobachtet, welche von den Autoren auf Prädationseffekte zurückgeführt wurden (Silvertown et al., 2011). Auch bei *Theba pisana* konnte in einigen Populationen eine unterschiedliche Einnischung von gebänderten und ungebänderten Morphen beobachtet werden, und diese konnte nicht nur von (mikro)klimatischen Effekten sondern auch von Räubervorkommen bzw. Fehlen von Räubern in bestimmten Habitaten abgeleitet werden (Heller & Gadot, 1984). Daher ist das Wirken von disruptiver und gerichteter Selektion durch Räuber bei *Cepaea nemoralis* und *Theba pisana* denkbar. Die bevorzugte Reproduktion gleicher oder ähnlicher Morphen, ein Umstand, der eine weitere Erklärung zur Erhaltung des Polymorphismus bieten könnte, konnte bisher hingegen nicht bestätigt werden, zumal dem auch die Dominanzverhältnisse bei der Vererbung von Bänderungen und Farben entgegen stehen (Goodhart, 1987). Wenngleich auch in manchen Populationen Veränderungen der Morphenzusammensetzung von Jahr zu Jahr beobachtet werden konnten (Cowie & Jones, 1987), konnte insgesamt jedoch durch eine Reihe von Untersuchungen (Goodhart, 1987) für *Cepaea* spp. festgestellt werden, dass der Schalenpolymorphismus in vielen Populationen auch über lange Zeiträume vergleichsweise

robust gegenüber Umwelteinflüssen ist. Auch bei *Theba pisana* konnte in einer dreizehnjährigen Studie von verschiedenen Populationen eine Stabilität der Genfrequenzen für Schalenmusterung beobachtet werden, wobei bestimmte Morphen in bestimmten Habitaten häufiger waren (Cowie, 1992). Als Ursachen für diese Stabilität des Schalenpolymorphismus kommen eine Reihe von Faktoren und Mechanismen in Frage, die Cowie (1992) beschreibt, und welche durch weitere Studienergebnisse ergänzt werden können:

1. Gerichtete, stabilisierende Selektion über Prädation und klimatische Einflüsse, wobei ein Zusammenhang mit den jeweiligen Habitatgegebenheiten nicht immer erkennbar ist, wie in diversen früheren Studien sowohl zu *T. pisana* als auch *Cepaea* spp. beschrieben wurde (Cain & Currey, 1963; Johnson, 1980; Johnson, 1981; Heller, 1981; Heller & Gadot, 1984; Cowie, 1990; Hazel & Johnson, 1990). Das Phänomen dieser (scheinbar) fehlenden Korrelation eines bestimmten Morphen mit einem Selektionsfaktor wird als „area effect“ bezeichnet (Cain & Currey, 1963; Johnson, 1976). Cowie (1992) räumt jedoch ein, dass es möglich ist, dass bestimmte selektiv wirkende Kräfte noch gar nicht erkannt worden sein könnten, und Cain & Currey (1963) merken an, dass die Habitatsunterschiede möglicherweise subtil und schwer identifizierbar sein können. Auch eine kürzlich veröffentlichte Studie zur Morphenverteilung bei *T. pisana* über 34 Jahre (Johnson, 2011) kommt zu dem Schluß, dass das habitatsspezifische Wirken von Prädation und klimatischen Faktoren eine sehr wahrscheinliche Ursache für eine, insgesamt gesehen, nur geringe Veränderung der Morphfrequenzen über drei Jahrzehnte sein kann, wenngleich auch das Potential für schnelle genetische Veränderungen erkennbar war, da die Varianz stets sehr groß war und schnelle Richtungswechsel bezüglich der in den jeweiligen Jahren wirkenden Selektion beobachtbar waren.
2. Gründereffekte, d.h. es kommt relativ häufig zum Aussterben lokaler Populationen, wonach dann eine Wiederbesiedlung stattfindet. Bei Vorhandensein einer gerichteten Selektion kommt es dabei nicht zu einer Gleichverteilung der Morphen.
3. Gründereffekte wie unter 2. beschrieben, jedoch mit dem Unterschied, dass keine Selektion wirkt bzw. dass die ausgeprägte Populationsgröße einen wirksamen genetischen Drift (zufällige Selektion) verhindert. Das Fehlen von Selektion kann jedoch als unwahrscheinlich erachtet werden, da es sich bei der Schalenfärbung von Schnecken um einen genetischen Polymorphismus handelt, welcher definitionsgemäß ein Resultat von Selektionsvorgängen ist (Ford, 1945; Goodhart, 1987). Ausgeprägte

Populationsgrößen können jedoch tatsächlich insbesondere bei *T. pisana* beobachtet werden (Cowie, 1984b; eigene Beobachtungen).

Ein weiterer Punkt, der bei der Generierung und Erhaltung des Schalenpolymorphismus eine Rolle spielt, ist möglicherweise die Heterosis, also ein durch Heterozygotie bedingtes gesteigertes Vermögen, mit wechselnden Bedingungen umgehen zu können (Goodhart, 1987; Cook, 1998), auch wenn der durch die Heterosis bedingte Vorteil möglicherweise nicht immer identifiziert werden kann (Goodhart, 1987). Dieses Fehlen einer eindeutigen Zuordnung ist deswegen denkbar, da verschiedene Schalenfarben und Bänderungsmuster auf eine Reihe von Gen-Loci zurückzuführen sind, welche darüber hinaus auch miteinander und mit weiteren, auch nicht auf die Färbung wirkenden Loci verknüpft sein können (Jones et al., 1977; Goodhart, 1987; Johnson, 2012). Zudem müssen nicht alle Loci einen sichtbaren Polymorphismus begründen und manche können außerdem epistatisch wirken (Goodhart, 1987; Johnson, 2012). Obwohl das vollständige Fehlen von Selektion aus bereits oben erläuterten Gründen als Ursache für den Polymorphismus *per se* nicht in Frage kommt, bestehen laut Goodhart (1987) dennoch nachvollziehbare Überlegungen darüber, dass der sichtbare Polymorphismus, zumindest teilweise, nicht-adaptiv bedingt sein könnte. Es ist denkbar, dass der sichtbare Polymorphismus lediglich ein Nebenprodukt einer Selektion auf nicht-sichtbare Eigenschaften sein könnte, wobei die Loci für den sichtbaren Polymorphismus mit denen der nicht-sichtbaren Eigenschaften verknüpft sein könnten. Diese Überlegung wird gestützt von der Beobachtung, dass es für einige mit *Cepaea hortensis* (MÜLLER, 1774) und *T. pisana* verwandte Helicoiden wie *Helix pomatia* (LINNAEUS, 1758) und *Arianta arbustorum* (LINNAEUS, 1758), die zudem ähnliche Habitate besiedeln, zwar Indizien für ein Bänderungsmuster gibt, dieses jedoch durch ein kryptisches Muster überlagert wird, so dass die verschiedenen Allelomorphen phänotypisch ähnlich erscheinen, also mehr oder weniger monomorphisch sind (Goodhart, 1987). Wenn also nun beispielsweise bei *C. hortensis* eine Selektion auf nicht-sichtbare Eigenschaften bestünde, an die ein sichtbarer Schalenfarb- und / oder Schalenbänderungs- Polymorphismus geknüpft wäre, so wäre es mehr als plausibel, dass, wenn einer der Allelomorphen einen durch den sichtbaren Polymorphismus bedingten Nachteil erfahren würde, dieser sichtbare Polymorphismus epistatisch überlagert werden würde, wie z.B. bei *H. pomatia* oder *A. arbustorum* durch kryptische Überlagerung geschehen (Goodhart, 1987). Goodhart (1987) erläutert weiterhin, dass eine solche Krypsis jedoch weder für *C. hortensis* noch *Cepaea nemoralis* (LINNAEUS, 1758) der Fall ist, so dass es denkbar ist, dass keiner der Schalenmorphen einen auf dem Schalenpolymorphismus basierten selektiven Vorteil besitzt, und eine Unterdrückung des sichtbaren Polymorphismus

nicht notwendig ist. Dies muß nicht bedeuten, dass die sichtbar verschiedenen Phänotypen beispielsweise keinerlei unterschiedliche Prädation erfahren - diese Prädation ist, wie auch bereits erläutert, vielfach beobachtet worden - jedoch liegt nach obigen Überlegungen nahe, dass die aus der Prädation resultierende Selektion ungleich schwächer ist als die Selektion auf die verknüpften nicht-sichtbaren Parameter (Goodhart, 1987). Des weiteren stellt Goodhart (1987) fest, dass diese Überlegungen für *T. pisana* ebenso gelten müssten; er spricht hier sogar von einem „Pseudopolymorphismus“, da bei dieser Art die meisten Phänotypen im für Theba typischen trockenen Habitat visuell sehr ähnlich erscheinen würden (dem kann jedoch für die in der vorliegenden Arbeit verwendeten Theba-Populationen widersprochen werden, da diese ausgeprägte visuelle Unterschiede in der Schalenfärbung aufwiesen). Zudem gibt es Hinweise darauf, dass die Variation der Schalenmorphologie über einen nicht selektionsabhängigen, ungerichteten Mechanismus auf der Basis unterschiedlicher Stressproteinlevel reguliert werden könnte, wobei eine hohe phänotypische Variation mit niedrigen Stressproteinleveln (Hsp70) korreliert, die Stressproteine also eine Pufferwirkung bezüglich der phänotypischen Ausprägung von Schalenmorphen haben und damit kanalisierend wirken (Köhler et al., 2009). Die Ergebnisse dieser Studie von Köhler et al. (2009) deuten auch darauf hin, dass Stressproteine einen Bestandteil des bereits 1942 von Waddington formulierten „Waddington’s Widget“, einem unbekannten Mechanismus, welcher phänotypische Variation in Organismen maskiert bis ein Stresszustand auftritt, darstellen könnten.

Insgesamt wird deutlich, dass bisher keine Einigkeit darüber besteht, warum der bei einigen Helicoiden beobachtete Schalenpolymorphismus vorliegt und wie er erhalten bleibt (Jones et al., 1977; Goodhart, 1987; Cowie, 1992; Johnson, 2012). Es wird aus der o.g. Literatur auch klar, dass möglicherweise noch gar nicht alle potentiell selektiv wirkenden Faktoren identifiziert und analysiert worden sind, ein Umstand, den Cowie bereits 1992 bemerkt hat, der jedoch bisher kaum zu einer Erweiterung des Wissenstands zu weiteren potentiell selektiv wirkenden Faktoren geführt hat. Somit besteht Forschungsbedarf zur Überprüfung von Hypothesen zu weiteren möglichen Selektionsfaktoren.

Falls man, bedingt durch die bisherigen Studien zur Erwärmungskapazität (Jones, 1973; Heath, 1975; Cook & Freeman, 1986), von einer höheren Erwärmung dunkler Morphen und somit einem Selektionsnachteil in heißen Gebieten ausgeht, so kann die Frage gestellt werden, ob die dunklere Färbung auch einen Vorteil mit sich bringt, der die Folgen der postulierten höheren Erwärmung wiederum ausgleicht. So ist bekannt, dass bei Vorliegen von erhöhten Temperaturen bei den meisten Invertebraten auch die Stoffwechselraten erhöht sind (Pörtner,

2001). Erhöhte Stoffwechselraten können wiederum zu einer Erhöhung der Bildungsrate von freien Radikalen führen (Abele et al., 1998), welche wiederum z.B. zu Membranschädigungen führen (Gutteridge & Halliwell, 1990). Interessanterweise ist Melanin ein Radikalfänger (Riley, 1997), und dabei auch ein Pigment, welches für dunkle Schalenfärbung bei Schnecken mitverantwortlich ist (Comfort, 1951). Daher ist denkbar, dass, ähnlich wie z.B. für dunkler gefärbte Krallenfrösche (*Xenopus laevis* (DAUDIN, 1802)) bestätigt (Corsaro et al., 1995), dunklere Schnecken mit den Folgen von oxidativem Stress besser umgehen können. Zur Quantifizierung des oxidativen Stress-Status ist der sogenannte *ferrous-oxidation-xylenolorange (FOX) assay* ein gut geeigneter Test (Hermes-Lima et al., 1995), welcher auch bei Gastropoden bereits erfolgreich zum Einsatz gekommen ist (Ramos-Vasconselos & Hermes-Lima 2003).

Des weiteren konnte festgestellt werden, dass Melanisierung und Immunität bei Invertebraten den Melanin - produzierenden Stoffwechselweg teilen, die sogenannte Phenoloxidase - Kaskade (Söderhäll & Cerenius, 1998; Rolff & Siva-Jothy, 2003). Die Phenoloxidase - Aktivität kann somit als Proxy für die Stärke der Immunabwehr genutzt werden und wurde als solches auch bereits erfolgreich bei Mollusken eingesetzt (Smith & Söderhäll, 1991; Barracco et al., 1999; Bahgat et al., 2002; Jordan & Deaton, 2005; Munoz et al., 2006; Seppälä & Jokela, 2010; Seppälä et al., 2011). Die oben beschriebene Verknüpfung von Immunität und Melanisierung auf Stoffwechselebene (Söderhäll & Cerenius, 1998; Rolff & Siva-Jothy, 2003) wirft somit die Frage nach einer möglichen Korrelation von Melanisierungsgrad (im Falle der Schnecken also Schalendunkelfärbung / - bänderung) und Stärke der Immunabwehr (ermittelt über Bestimmung der Phenoloxidase - Aktivität) auf, wie sie z.B. bei verschiedenen Insektenarten bereits nachgewiesen wurde (Wilson et al., 2001, Armitage & Siva-Jothy, 2005). Die Tatsache, dass höhere Parasitierungsraten durch Nematoden bei hellen Schalenmorphen im Vergleich zu dunklen Morphen bestimmter Helicidenarten gefunden wurden (Cabaret, 1983; Cabaret, 1988; Lahmar et al., 1990), unterstreicht dabei die Notwendigkeit, den potentiellen Zusammenhang von Schalenmorphologie und Toleranz gegenüber Pathogenen zu untersuchen.

2.2 Fragestellungen

Im Rahmen der vorliegenden Arbeit sollen mögliche Erholungsvorgänge auf zellulärer (Histopathologie des Mitteldarmdrüsengewebes) und biochemischer (Stressproteinantwort (Hsp70)) Ebene nach Hitzeexposition bei helicoiden Schneckenspezies aus dem Mittelmeerraum untersucht werden. Es soll überprüft werden, ob Unterschiede zwischen den

Testarten *X. derbentina* und *T. pisana* bezüglich ihrer Hitzetoleranz und ihrer Erholungskapazität nach Hitzebelastung zu finden sind und wie diese begründet sein können, um neue Erkenntnisse zu biochemischen und zellulären Mechanismen, die die Thermotoleranz dieser Arten begründen, zu gewinnen.

Im nächsten Teil soll überprüft werden, ob Unterschiede in der Kapazität zur Abwehr von oxidativem Stress zwischen hellen und dunklen Schalenmorphen (vermittelt über Melanin als Radikalfänger) von *T. pisana* sowohl konstitutiv als auch nach Hitzebelastung vorliegen, um einen möglichen Vorteil von dunkleren Morphen in heißen Habitaten zu identifizieren, der den Nachteil der postulierten höheren Erwärmung dieses Morphs ausgleichen könnte.

In einem berührungsfreien, reproduzierbaren Testverfahren soll dann die thermische Kapazität heller und dunkler Schalen von *T. pisana* ermittelt werden, was Aufschluß über die Frage geben soll, ob die Erwärmung dunkler Schalenmorphen tatsächlich höher ausfällt als die Erwärmung heller Morphen, wie es bisher aufgrund von Ergebnissen technisch weniger ausgereifter Arbeiten angenommen wurde.

Im nächsten Schritt soll dann die Frage bearbeitet werden, ob eine Verknüpfung von Melanisierung (Schalenfärbung) und Immunkompetenz existiert, hierzu sollen helle und dunkle Morphen verschiedener Helicoiden auf ihre Phenoloxidase-Aktivität untersucht werden, und zwar sowohl konstitutiv als auch nach Verabreichung eines Immunstimulans bzw. nach Exposition gegenüber einem parasitischen Nematoden. Des Weiteren soll die Toleranz unterschiedlicher Schalenmorphen gegenüber einem parasitischen Nematoden über Ermittlung der Mortalitätsraten nach Nematodeninfektion untersucht werden. Die Ergebnisse sollen dann sowohl für sich diskutiert als auch mit bisherigen Arbeiten in Verbindung gebracht werden, um weitere neue Erkenntnisse zu potentiell auf den Schalenpolymorphismus selektiv wirkenden Faktoren beitragen zu können.

3. Material und Methoden

3.1 Untersuchte Organismen

In der vorliegenden Arbeit wurden verschiedene Spezies der Überfamilie Helicoidea untersucht. Die Helicoidea stellen eine der insgesamt sieben Überfamilien, welche die Gruppe der nicht- achatinoiden Stylommatophora (Unterklasse: Pulmonata, Klasse: Gastropoda) bilden (Wade et al., 2006). Innerhalb dieser nicht-achatinoiden Stylommatophora gruppieren Wade et al. (2006) die Helicoidea zwischen die Orthalicoidea und Clausilioidea. Die Helicoidea selbst wiederum schließen die Familien Helicidae, Bradybaenidae,

Helminthoglyptidae, Hygromiidae, Camaenidae, Polygyridae und Sagdidae ein (Wade et al., 2006), wobei Verwandtschaftsbeziehungen und Stellungen der verschiedenen Arten innerhalb dieser Familien kritisch diskutiert worden sind (Steinke et al., 2004; Groenenberg et al., 2011). Für die vorliegende Arbeit wurden nur Arten der Familien Helicidae und Hygromiidae eingesetzt: *Theba pisana* (MÜLLER, 1774; Familie: Helicidae), *Cepaea hortensis* (MÜLLER, 1774; Familie: Helicidae), *Xeropicta derbentina* (KRYNICKI, 1836; Familie: Hygromiidae), *Cornu aspersum maximum* (Unterart von *Cornu aspersum* (MÜLLER, 1774), Nomenklatur nach Falkner et al., 2001; Familie Helicidae) und *Cernuella virgata* (DA COSTA, 1778; Familie: Hygromiidae). Alle untersuchten Schneckenarten weisen einen farblichen Schalenpolymorphismus auf, wobei sich nur die Bänderungen unterscheiden können, wie z.B. bei *T. pisana* (Hazel & Johnson, 1990), *C. virgata* (Baker, 1988) und *X. derbentina* (Di Lellis et al., 2012), oder es können sowohl die Schalengrundfarbe als auch die Bänderungen unterschiedlich ausfallen, wie z.B. bei *C. hortensis* (Jones et al., 1977) und *C. aspersum maximum* (Albuquerque de Matos, 1984, für *C. aspersum*; eigene Beobachtungen für die Unterart *C. aspersum maximum*).

3.2 Experimenteller Aufbau

Die für die in Kapitel 1 beschriebenen Arbeiten untersuchten Schnecken, *T. pisana* und *X. derbentina*, wurden im Department Vaucluse, Südfrankreich, in der Nähe von L'Isle-sur-la-Sorgue bzw. La-Roque-sur-Pernes Anfang Juli 2007 gesammelt. Nach einer zweiwöchigen Akklimatisationsphase im Labor sowie nach einer bis zu achtstündigen Kontrollhälterung in Wärmeschränken bei 24,7°C wurden Ganzkörperproben sowohl für histopathologische Analysen als auch für Hsp70-Analysen aufbereitet, um Aussagen zum Kontrollzustand der Tiere treffen zu können. Zur Untersuchung der Thermotoleranz und des Erholungsvermögens erfolgten des Weiteren histopathologische Analysen und Hsp70-Analysen an Individuen, welche einer subletalen Hitzeexposition (45°C für *X. derbentina*, 43°C für *T. pisana*) über bis zu acht Stunden ausgesetzt wurden, sowie einem direkt anschließenden bis zu 16 Stunden dauernden Erholungszeitraum, in dem die Tiere bei 24,7°C gehältert wurden.

Für die in Kapitel 2 beschriebene Untersuchung zur Kapazität der oxidativen Abwehr wurden Schnecken der Art *T. pisana* im Department Vaucluse, Südfrankreich, in der Nähe des Flughafens von Avignon, Caumont, in der ersten Augustwoche 2009 gesammelt. Nachdem die Tiere für zwei Wochen im Labor akklimatisiert worden waren, erfolgte eine Kontrollexposition (24,7°C) sowohl heller, ungebänderter als auch dunkler, gebänderter Schalenmorphen, über bis zu acht Stunden. Parallel wurden weitere Schnecken beider

Morphen für bis zu acht Stunden gegenüber subletaler Hitze (43°C) exponiert. Sowohl Kontrollen als auch hitzeexponierte Individuen wurden über Herstellung von Methanol - Ganzkörperhomogenaten für die Analyse von Lipidperoxiden mittels FOX (ferrous oxidation xylenolorange) Assay aufbereitet.

Das in Kapitel 3 beschriebene Experiment zur Ermittlung der thermischen Kapazität unterschiedlicher Schalenmorphen erfolgte an leeren gebänderten (‚dunklen') und leeren ungebänderten (‚hellen') Schalen von Schnecken der Art *T. pisana*, welche im Department Vaucluse in der Nähe von L'Isle-sur-la-Sorgue und Fontaine-de-Vaucluse gesammelt worden waren. Die Beschränkung der Untersuchung auf leere Schalen erfolgte aus Gründen der besseren Standardisierbarkeit, da hiermit Einflüsse des Weichkörpers (insbesondere Verdunstungsvorgänge) ausgeschlossen werden konnten. Die Untersuchung erfolgte in zwei Schritten, dabei wurde zunächst eine Erwärmung der unterschiedlichen Schalenmorphen mittels Strahlung (Vollspektrumlampe) erzeugt und die Aufheizung über thermographische Aufnahmen ermittelt. Im zweiten Schritt wurden die Schalen gleichmäßig im Wärmeschrank erwärmt, nach Aussetzen weiterer Erwärmung wurde dann die Wärmeabgabe von Individuen der verschiedenen Morphen an die Umgebung über thermographische Aufnahmen verfolgt.

Für die in Kapitel 4 beschriebenen Versuche wurden die Arten *C. hortensis*, *T. pisana* und *C. aspersum maximum* eingesetzt. *C. hortensis* wurde einem privaten Garten in Tübingen-Lustnau, Baden-Württemberg, entnommen, *T. pisana* wurde in der Nähe von Les Paluds de Noves im Department Bouches du Rhône in Südfrankreich gesammelt. Schnecken der Art *C. aspersum maximum* wurden vom Schneckengarten Munderkingen in Munderkingen, Baden-Württemberg, bezogen. Da sich der Bezug von geeigneten parasitischen Nematoden, welche in der Lage sind, alle Testarten zu infizieren, als schwierig erwies, wurden die Versuche auf Zymosan A, einem Präparat aus Hefezellwandbestandteilen, welches bereits erfolgreich zur Immunstimulation bei Mollusken eingesetzt worden ist (Coles & Pipe, 1994; Aladaileh et al., 2007; Hellio et al., 2007; Lacoue-Labarthe et al., 2009), als Immunstimulans beschränkt. Sowohl nach Kontrollhälterungen als auch nach designierter Immunstimulation mittels Zymosan A wurde hellen und dunklen Schnecken Hämolymphe entnommen, welche dann auf Phenoloxidase-Aktivität untersucht wurde, wobei die Phenoloxidase-Aktivität Rückschlüsse auf die Immunkompetenz erlaubt.

Die in Kapitel 5 dargestellten Experimente erfolgten an *C. hortensis*, welche in Tübingen-Derendingen, Süddeutschland, gesammelt wurden, und *C. virgata*, welche aus der Nähe von Volterra, Toskana, Norditalien, stammten. Neben kontrollgehälterten Tieren mit hellen und dunklen Schalen wurden auch mit verschiedenen Konzentrationen von *Phasmarhabditis*

hermaphrodita (SCHNEIDER, 1859), einem parasitischen Nematoden, infizierte Schnecken beider Morphen auf Mortalität und Phenoloxidase-Aktivität untersucht. Diese Parameter erlauben Rückschlüsse auf eine mögliche Toleranz und dieser Toleranz möglicherweise zugrundeliegenden Mechanismen verschiedener Schalenmorphen gegen den Parasiten *P. hermaphrodita* und das daran assoziierte Bakterium *Moraxella osloensis* (BOVRE & HENRIKSEN, 1967).

3.3 Histopathologische Untersuchungen

Die aus den Schalen präparierten Schnecken wurden für mindestens eine Woche bei 4°C in einer zweiprozentigen Glutardialdehyd-Lösung (gelöst in 0,01M Cacodylat-Puffer) fixiert. Danach erfolgte eine Dekalzifizierung in 1:2 Ameisensäure-Ethanol und eine schrittweise Entwässerung der Proben in Ethanol mit anschließender Einbettung in Histowax (Paraffin).
Anschließend wurden Dünnschnitte (7µM) der Mitteldarmdrüse mittels Mikrotom angefertigt, welche mit Hämatoxylin-Eosin-Lösung gefärbt und lichtmikroskopisch ausgewertet wurden. Die Auswertung erfolgte dabei qualitativ und semiquantitativ anhand einer Kategorisierung der Schädigungsgrade der verschiedenen Zelltypen und Strukturen wie sie bereits in Dittbrenner et al., 2009, erfolgreich angewandt wurde. Des Weiteren wurde die Anzahl der Kalkzellen und ihr Flächenanteil am Mitteldarmdrüsengewebe randomisiert erfasst.

3.4 Stressprotein (Hsp70)-Analysen

Nachdem die Tiere einzeln in flüssigem Stickstoff schockgefroren und bis zur weiteren Bearbeitung bei -80°C aufbewahrt worden waren, wurden die Ganzkörperproben in einer gewichtsbezogenen Menge Extraktionspuffer (80mM Kaliumacetat, 5mM Magnesiumacetat, 20mM Hepes) auf Eis homogenisiert und für 10min bei 20 000g und 4°C abzentrifugiert. Die Bestimmung der in den Proben enthaltenen Gesamtproteinmengen erfolgte nach Bradford (1976). Mittels SDS (*Sodium Dodecyl Sulfate*)-PAGE wurden konstante Proteinmengen von jeweils 40µg aufgetrennt und anschließend mittels Semi-dry Elektrotransfer auf eine Nitrocellulosemembran transferiert. Die Banden wurden dann über eine Peroxidasefarbreaktion (erster Antikörper: mouse anti-human Hsp70, zweiter Antikörper: goat anti-mouse IgG konjugiert an Peroxidase) sichtbar gemacht und densitometrisch ausgewertet.

3.5 Analyse von Lipidperoxidations-Leveln mittels FOX (ferrous oxidation xylenolorange) Assay

Der FOX Assay und die Ermittlung der Ergebnisse erfolgte nach Hermes-Lima et al. (1995). Individuen wurden nach Entfernung der Schalen in flüssigem Stickstoff schockgefroren und bis zur weiteren Bearbeitung bei -80°C aufbewahrt. Zur Aufarbeitung der Proben wurden diese auf Eis im Verhältnis 1:2 in Methanol homogenisiert (Festlegung des Verdünnungsgrads anhand von Vorversuchen) und anschließend bei 15 000g und 4°C über 5min zentrifugiert. Jeweils 50µL des resultierenden Überstands wurden in 1mL Reaktionsgemisch (0,25mM $FeSO_4$, 25mM H_2SO_4, 0,1mM Xylenolorange) über 24h bei Raumtemperatur inkubiert und anschließend spektrometrisch bei 580nm vermessen. Nach Zugabe von 5µL einer 1mM Cumolhydroperoxid-Lösung und weiterer Inkubation für 30min wurden die Proben nochmals bei 580nm spektrometrisch vermessen. Die Berechnung der Cumolhydroperoxid-Äquivalente als Maß für die durch Lipidperoxidation gebildeten Produkte pro g Naßgewicht (CHPE / g wet weight) erfolgte anhand der folgenden Formel:

$CHPE/g_{wet\text{-}weight}=(A_{580nm}/A_{580nm+CHP})*5\mu L\ CHP_{5nmol}\ *1000/V1*2$

wobei V1=das Probenvolumen (50 µL) darstellt und der Faktor 2 aus der Verdünnung (1:2 mit Methanol, Dichte 0.791 g/cm³) resultierte.

3.6 Thermographische Untersuchungen

Die thermographischen Aufnahmen und Messungen wurden in zwei Teilen mithilfe einer Thermokamera (*TVS 100, Goratec Technology, Erding, Deutschland*) durchgeführt. Für beide Teile wurden die folgenden Kameraeinstellungen gewählt, welche in Vortests als für optimale Bildauflösung geeignet ermittelt worden waren: Temperaturspanne 20°C-45°C, Average (AVE; reduziert das Rauschen durch angepasste Reduzierung der Aufnahmefrequenz)= 8, Emissionsgrad= 1,0. Der Emissionsgrad wurde bei der Berechnung der Ergebnisse auf 0,93 (Wert für rauen Kalk gemäß BARTEC, 2001) korrigiert. Sowohl im ersten als auch im zweiten Versuchsteil wurden helle, ungebänderte und dunkle, gebänderte Schalen getestet. Im ersten Versuchsteil erfolgte die Erwärmung mittels einer Vollspektrumlampe in einem speziell für diese Messungen entwickelten und konstruierten Holzschrank zur Reduktion von Luftverwirbelungen. In diesem Schrank wurden die an Holzstäben fixierten Schalen in reproduzierbaren Positionen angebracht und ihre Erwärmung über definierte Zeiträume vermessen. Es wurde die Gesamterwärmung der Schalen sowie die Kinetik ihrer Erwärmung ermittelt, dabei wurden die Schalen an jeweils drei Punkten (oben, Mitte, unten) thermographisch vermessen. Diese Meßwerte wurden sowohl isoliert als auch in einem für die

jeweilige Schale vereinigten Gesamtwert betrachtet. Im zweiten Versuchteil erfolgte die Erwärmung der definiert fixierten Schalen im Wärmeschrank; die Reduzierung von Luftverwirbelungen erfolgte unter Zuhilfenahme einer Styropordämmung. Nach Aussetzen der Erwärmung wurde die Temperaturabnahme der Schalen über definierte Zeiträume jeweils am Mittelpunkt der Schalenoberseite für jede Schale mehrfach gemessen. Schließlich wurden die Gesamt-Temperaturabnahme der jeweiligen Schalen sowie die Kinetik ihrer Temperaturabnahme ermittelt.

3.7 Untersuchung der Immunantwort mittels Phenoloxidase (PO) Assay und Ermittlung der Mortalitätsraten nach Nematodeninfektion

Für die in Kapitel 4 beschriebenen Versuche wurde eine Immunstimulation mittels Injektion von Zymosan A, einem Präparat aus Hefezellwand-Bestandteilen, welches zur Immunstimulation bei verschiedenen Invertebraten bereits eingesetzt worden ist (Cárdenas & Dankert, 1997; Vetvicka & Sima, 2004; Pang et al., 2010), angestrebt. Hierzu wurden ca. $4 * 10^7$ Partikel Zymosan A pro 1mL sogenannter Schneckensaline (Salzlösung nach Chiarandini, 1964; Zymosan A Konzentration nach Matricon- Gondran & Letocart, 1999) suspendiert, pro Schnecke wurden 100µL dieser Suspension in die obere bis mittlere Kopffußregion (cranialer Bereich des Fußes) subepithelial injiziert. Sowohl Kontrolltieren als auch behandelten Tieren wurden mit sterilen Spritzen und Kanülen definierte Hämolymphmengen nach der Methode von Renwrantz et al. (1981) entnommen, wobei auf das anschließende Zukleben der punktierten Gewebe mit Histoacryl aufgrund starker Schleimentwicklung, die in Vortests beobachtet worden war, verzichtet wurde. Für *C. aspersum maximum* war zudem ein Vorbohren der Schalen vor Hämolymphentnahme erforderlich. Die Analyse der Hämolymphe auf Phenoloxidase-Aktivität erfolgte gemäß Seppälä & Jokela, 2010, wobei die Inkubationszeiten artspezifisch angepasst wurden. Die Hämolymphe wurde in definierten Schritten mit Phosphatpuffer (pH 7,4), Aqua bidest. und L-Dopa-Lösung (4mg/mL) in Mikrotiterplatten versetzt und sofort (0h) bei 490nm spektrophotometrisch gemessen ($A_{490nm\ 0h}$). Danach erfolgte eine Inkubation bei 30°C über einen artspezifischen, in Vortests ermittelten Zeitraum, anschließend wurden die Proben ein zweites Mal spektrophotometrisch bei 490nm vermessen ($A_{490nm\ end}$). Die Phenoloxidase (PO) Aktivität in milliunits wurde nach folgender Formel berechnet:

PO Aktivität= $A_{490nm\ end}$-$A_{490nm\ 0h}$-$\Delta A_{490nm\ control}$

(wobei $\Delta A_{490nm\ control}$=gemittelte Absorptionsänderungen in Kontrollen).

Die in Kapitel 5 dargestellte Infektion von Schnecken mit dem Nematoden *P. hermaphrodita* erfolgte über direkte Infektion der Schnecken und zum Teil auch ergänzend über das Versetzen der Böden (Terrarienerde) der Versuchsgefäße mit Nematodensuspension. Die direkte Infektion der Schnecken wurde mittels Eintropfen der Nematodensuspension in die Schalenöffnungen individuell fixierter Schnecken erreicht. Zur Ermittlung der Mortalitätsraten wurden die Schnecken täglich mit einer stumpfen Nadel gereizt, ein Ausbleiben von Reaktionen auf den Reiz wurde als Proxy für Mortalität definiert. Die Hämolymphentnahme und die Analyse der Phenoloxidase-Aktivität erfolgten wie für Kapitel 4 beschrieben.

4. Ergebnisse und Diskussion

4.1 Kapitel 1: *Scheil, AE, Köhler, H-R, Triebskorn, R, 2011, Heat tolerance and recovery in Mediterranean land snails after pre-exposure in the field,* Journal of Molluscan Studies, *77 (2): 165- 174.*

Die Ergebnisse dieser Studie zeigen, dass *X. derbentina* im Gegensatz zu *T. pisana* zu einer gewissen, wenn auch nicht vollständigen, Erholung des Mitteldarmdrüsengewebes und der Hsp70-Antwort von natürlichem wie auch im Labor erzeugten Hitzestress innerhalb des Studienzeitraums (maximale Hitzebelastung von 8h, daran anschließend ein maximaler designierter Erholungszeitraum von 16h) befähigt ist. Es konnte auch gezeigt werden, dass *X. derbentina* im Vergleich zu *T. pisana* weniger stark durch Hitzestress beeinträchtigt wird. Bereits in den Vortests ergaben sich Hinweise auf eine höhere Toleranz von *X. derbentina* gegenüber erhöhten Temperaturen, da eine achtstündige Hitzeexposition bei 45°C als die höchste subletale Temperaturexposition für diese Art ermittelt werden konnte. Für *T. pisana* wurden 8h bei 43°C hingegen als höchstmögliche subletale Temperaturexposition festgestellt. Dies stimmt mit in vorhergehenden Studien ermittelten Werten überein (Dittbrenner et al., 2009; Köhler et al., 2009). Das vergleichsweise größere Regenerationssvermögen nach und die höhere Toleranz gegenüber Hitzestress bei *X. derbentina* ist wahrscheinlich u.a. auf die Fähigkeit dieser Art, vermehrt Kalkzellen im Mitteldarmdrüsengewebe bilden zu können (Hyperplasie) zurückzuführen. Des weiteren konnte eine Hypertrophie (Vergrößerung) dieser Kalkzellen bei Hitzebelastung beobachtet werden. Es ist bekannt, dass erhöhte Temperaturen zu einer Störung des Säure-Base-Haushalts bei Schnecken führen können (Barnhart, 1986), außerdem kann davon ausgegangen werden, dass die Tiere unter Hitzebelastung osmotischen Stress erfuhren, da die dabei auftretende Evaporation von Wasser zu einer Austrocknung

führen kann, bei der sich osmotisch aktive Substanzen wie z.B. Chlorid in der Extrapallialflüssigkeit anreichern (Arad, 2001). Weiterhin ist bekannt, dass die Kalkzellen der Mitteldarmdrüse eine große Rolle bei der Regulierung des Säure-Base-Haushalts und des osmotischen Gleichgewichts spielen (Burton, 1976; Taieb & Vicente, 1998). Die unter Hitzebelastung beobachtete Aktivität dieses Zelltyps (indiziert durch das Auftreten von Hyperplasie und Hypertrophie 2h bzw. 4h nach Hitzebelastung mit 45°C) deutet mit der ebenfalls nachgewiesenen Thermotoleranz bei *X. derbentina* darauf hin, dass die verstärkte Aktivität der Kalkzellen eine Strategie dieser Schneckenart darstellen kann, dem Hitzestress erfolgreich zu begegnen. Bei *T. pisana* konnte weder eine Hyperplasie noch eine Hypertrophie von Kalkzellen nachgewiesen werden, jedoch konnten schwerwiegende Anzeichen einer Übersäuerung auf zellulärer Ebene festgestellt werden. Dies unterstreicht, zusammen mit der vergleichsweise geringeren Thermotoleranz dieser Art und den Beobachtungen bei *X. derbentina*, die Bedeutung der Kalkzellen bei der Bewältigung von Hitzebelastung. Auch bezüglich der Induktion des Stressproteins Hsp70 durch Erwärmung unterschieden sich die beiden getesteten Arten voneinander. Hitzeschock- oder Stressproteine werden nicht nur durch erhöhte Temperaturen sondern auch von anderen proteotoxisch wirkenden Stressoren induziert (Sanders & Dyer, 1994). Auch Übersäuerungen (deren zelluläre Effekte, wie oben bereits erläutert, in der histopathologischen Analyse festgestellt werden konnten) können eine Induktion bewirken (Kregel, 2002). Dabei folgt die Hsp70-Antwort einer typischen Optimumskurve mit einer verstärkten Induktion bei ansteigender Stressbelastung (Kompensation) und anschließendem Abfall des Hsp70-Levels bei zu starker Belastung (Destruktion) (Eckwert et al., 1997). Auch bei *X. derbentina* konnte im zeitlichen Verlauf der Hitzebelastung zunächst eine verstärkte Induktion von Hsp70 beobachtet werden (Kompensation), im Weiteren kam es dann jedoch zu einem Absinken des Hsp70-Levels (Destruktion). Innerhalb des designierten Erholungszeitraums konnte jedoch eine erneute Induktion von Hsp70 bei dieser Art verzeichnet werden (erneute Kompensation bzw. Erholung). Bei *T. pisana* war in dieser Studie im Vergleich zu *X. derbentina* eine auffallend niedrigere Induktion von Hsp70 unter Hitzebelastung zu verzeichnen, auch konnte keine erneute Kompensation im designierten Erholungszeitraum festgestellt werden. Diese offenbar niedrige Reaktionskapazität des Stressproteinsystems lässt zusammen mit der Tatsache, dass auch bei Kontrolltieren der Art *T. pisana* Anzeichen für Übersäuerung auf zellulärer Ebene gefunden wurden, den Schluss zu, dass es möglich ist, dass *T. pisana* bereits im Feld eine Hitzebelastung erfahren hatte, von der innerhalb des Akklimatisationszeitraums keine ausreichende Erholung erfolgen konnte. Die Stressproteinantwort von *X. derbentina* erscheint

robuster und stellt wahrscheinlich einen weiteren Parameter, neben der Hypertrophie und Hyperplasie von Kalkzellen, dar, der die höhere Thermotoleranz dieser Art begründet.

4.2 Kapitel 2: *Scheil, AE, Scheil, V, Triebskorn, R, Capowiez, Y, Mazzia, C, and Köhler, H-R, 2012, Shell colouration and antioxidant defence capacity in* Theba pisana *(O.F. Müller, 1774),* Molluscan Research, *32 (3): 132-136.*

In dieser Studie konnte gezeigt werden, dass helle, ungebänderte und dunkle, gebänderte Morphen von *T. pisana* sowohl konstitutiv als auch nach Hitzebelastung bei 43°C über bis zu 8h (maximale subletale Hitzeexposition für diese Schneckenart (Dittbrenner et al., 2009; Köhler et al., 2009; Scheil et al. 2011)) keine signifikanten Unterschiede hinsichtlich ihrer Lipidperoxidations-Level aufweisen. Da u.a. die Menge an Lipidperoxidations-Produkten Aufschluß über die Kapazität eines Organismus' mit oxidativer Stressbelastung umzugehen gibt (Hermes-Lima et al., 1995), können die Ergebnisse die Hypothese einer besseren Abwehrkapazität gegen durch Hitzebelastung generierten oxidativen Stress bei dunkleren Individuen der Art *T. pisana* nicht bestätigen. Damit scheinen die Vermeidung oder Bekämpfung von oxidativem Stress über Radikalfänger wie beispielsweise Melanin (Riley, 1997), welches u.a. auch für die Färbung von Schneckenschalen verantwortlich ist (Comfort, 1951; Jones, 1973b), vermutlich keine Rolle als Selektionsfaktoren bei der Erhaltung des Schalenpolymorphismus bei *T. pisana* zu spielen. Darüber hinaus konnten bei beiden Morphen signifikant ansteigende Lipidperoxidations-Level bei ansteigender Hitzebelastung beobachtet werden. Somit untermauert die Studie die Eignung von Lipidperoxidations-Leveln als Biomarker für die Bestimmung des Stresszustands auch von terrestrischen Mollusken, so wie es z.B. auch von Jena et al. (2009) bereits für marine Spezies gezeigt worden ist.

4.3 Kapitel 3: *Scheil, AE, Gärtner, U, Köhler, H-R, 2012, Colour polymorphism and thermal capacities in* Theba pisana *(O.F. Müller 1774),* Journal of Thermal Biology, *37 (6): 462- 467.*

Die thermographische Untersuchung von hellen, ungebänderten und dunklen, gebänderten Schalenmorphen von *T. pisana* zeigte, dass keine bedeutsamen Unterschiede zwischen den beiden Morphen bezüglich ihrer thermischen Kapazität (Erwärmung der Schale, Abgabe von Wärme) feststellbar sind. Zwar konnte eine höhere Erwärmung im Zentrum der dunklen Schalenmorphen beobachtet werden, jedoch hatte dies zum einen keine Auswirkungen auf die Gesamterwärmung der Schalen, zum anderen fiel dieser Unterschied bei Betrachtung der absoluten Zahlenwerte sehr gering aus, womit die zu erwartenden Auswirkungen auf den Weichkörper des Tieres auch nur gering sind. Außerdem ergab eine Power Analysis (über

G*Power, Erdfelder et al., 1996), in der die Anzahl der Stichproben, welche für das Erreichen von möglicherweise signifikanten Unterschieden bezüglich der Gesamterwärmung erforderlich wäre, ermittelt wurde, dass die Stichprobenzahl pro Morphgruppe n=3000 überschreiten müßte. Sehr große n-Zahlen bergen jedoch wiederum die Gefahr, dass es zum Auftreten von sogenannten „trivial effects“ kommt (Quinn & Keough, 2003), d.h. dass geringfügigste Unterschiede als signifikant detektiert werden, welche jedoch effektiv bedeutungslos sind (Sachs, 2004). Erklärt werden kann das Fehlen von Unterschieden in der thermischen Kapazität verschiedener Morphen zum einen darüber, dass der visuelle Eindruck einer Farbe über die Reflektion von Teilen des sichtbaren Wellenlängenspektrums zustande kommt und diese *per se* wenig mit jenen Wellenlängen gemein haben müssen, welche für Erwärmung oder Abkühlung verantwortlich sind (Gunn, 1942). Zum anderen ist bekannt, dass die thermische Kapazität materialabhängig ist (Eichler et al., 2005; Heuberger & Fels, 2007). Da die Schalen pulmonater Landschnecken zu 97% aus Calciumcarbonat und nur zu 3% aus einer organischen Matrix, in die Pigmente eingelagert sind, bestehen (Heller & Magaritz, 1983), ist es plausibel, dass die thermische Kapazität des Calciumcarbonats überwiegt und der mögliche Einfluß von Farbpigmenten nur eine untergeordnete Rolle bei der Erwärmung und Abkühlung spielt. Die Ergebnisse der vorliegenden thermographischen Untersuchung unterschiedlicher Schalenmorphen implizieren darüber hinaus, dass die Hypothese einer durch unterschiedliche thermische Kapazitäten von verschiedenen Schalenmorphen bedingte Verteilung von Schnecken in verschiedenen Habitaten, wie sie durch einige frühere Studien aufgestellt worden ist (u.a. für *Cepaea sp.* Jones, 1973a; für *C. nemoralis* Heath, 1975; für *T. pisana* Heller & Gadot, 1984), kritisch überdacht werden sollte.

4.4 Kapitel 4: *Scheil, AE, Hilsmann, S, Triebskorn, R, and Köhler, H-R,* zur Veröffentlichung vorbereitet*, Shell colour polymorphism, injuries and immune defense in three helicid snail species*, Cepaea hortensis, Theba pisana *and* Cornu aspersum maximum.

Die Ergebnisse der diesem Kapitel zugrundeliegenden Versuche zeigten, dass in allen drei untersuchten Schneckenarten keine Unterschiede zwischen verschiedenen Schalenmorphen bezüglich der konstitutiven Phenoloxidase-Aktivität festzustellen sind. Diese Befunde widersprechen den Beobachtungen, welche z.B. für unterschiedliche Farbmorphen bei Insekten getätigt worden sind (Wilson et al., 2001; Armitage & Siva-Jothy, 2005). In diesen Arbeiten konnte eine positive Korrelation zwischen stärkerer Melanisierung der Kutikula, höherer konstitutiver Phenoloxidase-Aktivität und daraus resultierender höherer Pathogenresistenz festgestellt werden. Da Phenoloxidase jedoch auch cytotoxische

Eigenschaften besitzt (Ballarin et al., 1998) und des weiteren auch mit oxidativem Stress in Verbindung gebracht werden konnte (Kumar et al., 2003), erscheint eine Beschränkung auf Induktion einer höheren Phenoloxidase-Aktivität bei tatsächlichem Vorliegen von Pathogenen anstelle der Erhaltung eines prophylaktisch hohen Phenoloxidase-Levels biologisch sinnvoll.

Darüber hinaus wurde in der vorliegenden Arbeit beobachtet, dass Zymosan A im vorliegenden Testdesign bei allen drei untersuchten Heliciden-Arten und beiden getesteten Morphen (hell, ungebändert und dunkel, gebändert) nicht zu einer Induktion einer höheren Phenoloxidase-Aktivität führte. Dadurch bedingt konnten auch keine morphspezifischen Unterschiede in der Phenoloxidase-Induktion beobachtet werden. Das Ausbleiben der Phenoloxidase-Induktion ist eher ungewöhnlich, da eine Immunstimulation und Phenoloxidase-Induktion über β-1,3 Glucane, welche eine Hauptkomponente von Hefezellwänden, wie sie für Zymosan A verwendet werden, darstellen, bereits bei einer Reihe von Invertebraten (Cárdenas & Dankert, 1997; Vetvicka & Sima, 2004; Pang et al., 2010), darunter auch Mollusken (Coles & Pipe, 1994; Aladaileh et al., 2007; Hellio et al., 2007; Lacoue-Labarthe et al., 2009), erfolgreich durchgeführt worden ist. Tatsächlich wurde das Ausbleiben der Phenoloxidase-Induktion nach Applikation von Zymosan A auch schon beobachtet, jedoch war dies seltener der Fall und Erklärungen für dieses Phänomen sind eher spekulativer Natur (Arizza et al., 1995; Brivio et al., 1996).

Dennoch konnte in der vorliegenden Studie ein interessanter Unterschied zwischen hellen und dunklen Schalenmorphen bei allen drei getesteten Schneckenspezies festgestellt werden: Der Entzug einer definierten Hämolymphmenge führte bei allen Testarten innerhalb von 24h zu einer signifikanten Erniedrigung des Phenoloxidase-Levels bei hellen Morphen, jedoch nicht bei den dunklen Morphen. Da durch die Bestimmung der konstitutiven Phenoloxidase-Level eine bereits grundsätzlich höhere Phenoloxidase-Aktivität bei dunklen Morphen ausgeschlossen werden kann, kann davon ausgegangen werden, dass dieser Unterschied tatsächlich von der Hämolymphentnahme verursacht wird. Auch wenn die genauen Mechanismen, die diesem Phänomen zugrunde liegen, in dieser Arbeit noch nicht geklärt werden konnten, so kann doch festgehalten werden, dass dieser erstmals beobachtete Unterschied zwischen hellen und dunklen Morphen bei drei verschiedenen Heliciden-Arten eine Rolle bei Selektionsvorgängen spielen könnte. Es ist bekannt, dass Phenoloxidase u.a. auch bei der Sklerotisierung von Molluskenschalen eine wichtige Rolle spielt (Waite & Wilbur, 1976; Nellaiappan & Kalyani, 1989), sowie bei der Wundheilung (Ratcliffe et al., 1985), beide Vorgänge sind insbesondere nach Verletzungen, wie sie z.B. durch die Hämolymphentnahme erfolgt sind, und wie sie unter natürlichen Umständen auch durch

Räubereinwirkung usw. entstehen können, wichtig. Für Sklerotisierungs- und Wundheilungsvorgänge nach Verletzungen, sowie zur Abwehr möglicher Pathogene, welche nach Verletzungen der physikalischen Barrieren Schale und Haut leichter eindringen können (Gliński & Jarosz, 1997), steht dunklen Morphen also vergleichsweise mehr Phenoloxidaseaktivität zur Verfügung.

4.5 Kapitel 5: *Scheil, AE, Hilsmann, S, Triebskorn, R, and Köhler, H-R,* zur Veröffentlichung vorbereitet, *Shell colouration and parasite tolerance in two helicoid snail species* (Cepaea hortensis *and* Cernuella virgata).

In dieser Studie konnte festgestellt werden, dass zum einen Schnecken der Art *C. virgata* stärker durch eine Infektion mit *P. hermaphrodita* beeinträchtigt werden als die ebenfalls untersuchte Art *C. hortensis*. Dies äußerte sich in einer deutlich höheren Mortalitätsrate für *C. virgata* bereits nach Infektion mit vergleichsweise geringeren Nematodenkonzentrationen als sie bei *C. hortensis* appliziert wurden. Zum anderen konnte beobachtet werden, dass helle, ungebänderte Morphen der Art *C. virgata* eine deutlich höhere (90%) Mortalitätsrate nach Infektion mit *P. hermaphrodita* aufwiesen als dunkle, gebänderte Morphen (50%). Bei *C. hortensis* war der Unterschied zwischen den Morphgruppen sehr viel geringer ausgeprägt (30% für helle und 20% für dunkle Morphen); dieser Umstand ist wahrscheinlich auf die insgesamt höhere Toleranz dieser Art gegenüber *P. hermaphrodita* zurückzuführen. Die Ergebnisse der Phenoloxidase Assays zur Bestimmung der Phenoloxidase-Aktivität erlauben jedoch nur eine vage Aussage zum Zusammenhang dieser biochemischen Reaktion mit der bei *C. virgata* beobachteten morphspezifischen Toleranz. Zwar konnte in infizierten dunklen *C. virgata* eine tendenziell höhere Phenoloxidase-Aktivität an den meisten Testzeitpunkten beobachtet werden, jedoch ergaben post-hoc Tests zu keinem Zeitpunkt signifikante Unterschiede zwischen den getesteten Morphen. Bei *C. hortensis* konnte keine (innerhalb von 24h nach Infektion) bzw. nur eine geringe (innerhalb von 7d nach Infektion) Induktion einer höheren Phenoloxidase-Aktivität in mit *P. hermaphrodita* infizierten Tieren beobachtet werden, zudem waren weder hinsichtlich der konstitutiven Phenoloxidase-Aktivität noch hinsichtlich der nach Nematodeninfektion beobachteten Phenoloxidase-Aktivität Unterschiede zwischen den Morphen detektierbar. Für *C. hortensis* kann somit morphenunabhängig eine vergleichsweise hohe Toleranz gegenüber *P. hermaphrodita* festgestellt werden, welche jedoch nicht über eine erhöhte Phenoloxidase-Aktivität erklärt werden kann. Bei *C. virgata* kann die Hypothese einer höheren Toleranz von dunklen Morphen gegen Infektion mit *P. hermaphrodita* bestätigt werden. Die Hypothese, dass diese

Toleranz über eine erhöhte Phenoloxidase-Aktivität in dunklen Morphen im Vergleich zu hellen Morphen vermittelt wird, wird jedoch durch die Ergebnisse der Phenoloxidase Assays nicht gestützt.

Abschließende Betrachtungen

Die vorliegende Arbeit zeigt, dass die Thermotoleranz der beiden helicoiden Schneckenarten *X. derbentina* und *T. pisana* unterschiedlich hoch ausfällt, wobei *X. derbentina* die gegenüber höheren Temperaturen tolerantere Art darstellt. Begründet wird diese höhere Thermotoleranz u.a. von der Fähigkeit dieser Art, vermehrt und vergrößerte Kalkzellen in der Mitteldarmdrüse bilden zu können, dieser Zelltyp spielt eine wichtige Rolle bei Belastungen des Säure-Base-Haushalts, wie sie unter Hitzestress auftreten können (Burton, 1976; Barnhart, 1986; Taieb & Vicente, 1998). Auch der Besitz einer vergleichweise robusteren Hsp70-Antwort trägt wahrscheinlich zu der hohen Thermotoleranz bei, da Hitzeschockproteine eine wesentliche Rolle bei der Bewältigung von proteolytischem Stress, wie er z.B. durch Hitze bzw. deren Effekte ausgelöst werden kann, spielen (Sanders & Dyer, 1994; Kregel, 2002). Die Hypothese einer höheren Abwehrkapazität gegenüber durch Hitzebelastung verursachtem oxidativen Stress bei dunklen Schalenmorphen von *T. pisana* konnte durch die vorliegende Arbeit nicht bestätigt werden. Es ist daher nicht davon auszugehen, dass dunklere Schalenmorphen eine durch Melanin als Radikalfänger (Riley, 1997) verliehene höhere Toleranz gegenüber oxidativem Stress besitzen. In der vorliegenden Arbeit konnte des Weiteren die nach früheren Studien mit geringerem technischen Aufwand postulierte Annahme einer höheren Erwärmungskapazität von dunkleren Schalenmorphen (Jones, 1973a; Heath, 1975; Cook & Freeman, 1986) für die Art *T. pisana* stark relativiert werden. Diese Erkenntnis sollte in zukünftige Überlegungen zu Erklärungsmodellen für die Entstehung und Erhaltung des Schalenpolymorphismus bei Helicoideen mit einfließen. Die Hypothese eines thermisch bedingten Nachteils dunklerer, gebänderter Schneckenmorphen in wärmeren und / oder offeneren (weniger beschatteten) Habitaten und wärmeren Klimaten (Johnson, 1981; Hazel & Johnson, 1990; Johnson, 2011) sollte neu betrachtet werden. Dabei soll die z.B. von Johnson (2011) festgestellte Korrelation von Farbmorphverteilungen und Klimabedingungen nicht angezweifelt werden, jedoch sollten alternative Faktoren und Korrelationen in die Neubetrachtung miteinbezogen werden. So haben Morley & Lewis (2008) festgestellt, dass feuchtere Klimabedingungen das Auftreten von Parasiten in Schnecken begünstigen können. Eine Reihe weiterer Studien konnte zeigen, dass die Belastung mit parasitischen Nematoden in dunklen (gebänderten) helicoiden Schnecken deutlich geringer ausfällt als in

ungebänderten Individuen (Cabaret, 1983; Cabaret, 1988; Lahmar et al., 1990). In der vorliegenden Arbeit konnte die Hypothese einer Verknüpfung von dunklerer Schalenfärbung und höherer Toleranz gegenüber Nematodeninfektion bestätigt und durch die Beobachtung einer höheren Überlebensfähigkeit der dunklen Morphen für *C. virgata* ergänzt werden. Diese Toleranz und diese Überlebensfähigkeit stellen möglicherweise bisher nicht berücksichtigte Faktoren dar, welche die Morphenverteilungen von Helicoideen beeinflussen. Unterschiede in der Phenoloxidase-Aktivität der verschiedenen Morphen nach Nematodeninfektion konnten jedoch nicht gemessen werden - somit kann die beobachtete Toleranz nicht auf diesen Parameter zurückgeführt werden. Es ist jedoch plausibel, dass die sowohl bereits in früheren Studien beobachtete niedrigere Nematodeninfektionsrate von dunklen Morphen bestimmter Helicoidea, darunter auch *T. pisana* und *C. virgata* (Cabaret, 1983; Cabaret, 1988; Lahmar et al., 1990), und die in der vorliegenden Arbeit nach Nematodeninfektion beobachtete höhere Überlebensfähigkeit von dunklen Morphen der Art *C. virgata* in feuchteren Habitaten vorteilhaft sein könnte. Nach der Köppen-Geiger-Klima-Klassifizierung (Kottek et al., 2006) ist der mediterrane Raum als deutlich trockener einzustufen, als beispielsweise Mitteleuropa oder die britischen Inseln. Die Tatsache, dass sich Feuchtigkeit unter Vegetation länger erhält als in offenem Terrain ist allgemein bekannt. Somit wäre also eine Beeinflussung der z.B. für die Helicoide *T. pisana* bekannten Morphenverteilung, bei welcher die dunkleren Morphen tendenziell häufiger in nördlicheren (und feuchteren) Regionen des artspezifischen Verteilungsgebiets vorkommen und hellere Morphen im mediterranen Raum stärker vertreten (Cowie, 1984a) bzw. häufiger in offenerem Terrain zu finden sind (Hazel & Johnson, 1990), wie auch der für *C. virgata* in bestimmten Habitaten bekannten Verteilung von Schalenmorphen (höhere Prävalenz von dunklen Morphen in dichteren Straßenrandvegetationen; Baker, 1988) über die unterschiedlichen Feuchtigkeitsverhältnisse und den daraus resultierenden unterschiedlichen Parasitendruck vorstellbar.

Des Weiteren konnte in der vorliegenden Arbeit die Hypothese einer Verknüpfung von Melanisierung (Schalenfärbung) und Voraussetzungen zu erfolgreicher Wundheilung (vermittelt über Phenoloxidase) für drei Heliciden-Arten, *T. pisana*, *C. hortensis* und *C. aspersum maximum*, bestätigt werden. Dabei ist wahrscheinlich davon auszugehen, dass dunklere Schalenmorphen bei Verletzungen und Hämolymphverlust einen selektiven Vorteil besitzen, da ihnen vergleichsweise mehr Phenoloxidase-Aktivität zur Verfügung steht, welche eine Rolle bei Sklerotisierungs- und Wundheilungsprozessen spielt (Waite & Wilbur, 1976; Ratcliffe et al., 1985; Nellaiappan & Kalyani, 1989). Eine Verknüpfung von Schalenfärbung

und Phenoloxidase-Induktionsfähigkeit nach Immunstimulation durch Zymosan A konnte indes aufgrund ausbleibender Phenoloxidase-Induktion nicht beobachtet werden.

5. Literatur

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Kapitel 5: *Scheil, AE, Hilsmann, S, Triebskorn, R, and Köhler, H-R,* zur Veröffentlichung vorbereitet, *Shell colouration and parasite resistance in two helicoid snail species (*Cepaea hortensis *and* Cernuella virgata*).*

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Kapitel 1: Heat tolerance and recovery in Mediterranean land snails after pre-exposure in the field

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Abstract

Mediterranean land snails such as *Xeropicta derbentina* (Hygromiidae) and *Theba pisana* (Helicidae) are known for their remarkable tolerance to elevated temperatures and desiccation, yet the biochemical and cellular mechanisms underlying this tolerance are relatively unknown. We investigated the effects of increased temperatures and the ability of these snail species to recover from heat stress, examining the condition and amount of different types of hepatopancreatic cells (histopathology) and heat-shock protein 70 (stress protein) levels. Snails were exposed to defined control and increased temperatures and allowed a postheat phase for possible recovery. Results indicate the ability of *X. derbentina* to recover from natural and experimental temperature stress to some extent within the set time, whereas no such ability was found in *T. pisana*. *Xeropicta derbentina* is more heat-tolerant in general and less affected by heat stress than *T. pisana*. This is probably due to its greater ability to increase size and number of hepatopancreatic calcium cells that are essential for osmoregulation. The hypertrophy and hyperplasia of calcium cells can be regarded as a general response of molluscs to osmotic stressors, e.g. heat, as long as the organism is not overwhelmed by the stressor. Additionally, it can be assumed that the robust stress-protein system of *X. derbentina* plays a crucial role in this species' high thermotolerance.

Introduction

Terrestrial snails are animals which usually are not associated with tolerating hot and dry habitats, especially since desiccation is regarded as a severe threat to these organisms due to their water-permeable skin (Machin, 1964). However, it has been known at least since the 1970s that there are quite a number of snails living even in hot and dry deserts (Schmidt-Nielsen et al., 1971). From these times, there are studies describing the heat tolerance and associated behavioural adaptations of distinct snail species (Pomeroy, 1968; Yom-Tov, 1971; McQuaid et al., 1979). Studies on the physiological, cellular and molecular mechanisms underlying this thermotolerance are sparse, though, and one of the crucial aspects furthering such thermotolerance, namely recovery of molecular or cellular stress response systems, has not been investigated yet in terrestrial snails. The Mediterranean, being characterised by a subtropic climate with dry summers demanding special adaptations of animals to heat and desiccation (Blondel & Aronson, 1999), is home to the test organisms of the present study, *Xeropicta derbentina* (Krynicki, 1836) and *Theba pisana* (Müller, 1774). These snails, being highly adapted to their habitat, therefore qualify ideally for studies on snail thermotolerance, and have already proven their suitability for such tests (Dittbrenner et al., 2009; Köhler et al., 2009). In this study, their heat tolerance and ability to recover from heat stress were investigated using Heat shock protein 70 (Hsp70) analysis and qualitative and semi-quantitative histopathology of the hepatopancreas. The hepatopancreas plays a central role in the metabolism of snails (Storch & Welsch, 2004). It is crucial for osmoregulation and regulating the acid-base balance (Burton, 1976; Taieb & Vicente, 1998) which both can be affected by heat stress (Barnhart, 1986; Ryan & Gisolfi, 1995; Michaelidis et al., 1999; Arad, 2001). Therefore, it is the ideal monitor organ in conducting heat tolerance experiments. Particularly the number and condition of hepatopancreatic calcium cells are useful parameters for the analysis of heat tolerance in snails (Dittbrenner et al., 2009).

Even though heat shock or stress proteins are induced not only as a consequence of heat stress but also due to other proteotoxic stressors (Sanders & Dyer, 1994), it was their increased expression after heat exposure they were discovered for and named after (Ritossa, 1962; Tissiéres, 1974). Hence, stress proteins are ideal biomarkers for investigating the effects of heat stress, and, in case of Hsp70, were successfully used in heat tolerance experiments with Mediterranean land snails (Köhler et al., 2009). The combination of structural (histopathology) and biochemical (Hsp70-analysis) markers counterbalances the advantages and disadvantages of these parameters concerning sensitivity, variability and response

kinetics, and renders a comprehensive illustration of a stressed organism's condition possible (Adams et al., 2001; Triebskorn et al., 2001; Köhler & Triebskorn, 2004).

In our study, we wanted to investigate the heat tolerance and recovery capacity of snails after pre-exposure in the field. Therefore we chose to sample animals in midsummer, as by that time snails have experienced weeks or even months of extreme temperature in their natural habitat. We then wanted to analyse the histopathological status and stress protein response of snails offered a two-week acclimatisation phase under controlled laboratory conditions only (´controls´) and snails exposed to artificially induced heat stress followed by a "normally"-tempered post-heat phase (´heat-exposure-group´) in order to answer the following questions: Is recovery possible after pre-exposure to extreme temperatures in the field? Do *Xeropicta derbentina* and *Theba pisana* have different strategies to cope with elevated temperatures and, if so, what are these strategies? And, finally, are these snails able to recover from artificially induced heat stress under laboratory conditions?

Materials & Methods

Test animals, sampling and laboratory maintenance

Animals of two different species were collected in Southern France (Vaucluse) in the first week of July 2007: *Xeropicta derbentina* (Krynicki, 1836) was sampled from a site in La-Roque-sur-Pernes (43° 59' N, 5° 6.5' E) at 10.45am, the ground temperature was 20.4°C and the air temperature 1m above ground was 21.1°C. *Theba pisana* (Müller, 1774) was sampled between L'Isle-sur-la-Sorgue and Fontaine-de-Vaucluse (43° 55.5' N, 5° 6' E) at 11.30am when the ground temperature was 27.1°C and the air temperature (1m above ground) was 24.6°C. Both sampling sites are dry, open and sun-exposed habitats and resemble each other concerning structure and vegetation. In the Vaucluse (Avignon), the temperatures (average maximums) range from 18°C to 30°C in July and August and from 2°C to 10°C in January (http://meteo.msn.com/). A daily maximum (air temperature) of more than 40°C in the Vaucluse could be recorded (data provided by AgroClim, INRA Avignon, France) and it has been shown that the body temperature of snails in this region can exceed 40°C (Köhler et al., 2007).

Species identity of the test animals was determined by Dr. Wolfgang Rähle, University of Tübingen, Germany, according to morphological criteria. Laboratory maintenance was as follows: Sorted by species, animals were kept in plastic containers (24.2 x 20.7 x 6.4cm) laid-out with moistened paper towels and cleaned every other day. The humidity within the containers ranged from 80% directly after cleaning to 45% the following day. Also, every

other day snails were fed a standardized diet of organic carrots *ad libitum*. All animals were allowed a two week acclimatisation phase before starting experiments whereas the ambient (laboratory) temperature was 24±1°C.

Experimental set up and conditions

The temperature experiments were conducted in heating cabinets (*noctua IH50* / *Lovibond ET619-4*) implanting the plastic containers used for maintenance. Snails were divided in groups and either exposed to a control temperature (24.7°C) or the highest respective sublethal temperature (8h at 45°C for *X. derbentina*, 8h at 43°C for *T. pisana*). The chosen sublethal temperatures reflect the results of pre-tests (data not shown) and previous experiments with specimens of these snail populations (Dittbrenner et al. 2009; Köhler et al. 2009, Lazzara, 2007). During the 8h exposure time, respective 10 replicate individuals from both control and heat exposure were sampled independently for both methods used, histopathology and stress protein analysis, at 0h, 0.5h, 2h, 4h and 8h. After 8h, heat-exposed snails and controls underwent a "post-heat phase" for another 16h at 24.7°C. Within these 16h, additional samples were taken as described above at 10h (8h heat plus 2h post-heat), 16h (8h heat plus 8h post-heat) and 24h (8h heat plus 16h post-heat).

As a proxy for mortality the possible immobility of each snail was determined by poking the soft body with a blunt needle.

Histopathology

Following shell removal snails were fixed in 2% glutardialdehyde dissolved in 0.01M cacodylate buffer at 4°C for at least one week, and then decalcified using a 1:2 mixture of concentrated formic acid and ethanol (70%) overnight. Samples were subsequently dehydrated in a graded series of ethanol and embedded in *Histowax* (paraffin).Thin tissue sections (7µm) of the hepatopancreas were obtained using a *Leitz* microtome, spread on albumized microscope-slides, stained by haematoxylin-eosin (HE) and examined using light microscopy (*Zeiss Axioskop 2*).

The condition of hepatopancreatic cells (calcium cells, digestive cells and excretory cells), the shape of the tubules and their integrity was qualitatively and semi-quantitatively assessed on the basis of histopathological symptoms according to Dittbrenner et al. (2009) and Triebskorn & Köhler (2003) using five different categories reflecting the status of histopathological damage: category 1 was control status, category 3 was status of cellular reaction and category 5 was status of cellular destruction, whereas categories 2 and 4 represented intermediate

stages between categories 1 and 3, or 3 and 5, respectively. Results from ten randomly chosen tubules per snail were fused and means were calculated to express the overall condition of the respective cell type or tubular shape / integrity ("tubules" in the following) in the particular animal. Furthermore, the "general" condition of the entire hepatopancreas was assessed for each individual: Subsequently, all individual assessment values for the ten individuals per time point and exposure group were averaged to result in the mean assessment value (MAV) for each cell type and the general condition of the hepatopancreas, respectively. The means were taken from these results for each cell type and appearance of tubules of each snail resulting in the mean assessment value (MAV) which expresses the status of the respective exposure group.

To determine the proportion of distinctive cell types in the hepatopancreatic tubules the number of respective cells in 10 randomly chosen tubules per animal were counted and their surface ratio (proportion of respective cell type related to the entire tubule area) was estimated and compared with the countings.

Stress protein (Hsp70-) analysis

Hsp70-analysis was conducted according to Köhler et al. (2009): Snails were individually shock-frozen in liquid nitrogen (-196°C) and stored at –80°C until further sample processing. Animals were then homogenized on ice in 300-1000µL (depending on snail size) of extraction buffer (80mM potassium acetate, 5mM magnesium acetate, 20mM Hepes) and centrifuged for 10 minutes at 20000g and 4°C. The total protein content in each sample was determined according to the method of Bradford (1976). Using minigel SDS-PAGE (12% acrylamide, 0.12% bisacrylamide (w/v), 15min at 80V, 90min at 120V) constant protein contents (40µg) were separated and then transferred to nitrocellulose membranes by semi-dry blotting. Filters were blocked in a 1:2 mixture of horse serum and TBS (50mM Tris ph 5.7, 150mM NaCl) for one hour at room temperature. After washing in TBS (5min.) filters were incubated in the first antibody solution (mouse anti-human Hsp70, *Dianova*, Hamburg, Germany, in a 1:5000 dilution in 10% horse serum in TBS) on a lab shaker at room temperature overnight. After further washing in TBS filters were incubated in the second antibody solution (goat anti-mouse IgG conjugated to peroxidase, *Jackson Immunoresearch*, West Grove, PA, USA, dilution 1:1000 in 10% horse serum / TBS) on the shaker at room temperature for 2h. Following the next washing in TBS, the antibody-complex was detected by staining with 1mM 4-chloro(1)naphtol and 0.015% H_2O_2 in 30mM Tris pH 8.5 and 6% methanol. As in Köhler et al. (2009), quantification of resulting protein bands was conducted using the

densitometric image analysis system *E.A.S.Y. Win 32* of *Herolab*, Wiesloch, Germany. The applied standard sample (total homogenate of *Cepaea hortensis*) was identical to the standard sample used in the study of Köhler et al. (2009) facilitating comparison of results.

Statistical analysis

Histopathological data were statistically analysed using JMP® 4.0. As distribution was not normal (according to Shapiro-Wilks-test) for histological results of both populations as well as for Hsp70-analysis results for the *Theba*-population, significant differences were detected using the nonparametric Wilcoxon U-test. Data obtained from the Hsp70- analysis of the *Xeropicta*- population followed normal distribution (Shapiro-Wilks-test) and were analysed for significant differences using the Mann-Whitney-U-test (implanting JMP® 4.0). For all results the levels of significance were set to: $0.01<P\leq0.05$: * (slightly significant); $0.001<P\leq0.01$: ** (significant); $P\leq0.001$: *** (highly significant).

Results

General observations

Both snail species retracted into their shells at least 1h after the beginning of the exposure experiments. However, they did not form epiphragms. No mortalities were recorded during the experiments.

XEROPICTA DERBENTINA

Histopathology- Qualitative assessment of X. derbentina

Control

The control group individuals of the *Xeropicta*-population showed hepatopancreatic tubules with relatively smooth bases, narrow lumina and only slightly convex apices (Fig. 1a).

The analysis of the digestive cells of these individuals revealed a dense cytoplasm as well as minor irregularities concerning vacuolisation and compartmentalisation (dark vesicles and granules at the apices). An increased appearance of convex and in few cases also ruptured apices could be detected at the end of the test period. Calcium cells of controls (0.5h – 24h) often showed enlarged and apically situated nuclei which were found to be stained darkly blue indicating a low pH. The cytoplasm appeared moderately dense (Fig. 1b). Few calcium cells

showed ruptured plasma membranes after 16h. Regarding excretory cells, which were moderately filled with brown to greenish granules, no irregularities could be detected.

Heat-exposure and post-heat-phase

Tubules of animals from the temperature exposure groups of the *Xeropicta*- population resembled those of the controls. The overall number of partly ruptured cell apices appeared to be increased, and cellular debris could be found in the lumen in isolated cases (Fig. 1c). Particularly at 10h (2h post-heat phase) and 24h (16h post-heat phase) the integrity of tubules deteriorated due to increased appearance of ruptured apices and dilated lumina, and in few cases even total disintegrations of tubules occurred (Fig. 1d). The digestive cells revealed convex or partly ruptured apices and irregularly shaped and enlarged nuclei. As vesicles and granules increasingly occurred at the apical areas of these cells over test time, the compartmentalisation of this cell type deteriorated. The density of the cytoplasm decreased during the test, however green and brown granules could be detected in the cytoplasm at all time points.

The condition of the calcium cells deteriorated over test time due to disturbed compartmentalisation (indicated by apically situated nuclei) and lysis of membranes (Fig. 1e). As in the controls, nuclei were stained blue indicating a low (acid) pH . The cytoplasm of these cells was moderately dense at the beginning and continued to fade over test time.

Excretory cells in individuals of these groups showed few brown granules at the beginning, however the occurence of these granules slightly increased over test time.

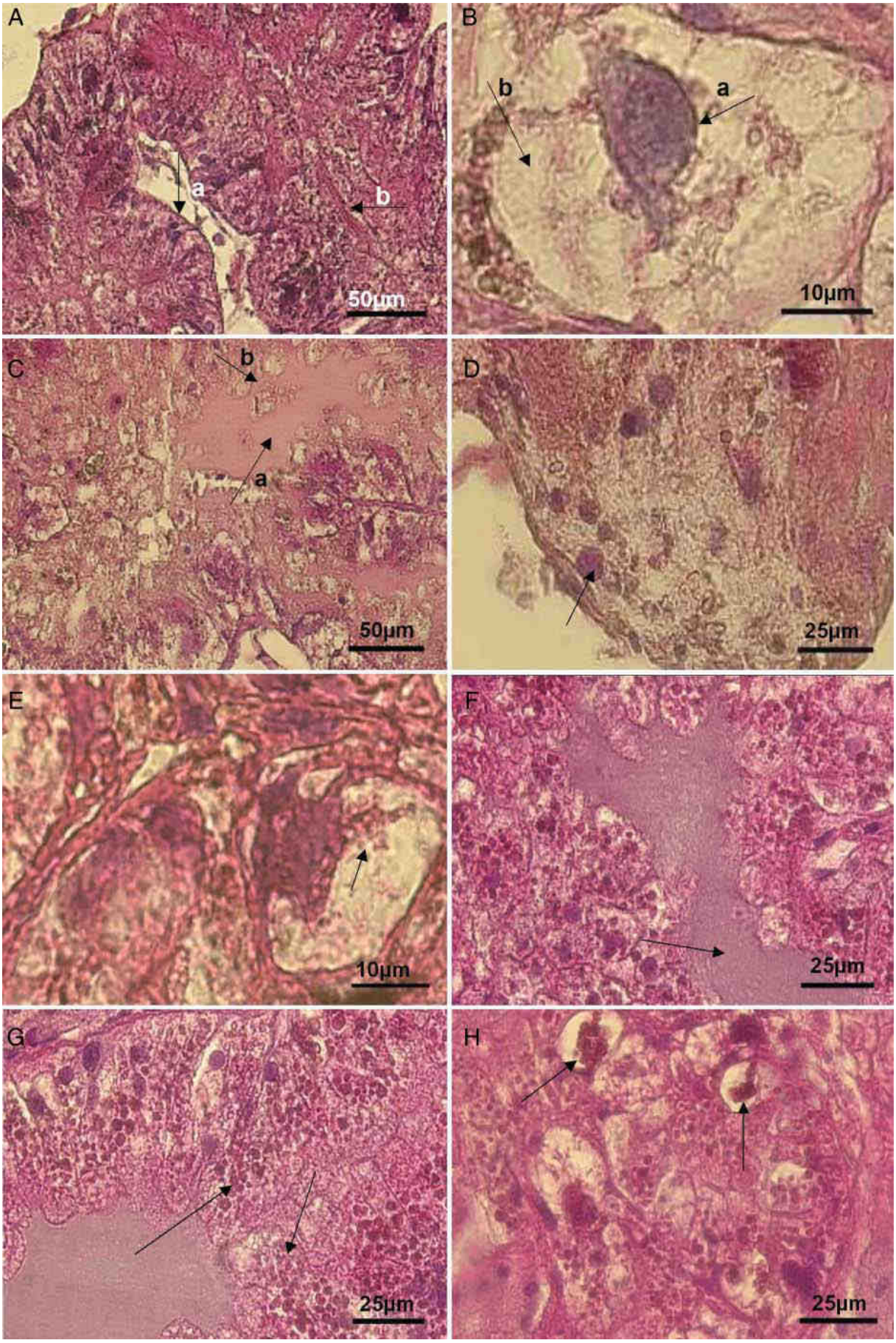
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Fig. 1:
a: Tubules of a control animal of the *X. derbentina*-population at 0h with smooth bases (arrow a) and narrow lumina (arrow b).
b: Calcium cell of a control animal of the *X. derbentina*-population at 24h. a) indicates the strong blue stain of the nucleus, b) shows the sparse cytoplasm.
c: Tubules of a heat-exposed animal of the *X. derbentina*-population at 16h. a) indicates a widened lumen and b) displays immigrating cells.
d: Detail of a tubule of a heat-exposed animal of the *X. derbentina*-population at 24h. The picture shows total disintegration of a tubule (vanishing cell barriers), the arrow points at a strongly blue-stained cell nucleus.
e: Calcium cell of a heat-exposed animal of the *X. derbentina*-population at 10h showing the beginning of lysis of the nucleus' membrane (arrow).
f: Detail of a tubule of a control animal of the *T. pisana*-population at 8h. The lumen is strongly blue-stained (arrow) and of moderate width.
g: Detail of a tubule of a control animal of the *T. pisana*-population at 2h showing dark, large and apically situated vesicles in the digestive cells (arrows).
h: Detail of a tubule of a heat-exposed animal of the *T. pisana*-population at 0.5h. Arrows point at strongly filled excretory cells.

Histopathology- Semi-quantitative assessment of X. derbentina

Control, heat-exposure and post-heat-phase

Mean assessment value (MAV) - Overall integrity

Concerning the overall integrity of the hepatopancreas in *X. derbentina* slightly significant differences could be detected between control and heat-exposure group from 4h exposure onwards (Fig. 2a). Within the heat-exposure group all mean assessment values (MAVs) obtained for t≥4h were highly significantly higher than the initial value (0h). Whereas the mean value for the control group varied around 3, values within the heat-exposure group varied from 3.44 (lowest) at 0h and 4.5 (highest) at 24h (Fig. 2a).

Tubules (tubular shape & integrity)

From 4h onwards until the end of test time values for the condition of the tubules differed either highly significantly or slightly significantly between control and heat-exposure group whereas MAVs for the heat-exposure group were higher (Fig. 2b).

Digestive cells

Regarding the condition of the digestive cells significantly higher values could be found in the heat-exposure group compared to the control group at 8h and 24h (Fig. 2c). For both groups, control and heat-exposure, values increased with test time (Fig. 2c).

Calcium cells

Significant differences between control and heat-exposure group occurred at 24h only where the heat-exposure group revealed a higher value (Fig. 2d). Within the heat-exposure-group values increased over test time as well (Fig. 2d).

Excretory cells

No significanct differences were found regarding the condition of excretory cells within and between control and heat-exposure group.

Surface ratio of cell types & calcium cell countings

The analysis of the ratio of digestive and excretory cells' surface in the control group revealed no relevant differences. The surface ratio of calcium cells in this group was slightly significantly different at 10h versus 0h and 8h (Fig. 3a).

The surface ratio of the digestive cells in the heat-exposure group first decreased but then reached the starting-level again at 24h (Fig.3b). The ratio of calcium cells and excretory cells revealed only minor differences throughout the test (Fig. 3a+b). When comparing the control with the heat-exposure group significant differences were found at 10h for calcium cells (Fig. 3a).

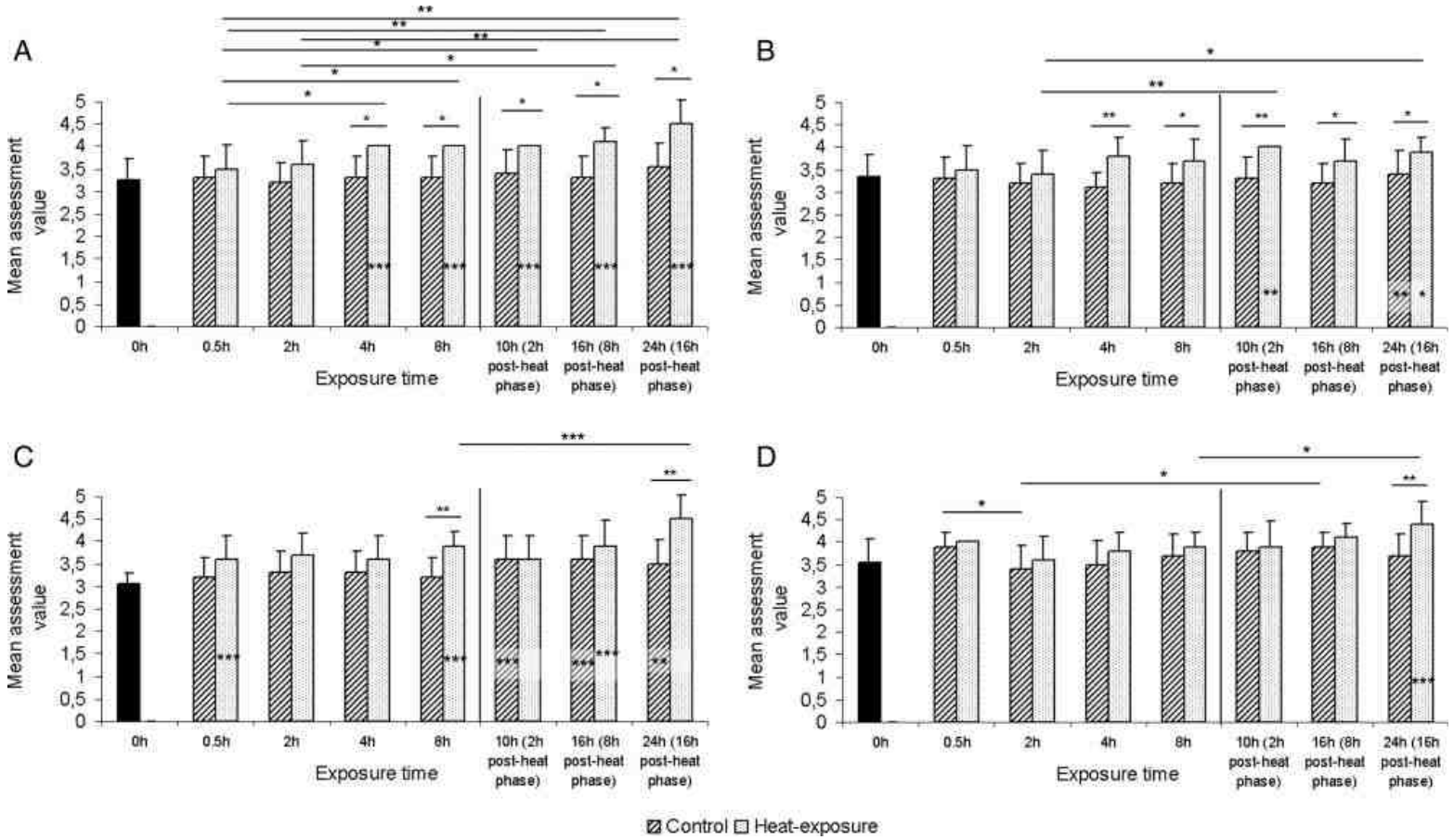

Fig. 2: Results for the histopathological analysis of control group versus heat-exposure group of the *X. derbentina*-population (mean + sd; n=10 except for 0h (t0): n=20). Significant differences are indicated by asterisks (0.01<P≤0.05: * (slightly significant); 0.001<P≤0.01: ** (significant); P≤0.001: *** (highly significant)). Significances versus 0h are shown in the respective bars.
a: Mean assessment values for the overall condition of the hepatopancreas.
b: Mean assessment values for the tubules' condition.
c: Mean assessment values for the digestive cells' condition.
d: Mean assessment values for the calcium cells' condition.

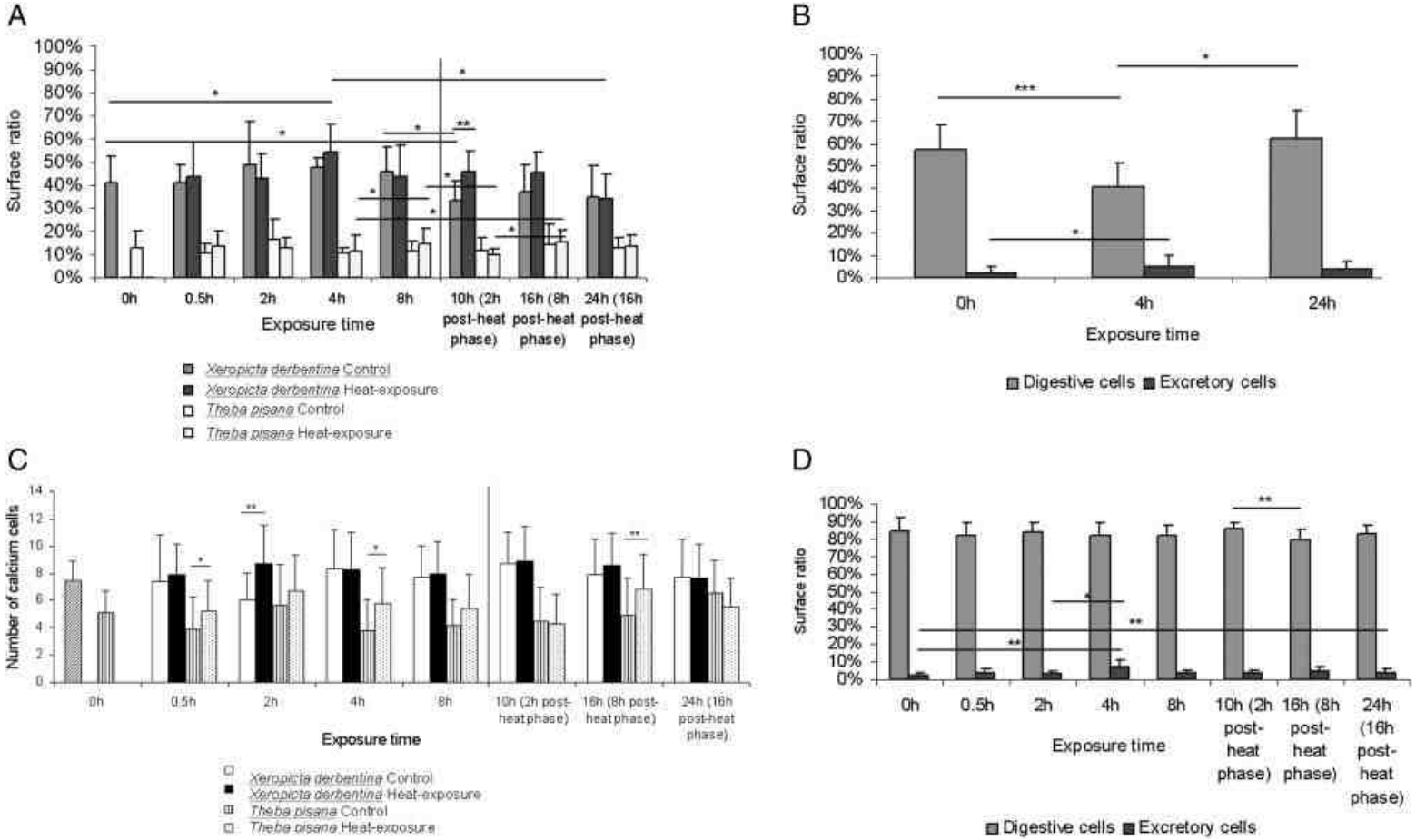
A
Surface ratio
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
0h
0.5h
2h
4h
8h
10h (2h post-heat phase)
16h (8h post-heat phase)
24h (16h post-heat phase)
Exposure time
Xeropicta derbentina Control
Xeropicta derbentina Heat-exposure
Theba pisana Control
Theba pisana Heat-exposure
B
Surface ratio
0h
4h
24h
Exposure time
Digestive cells
Excretory cells
C
Number of calcium cells
0h
0.5h
2h
4h
8h
10h (2h post-heat phase)
16h (8h post-heat phase)
24h (16h post-heat phase)
Exposure time
Xeropicta derbentina Control
Xeropicta derbentina Heat-exposure
Theba pisana Control
Theba pisana Heat-exposure
D
Surface ratio
0h
0.5h
2h
4h
8h
10h (2h post-heat phase)
16h (8h post-heat phase)
24h (16h post-heat phase)
Exposure time
Digestive cells
Excretory cells

Fig. 3
a: Surface ratio of calcium cells within tubules of control groups and heat-exposed groups of *Xeropicta derbentina* and *Theba pisana* at different exposure times (mean & sd; n=10, except for 0h: n=20). Significant differences are indicated by asterisks in the graph (0.01<P≤0.05: * (slightly significant); 0.001<P≤0.01: ** (significant); P≤0.001: *** (highly significant)).
b: Surface ratios of digestive and excretory cells within the heat-exposure group of the *X. derbentina*-population (mean + sd; n=10 except for 0h (t0): n=20) at 0h, 4h and 24h. Significant differences are indicated by asterisks (0.01<P≤0.05: * (slightly significant); 0.001<P≤0.01: ** (significant); P≤0.001: *** (highly significant)). Digestive cells also differed slightly significantly at 0h vs. 10h and 2h vs. 4h. Excretory cells also differed slightly significantly at 0h vs. 8h and 10h.
c: Number of calcium cells within tubules of control-groups and heat-exposed groups of *Xeropicta derbentina* and *Theba pisana* at different exposure times (mean & sd; n=10, except for 0h: n=20)). Significant differences between results within the populations are indicated by asterisks in the graph (0.01<P≤0.05: * (slightly significant); 0.001<P≤0.01: ** (significant); P≤0.001: *** (highly significant).
The comparison of results of the control-groups of the different populations reveals highly significant differences at all times (** for 16h / * for 24h, respectively) except for 2h. Comparison of heat-exposure-groups of the different populations resulted in slightly significant differences at 0h, 2h, 4h and 16h whereas results at 0.5h and 24h were significantly different and results at 8h and 10h differed in a highly significant way.
d: Surface ratios of digestive and excretory cells within the heat-exposure group of the *T. pisana*-population (mean + sd; n=10 except for 0h (t0): n=20). Significant differences are indicated by asterisks (0.01<P≤0.05: * (slightly significant); 0.001<P≤0.01: ** (significant); P≤0.001: *** (highly significant))

Stress protein (Hsp70-) analysis of X. derbentina

In all immunoblots one Hsp70 band was identified for each sample.

Control, heat-exposure and post-heat-phase

In the control group of *X. derbentina* the Hsp70-level was elevated in a highly significant way at 4h compared to all other test points of this group. No further significant differences occurred in the control group (Fig. 4a).

Regarding the heat-exposure group of this population the Hsp70-level rose in a highly significant manner (vs. 0h) as early as 0.5h after beginning of the test and showed a peak at 2h. The Hsp70-level then decreased until it reached the base level again at 8h and 10h, however increased once more reaching a second significant peak after 8h of recovery (at hour 16). At the end of test time (24h) the Hsp70-level reached the base level again (Fig. 4a).

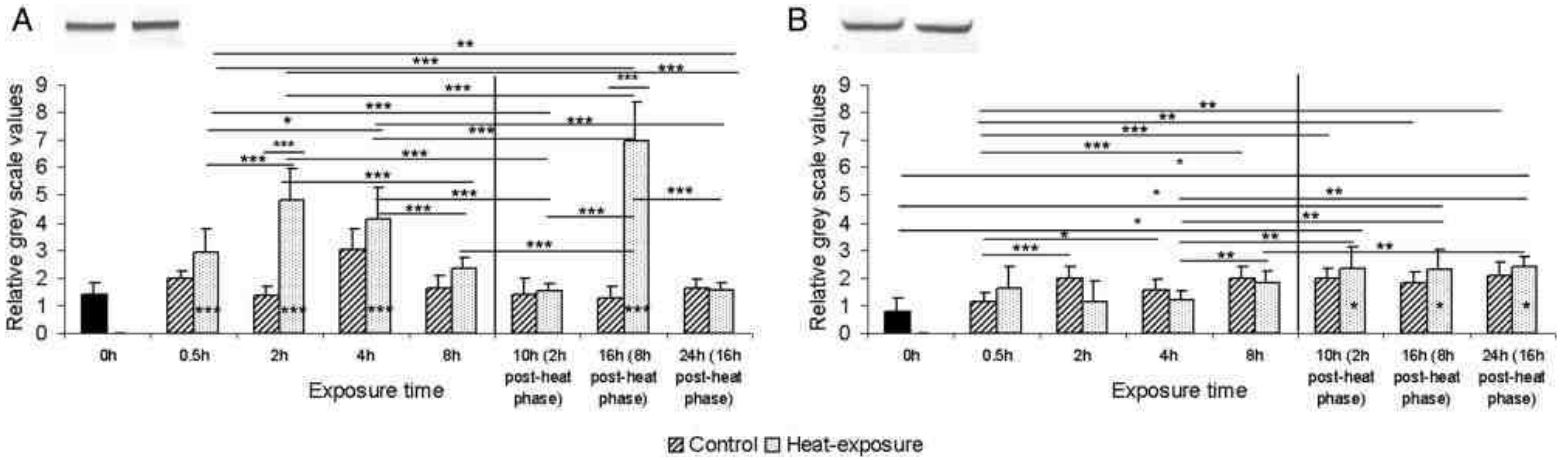

Fig.4: Results of the Hsp70- analysis, mean + sd; n=10 except for 0h (t0): n=20). Significant differences are indicated by asterisks (0.01<P≤0.05: * (slightly significant); 0.001<P≤0.01: ** (significant); P≤0.001: *** (highly significant)). Significances against 0h are shown within the respective bars.
a: Hsp70 levels of control group versus heat-exposure group of *X. derbentina*, shown with representative immunoblots of two samples. Significances within the control group were restricted to 4h being highly significant to all time points (significant to 0.5h).
b: Hsp70 levels of control group versus heat-exposure group of *T. pisana*, shown with representative immunoblots of two samples.

Histopathology- Qualitative assessment of T. pisana

Control

Tubules of *T. pisana* from the control group were characterized by smooth bases, mostly convex apices which appeared to be ruptured in few cases, and lumina of moderate width. The most distinctive feature in nearly all tubules of these animals was a blue stain of the lumina (Fig. 1f) indicating a low (acid) pH.

The compartmentalisation of digestive cells in these groups appeared to be irregular from the beginning of the test due to the appearance of large, dark vesicles in the apices (Fig. 1g). The nuclei of these cells were in good condition at the beginning of the test, however deteriorated at later time points as their shape became slightly irregular. Apices were mostly convex and in few cases ruptured. Vacuoles were very dark, and the cytoplasm was of moderate density.

The calcium cells of these groups were of regular shape, however their nuclei appeared to be caryolytic in most cases and the nuclei were situated in the apical areas of the cells. The cytoplasm was fading.

From the beginning of the experiment the excretory cells were strongly filled with dark granules slightly increasing in number after 4h onwards.

Heat-exposure and post-heat phase

The analysis of hepatopancreatic cells and tubules of the exposure groups of *T. pisana* revealed results similar to the controls. Additionally, the integrity of tubules particularly deteriorated during the post-heat phase (10h to 24h) compared to 0h (t0) due to a intensified dilation of lumina, increased numbers of ruptured apices and total disintegration of structures in some cases. The strong filling of excretory cells became more pronounced during the post-heat phase (Fig. 1h).

***Histopathology- Semi-quantitative assessment of* T. pisana**

Control, heat-exposure and post-heat-phase

Mean assessment value (MAV)- Overall integrity

For the overall integrity of the hepatopancreas in the control group of the *T. pisana* population differences could be detected when comparing 0h and 8h versus post-heat phase values and within the post-heat phase (Fig. 5a).

Within the heat-exposure group values increased within the post-heat phase (Fig. 5a).

The comparison of control and heat-exposure group revealed only a few minor differences (Fig. 5a). The highest value, 4.1, was found in the heat-exposure group at 10h whereas the lowest values, 3.1, occured in the control group at 0.5h and 8h (Fig. 5a).

Tubules (tubular shape & integrity)

No significant differences occured within the control group regarding the shape and condition of tubules (Fig. 5b). However, in the heat-exposure group, values increased within the post-heat-phase (Fig. 5b). The analysis of control versus heat-exposure group revealed one slightly significant difference only (Fig. 5b).

Digestive cells

Regarding the condition of digestive cells the control group exhibited few differences within test time (Fig. 5c). Within the heat-exposure group values first decreased until 8h, then reached the base-level again during the post-heat-phase (Fig. 5c). Comparing control and heat-exposure group, only a single slightly significant difference was found (Fig. 5c).

Calcium cells

The condition of calcium cells did not differ within or between control and heat-exposure groups.

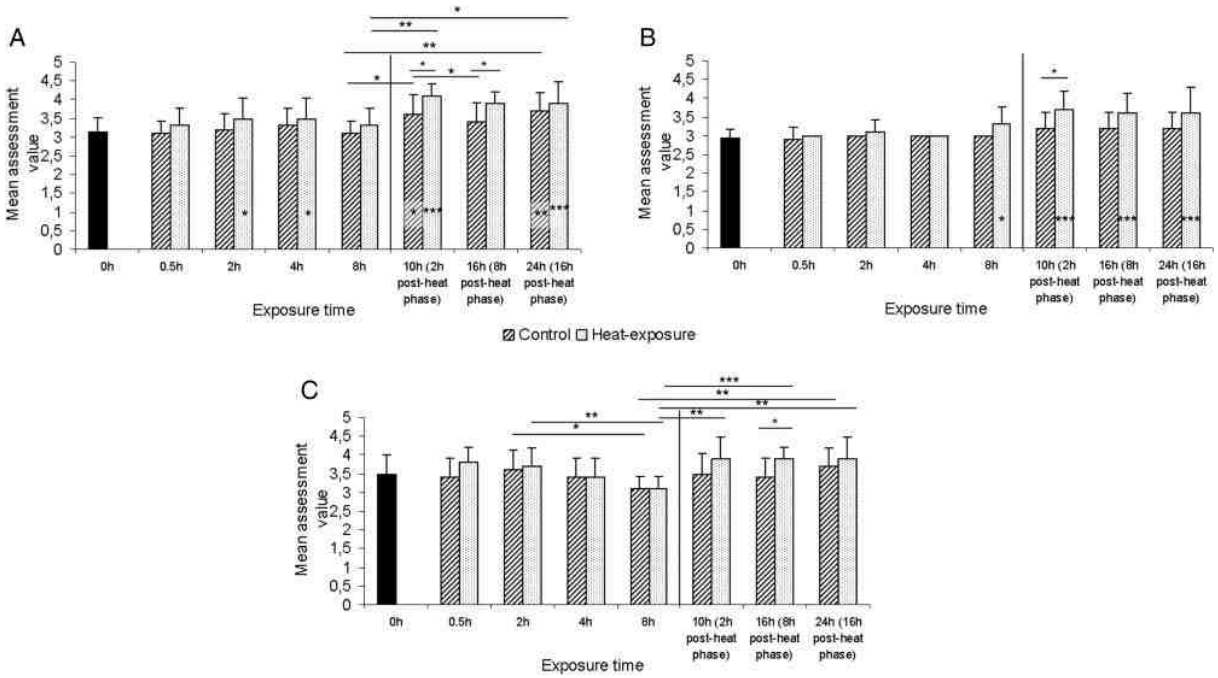

Fig. 5: Results for the histopathological analysis of control group versus heat-exposure group of the *T. pisana*-population (mean + sd; n=10 except for 0h (t0): n=20). Significant differences are indicated by asterisks (0.01<P≤0.05: * (slightly significant); 0.001<P≤0.01: ** (significant); P≤0.001: *** (highly significant)). Significances versus 0h are shown in the respective bars.
a : Mean assessment values for the overall condition of the hepatopancreas.
b: Mean assessment values for the tubules' condition.
c: Mean assessment values for the digestive cells' condition.

Excretory cells

The assessment values for the condition of excretory cells within the control group were all slightly significantly higher from 4h onwards compared to 2h (data not shown). However, no differences occured within the heat-exposure group and no differences were found when comparing the two different treatment groups as well.

Surface ratio of cell types & calcium cell countings

The analysis of the control group regarding surface ratio of hepatopancreatic cell types revealed no relevant differences.

Within the heat-exposure group an increase of the surface ratio of digestive cells became evident between 10h and 16h (Fig. 3d). Furthermore the surface ratio of calcium cells first slightly increased (8h), then decreased, but reached the base-level again during the post-heat-phase (Fig. 3a). The surface ratio of excretory cells increased up to 4h, then decreased again (Fig. 3d).

Concerning the comparison of control and heat-exposure groups no differences could be detected.

Analysing the calcium cell countings for this population and comparing control with heat-exposure group the heat-exposure group showed slightly higher numbers at three time points (Fig. 3c).

When comparing the calcium cell countings of the different populations, *X. derbentina* and *T. pisana*, *X. derbentina* generally revealed higher numbers at all time points in all treatment groups except for 2h for the controls (Fig. 3c).

Stress protein (Hsp70-) analysis of T. pisana

Control, heat-exposure and post-heat-phase

The induction of Hsp70 in *T. pisana* was strikingly lower than in *X. derbentina*. In the control group, the 0.5h differed from all time points except from 0h (Fig. 4b). Significant induction was detectable in the heat-exposure group at the end of the exposure period and persisted throughout the post-heat phase (Fig. 4b).

Discussion

Xeropicta derbentina

The overall condition of the hepatopancreas (mean assessment value) in the investigated *X. derbentina* population deteriorated after 4h of heat-exposure at 45°C. Event though snails were offered a 16h post-heat phase at 24.7°C after 8h of heat-exposure, no recovery of the overall condition of the hepatopancreas could be detected. The deterioration of the hepatopancreas was mainly characterised by a dilation of the lumina and ruptures of cell apices, as well as by impairment of the condition of digestive and calcium cells. Additionally, changes in surface ratio and numbers of cells occured.

An elevation of the metabolic rate due to high temperature exposure probably caused the dilation of the lumina. Generally, elevated temperatures result in an increase of metabolic rates in heterothermic animals (Gillooly et al., 2001). Yet, Mediterranean land snails can enter dormancy and drop their metabolic rates when facing elevated temperatures thereby forming a characteristic epiphragm (Arad & Tzameret, 1998). However, as no epiphragms were built during the course of exposure experiments we conclude that our test animals were not in a dormant state, and would therefore experience an elevated metabolic rate rather than a decrease. As hepatopancreatic cells are usually strongly involved in metabolic processes even under normal conditions (Thiele, 1953; Abolins-Krogis, 1961; Sumner, 1965; Porcel, 1996; Taieb & Vicente, 1998) an increased metabolic rate could have led to a stronger supply of the hepatopancreas with digestive liquids causing a dilation of hepatopancreatic lumina.

The increased abundance of ruptured cell apices can probably be linked to increased release and activity of lysosomal enzymes. Moeller et al. (1976) showed that elevated temperatures can lead to a disruption of lysosomal membranes. Being released into extracellular matrices lysosomal enzymes destroy cell membranes (Poste, 1971). As found in the qualitative assessment of the digestive cells, the compartmentalisation of this cell type was disturbed in control and, to a greater extent, in the heat-exposure group due to a number of apically situated vesicles some of which could have likely been lysosomes. The occurrence of such lysosomes in the control group was probably due to the high temperatures the snails had already encountered in the field during summer.

Furthermore, we detected a highly significant decrease of the surface ratio of digestive cells after 4h of heat exposure. This can also be seen as a consequence of the above mentioned disintegration of cell membranes due to activity of lysosomal enzymes. At the end of the test,

the surface ratio of digestive cells reached the starting level again; this may indicate some recovery processes.

The impairment of the condition of the calcium cells in the heat-exposure group was due to a disturbed acid-base-status and osmotic stress caused by the elevated temperature. Elevated temperatures can lower the pH in snail tissues (Barnhart, 1986). This leads to an accumulation of acid metabolic end products, such as succinate and D-lactate, which in turn can cause metabolic acidosis (Ryan & Gisolfi, 1995; Michaelidis et al., 1999). Furthermore, it is likely that the snails counteracted heat exposure by increased evaporation of water which in turn likely resulted in osmotic stress. Arad (2001) found that with increasing desiccation osmotically active substances such as chloride accumulate in extrapallial fluids of *Theba pisana*. As calcium cells are strongly involved in regulation of the acid-base-status and osmotic balance (Burton, 1976; Taieb & Vicente, 1998), and as this cell type showed almost karyolitic, strongly blue-stained nuclei (indicating a low pH), a disturbed compartmentalisation with apically situated nuclei and faded cytoplasm, it is very likely that these cells suffered from overcharge caused by heat stress, associated osmotic stress and acidosis. This explanation becomes even more plausible when concurrently considering the characteristics of the Hsp70-response of this group, which clearly breaks down twice during test time (at 8h and 24h). Obviously, the post-heat phase did not offer enough time for cellular recovery in our case as the condition of calcium cells did not improve during the post-heat phase. The condition of calcium cells in *X. derbentina* under heat exposure can also be linked to the alterations in surface ratio and absolute cell numbers of this cell type. Concerning the absolute number of calcium cells under heat exposure, we found a significant increase after 2h of test time which can be interpreted as hyperplasia. This early response of the calcium cells can be regarded as an attempt of the snails to regulate thermal stress and possibly also to supply replacement for heat-impaired cells. However, the slightly significant increase of the surface ratio of this cell type after 4h also suggests a hypertrophy of calcium cells. Both, hyperplasia as well as hypertrophy, in conjunction with our findings for the general condition of the hepatopancreas and for the calcium cells in particular, show that the calcium cells are very active in consequence of heat exposure. Marigómez et al. (1990) and Najle et al. (2000) also found hypertrophy of calcium cells in *Littorina littorea* and *Nacella concinna* following cadmium-exposure. In this context, it becomes clear that this effect could represent a general response of molluscs to osmotically active stressors.

The fact that certain symptoms of heat stress were not only found in the exposure group but, to a minor extent, in the control animals as well, suggests that the snails had already

encountered a substantial heat stress in the field. However, this is a consequence of the sampling date which was midsummer for the experiments here. In contrast, sampling took place in early spring for the test of Dittbrenner et al. (2009) who used the same sampling methods, laboratory maintenance and similar test set-up but did not find symptoms of heat stress to the same extent in their control individuals as in our case. This also makes clear that these snails probably did not fully recover from previous field exposure.

All in all, the histopathological results for *X. derbentina* showed that only little evidence for recovery processes was found at the cellular level during the laboratory test. Furthermore, we also did not find evidence for full recovery from heat stress encountered in the field. However, it also became evident that *X. derbentina* is able to respond to extreme heat stress with hyperplasia and hypertrophy of calcium cells.

Concerning the results of the Hsp70 analysis, the highly significant peak at 4h in the control group of *X. derbentina* provides evidence for circadian variation. Such variations are common in other molluscs, e.g. chitons, conferring high thermotolerance even at fluctuating temperatures throughout the day (Schill et al., 2002). Interestingly, the 4h test point was at 12 a.m., which represents a time when there is usually high radiation in the snails' natural habitat.

Both peaks found in the heat-exposure group indicate a typical Hsp70 optimum kinetic (Eckwert et al., 1997) as in both cases the Hsp70-level decreased again after reaching the maximum. The second peak indicates recovery processes. The decrease of the Hsp70-level after the first peak can be interpreted as an Hsp70-response in the non-compensation (=destruction) phase (Eckwert at al., 1997) caused by impairment of the stress protein apparatus. This is corroborated by the corresponding histopathological data. Similar kinetics have already been described for *X. derbentina* after 8h of heat-exposure by Lazzara (2007) as well. Therefore, the second peak occurring after this decompensation phase can be regarded as the result of a re-induction of Hsps. However, the decrease after the second peak indicates yet another breakdown of the stress protein apparatus within test time. Different factors could have contributed to this second breakdown: Since the snails were not fed during the test for practical reasons and as the Hsp70 response is very energy-comsuming (Currie et al., 1999), this could have led to energy depletion. This, in turn, can lead to reduction or even shortfall of the Hsp70 response (Hand & Hardewig, 1996). Again, one must also keep in mind that, as mentioned before, these snails had encountered heat stress in the field already. This pre-exposure in the field probably affected the overall reaction capacity of the stress protein system. Krais (2008) showed that *X. derbentina* sampled in summer and exposed to elevated

temperatures and chemicals in the laboratory hold a Hsp70 stress protein reaction capacity three times lower than equally treated snails sampled in spring. These reactions also point up the different kinetics of different biomarkers: Whereas the stress protein apparatus offers a quick response, cellular reactions usually take more time (Triebskorn et al., 2001).

In summary, these results imply that *X. derbentina* is able to recover even from severe heat stress at the Hsp70 level if no energy shortage occurs.

Theba pisana

In the investigated *T. pisana* population, the overall condition of the hepatopancreas deteriorated over test time under heat exposure, a trend that even continued within the post-heat phase. This deterioration was mainly due to an impairment of tubuli shape and condition of the digestive cells, and accompanied by a generally poor condition of the calcium cells.

As in the control group, we observed a strong blue staining of the lumina in nearly all heat-exposed animals at all times. This stain indicates a low pH and acidosis, which is a consequence of heat stress as described for *X. derbentina* in this paper as well. However, the fact that whole lumina suffered from acidosis in *T. pisana* implies that this species is more severely affected by the heat exposure than *X. derbentina*. This can probably be linked to the fact that, in contrast to *X. derbentina,* we found no hypertrophy and only slight hyperplasia of calcium cells in *T. pisana* indicating a lower activity of this cell type in this species. Furthermore, the surface ratio as well as the absolute numbers of calcium cells were generally much lower in *T. pisana,* even in controls, when compared to *X. derbentina*. Concerning the surface ratio this has also been shown by Dittbrenner et al. (2009). Obviously this species possesses less and smaller calcium cells than *X. derbentina* even under natural conditions, and is not able to respond to heat stress with enlargement and / or upregulation of this cell type combating the effects of heat-induced acidosis by adjusting the acid-base balance to the same extent as *X. derbentina*.

Also, there were notably little differences between the condition of cells in control versus heat-exposure group at the different time points. This may indicate, in combination with the observed symptoms of an acidosis, that these snails had encountered and were affected by the heat stress they had experienced in the field already. As in *X. derbentina,* this can be explained by the sampling date in summer, however *T. pisana* was obviously more severely affected not only by the experiment but by the preceeding natural heat stress since the characteristics of acidosis in the control group were more prominent than in *X. derbentina*. Furthermore, none of the histopathological results (control and heat-exposure) indicated any recovery processes, which also may be a consequence of the aforementioned strong effects of

the encountered natural heat exposure. It is probable that the damage caused by natural heat stress in combination with heat stress applied during the experiment was too severe to be repaired within the 16h post-heat-phase which might have been too short. It is also known that, for example, slugs affected in hepatopancreatic cell composition by metal pollution exposure require 1-2 weeks for recovery (Zaldibar et al., 2008). These findings also may explain why no hypertrophy and / or hyperplasia of hepatopancreatic calcium cells could be detected: It is very likely that the whole hepatopancreatic tissue was overwhelmed by the combination of the preceeding natural and following experimental heat stress.

The results of the Hsp70-analysis of the control group of *T. pisana*, as implied by the histopathological results already, indicate that these snails suffered from heat stress and its effects in the field and that as a consequence their stress protein response's reaction capacity was already negatively affected. Heat stress itself can cause elevated Hsp70 levels, however acidosis can also result in an elevated stress protein response (Kregel, 2002). Together, heat stress and resulting acidosis can have overwhelmed the stress protein system in *T. pisana* in the field already. Also, the measured Hsp70 levels in this study are notably lower than those detected by Köhler et al. (2009) in spring-sampled Mediterranean snails. This has to be seen as a consequence of the different sampling dates and different conditions encountered in the field and also points to an affected stress response capacity in midsummer.

Furthermore, observing the results in the heat-exposure group substantiates the aforementioned assumption of an overwhelmed stress protein system. As we detected only slight Hsp70 level variation within the heat-exposure group and no differences between control and heat-exposure group, and as it is unlikely that the animals had not reacted to the applied heat stress concerning the histopathological results and the fact that almost all known organisms respond to elevated temperatures with an induction of stress proteins (Craig, 1985; Welch, 1993; Parsell & Lindquist, 1994), we may conclude that the stress protein response of *T. pisana* was already depleted in the field and therefore no variations in Hsp70 levels could be detected during our test. It is also unlikely that we were not able to record the stress protein response for temporal reasons as the induction of stress proteins usually takes place within 10 to 15 minutes after stress occurrence (Bukau, 1993).

However, as *T. pisana* still occurs in masses in Mediterranean fields, it can be assumed that this species possesses other (still unknown) protective biochemical mechanisms or behavioural characteristics which ensure its survival in its natural hot and dry habitat.

Comparison of X. derbentina *and* T. pisana

Altogether, for the aforementioned results we assume that *T. pisana* is more sensitive to heat stress than *X. derbentina*, additionally this is supported by the necessity of choosing a lower sublethal temperature for *T. pisana* as well. Also, we found no signs of recovery, neither from naturally experienced nor artificially induced heat stress in this species.

Due to the estival sampling date it can be assumed that both populations had encountered massive heat stress in the field already, this affected both the histopathological as well as Hsp70-analysis results in controls and heat-exposure groups.

The high thermotolerance of *X. derbentina* can be explained by the ability of this species to produce more and larger calcium cells in case of disturbance of the acid-base balance which can be a consequence of heat stress. As the hypertrophy of calcium cells has been observed in other molluscan species as a response to heavy metal stress as well (Marigómez et al., 1990; Najle et al., 2000) it could be assumed as a general response of molluscs to osmotically active stressors. Furthermore, *X. derbentina* proved to hold a robust stress protein system capable of quick recovery if no energy shortage occurs.

Compared to *X. derbentina*, *T. pisana* proved to be more sensitive to natural and artificially induced heat stress. This was manifested in a lower sublethal temperature, the complete absence of recovery processes and symptoms of a severe acidosis and an overwhelmed stress protein response in *T. pisana*. However, *T. pisana* still has a considerable thermotolerance in comparison to other snail species such as *Cepaea hortensis* (Lazzara, 2007), for example. This thermotolerance is probably to be explained by other physiological / cellular mechanisms or behavioural adaptations, though. Nevertheless, these mechanisms seem to be less effective in protecting from heat stress than those found in *X. derbentina*.

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Kapitel 2: Shell colouration and antioxidant defence capacity in *Theba pisana* (O.F. Müller, 1774)

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Abstract

Theba pisana (Müller, 1774), a Mediterranean snail, is known for its heat tolerance and its remarkable shell colour polymorphism ranging from pale white to darkly striped, although darker morphs are considered less vital in hot habitats due to presumably stronger absorption of radiation. Melanin, the black pigment of these snails, is known as an effective antioxidant, and elevated temperatures can increase oxidative stress. By analysing oxidative waste products via the ferrous oxidation xylenol orange (FOX) assay as a marker for the lipid peroxidation level, we aimed at investigating possible links between heat stress, colouration and antioxidant defence capacity in *T. pisana*. Although we found increasing levels of peroxidation products with increasing heat exposure duration, there was no difference in antioxidant defence capacity observable between different morphs of *T. pisana*. Hence the avoidance of lipid peroxidation as an environmentally relevant factor for the maintenance of melanistic morphs in strongly illuminated habitats can be considered improbable.

Additional keywords: Polymorphism, Mediterranean land snail, heat stress, oxidative stress, lipid peroxidation

Introduction

Some pulmonate snails such as *Xeropicta derbentina* (Krynicki, 1836), *Theba pisana* (Müller, 1774) and some *Cernuella* (Schlüter, 1838) species are known for their remarkable ability to live in hot and sun-exposed habitats such as the Mediterranean (Arad et al. 1993; Aubry et al. 2006). Behavioural and metabolic adaptations of these species to their habitats are well investigated and include, for example, climbing vertical objects to avoid higher ground temperatures, as well as aestivation (Pomeroy 1968; Yom-Tov 1971; McQuaid et al. 1979; Cowie 1985). However, our understanding of biochemical mechanisms furthering their thermotolerance is rather marginal. Furthermore, in the case of *T. pisana*, which represents a highly polymorphic species in terms of shell colouration (Cowie 1984), one can observe that even dark banded morphs can be found in hot habitats in relatively high numbers, though darker pigmentation is usually regarded as disadvantageous in such environments due to (postulated) higher absorption of sunlight (Heath 1975; Hazel and Johnson 1990). Nevertheless, darker pigmentation could also be beneficial in this snail species. It has been shown that banded morphs, in being more cryptic, can be less prone to predation (Heller 1981; Heller and Gadot 1984). Furthermore, darker pigmentation is caused by melanin (Comfort 1951), and melanin is known to absorb free radicals generated by oxidation of primary metabolites (Riley 1997). It was also found that tissues of pigmented individuals of *Xenopus laevis* (Daudin, 1802) frogs were less susceptible to oxidative damage than tissues of albinos (Corsaro et al. 1995). This raises the question whether similar correlations between pigmentation and antioxidant defence capacity can be found in snails, e.g. *T. pisana*, that can help to explain the maintenance of colour polymorphism in environments exposed to hot sun.

Oxidative damage can occur due to exposure to elevated temperatures, as increasing temperatures cause elevated metabolic rates in most invertebrates (Pörtner 2001). Higher metabolic rates in turn favour the generation of so-called reactive oxygen species (ROS) (Abele et al. 1998), which can attack not only DNA and proteins, but also lipids by peroxidation leading to an impairment of biomembranes and generating lipoperoxides (Gutteridge and Halliwell 1990). Also, heat stress can lead to alterations in the conformation of enzymes which facilitate the production of the above mentioned ROS (Gutteridge and Halliwell 1990).

The amount of oxidative waste products, such as lipoperoxides, can be used for analysing an organism's ability to cope with oxidative stress and can be quantified using the ferrous oxidation xylenol orange method (FOX) assay (Hermes-Lima et al. 1995). Another method

for detecting lipid peroxidation products in organisms is the malondialdehyde (MDA) assay, however, this assay has been criticized for being insufficiently accurate and specific (Halliwell and Gutteridge 1985; Smith and Anderson 1987; Janero 1990; Ceconi et al. 1991; Bonnes-Taourel et al. 1992). The FOX assay can be considered as a sensitive alternative (Hermes-Lima *et al.* 1995), and has been successfully applied on tissue extracts (Hermes-Lima et al. 1995), membrane preparations (Jiang et al. 1991; Jiang et al. 1992; Hunt et al. 1993) and plasma samples (Frank 1992), in vertebrates (Frank 1992; Hermes-Lima et al. 1995) as well as in invertebrates (Monserrat et al. 2003; Ramos-Vasconselos and Hermes-Lima 2003).

For this study, we decided to investigate possible correlations between pigmentation and antioxidant defence capacity in *T. pisana* snails. By analysing two clearly different colour morphs (banded and unbanded) of *T. pisana* for their content in lipoperoxides under control as well as heat-exposure conditions, we endeavoured to demonstrate the possibility of a higher antioxidant defence capacity in darker snails that possibly counterbalances their postulated disadvantage of stronger warming in open, strongly illuminated environments.

Materials and methods

Two morphs of *Theba pisana* snails, banded and unbanded, both with pale soft bodies (Fig. 1) from a natural meadow site near Avignon in Vaucluse, Southern France (N 43° 53' 50.88", E 4° 54' 7.10"), were sampled in the first week of August 2009. Animals were transported in plastic containers (24.2 x 20.7 x 6.4 cm) and then transferred to plastic terraria (30 x 19.5 x 20.5 cm) in the laboratory. The terraria contained a 2 cm layer of *JBL Terra Basis ground covering for terraria* (JBL GmbH & Co. KG, Neuhofen, Germany) and were cleaned and moistened with tap water every other day. Snails were fed a diet of *Hipp Bio-Milchbrei* (Hipp GmbH & Co. Vertrieb KG, Pfaffenhofen, Germany) (organic baby food, prepared according to package instructions) *ad libitum* every other day. Animals were allowed a two-week acclimatisation phase at 24±1°C before the experiments were started.

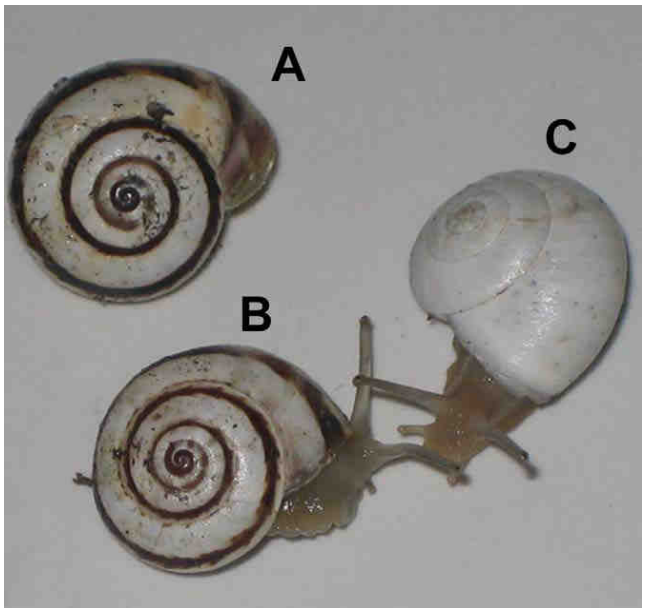

Figure 1. *Theba pisana* – individuals with striped 'dark' shells (A + B) and a white, 'pale' shell (C).

In order to determine whether the unbanded and banded snails were indeed of significantly different colour intensity, we conducted a densitometric analysis of shells of the different morphs similar as in Dittbrenner et al. (2009) or Köhler et al. (2009). For this analysis, 8 shells of each morph were individually and identically illuminated on a standardised grey background plate and photographs of both apex and umbilicus sides of their shells were taken using a digital single lens reflex camera (*Sony α100*) with a fixed aperture and fixed shutter speed. An average grey value of each shell integrating both apex and umbilicus sides was calculated using ImageJ1.45 (National Institutes of Health, Bethesda, MD, USA), referenced to the background plate grey scale to standardise comparison between the images.

Adult snails of similar size range were divided into two morphological groups, pale-shelled unbanded animals ('pale') and dark-shelled animals with clear dark-brown banding ('dark') (Fig. 1). Each morphological group was subdivided into two exposure groups, control and heat-exposure. Control temperature was 24.7°C, whereas the highest sublethal temperature for 8h for *T. pisana*, 43°C (Dittbrenner et al. 2009; Köhler et al. 2009; Scheil et al. 2011), was chosen for heat-exposure. The experiments were conducted using the maintenance terraria in heating cabinets (*noctua IH50* (Noctua GmbH Mössingen, Germany) / *Lovibond ET619-4* (Tintometer GmbH, Lovibond Water Testing, Dortmund, Germany)), ensuring that all snails were heated to the same degree irrespective of colouration. From each morphological group, 10 individuals were sampled at the beginning of the tests (0h). Within the overall 8h exposure time, 10 replicate animals were sampled from each morphological group and respective exposure group after 2h, 4h and 8h of test time. All animals were tested for mortality by

poking with a blunt needle to reveal possible lack of response which was interpreted as a proxy for mortality.

All chemicals were purchased from Roth (Carl Roth GmbH & Co. KG, Karlsruhe, Germany), Sigma Aldrich (Sigma Aldrich Chemie GmbH, Steinheim, Germany) or Merck Chemicals (Merck KG aA, Darmstadt, Germany). The FOX assay was conducted and results calculated according to Hermes-Lima et al. (1995). Snails were individually shock frozen in liquid nitrogen (-196°C) immediately after shell removal and stored at –80°C until further processing. Individuals were then homogenised in ice-cold HPLC-grade methanol with a density of 0.791 g/cm³ (dilution 1:2, the appropriate amount of methanol was calculated following quantification of the wet weight of the specimen), centrifuged at 15.000 *g* and 4°C for 5 min and the resulting supernatants were stored at –80°C. The assay mixture was then prepared: a 50 µL sample of each supernatant (in blanks: aqua bidest., as a substitute for the supernatant) was added to 1 mL of assay mixture (0.25 mM $FeSO_4$, 25 mM H_2SO_4 and 0.1 mM xylenol orange) in *Roth single-use polystyrol cuvettes* (1.5 mL) (Carl Roth GmbH & Co. KG, Karlsruhe, Germany) and incubated at room temperature for 24h. Measurements were then taken by using a spectrometer (*Spectronic Unicam Hελios Gamma*, Spectronic Unicam, Cambridge, UK) at 580 nm ($A_{580\ nm}$). Then 5 µL of 1 mM cumenehydroperoxide (CHP) solution was added to each sample, and samples were incubated for a further 30 min. After that, samples were re-measured at 580 nm ($A_{580\ nm+CHP}$).

Cumenehydroperoxide- equivalents per gram wet weight of snails (CHPE / g wet weight) were then calculated according to the equation of Hermes-Lima et al. (1995):

$CHPE/g_{wet\text{-}weight}=(A_{580nm}/A_{580nm+CHP})*5\mu L\ CHP_{5nmol}\ *1000/V1*2$

where V1=sample volume (50 µL) and 2 results from dilution (1:2 with methanol, density of 0.791 g/cm³.

All data were statistically analysed implanting JMP® 9.0 (*SAS Institute Inc.*, Cary, USA). Densitometric data were normally distributed according to the Shapiro-Wilks-test and analysed for significant differences via the Tukey-Kramer-HSD-test ($p \leq 0.001$: ***). The data resulting from the FOX assay were split in two groups, controls (unheated; 24.7°C) and heat-exposure (heated to 43°C), and a two-factor analysis of variance (ANOVA) was applied to each group to investigate the effects of shell colour over time. Since not all of these data were normally distributed according to the Shapiro-Wilks-test, and also did not show equal variances according to Levene's test in all cases, several transformations (log, log10, root, power (x^2)) were conducted, however, none resulted in a consistent normally-distributed outcome with equal variances. To account for this, using the original untransformed data the

p-level was set to 0.01. As only the model for the heat-exposure group is significant and time proved to be the only significant factor in the corresponding effect tests, the data of different morphs were fused and analysed via a post-hoc Wilcoxon-test for significant differences between time points. After Bonferroni-correction for 6 pairwise comparisons the p-levels were set to $0.016<p\leq0.0083$: * (significant); $0.00016<p\leq0.016$: ** (highly significant); $p\leq0.00016$: *** (most significant).

RESULTS

No mortalities occurred during this experiment.

The densitometric analysis of differently coloured shells revealed that unbanded shells are significantly lighter than banded shells (Fig. 2, $p\leq0.001$: ***).

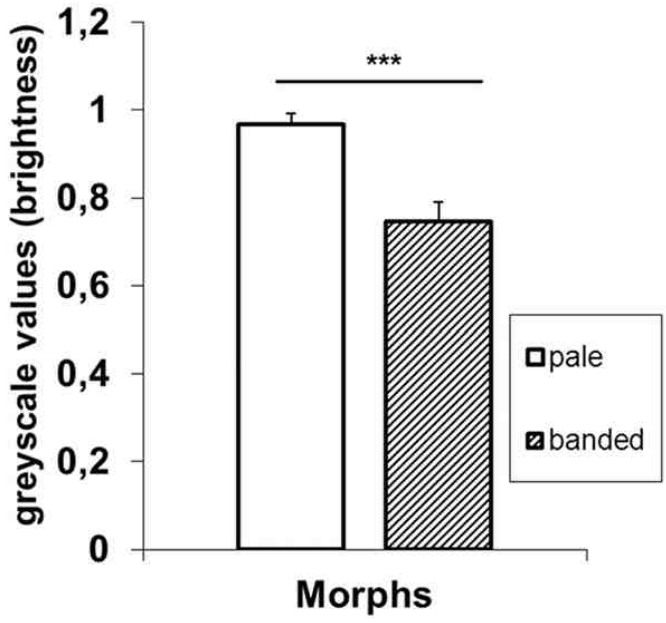

Figure 2. Densitometric analyis of unbanded (pale) vs. banded morphs. Comparison of lightness of shells (grey scale values) of unbanded (pale) vs. banded morphs (means + sd, n=8). The significant difference is indicated by an asterisk ($p\leq0.001$: ***).

Concerning the analysis of lipid peroxidation, within the controls (Fig. 3a; maintained at 24.7°C) no trends in increase or decrease of CHPE / g wet weight could be observed and correspondingly no significant effects of shell colour, time or shell colour and time interaction on lipid peroxidation were found during the course of the experiment according to a two-factor ANOVA and corresponding effect tests (Table 1a, b; $p\leq0.01$).

Table 1. Two factor ANOVA & Effect tests. Two factor ANOVA (A) for unheated controls and corresponding effect tests (B); p≤0.01. Two factor ANOVA (C) for heat-exposure and corresponding effect tests (D); p≤0.01.

A

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	7	9625,774	1375,11	1,6041
Error	69	59148,904	857,23	**Prob > F**
C. Total	76	68774,678		0,1489

B

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Colour	1	1	4687,7846	5,4685	0,0223
Time	3	3	3819,7609	1,4853	0,2262
Colour*Time	3	3	4785,1969	1,8607	0,1444

C

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	5	62675,66	12535,1	3,6610
Error	54	184891,90	3423,9	**Prob > F**
C. Total	59	247567,56		0,0063*

D

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Colour	1	1	381,643	0,1115	0,7398
Time	2	2	56227,523	8,2110	0,0008*
Colour*Time	2	2	4458,178	0,6510	0,5256

In heat-exposure groups (Fig. 3b; exposed to 43°C) no increase or decrease was found after 2h heat exposure, however, a clear increase of CHPE / g wet weight was observable from 4h exposure time onwards. At 8h exposure time, the level of lipid peroxidation remained similarly high as that at 4h (Fig. 3b). No significant effects of shell colour or shell colour and time interaction on lipid peroxidation were observed within this experiment, however, two-factor ANOVA and corresponding effect tests revealed time as a significant factor influencing lipid peroxidation (Table1c, d; p≤0.01). The subsequent post-hoc Wilcoxon-test with the data of both colour morphs pooled revealed the amount of CHPE / g wet weight at 4h to be most significantly higher than at 0h and 2h (Fig. 3b). Also, the value for 8h exposure was highly significantly increased compared to 0h, and most significantly increased versus 2h exposure (Fig. 3b).

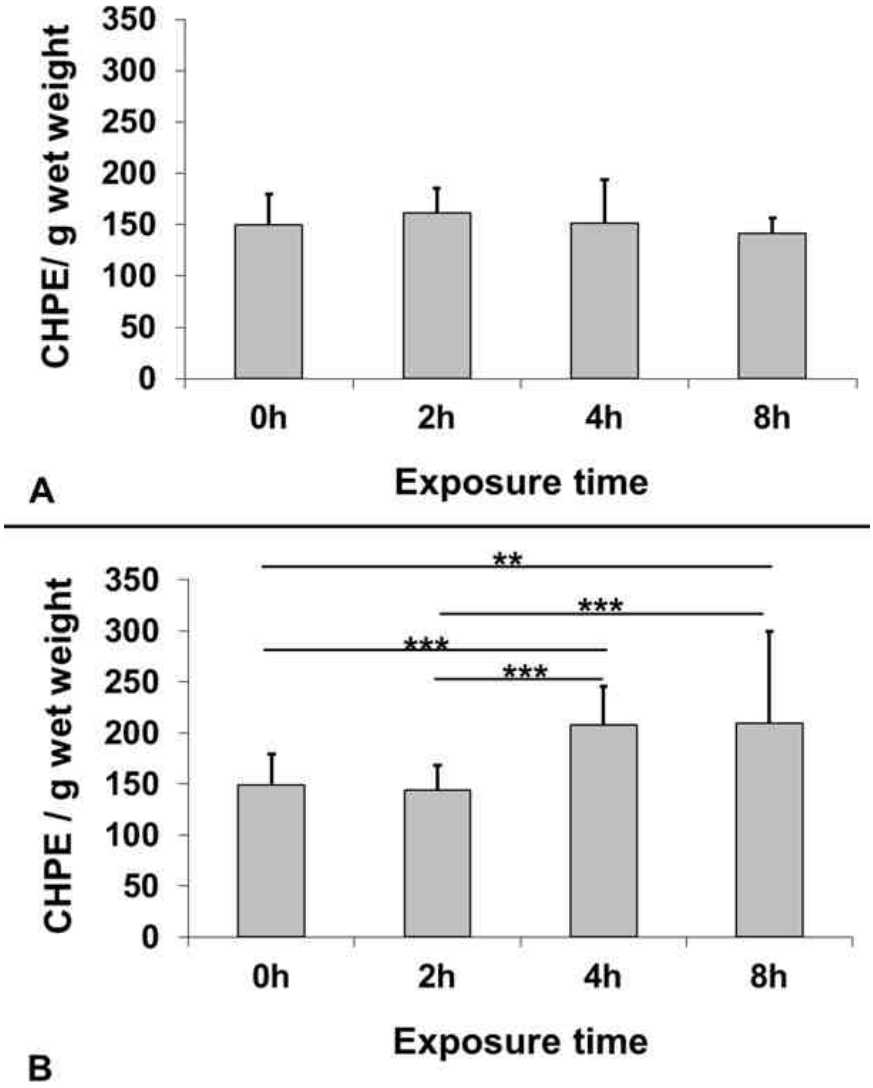

Figure 3. Comparison of CHPE/g wet weight in pale-shelled (PS) vs. dark-shelled (DS) snails, in unheated (24.7°C) controls (A) and heat-exposed (43°C) snails (B). Means + sd, n=10. Significant differences are indicated by asterisks; $0.016<p\leq0.0083$: * (significant); $0.00016<p\leq0.016$: ** (highly significant); $p\leq0.00016$: *** (most significant) after Bonferroni-correction for 6 pairwise comparisons.

Discussion

It became evident that increasing duration of heat exposure elevated the lipid peroxidation level, determined via the amount of lipohydroperoxides, in both morphs. Consequently, this lipid peroxidation level proved to be a good biomarker for heat stress in *T. pisana*. Our findings further substantiate the suitability of lipid peroxidation as a biomarker for assessing the condition of terrestrial molluscs, as it has already been described for marine species (Jena et al. 2009). Additionally, the fact that no mortalities were recorded throughout the experiment corroborates 43°C as the sublethal temperature over a time span of 8h for *T. pisana*, as it has also been observed in previous studies (Dittbrenner et al. 2009; Köhler et al. 2009 and Scheil et al. 2011).

Concerning the densitometric analysis of differently coloured shells, the method applied again proved to be an efficient way to analyse and objectively quantify colour differences as it has already been shown by Dittbrenner et al. (2009) or Köhler et al. (2009). As we found unbanded snails to be significantly lighter than banded ones, we can exclude the possibility that absence of significant differences between the different morphs' lipid peroxidation levels are due to only minor differences in coloration and melanisation.

At this point one could also argue that shell pigmentation might not be linked to the tissue or the organism's general melanin content. However, it has been shown for other gastropods that melanin causing shell pigmentation is derived from cells of the mantle epithelium (Timmermans 1969). Additionally, in *Cepaea nemoralis*, another helicid snail, it is known that banded shells contain a considerably higher amount of melanin than unbanded shells (Jones 1973). Therefore, darker-shelled morphs of *T. pisana* also may contain more melanin than pale-shelled morphs, and they may have a generally higher ability to produce melanin than pale individuals. It is known that, for example, insects with darker cuticles can have a higher phenoloxidase activity than lighter-coloured insects (Armitage and Siva-Jothy 2005). And it is known that both melanism and physiological parameters, such as phenoloxidase activity, share the same pathway (Rolff and Siva-Jothy 2003), so a morphological trait such as shell colour and a physiological parameter such as antioxidant defence capacity obviously can be correlated, especially when both are based on the same metabolite, melanin. Interestingly, it is also known for molluscs that melanin is produced via the phenoloxidase pathway (Malanga and Young 1978; Aladaileh et al. 2007).

However, as no effects of shell colour could be detected in the heat-exposure groups of the two different morphs, one can conclude that dark morphs of *T. pisana* are not able to combat the effects of oxidative stress resulting in lipid peroxidation to a greater extent than pale morphs, at least not within the ecologically relevant time span investigated in this study. Consequently, a possible advantage of being dark, causing the maintenance of polymorphism in this snail species, must be attributed to factors other than fighting lipid peroxidation, for example predation and microhabitat effects, as it has been described by Heller 1981; Heller and Gadot 1984 or Hazel and Johnson 1990.

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Kapitel 3: Colour polymorphism and thermal capacities in *Theba pisana* (O.F. Müller 1774)

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Abstract

Several land snail species are highly polymorphic regarding their shell colouration. This polymorphism has been related to predatory effects as well as climatic reasons, assuming that dark morphs benefit from being more cryptic and therefore less prone to predation, whereas pale morphs are at an advantage under solar radiation, as they are suspected to heat up less. However, the assumption of different thermal capacities of these morphs is based on experiments with little standardisation or little environmental relevance. In this study, we aimed at measuring thermal capacities of two different morphs (pale versus dark-brown banded) of the Mediterranean land snail *Theba pisana*, applying a standardised and environmentally relevant test set-up, in order to prove whether darker morphs indeed do heat up more than lighter coloured morphs. We did not find any differences in the thermal capacity of the different morphs and conclude that thermal capacity of the shell is predominantly defined by its material rather than its coloration. These results are discussed with regard to previous studies on thermal characteristics of different land snail morphs and correlations between climate and morph distribution.

Key words

Land snail, Shell Colour, Heating Capacities, Morph Distribution, Climate

Introduction

Shell colour polymorphism is a widespread phenomenon in various land snail species such as *Cepaea* sp., *Cernuella virgata* or *Theba pisana* (Baker, 2002; Cook, 1998). Explanations for the maintenance of this polymorphism are manifold, among which predation susceptibility and direct climatic effects are very popular. Regarding predation, darker (banded) morphs are supposed to be more cryptic and less prone to being discovered by predators, yet they are presumably at a disadvantage in terms of solar heating (Heller and Gadot, 1984). It is assumed that the darker morphs heat up to a greater extent in the sunlight and therefore are more frequently found in northern (colder) and sheltered (grown-over) habitats (Cowie, 1990; Heller, 1981; Heller and Gadot, 1984; Johnson, 1981). This assumption of a greater thermal capacity of the dark morphs is based on studies in which the heating characteristics of different snail morphs were tested using either natural sunlight or artificial radiation (Cook and Freeman, 1986; Heath, 1975; Jones, 1973). However, all of these studies have their shortcomings concerning standardisation or environmental relevance of test conditions, as they were either conducted outdoors on different days in different seasons with no consideration of windspeed, or implanted an artificial light source not covering the full natural light spectrum. Hence, we decided to develop an experimental set-up as standardised as possible in order to test the heating characteristics of pale, unbanded shell morphs versus dark, strongly-banded shell morphs of *Theba pisana*. To test the sole influence of different shell colours, we also restricted the experiments to empty shells, therefore preventing any other animal factors (e.g. water content of the soft body) to interfere with the experiment. Thus, the aim of our study was to reveal whether or not thermal characteristics differ between dark and pale shell morphs of *T. pisana* when treated under standardised conditions.

Materials & Methods

2.1 Test shells, sampling sites and preparation of shells

All tested shells were equally-sized (range 15-20mm diameter) and obtained from adult *T. pisana* snails.

The snails were sampled in the Departement Vaucluse, Southern France, from a site near L'Isle-sur-la-Sorgue and Fontaine-de-Vaucluse (43° 55.5' N, 5° 6' E). Animals were kept in the laboratory as described in Scheil et al. (2011), and after the animals had died, soft body tissues were entirely removed from the shells. To remove any dirt particles and remains of the

soft bodies, the shells were carefully cleaned with a soft toothbrush and deionized water, and treated in an ultrasonic bath (*Sonorex TK 52*, Bandelin electronic GmbH & Co. KG, Berlin, Germany) filled with deionized water for 15min.

2.2 Test set-up and procedure

All measurements and thermographic pictures were taken with a heat-sensing thermographic camera (*TVS 100*, Goratec Technology, Erding, Germany). The camera was adjusted for optimal picture resolution. The temperature span was 20°C- 45°C. An Average (AVE) of 8 was set. The Average (AVE) is a setting for noise reduction in thermographic cameras. It secures that not every single and very small temperature measurement is recorded, resulting in a more stable and reliable display. According to pre-tests we found an AVE of 8 to result in the most precise pictures. An emission ratio (E) of 1.0 was defined for the measurements. The emission ratio (E) was corrected to 0.93 for the calculations. (In engineering, different types of chalk are distinguished. This E value refers to coarse chalk, according to BARTEC, 2001, since chalk, $CaCO_3$, is the main compound of snail shells, according to Heller and Magaritz, 1983.) Room temperature where experiments were conducted was 22°C.

Two colour morphs were tested, a dark morph with bold brown bands (“dark”) and a pale white morph with no banding at all (“pale”) (Fig. 1). Visual inspection of the shells did not give any indication that shell thickness differs between the two morphs. Additionally, these morphs were checked for differences in pigmentation intensity by densitometric analysis (in the style of Köhler et al., 2009) as follows: Eight shells of each morph were individually and identically illuminated on a standardized grey background plate and photographs of both apex and umbilicus sides of their shells were taken using a digital single lens reflex camera (*Sony α100*) with a fixed aperture and fixed shutter speed. An average greyscale value (brightness) of each shell integrating both apex and umbilicus sides was calculated using ImageJ1.45 (National Institutes of Health, Bethesda, MD, USA), whereas the greyscale value of the background plate surrounding the shell was used as a standard.

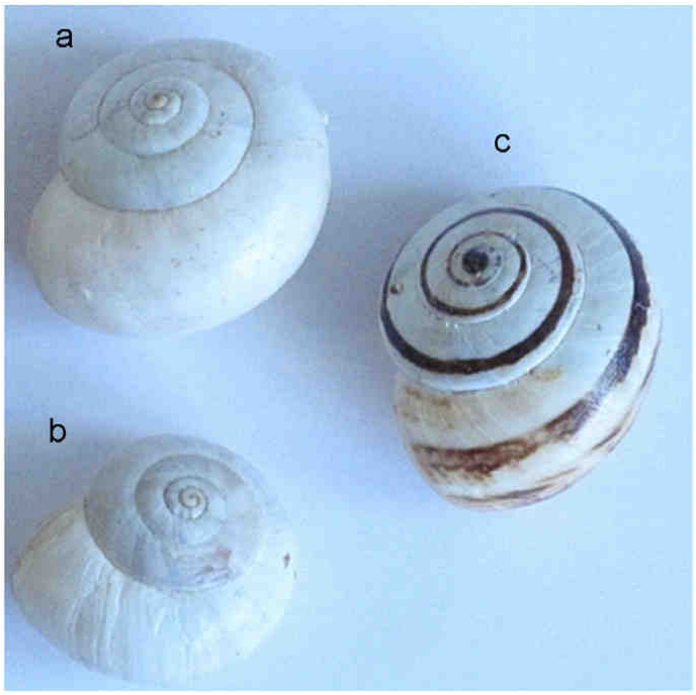

Figure 1. Shell examples (*Theba pisana*) for the two tested morph groups,pale (a,b) and dark (c).

For the thermographic measurements we conducted two different test schemes using either a full spectrum light bulb (*Ultra Vitalux*, Osram GmbH, München, Germany) or a heating cabinet (*ST2A60*, STL-Neckarwestheim, Neckarwestheim, Germany) to heat up the shells. In the light bulb experiment we tested the illumination-induced heating of the shells. The heat loss of shells was analysed using the heating cabinet. Of each morph, 14 individuals were tested (n=14) in the light bulb experiment, and 8 shells of each morph were used in the heating cabinet test (n=8). For both test schemes the shells were individually glued to the tips of wooden skewers and mounted on a foam block. This allowed stable but flexible positioning of shells in the heating cabinet or under the light bulb, respectively.

For the light bulb experiment each shell was individually fixed to the foam block and placed under the centre of the light bulb in a defined position with a space of 30cm between the shell and the light bulb. The light bulb was suspended from the top in a wooden board specially constructed for this experiment to minimize turbulences. The board was equipped with a 5cm slit confining heat loss during camera measurements. When the internal temperature of the board thoroughly reached room temperature (22°C, monitored by two thermometers), the light bulb was switched on and measurements and thermographic pictures were taken 10s, 20s, 30s, 40s, 50s, 60s, 90s, 120s, 150s and 180s afterwards. The 180s time point was chosen as a maximum as it became clear in pre-tests (data not shown) that shells exceeded ecologically relevant temperatures after that time span. It was ensured that the internal board temperature reached room temperature again each time before starting any further test. The

measuring points at each shell were top, centre and bottom (Fig. 2). The temperatures taken were converted to Kelvin and the corrected emission-ratio for coarse chalk (0.93; BARTEC, 2001) was applied to the results. For each measuring point the average warming per second was calculated, and, to obtain an estimate of the general warming of the whole shell, the results for all three measuring points were fused for each shell. Also, the kinetics of warming (differences between the time points) for each measuring point and the whole shells (all measuring points fused for each shell) were analysed.

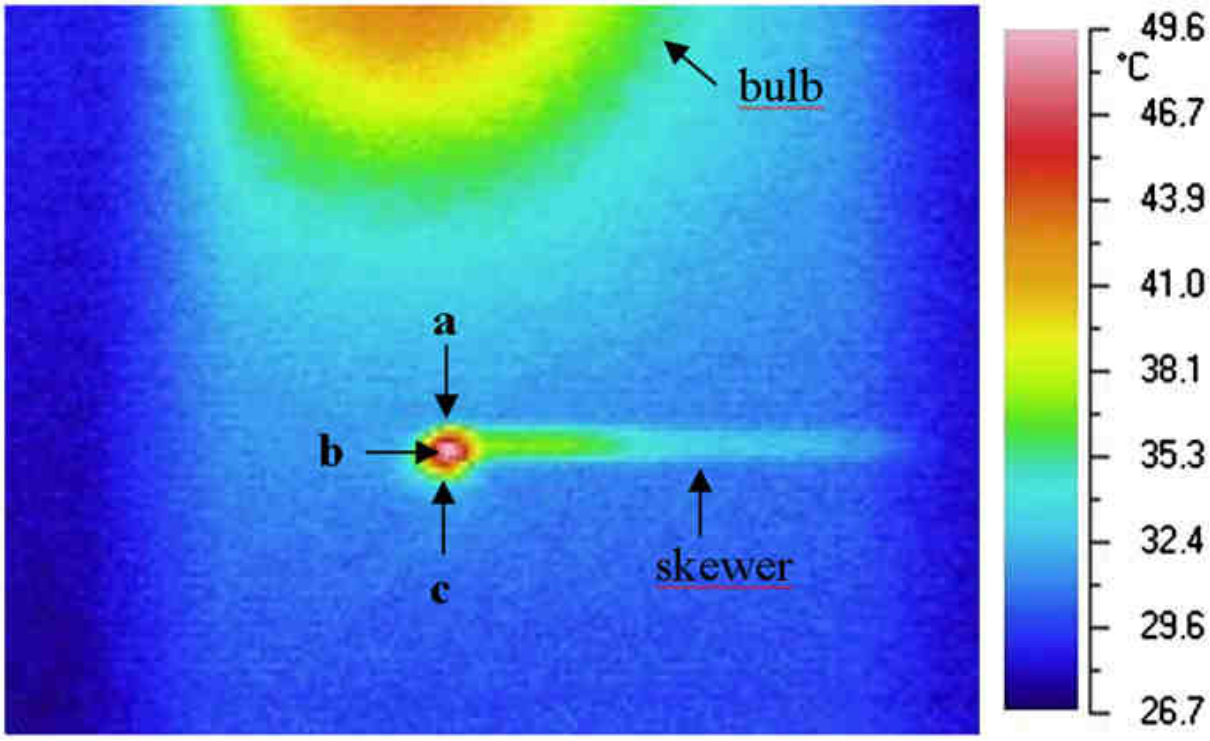

Figure 2. Thermographic picture of a shell demonstrating the test set-up and measurement points, a= top of shell, b= centre of shell, c= bottom of shell.

In order to test the heat loss of shells in the most standardised way possible, we chose to heat up the shells uniformly in a heating cabinet. For the heating cabinet test three positions were defined for the snail skewers on the foam block from left to right: a, b and c. Each shell was measured twice on each position, therefore resulting in 6 runs for each shell. The foam block was mounted to a defined position in a styrofoam box with the opening turned forward. The Styrofoam box was fixed to a defined position in the heating cabinet. The heating cabinet was then set to 50°C, and when the internal temperature, monitored by two mobile thermometers put into defined positions and the heating cabinet's own thermometer, reached 45°C, further heating was stopped and measurements were started. The styrofoam box ensured a slower loss of heat and therefore better conditions for taking pictures and measurements of the heated shells, as it was essential for correct measurement to open the heating cabinet (pre-tests, data

not shown). The temperature of 45°C was chosen as this represents an ecologically relevant temperature for these snails (Köhler et al., 2009; Scheil et al., 2011). Pictures and measurements were taken immediately after opening of the cabinet ("start", 0s), and 5s, 10s, 15s, 20s, 25s afterwards. The measuring point for each shell was its centre. Again, for the results the measured temperatures were converted to Kelvin and a corrected emission ratio (0.93 for coarse chalk, according to BARTEC, 2001) was applied. We calculated the average heat loss per second and the kinetics of heat loss (differences between the time points).

2.3 Statistical analysis

All data were analysed implanting JMP®8.0 (SAS Institute Inc., Cary, USA). Data were tested for normality with the Shapiro-Wilks-test. Normally distributed results were tested for significant differences using the Tukey-Kramer-HSD-test, whereas not normally distributed data were tested for significances implanting the nonparametric Wilcoxon-U-test. The levels of significance were set to $0.01<P\leq0.05$: * (slightly significant); $0.001<P\leq0.01$: ** (significant); $P\leq0.001$: *** (highly significant). For the results of the average warming of entire shells we applied a power analysis, computing the required sample size for possibly achieving significant results, via G*Power 3 (freeware, developed by Erdfelder et al., 1996).

Results

3.1 Densitometric analysis of different morphs

The densitometric analysis of the two different morphs revealed that the banded, dark morphs are highly significantly darker than the unbanded pale morphs (dark morphs greyscale value (brightness) means = 0.74696154, sd= 0.04378491; pale morphs greyscale value (brightness) means= 0.96727697, sd= 0.02543142; $P\leq0.001$).

3.2 Light bulb experiments- warming of shells

Concerning the average warming detected at the top of shells and bottom of shells (Figs. 3a) and 3c) no significant differences were found in pale morphs versus dark morphs. Measuring the centre of shells revealed a significant difference between pale and dark morphs (Fig. 3 b). However, the average warming of entire shells (all measurement points, Fig. 3d) was not significantly different in the two morphs. The results of the power analysis (Fig. 6) show that the required sample size concerning the warming of entire shells would be n=3330 for each morph group and n=6660 in total.

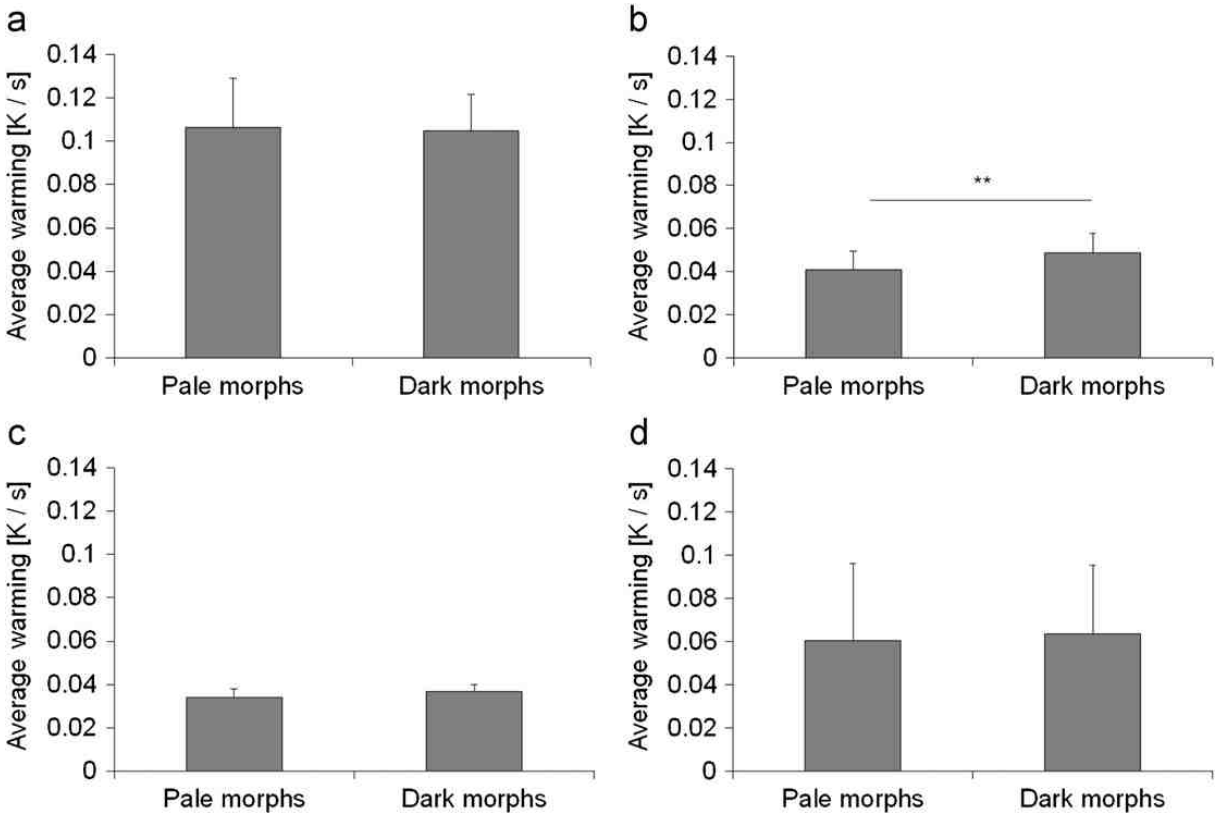

Figure 3. Average warming of a) top of shells b) centre of shells c) bottom of shells d) all measurement points (means+sd, n= 14; ** P=0.0100).

Analysing the kinetics of warming in both shell morphs, no significant differences could be found regarding all measurement points (single and fused) at all tested times (Fig. 4).

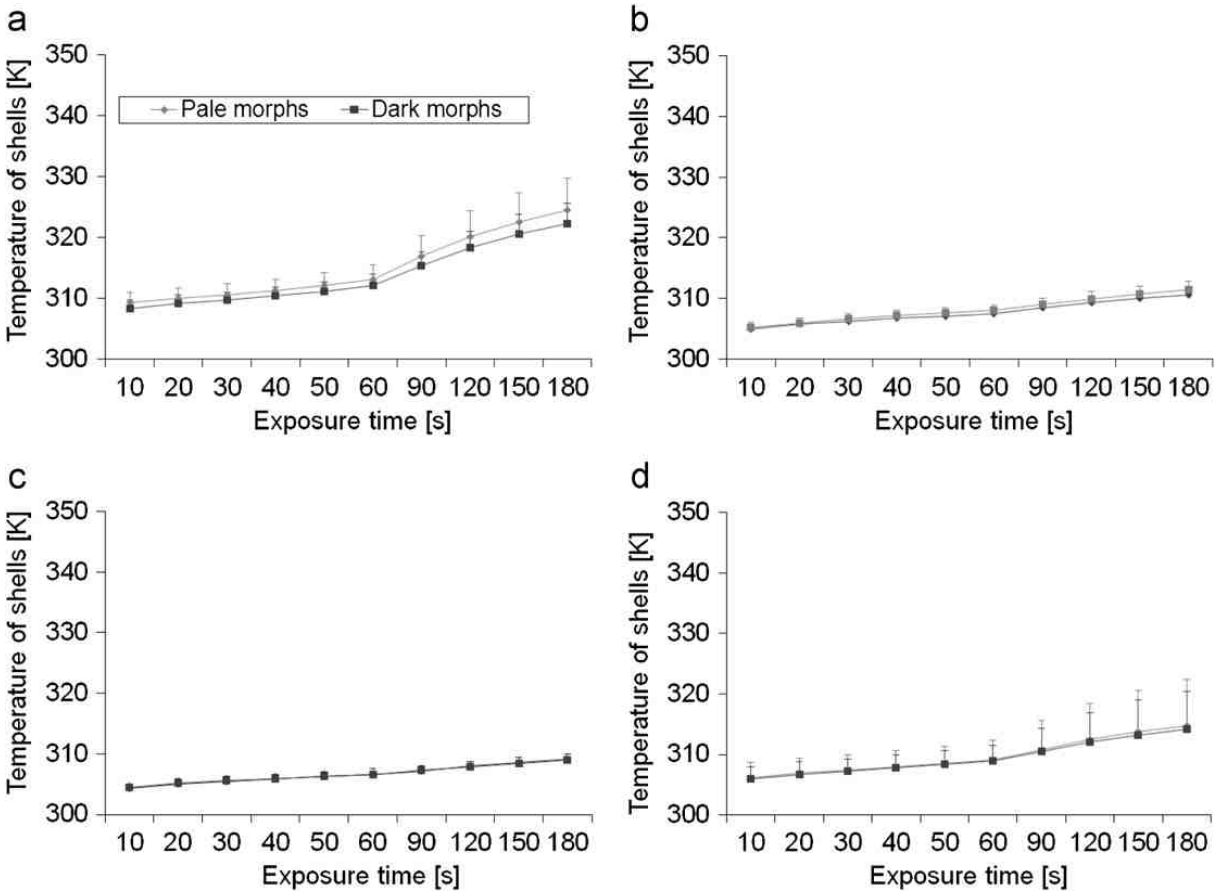

Figure 4. Kinetics of warming of a) top of shells b) centre of shells c) bottom of shells d) all measurement points (means+sd, n= 14).

3.3 Heating cabinet experiments- heat loss of shells

No significant differences between pale and dark morphs were found regarding the average heat loss of shells (Fig. 5a). Furthermore, no significant differences between pale and dark morphs could be detected when analysing the kinetics of heat loss of the shells (Fig. 5b).

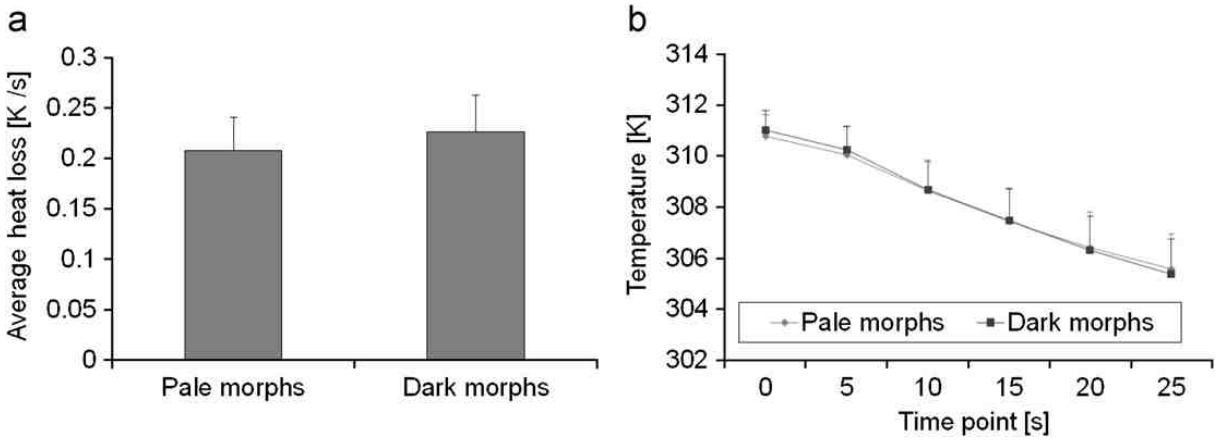

Figure 5. Heat loss of pale vs. dark morphs a) average heat loss b) kinetics (means+sd, n=8).

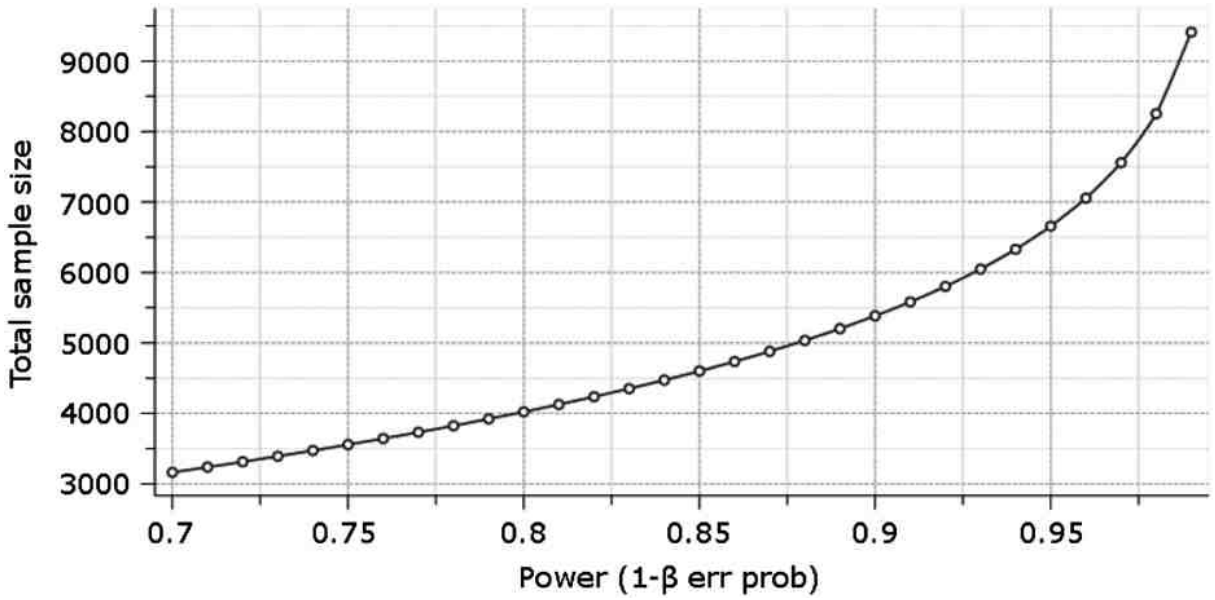

Figure 6. Power analysis of the required sample size for the average warming of entire shells, computed via G*Power 3; t-tests- Means: Wilcoxon-Mann-Whitney test (two groups) Tail(s)= Two, Parent distribution= Normal, Allocation ratio N2/N1=1, α err prob = 0.05, Effect size d= 0.0904247.

Discussion

The results of our warming and heat loss experiments with two colour morphs (pale and dark) of *T. pisana* show that no differences regarding warming or heat loss of entire shells can be observed under highly reproducible conditions. The significantly higher warming of dark shells when considering the centre of shells only, does not result in a generally higher warming of entire shells as well. Additionally, it has to be taken into account that this difference of warming at the centre of shells is very small in absolute terms. Therefore, the probability of consequences for the animals resulting from this difference can be considered very low. Furthermore, the results of the power analysis for the measurement of the warming of entire shells demonstrate that a very large sample size would be required, i.e. only with sample sizes exceeding 3000 shells for each morph group and 6000 shells in total there is a probability of achieving significant differences. However, it is known that very large sample sizes can produce a significant outcome due to trivial effects only (Quinn and Keough, 2003). This means that very large sample sizes may detect tiny differences which are practically meaningless (Sachs, 2004). We therefore conclude that the probability of the occurrence of significant differences between pale and dark shells concerning heating properties under highly standardised conditions is very low.

Additionally, there were no differences detectable between the colour morphs in terms of warming or heat loss kinetics.

Several studies on differently-coloured snails suggest a higher warming capacity of the darker morphs (Cook and Freeman, 1986; Heath, 1975; Jones, 1973). However, all of these studies were conducted under less- standardised conditions than our experiments, and often with little environmental relevance. Concerning Jones' experiments (1973), the exact conditions of the tests, e.g. placement and orientation of shells, eventual windspeed, humidity, season when tests were conducted are not mentioned . Cook and Freeman (1986) conducted tests implanting a microscope light bulb for radiation, so they did not use a natural or close-to-natural light source. Heath (1975) did place the shells under natural sunlight in defined positions, however, these experiments were conducted outdoors with no consideration of windspeed and over a rather broad time period from May to August. As quality, intensity and duration of solar radiation can greatly vary in different seasons (Burkholder, 1936), this set-up cannot be considered applicable for yielding results as optimal as possible, too. In our experiments, we were able to standardise the whole test procedure by exact and equal positioning of each test shell using the same full spectrum light bulb or heating cabinet with as little turbulences as possible during each test run.

To explain our results one must consider that, contrary to the common-sense assumption that darker colours absorb more solar radiation than pale colours, the visual impression of colour is generated via reflection of parts of the visible light spectrum, and this does not reveal any information about absorption or reflection of other wavelengths, e.g. infra-red, which are accountable for heating or cooling (Gunn, 1942). Therefore, a higher warming of darker shells is theoretically not mandatory and our test results corroborate this as well. In fact, there is other work showing that differently coloured morphs of a species may not necessarily have different warming capacities as well (Pepper and Hastings, 1952; Stower and Griffiths, 1966).

Additionally, it is also known that thermal or heat capacity is material-dependent (Eichler et al., 2005; Heuberger and Fels, 2007). Taken together with the fact that shells of pulmonate land snails are made up of 97% calcium carbonate and only 3% organic matrix (Heller and Magaritz, 1983), this also helps explaining the uniform heating properties of different colour morphs that we observed. Moreover, our findings also offer a possible explanation for the absence of differences in colour morphs concerning thermal behaviour as it was observed by Cowie (1992): as there is no difference between shell colour morphs regarding thermal capacity, there is no necessity for the assumption that different thermal behaviour strategies have evolved as well.

Furthermore, our findings also show that the shells' heat loss properties are equal in both tested morphs. This, of course, is plausible when taking into account the above mentioned

facts on visual impact of colour versus absorption or, in case of heat loss, reflection of, for example, infra-red rays (material dependency rather than colour dependency). It is also in accordance with Jones' (1973) considerations on heat loss of differently coloured snails, where he explains why an assumption of a more intense heat loss of darker morphs, when radiation is absent, cannot be true.

Thus, the question arises, why correlations between climate and morph distribution can be found when there is no difference in heat gain and loss properties of different *T. pisana* morphs. There is several work indicating such correlations, regarding either shell or body colouration, which all find the darker morphs to occur more frequently in northern, hence colder and wetter (climate) or more shaded and less open (microclimate, vegetation) habitats, whereas the paler morphs are more frequent in southern and open habitats (Johnson, 2012; Johnson, 2011; Cowie, 1990; Heller, 1981; Heller and Gadot, 1984; Johnson, 1981). Also, there is evidence that the paler morphs are more abundant in hotter summers (Johnson, 2012; Johnson, 2011). Yet, these correlations might, at least partly, also derive from rather indirect climate effects associated to the occurrence of parasites, for example nematodes. As nematodes require high moisture levels (Kaya, 2000-2001), it is possible that parasite stress is higher in northern and more humid regions, in more sheltered habitats that retain moisture over longer periods or in colder and wetter summers. Indeed, it has been observed that climatic conditions have an effect on parasite frequency in terrestrial molluscs (Morley and Lewis, 2008). Taken together with the results of Cabaret (1983), who found unbanded morphs of *T. pisana* to be more susceptible to nematode infection than banded morphs under both natural and experimental conditions, one could imagine that the differential shell morph distribution is a result of a higher parasitic stress in wetter habitats furthering the less-susceptible darker snails.

Additionally, it is also known that in terms of microhabitat choice predation plays an important role and that darker morphs are at an advantage in sheltered habitats for being more cryptic (Heller, 1981; Heller and Gadot, 1984). Heller and Gadot (1984) also state that white shells are advantageous in open habitats for protecting against solar radiation. However, regarding our findings this benefit of pale shells appears questionable, and our results might point to the consideration that predation effects could be the more important factors contributing to the observed differential morph distribution.

Concerning microhabitat and habitat choice, our results suggest that the idea of different thermal capacities being one of the driving forces of differential morph distribution should be regarded much more cautiously as before, and they indicate that correlations between climate,

morph distribution and polymorphism may be of a more indirect nature than previously assumed.

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Kapitel 4: Shell colour polymorphism, injuries and immune defense in three helicid snail species, *Cepaea hortensis, Theba pisana* and *Cornu aspersum maximum*

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Abstract

Shell colour polymorphism is a widespread feature of various land snail species, and evolutionary persistence has been attributed to a number of reasons among which climate impact due to different warming capacities of different morphs and predation effects are common. However, the assumption of morph-specific thermodynamics has been relativised by recent work, and more indirect climatic effects, namely the impact of higher humidity and associated higher pathogen stress in particular habitats have been proposed as possible factors influencing morph distribution. Indeed, previous studies have demonstrated that, for example, parasitic load can be different in differently coloured shell morphs, yet possible mechanisms underlying this phenomenon still remain unclear. In our study we aimed at elucidating the question whether there is a correlation between shell colouration and immune defense in three land snail species by comparing phenoloxidase (PO) activity levels of different morphs after immunostimulation via Zymosan A-injection. Since phenoloxidase is involved both in immune defense as well as in melanin production, the PO activity level is particularly interesting when trying to resolve this question. Even though Zymosan A failed to induce PO activity rendering a comparison of inducible PO activity impossible, an interesting and concomitant difference between pale and dark morphs of all tested species could be observed: dark snails were less affected by hemolymph withdrawal and were able to maintain or regenerate a significantly higher PO activity level after hemolymph withdrawal than pale snails. Possible implications of this observation are discussed.

Introduction

Shell colour polymorphism is a phenomenon which can be found in several land snail species (Cook, 1998; Albuquerque de Matos, 1984; Johnson, 1981), including *Cepaea hortensis* (O.F. MÜLLER, 1774), *Theba pisana* (O.F. MÜLLER, 1774) and *Cornu aspersum* (O.F. MÜLLER, 1774). Quite a number of studies attribute this polymorphism to predation or climatic effects (Johnson, 2011; Cowie, 1990; Heller, 1981; Heller & Gadot, 1984; Johnson, 1981), whereas climatic effects are often explained by a higher warming capacity in darker morphs furthering the paler morphs in sun-exposed, warmer habitats (Heath, 1975; Jones, 1973). However, recent work (Scheil et al., 2012) has shown that the assumption of a higher warming capacity in darker snail shells should be regarded with caution, and alternative factors and correlations have been proposed, among which are humidity and the higher occurrence of parasites under humid conditions in northern and / or sheltered habitats. And in fact, it was demonstrated that wetter conditions can further parasite stress on molluscs (Morley & Lewis, 2008). Interestingly, a correlation between shell colouration and parasitic load was observed in several snail species with the darker morphs being less parasitized than paler morphs (Cabaret, 1983; Cabaret, 1988). Taken together, this raises the questions whether there is a difference in pathogen resistance between dark and pale snail morphs, and which mechanism would be underlying such a correlation between shell colouration and immune defense. A correlation between colouration and phenoloxidase (PO)-mediated immunity has already been demonstrated in insects, whereas stronger melanisation and darker cuticle colour are linked to higher immunity (Barnes & Siva-Jothy, 2000; Cotter et al., 2004; Armitage & Siva-Jothy, 2005). In fact, it is known that melanism and immunity parameters are both based on the melanin-producing pathway, the so-called PO-cascade (Söderhäll & Cerenius, 1998; Rolff & Siva-Jothy, 2003). This cascade can be activated via β-1,3-glucans, peptidoglycans and lipopolysaccharides, which are derived from fungi or bacteria (Söderhäll & Cerenius, 1998). Such β-1,3-glucans can be found, for example, in Zymosan A, a yeast cell wall preparation that is commonly used for artificial PO activity stimulation in invertebrates (Vetvicka & Sima, 2004), and which was also chosen for immunostimulation in this study. To our knowledge, nothing is known about mechanisms underlying possible links between shell colouration and immunocompetence in molluscs even though hints to such links were found some decades ago (Cabaret, 1983; Cabaret, 1988). However, melanin has been shown to be a pigment which is also responsible for colouration of snail shells (Comfort, 1951), and PO is an important parameter in immune defense against microbial and parasitic pathogens in

molluscs (Aladaileh et al., 2007; Bahgat et al., 2007), also playing an important role in wound healing (Ratcliffe et al., 1985) and sclerotization of molluscan shells (Waite & Wilbur, 1976; Nellaiappan & Kalyani, 1989). Therefore it is imaginable that correlations between shell pigmentation and immune defense and between shell pigmentation and wound healing / sclerotization processes exist in molluscs as well.

In this study, we have focused on investigating correlations between shell colouration and constitutive as well as inducible PO activity in different shell colour morphs of *Cepaea hortensis*, *Theba pisana* and *Cornu aspersum maximum*, applying Zymosan A as an immunostimulant.

Materials & Methods

Test organisms, sampling and maintenance

Tests were conducted with three different land snail species, *Cepaea hortensis* (O.F. Müller, 1774), *Theba pisana* (O.F. Müller, 1774) and *Cornu aspersum maximum* (cultivated variety of *Cornu aspersum*, O.F. Müller, 1774; nomenclature according to Falkner et al., 2001). *Cornu aspersum maxium* was obtained from a local snail farm (Schneckengarten Munderkingen, Munderkingen, Germany) and acclimatised to laboratory conditions (20°C, 60-90% humidity) for at least two weeks before the experiments were started. *Cepaea hortensis* was sampled from a private garden in Tübingen-Lustnau, Germany, where no pesticides are applied, and *Theba pisana* was collected near Les Paluds de Noves (Dept. Bouches du Rhône) in Southern France. Before testing, *C. hortensis* and *T. pisana* were acclimatised to laboratory conditions (18°C for *C. hortensis*, 22°C for *T. pisana*, 50-90% humidity) for at least two weeks. All snails were fed a diet of organic carrots / cucumbers / zucchini / oats *ad libitum* once a week and organic baby food (*Hipp Bio-Milchbrei*, Hipp GmbH & Co. Vertrieb KG, Pfaffenhofen, Germany), prepared according to package instructions, *ad libitum* twice a week. Clean cuttlebone was provided *ad libitum* at all times. Animals were kept in ventilated plastic terraria (30 x 19.5 x 20.5cm) containing a moistened 2cm layer of *JBL Terra Basis ground covering for terraria* (JBL GmbH & Co. KG, Neuhofen, Germany). Terraria were re-moistened with tap water every other day and cleaned on a weekly basis.

Experimental set-up, general

To avoid bias through naturally existing parasitic infections such as nematodes, 10% of the sampled snails were tested by peptic digestion of pieces of the headfoot as described in Cabaret (1980). In all cases, no parasites could be detected.

All test snails were individually exposed to the respective exposure or control conditions in plastic boxes (9 x 6 x 9cm, with perforated transparent lids, in case of *C. hortensis* and *T. pisana*) or plastic terraria (16.5 x 16.5 x 19cm) with lid and wall perforations, in case of C. *aspersum maximum*) lined with a moistened 2cm layer of *JBL Terra Basis ground covering for terraria* (JBL GmbH & Co. KG, Neuhofen, Germany). The species-specific laboratory maintenance temperatures as mentioned above were sustained throughout the respective tests. Hemolymph collection was conducted according to Renwrantz et al., 1981, with the following modifications: hemolymph (HL) was withdrawn from each animal from the hemocoel of the upper to middle subepithelial region of the headfoot at a quantity of 20µL using sterile syringes (1mL) and 0.40 x 20mM gauge sterile hypodermic needles. In *C. aspersum maximum*, pre-drilling of a small hole into the shells was required due to the shells' hardness. Punctured veins were not glued as this resulted in strong mucus production during pre-tests.

For Zymosan A- injections the same types of syringes and needles, and the same puncture sites were used as for HL collection. The Zymosan A solution contained 5mg Zymosan A (Sigma Aldrich Chemie GmbH, Steinheim, Germany) in 1mL snail saline (prepared according to Chiarandini, 1964), equivalent to ± 4 * 10^7 particles / mL (as in Matricon-Gondran & Letocart, 1999). The Zymosan A solution was freshly prepared for each test.

Originally, we planned to re-sample Zymosan A- injected snails 6h and 24h after injection. The 24h time point was chosen as it has been shown in other molluscs that PO activity can increase two-fold within 24h after Zymosan A-injection (Aladaileh, 2007). The 6h time point was chosen in order to test for a possibly earlier PO activity induction. However, in *C. hortensis* and *T. pisana*, amendments to this schedule were necessary as described below.

Experimental set-up for C. hortensis

Snails were divided in two different morph groups: yellow (later referred to as 'pale'(p)) and strongly-banded with five brown bands on yellow base colour (later referred to as 'dark' (d)) (Fig. 1a). Of each morph group, 14 animals were sampled for HL collection at the beginning of the experiment (0h, base level). Then 100µL of the Zymosan A- solution were injected into each snail. After 24h, snails were sampled for HL collection again (24h Zymosan A exposure). In contrast to our later experiments with *C. aspersum maximum*, we avoided sampling hemolymph at 6h of test time, as this proved to be too stressful for *C. hortensis* in pre-tests, probably due to the short recovery time between 0h and 6h, and the relatively small size of *C. hortensis*.

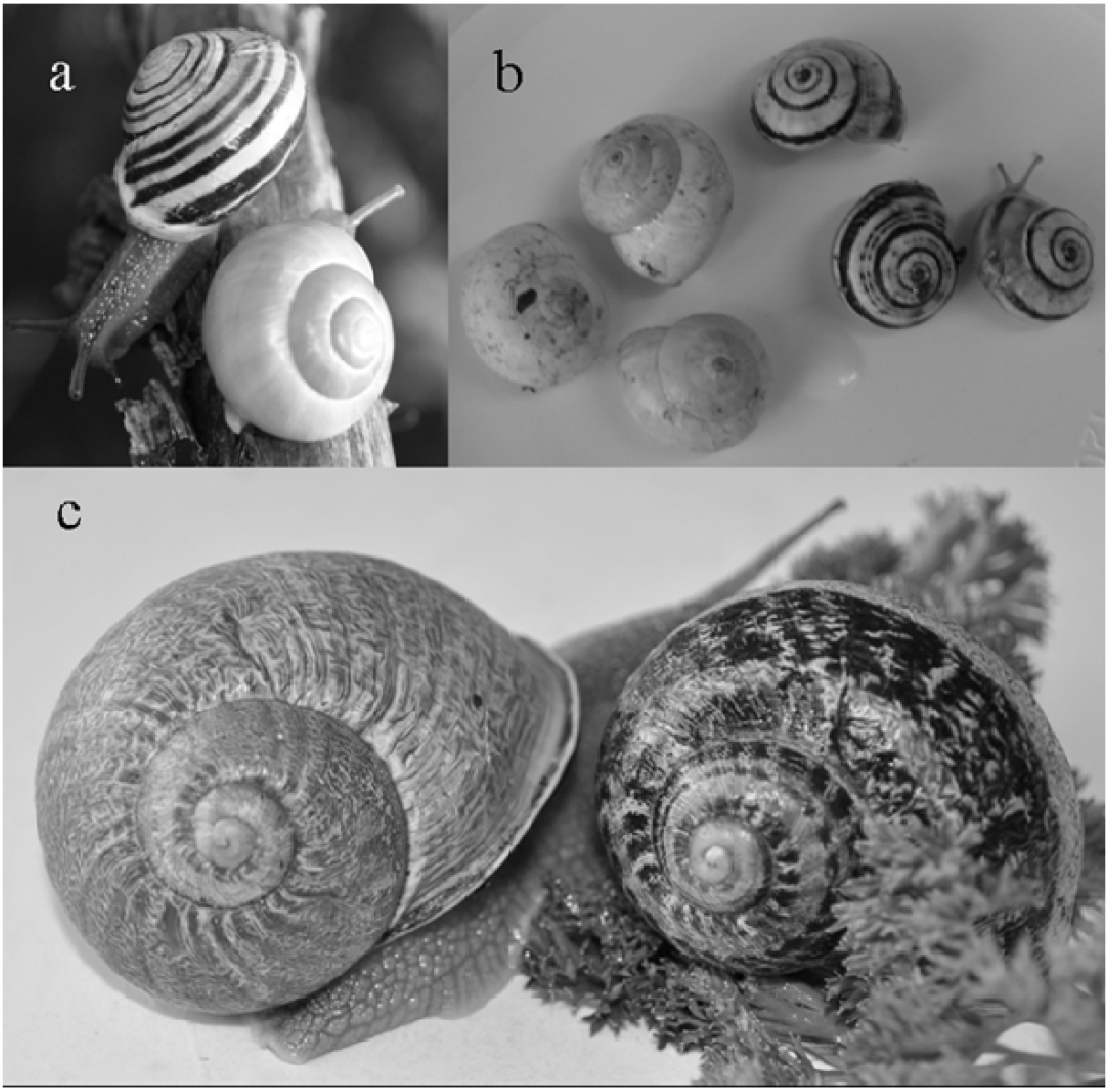

Figure 1. Different morphs of test snails; pale and dark morph of *C. hortensis* (a), pale and dark morphs of *T. pisana* (b), pale and dark morph of *C. aspersum maximum* (c).

Experimental set-up for T. pisana

Snails were divided in two different morph groups: pale white (referred to as 'pale' (p) in the following) and darkly- banded with distinct, dark- brown bands (referred to as 'dark' (d) in the following) (Fig. 1b). Tests were conducted in two runs for each morph: 1) 10 animals were sampled for HL collection at 0h (base level 1). After 24h, these animals were resampled for a further HL collection (24h HL withdrawal).

2) 10 animals were sampled for HL collection at 0h (base level 2). These animals were also injected an 100µL aliquot of the Zymosan A solution each after the 0h- HL collection. After 24h, these snails were resampled for HL collection (HL withdrawal + 24h Zymosan A exposure). Another 10 snails were injected 100µL of Zymosan A solution each at 0h without

prior HL collection. After 24h, they were sampled for HL collection (24h Zymosan A exposure). As in *C. hortensis*, we omitted a 6h hemolymph-sampling due to the short time span between 0h and 6h, and the small size of the snails.

Experimental set-up for C. aspersum maximum

Snails were divided in two different morph groups: pale brownish / yellowish without bands (referred to as 'pale' (p) in the following) and dark brown with bands (referred to as 'dark' (d) in the following) (Fig. 1c). Tests with this species were also conducted in two runs for each morph (Fig. 2): 1) 15 snails were sampled for HL collection at 0h, 6h and 24h (base level 1, 6h HL withdrawal and 24h HL withdrawal, the latter as corresponding controls to the respective Zymosan A exposure times). Another 15 animals were sampled for HL collection at 0h (base level 2), these animals were also injected 100µL of the Zymosan A solution each at 0h. They were resampled at 6h and 24h (HL withdrawal + 6h and 24h Zymosan A exposure).

2) 15 animals were sampled for HL collection at 0h (base level 3). Another 15 animals were sampled for HL collection at 6h (6h control), these animals were resampled at 24h (24h control). A further 15 snails were injected an 100µL aliquot of the Zymosan A solution each at 0h without prior HL collection. These animals were sampled for HL collection at 6h (6h Zymosan A exposure) and 24h (24h Zymosan A exposure).

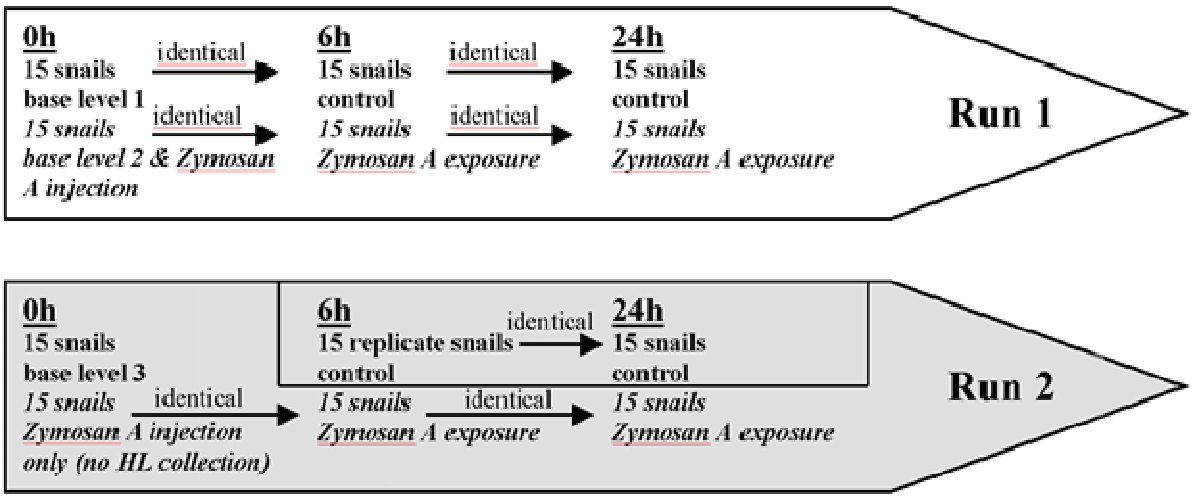

Figure 2. Experimental set-up for *C. aspersum maximum*; both runs (run 1 & run 2) were conducted for each morph, pale and dark.

Phenoloxidase (PO) Assay

The phenoloxidase assay was conducted with hemolymph (HL) samples. This restriction to HL samples was chosen as we aimed at depicting the immune-defense-related function of PO as a part of the humoral immune response of molluscs (as described in Gliński & Jarosz,

1997), avoiding possible bias through, for example, reproduction-related functions of the enzyme in other tissues (Bai et al., 1997; Kalyani et al., 1985). Furthermore, the analysis of PO in HL samples has already been successfully performed for a number of invertebrate species, including molluscs (Smith & Söderhäll, 1991; Barracco et al., 1999; Jordan & Deaton, 2005; Munoz et al., 2006; Seppälä & Jokela, 2010; Seppälä et al., 2011), and PO analysis in other tissue samples is rather regarded as an alternative in case HL collection is not feasible (Luna-González et al., 2003). The assay procedure was adapted from Seppälä & Jokela (2010) with slight modifications. In short, 20µL of hemolymph (HL) were mixed with 200µL of phosphate buffered saline (PBS, pH 7.4; Sigma Aldrich Chemie GmbH, Steinheim, Germany) and immediately shock-frozen in liquid nitrogen. The resulting samples were stored at -80°C until further processing. After thawing, 40µL sample aliquots were placed in 96-well microtiter plate wells which contained 140µL of cold aqua bidest. and 20µL of PBS each. Each sample was measured in triplicates. Additionally, four controls (sample aliquots replaced by aqua bidest.) per plate were set up. Then, 20µL of cold L-dopa (Sigma Aldrich Chemie GmbH, Steinheim, Germany) solution (4mg / mL aqua bidest.) were added to each well and plates were immediately measured photometrically at 490nm in a microplate reader (ELx800, Bio-Tek Instruments, INC., Vermont, USA) resulting in 0h values. The plates were then covered and incubated at 30°C in a thermocabinet (ST 2 A60, STL-Neckarwestheim, Neckarwestheim, Germany) for a species-specific time (30min. for *C. hortensis*, 6h for *T. pisana* and 3.5h for *C. aspersum maximum*, determined in pre-tests, data not shown) to ensure linearity of the absorbance increase allowing most accurate measurments. After incubation, the plates were re-measured photometrically at 490nm resulting in incubation time values. PO activity was then calculated according to the following equation

PO activity= incubation time values – 0h values – mean absorbance change in controls,

and expressed in milliunits.

Statistical analysis

The obtained data were statistically analysed implanting JMP® 9.0 (SAS Institute Inc., Cary, USA). Data were tested for normality using the Shapiro-Wilks- test, and when following normal distribution were analysed via Tukey-Kramer-HSD for significant differences. Not normally distributed data were analysed for significant differences using the non-parametric Wilcoxon U- test and in case of multiple comparisons, a Bonferroni correction was applied. Levels of significance were set to $0.01<P\leq0.05$: *; $0.001<P\leq0.01$: **; $P\leq0.001$: *** for normally distributed data and not normally distributed data used in single comparisons. For not normally distributed data analysed in multiple comparisons, the levels of significance

were calculated implanting a Bonferroni correction, they are shown in the respective figure legends.

Results

Phenoloxidase (PO) activity in C. hortensis

The base levels of the different morphs were not significantly different from each other, even though the level of dark morphs tended to be higher (Fig. 3). Also, the PO activity levels of the different morphs after HL withdrawal and Zymosan A exposure for 24h did not differ significantly (Fig. 3). However, when comparing the results within the morph groups it became evident that the PO activity level in pale morphs decreased significantly after HL withdrawal and Zymosan A exposure for 24h (Fig. 3). In the dark morphs no significant difference could be detected between base level and results after HL withdrawal and Zymosan A exposure for 24h (Fig. 3).

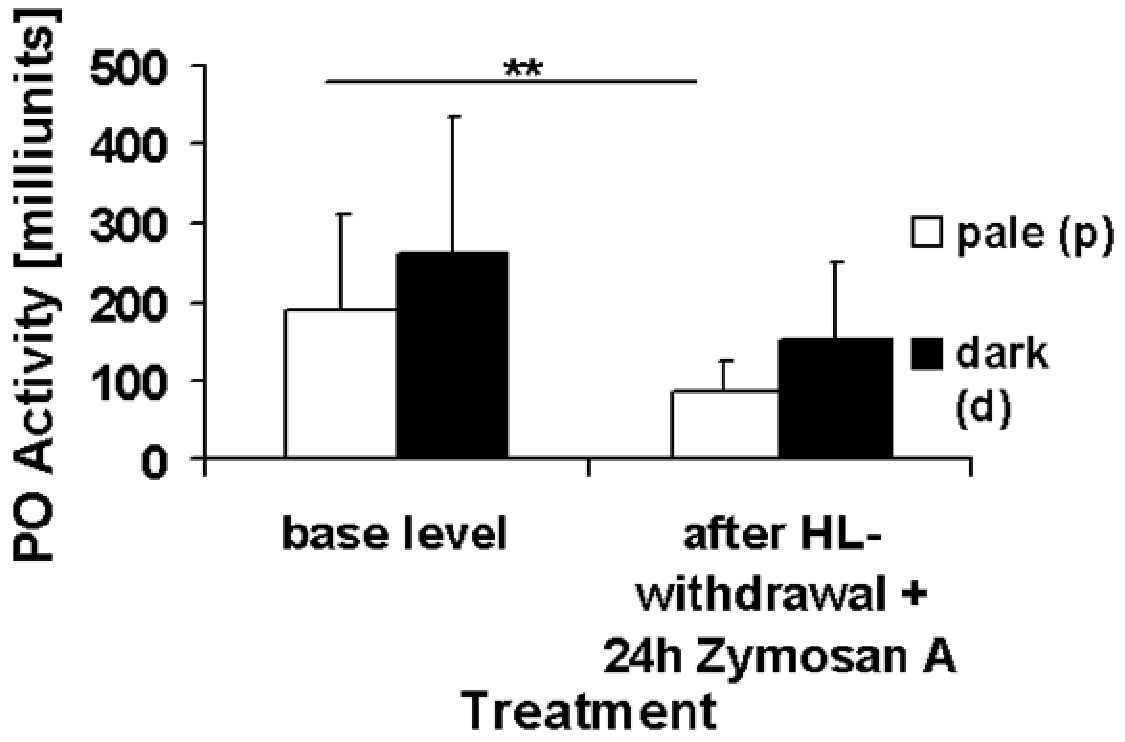

Figure 3. Phenoloxidase (PO) activity levels in different morphs of *C. hortensis*; base levels and levels after hemolymph withdrawal and 24h Zymosan A-exposure (means + sd; n=10; $0.001<p\leq0.01$: **).

Phenoloxidase activity in T. pisana

Run 1: No significant differences between the base levels (base level 1) were found between the two morphs, yet there was a tendency for a higher level in dark morphs (Fig. 4). 24h after

HL withdrawal no significant differences between the two morphs could be detected as well (Fig. 4). However, the 24h HL withdrawal results for the pale morphs had significantly decreased compared to the respective base level, whereas no such decrease could be found in the dark morphs (Fig. 4).

Run 2: When comparing the two morphs, no significant differences were found concerning the base levels (base level 2) even though, by trend, the level in dark morphs appeared higher, also there were no significant differences detectable between the base levels of run 1 and run 2 (Fig. 4). The HL withdrawal combined with a 24h Zymosan A exposure resulted in a significant decrease of the PO activity level compared to the base level in both morphs (Fig. 4). A 24h Zymosan A exposure without prior HL withdrawal did not result in significant differences compared to the base levels in both morphs (Fig. 4). However, in both morphs the 24h Zymosan A exposure results were significantly higher compared to the respective HL withdrawal + 24h Zymosan A exposure results (Fig. 4). Furthermore, the 24h Zymosan A exposure data recorded for the two morphs differed significantly from each other with the dark morphs showing a higher PO activity level.

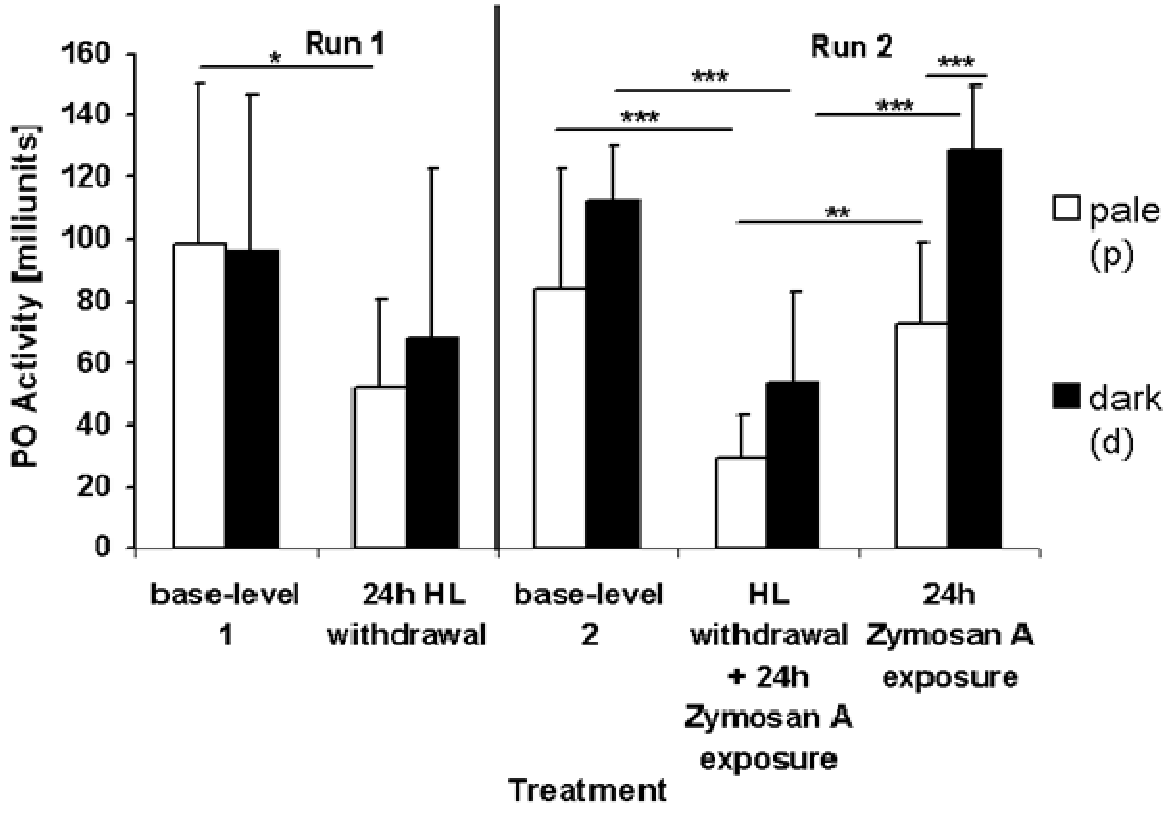

Figure 4. Phenoloxidase (PO) activity levels in different morphs of *T. pisana*; test run 1 with base levels and levels 24h after hemolymph withdrawal, and test run 2 with base levels, levels after hemolymph withdrawal and 24h Zymosan A-exposure and levels after 24h Zymosan A-exposure only (means + sd; n=10; $0.01<p\leq0.05$: *; $0.001<p\leq0.01$: **; $p\leq0.001$: ***).

Phenoloxidase activity in C. aspersum maximum

Run 1: The base levels (base level 1) of the two morphs did not differ significantly from each other (Fig. 5). Also, the 6h HL withdrawal results were not significantly different between the two morphs, and did not differ from their respective base levels as well (Fig. 5). However, the 24h HL withdrawal results in the pale morphs showed a significant decrease compared to the respective base level, whereas no such difference was found in the dark snails (Fig. 5). The base levels 2 of the different morphs did not differ from each other as well. Also, there were no significant differences between base levels 1 and 2 (Fig. 5). HL withdrawal combined with a 6h Zymosan A exposure resulted in a significantly decreased PO activity level for the pale morphs but not for the dark morphs (Fig. 5). After HL withdrawal + 24h Zymosan A exposure the PO activity levels were significantly decreased in both morphs (Fig. 5).

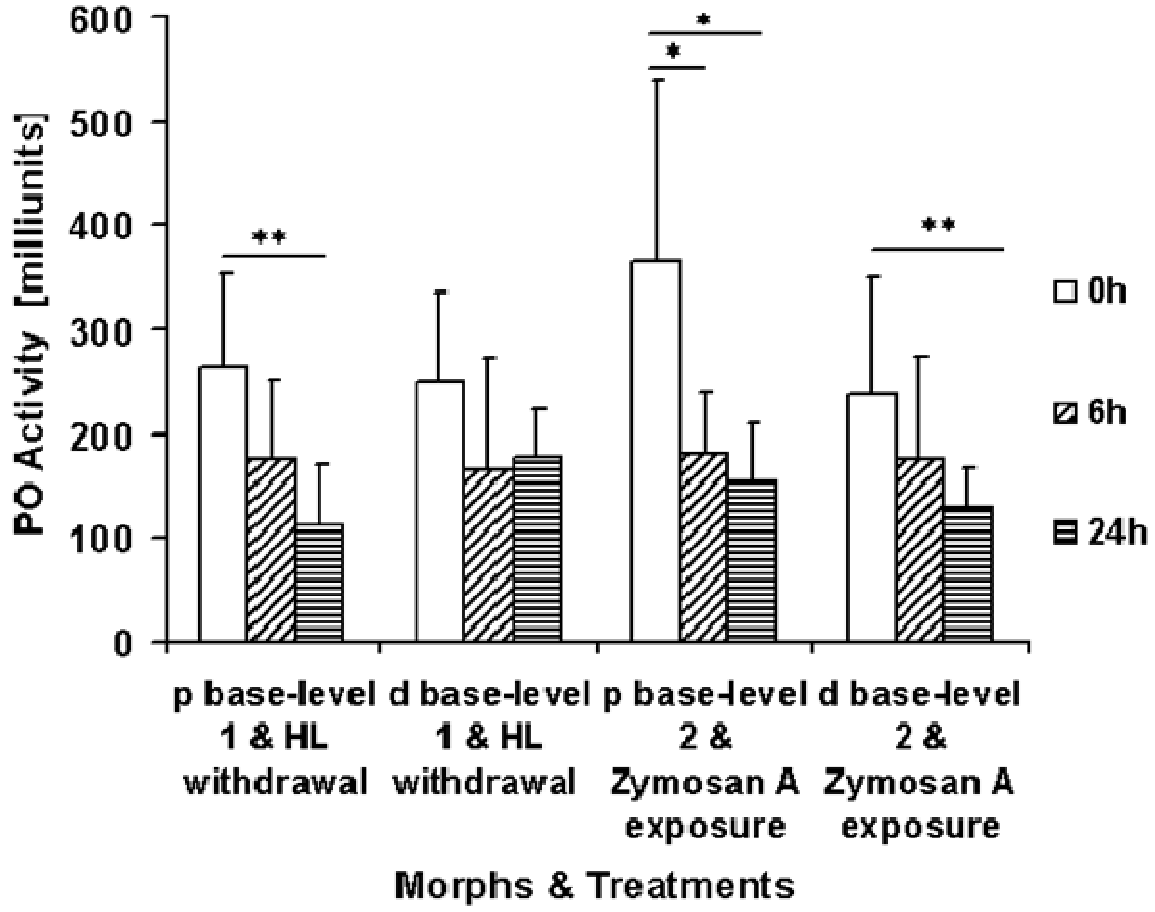

Figure 5. Phenoloxidase (PO) activity levels in different morphs of *C. aspersum maximum*; test run 1 with base-levels and levels 6h or 24h after hemolymph withdrawal considering the same individuals as for the base levels, and base levels and levels after hemolymph withdrawal plus 24h Zymosan A-exposure considering the same individuals as for the base levels (means + sd; n=15; $0.000435<p\leq0.0022$: *; $p\leq0.000435$: ** after Bonferroni-corrections for 23 comparisons).

Run 2: In run 2, no significantly different results could be detected, only a trend for slightly higher levels in base level and control snails was observable for the dark morphs (Fig. 6).

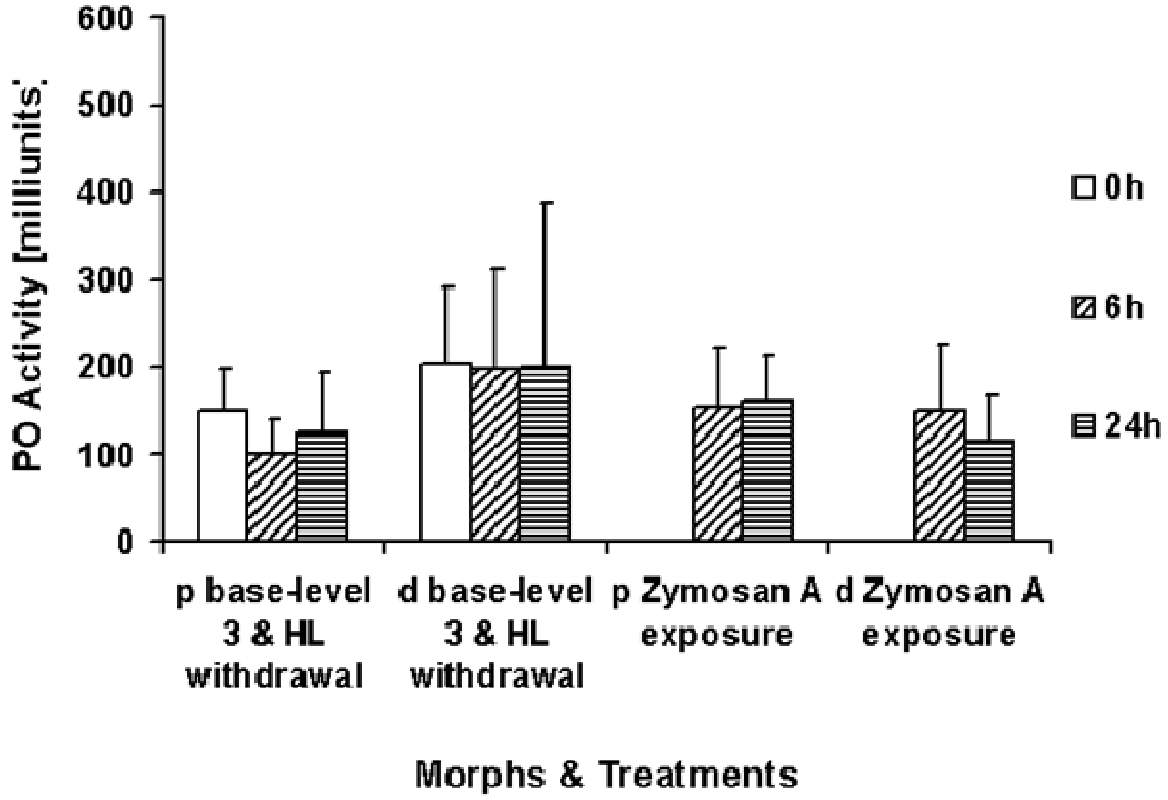

Figure 6. Phenoloxidase (PO) activity levels in different morphs of *C. aspersum maximum*; test run 2 with base levels and levels 6h or 24h after hemolymph withdrawal considering other individuals as for the base levels, and levels after 6h or 24h Zymosan A-exposure (means + sd; n=15; $p \leq 0.0024$:* after Bonferroni-corrections for 21 comparisons).

Discussion

Concerning the constitutive levels (base levels) of PO activity, it is remarkable that we did not find significant differences between different morphs in each of the species tested. This is contrary to what has been found in other invertebrate species, e.g. *Tenebrio* beetles (Armitage & Siva-Jothy, 2005), or other insects (Wilson et al., 2001). These studies revealed a positive relation between either stronger melanisation and higher constitutive PO activity levels leading to increased pathogen resistance in darker animals (Armitage & Siva-Jothy, 2005), or between melanism and disease resistance involving phenoloxidase (Wilson et al., 2001). However, concerning the three snail species tested in our study, we may exclude a higher pathogen resistance of darker snails based on differences in constitutive PO activity levels. Maintaining relatively high constitutive (or prophylactic) levels of phenoloxidase can provide animals with the benefit of higher resistance to pathogens, yet this may also be costly for the

respective organisms (Siva-Jothy & Thompson, 2002). Additionally, besides the nutrient-related cost there might be also another cost involved: For *Anopheles gambiae* being refractory to *Plasmodium* due to high melanisation and encapsulation capacities it has been shown that they are under permanent oxidative stress (Kumar et al., 2003). It is also known that phenoloxidase activity provides cytotoxic properties, this has, for example, been observed in ascidians (Ballarin et al., 1998) and is based on the generation of reactive oxygen metabolites during the PO-mediated conversion of phenols to *o*-quinones and then melanin. Therefore it is likely that maintaining a high constitutive PO activity level poses a considerable oxidative stress on the respective organism, in our case snails. A solution to the 'dilemma' of oxidative stress vs. pathogen resistance might come from relying on induced PO activity as it has been proposed for lighter-coloured beetles by Armitage & Siva-Jothy (2005), this restricts oxidative stress deriving from PO activity to periods of actual demand for immune response. However, in our experiments with *C. hortensis*, *T. pisana* and *C. aspersum maximum*, we did not observe any upregulation of PO activity following injection of Zymosan A in any of these three species. Consequently, no differences in inducible PO activity could be observed in different morphs. This might lead to the exclusion of a higher pathogen resistance based on different PO activity in darker morphs, rejecting our hypothesis. Yet, it has to be taken into account that the fact that the Zymosan A-injection failed to induce higher PO activity levels in all three species should be considered a rather unusual phenomenon. Immunostimulation via β-1,3 glucans, being a major component of yeast cell walls (Zymosan A), has been observed in a variety of invertebrates (Cárdenas & Dankert, 1997; Vetvicka & Sima, 2004; Pang et al., 2010), including molluscs (Coles & Pipe, 1994; Aladaileh et al., 2007; Hellio et al., 2007; Lacoue-Labarthe et al., 2009). However, there is also work showing that Zymosan A can appear ineffective in causing elevated PO activity levels (Arizza et al., 1995; Brivio et al., 1996), yet studies on this phenomenon are relatively scarce and no concluding explanation for this has been offered so far. Brivio et al. (1996) suggest the possibility of "self activation" of the proPO system upon injuries during the hemolymph collection process. In our case it is possible that a similar self activation took place during hemolymph collection as well as due to Zymosan A injection, and that this self activation might have masked the intended effects of the Zymosan A. However, this explanation remains relatively speculative so far. Another observation of Brivio et al. (1996) was that increasing Ca^{2+} concentration led to decreasing PO activity in the hemolymph of their test organisms (*Allogamus auricollis*), and they suggest that high calcium levels may protect against unwanted proPO activation in insects as this is also known for other arthropods

(Söderhäll, 1981; Ashida et al., 1983). As calcium ion levels in snail hemolymphs can be relatively high (Greenaway, 1971; Grospietsch et al., 2000), it is possible that a high calcium content might also form a barrier against undesired PO activity in our test snails. Again, although being a plausible explanation for the absence of Zymosan A-related PO activity increase in our snails, we cannot draw a final conclusion from this ruling out other, probably unknown factors that might have contributed to or even exclusively led to the observed phenomenon of PO activity decline. As a consequence, we find it difficult to conclude whether darker morphs of the three test species possess a stronger immunocompetence based on PO activity than paler conspecifics or not.

However, we did observe a remarkable and concomitant difference between dark and pale morphs in all three tested snail species: 24h after hemolymph-withdrawal the PO activity level was significantly decreased in the hemolymph samples of pale snails but not in the samples of dark animals. As this probably affected a possible PO activity induction due to Zymosan A, we repeated the experiments with *T. pisana* and *C. aspersum maximum* without prior hemolymph-withdrawal (run 2) to exclude bias through this, yet no Zymosan A-related PO activity increase was observable as discussed above. A plausible explanation for the significant decrease of PO activity following hemolymph withdrawal solely in pale snails could be that pale snails possibly cannot compensate for hemolymph and /or hemocyte loss as fast or as effectively as dark snails. As PO is produced by hemocytes (Butt & Raftos, 2008), and positive correlations between hemocyte density and PO activity levels have been found in insects as well as molluscs (Cotter et al., 2004; Seppälä & Jokela, 2010), it is possible that hemolymph-withdrawal causing a reduction of hemocyte numbers also results in reduced PO activity. Taking this into account, our results indicate that dark snails can either regenerate hemocyte numbers or PO content in the hemolymph within 6h (*C. aspersum maximum*) or 24h (*C. hortensis* & *T. pisana*) to a better extent than pale snails. One might also presume that hemocyte numbers were generally higher in dark snails, however, the absence of significantly different constitutive levels in different morphs contradicts this. As hematopoiesis in gastropods is generally only poorly understood (Loker, 2010), we find it difficult to speculate on possible mechanisms underlying such a regeneration of hemocyte numbers / hemolymph PO content and its plausible links to colour polymorphism in land snails. Nevertheless, considering our results it is plausible that dark snails benefit from being obviously less affected by hemolymph withdrawal concerning PO activity levels than pale snails. Such a benefit can, for example, occur following injuries involving shell and / or tissue impairment leading to hemolymph loss and requiring wound healing processes. This consideration gains

in importance when taking into account that phenoloxidase plays an important role in shell sclerotization of molluscs (Waite & Wilbur, 1976; Nellaiappan & Kalyani, 1989). As phenoloxidase is an important parameter in pathogen resistance in molluscs (Hellio et al., 2007; Butt & Raftos, 2008), and as tissue and / or shell impairment can facilitate pathogen infections since this affects the main physical barriers of molluscs (Gliński & Jarosz, 1997), it becomes even more plausible that darker snail morphs can be at an advantage compared to paler morphs when shell and body wall injuries occur.

Even though, due to lack of induction via Zymosan A, no differences in PO activity levels in different morphs could be observed in this experiment, another difference between pale and dark snail morphs concerning their immune response to hemolymph withdrawal stress was detected in our study. This is interesting and implies that the dark morphs may benefit under certain circumstances from being able to regenerate or preserve hemolymph PO content to a better extent than pale morphs.

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Kapitel 5: Shell colouration and parasite tolerance in two helicoid snail species

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Abstract

The polymorphism of shell colouration in helicoid snails is a well-known phenomenon attributed to different factors such as predation and climatic effects. Another aspect contributing to this polymorphism could be the interplay of melanin production and phenoloxidase-related immunity. Therefore, in this study we aimed at answering the questions whether there is a differential sensitivity of different snail shell colour morphs to nematode infection, and whether this can be related to differences in phenoloxidase (PO) activity levels using the two helicoid, polymorphic snail species *Cepaea hortensis* and *Cernuella virgata*. Snails of both species were artificially infected with the parasitic nematode *Phasmarhabditis hermaphrodita*, and analysed for mortality and PO activity levels. We found *C. virgata* to be more severely affected by *P. hermaphrodita* infection than *C. hortensis*, and the dark *C. virgata* morphs to be more resistant to lethal effects of this infection than pale morphs. However, these differences in sensitivity to the parasite could not clearly be related to different PO activity levels.

Key words

Phenoloxidase, Nemaslug®, *Phasmarhabditis hermaphrodita*, Melanin, Shell polymorphism

Introduction

Shell colour polymorphism is a phenomenon widely observed among several land snail species (Cook, 1986; Goodfriend, 1986), including helicoid snails such as *Cepaea hortensis* (O.F. Müller, 1774) and *Cernuella virgata* (Da Costa, 1778) (Jones et al., 1977; Gittenberger, 1993). This phenomenon has been related to a number of different factors, namely predation and climatic effects (Johnson, 2011; Cowie, 1990; Heller, 1981; Heller & Gadot, 1984; Johnson, 1981; Jones, 1977). However, recent work has shown that the idea of a different warming capacity in differently coloured morphs, which has been used to explain climatic effects, should be regarded with caution and probably does not apply to snail species with a constant primary shell colour and just different banding patterns (Scheil et al., 2012). Furthermore, it has been observed that under wet climatic conditions parasite stress is higher on snails than in dry climate as both parasite and snail development will benefit from wet ground conditions, and snail activity increases under wet conditions furthering parasite-snail encounters (Morley & Lewis, 2008). Additionally, it was found that in some helicid snails pale, unbanded morphs are more severely parasitized by nematodes than banded morphs (Cabaret, 1983; Cabaret, 1988, Lahmar et al., 1990), thus leading to the hypothesis that wet climate might favour dark morphs. A higher pathogen resistance of darker morphs has also been observed in other invertebrates previously, e.g. in insects (Barnes & Siva-Jothy, 2000; Cotter et al., 2004; Armitage & Siva-Jothy, 2005). It has been shown as well that this kind of resistance in insects is based on higher phenoloxidase (PO) levels in the hemolymph of dark morphs (Armitage & Siva-Jothy, 2005; Wilson et al., 2001), and that in invertebrates both melanism and immunity share the melanin-producing pathway, the so-called PO-cascade (Söderhäll & Cerenius, 1998; Rolff & Siva-Jothy, 2003). A stimulation of PO activity following injection of non-self molecules has been demonstrated in bivalves (Hellio et al., 2007), and PO activity has already been used as an immune parameter in a variety of different molluscs (Smith & Söderhäll, 1991; Barracco et al., 1999; Bahgat et al., 2002; Jordan & Deaton, 2005; Munoz et al., 2006; Seppälä & Jokela, 2010; Seppälä et al., 2011). However, in snails, to our knowledge, no studies on a possible correlation between colour polymorphism and parasite tolerance including analysis of the supposably underlying parameter PO activity have been conducted so far, even though it is known that melanin is a pigment accountable for molluscan shell colouration (Comfort, 1951). Hence, in this study, we chose to investigate the effects of the parasitic nematode *Phasmarhabditis hermaphrodita* (A. Schneider, 1859), a nematode which is considered widespread in Europe (Rae et al., 2007) and also commercially

available for slug and snail control (Nemaslug®, *Becker Underwood, Littlehampton, UK*), on mortality and PO activity levels in the two helicoid snail species *Cepaea hortensis* and *Cernuella virgata*, in order to shed some light on the following questions: Is there a differential tolerance of different morphs of these species to *P. hermaphrodita* and, if so, can this be explained by differences in PO activity?

Materials & Methods

Test Animals and Laboratory Maintenance

Adult *Cepaea hortensis* were collected from meadows and shrubs near the rail tracks of Tübingen-Derendingen, Baden-Württemberg, Germany, after nightly rainfalls in June, 2011. Two morphs, a yellow unbanded morph (later referred to as 'pale') and a yellow banded morph with five distinct brown bands (later referred to as 'dark') were sampled. *Cernuella virgata* (adult individuals) were sampled 5km south of Volterra, Tuscany, Italy, on shrubs, in August 2011. Again, two morphs were sampled, a pale white morph without banding (later referred to as 'pale'), and a morph with distinct brown banding (later referred to as 'dark').

Both species were kept in ventilated plastic terraria (30cm x 18cm x 19cm; *Exoterra medium, Hagen Deutschland GmbH & Co. KG, Holm, Germany*) laid-out with a 2cm cover of moistened *JBL Terra Basis ground covering for terraria* (*JBL GmbH & Co. KG, Neuhofen, Germany*). The ground covering was re-moistened with tap water every other day and terraria were thoroughly cleaned at weekly intervals. The snails were fed an *ad libitum* diet of organic carrots, zucchini, oats and cucumbers, and organic baby food (*Hipp Bio-Milchbrei, Hipp GmbH & Co. Vertrieb KG, Pfaffenhofen, Germany*) prepared according to package instructions twice a week. Clean cuttlebone was provided at all times. The snail terraria were installed in climate chambers and snails acclimatized to laboratory conditions (18°C for *C. hortensis* / 22°C for *C. virgata*; 50-90% humidity; 12h:12h light-dark cycle) for two weeks at least before starting experiments.

Experimental Set-up and Sampling

For experimental infection of snails, *Phasmarhabditis hermaphrodita* (Rhabditida, Nematoda) were obtained commercially as Nemaslug® (*Becker Underwood, Littlehampton, UK*) from a local distributor (*Sautter & Stepper, Ammerbuch, Germany*). For the PO assay experiment, the snails were individually infected using the following method: Nemaslug® was diluted in temperated (18°C) tap water to a nominal concentration of approximately 3000 nematodes / mL (resembles the single recommended application concentration for the soil surface area

provided in the test boxes- according to the supplier a package of approximately 6 million nematodes is to be diluted in 2-5L of water and applied to about $20m^2$; the ventilated plastic test boxes provide a bottom surface area of $81cm^2$). Since individual infections were shown to be more successful than mass-infections in other snails (Sauerländer, 1979) we applied 810µL of the Nemaslug® solution via the shell opening to each snail allotted for infection. During this process snails were individually fixed to the bottoms of the test boxes (9 x 6 x 9cm) for 15min using adhesive gum (*Reusable Adhesive Gum, Lyreco, Barsinghausen, Germany*). Subsequently, snails were freed from fixation and the plastic boxes filled with a 2cm layer of moistened ground covering. Additionally, the ground covering was infused with 810µL of the Nemaslug® solution (about the single recommended application concentration) per box. Control snails were left uninfected and the respective ground coverings were left uninfused. Snails were then kept under the same temperature, humidity, and light conditions as mentioned for the respective laboratory stocks and fed with organic carrots and cucumbers. Clean cuttlebone was provided at all times. At daily check-ups, snails which had attached to the lids or walls of the test boxes were placed back onto the ground coverings. For the PO assays, 15 *C. hortensis* of each morph (pale and dark) and each treatment group (control and infected) were sampled for hemolymph (HL) 0h (base level), 3d and 7d after infection (first experiment with *C. hortensis*), or 0h (base level), 6h and 24h after infection (second experiment with *C. hortensis*). For *C. virgata*, 10 snails of each morph (pale and dark) and each treatment group (control and infected) were sampled for HL 0h (base level), 24h, 3d and 5d after infection. Hemolymph (HL) collection was conducted according to the method of Renwrantz et al. (1981), with slight modifications: from the hemocoel located at the upper to middle subepithelial region of the headfoot (=anterior to middle part of the foot) of each test snail, hemolymph (HL) was withdrawn at a quantity of 20µL using sterile syringes (1mL) with 0.40 x 20mM gauge sterile hypodermic needles. The sampled HL was mixed with 200µL of phosphate buffered saline (PBS, pH 7.4; *Sigma Aldrich Chemie GmbH, Steinheim, Germany*) and immediately shock-frozen in liquid nitrogen. These HL samples were stored at –80°C until further processing.

Phenoloxidase Assays

The phenoloxidase (PO) assays were conducted using hemolymph (HL) samples. This method was chosen in favour of body tissue homogenates as we aimed at displaying the immune defense-related function of PO as part of the combined humoral and cellular (hemocyte-associated) molluscan immune defense as described in Gliński & Jarosz, 1997, avoiding possible bias through reproduction-related functions of PO in other tissues (Bai et

al., 1997; Kalyani et al., 1985). Additionally, the analysis of PO in HL samples has already been successfully performed in a variety of adult invertebrate species, including molluscs (Smith & Söderhäll, 1991; Barracco et al., 1999; Bahgat et al., 2002; Jordan & Deaton, 2005; Munoz et al., 2006; Hellio et al., 2007; Seppälä & Jokela, 2010; Seppälä et al., 2011), whereas the analysis of PO in tissue homogenates can be considered as an alternative for experiments on larvae or juveniles where hemolymph sampling is hardly feasible (Luna-González et al., 2003). Furthermore, considering the mode of infection and multiplication of *P. hermaphrodita* and its associated bacterium, *Moraxella osloensis*, (Richards et al., 2008; Tan & Grewal, 2001; Glen et al., 1996; Glen et al., 1994; Wilson et al., 1993a; Wilson et al., 1993b), the hemolymph of snails can be regarded as the tissue where defense reactions against the parasite are to be observed with high probability.

The PO-assays were conducted according to Seppälä & Jokela (2010) with slight modifications: After thawing on ice, 40µL aliquots of the HL samples were placed in 96-well microtiter plate wells containing 140µL of cold aqua bidest. and 20µL of PBS each. Measurements were done in triplicates. Four controls (sample aliquots replaced by aqua bidest.) per plate were set up. After adding 20µL of L-dopa (*Sigma Aldrich Chemie GmbH, Steinheim, Germany*) solution (4mg / mL aqua bidest.) to each well, plates were immediately measured photometrically at 490nm in a microplate reader (*Elx800, Bio-Tek Instruments, INC., Vermont, USA*) resulting in 0h values. The covered plates were then incubated at 30°C in a heating cabinet (*ST2 A60, STL-Neckarwestheim, Neckarwestheim, Germany*) for a species-specific time (30min. for *C. hortensis*; 7h for *C. virgata*; determined in pre-tests, data not shown) to ensure linearity of the absorbance increase. The plates were re-measured photometrically at 490nm resulting in incubation time values. According to the following equation

PO activity= incubation time values – 0h values – mean absorbance change in controls;

PO activity was calculated and expressed in milliunits.

Natural Nematode Infestation & Verification of Infection Success

In order to avoid bias through natural pre-infestation with nematodes, about 10% of the snails were checked for nematode infection before experiments started by peptic digestion of pieces of the headfoot as described in Cabaret (1980). The same method was applied to three snails of each species, morph and infection group to enable verification of the success of the artificial infection procedure.

Mortality in C. hortensis

For the first mortality experiment for *C. hortensis*, 15 snails of each morph were individually infected as described above using five times the supplier-recommended Nemaslug® application rate as done by Wilson et al. (2000). The treated snails were individually maintained in ventilated plastic boxes (9 x 6 x 9cm) with a moistened ground covering at the same temperature, light and humidity conditions as the *C. hortensis* laboratory stock for five weeks, and 15 untreated snails of each morph were individually kept as controls for the same time span. Snails were fed with organic cucumbers and carrots every other day, and clean cuttlebone was provided. As this experiment did not result in any mortalities, a second run was set up. For the second mortality experiment, 10 snails of each morph were individually infected with 1mL nematode suspension of five times the supplier-recommended Nemaslug® application rate, additionally, the ground covering of each snail box was infused with 5mL of Nemaslug® solution with a concentration of approximately five times the supplier-recommended application rate, resulting in exposure to about 30x the supplier-recommended application rate for each snail. 10 control snails per morph were set up. Snails were then kept as described above for three weeks (21 days) and mortalities were counted.

Mortality in C. virgata

Pre-tests revealed the single supplier-recommended Nemaslug® application rate to cause mortalities in *C. virgata* when the nematode solution was applied individually (data not shown). Therefore, for the mortality experiment, 10 snails of each morph were individually infected as described above using Nemaslug® solution at a concentration of approximately five times the supplier-recommended application rate. Additionally, the ground covering of each snail box was infused with 1mL of this solution as well, resulting in exposure to about 10x the supplier-recommended application rate for each snail. 10 control snails of each morph were set up. The snails were then kept at the same light, temperature and humidity conditions as the *C. virgata* laboratory stock for 14 days, and were fed with organic carrots and cucumbers every other day with clean cuttlebone provided at all times. Mortalities were counted throughout the experiment.

Statistical Analysis

All data obtained from the results of the PO assays were statistically analysed using JMP® 10.0 (*SAS Institute Inc.*, Cary, USA).

Statistical analysis was conducted as follows: the results were split in two groups, controls and infected, and a two-factor analysis of variance (ANOVA) was conducted for each group to investigate the effects of shell colour on PO activity over time. Since some data sets were

not normally distributed according to the Shapiro-Wilks-test, and some data did not hold equal variances according to the Levene's test as well, several transformations were run (log, log10, root, power (x^2)) with none resulting in a consistent normally-distributed outcome with equal variances. To account for this, the p-level was set to 0.01 while using the original untransformed data.

Whenever significancies were detected in the ANOVA, corresponding post-hoc Wilcoxon-tests including Bonferroni-corrections were applied to the respective data. The data for the first experiment with *C. hortensis* were fused for both morphs within each treatment group as no effects of colour had been detected in the effect tests. To reveal possible differences between the different treatments, a regression analysis was conducted with data of the control and infection group. No post-hoc tests were performed on data of the second experiment with *C. hortensis*, as the ANOVA had not resulted in a significant outcome. For the experiment with *C. virgata*, the data of both morphs in the control group were fused since no colour effects had been observed in the effect tests, however, the data of the infection group remained split for colour morphs as a significant colour*time interaction had been found. This interaction was carefully taken into account when interpreting the results of the respective post-hoc Wilcoxon test conducted on these data.

Results

Natural Nematode Infestation & Verification of Infection Success

For both species, *C. hortensis* and *C. virgata*, no natural pre-infestation with nematodes could be found. The analysis of snail tissue for verification of the infection success revealed that in all snails examined *Phasmarhabditis hermaphrodita* nematodes could be found, indicating that the artificial infection procedure had been successful.

Mortality in C. hortensis

The first mortality experiment with *C. hortensis* did not lead to any mortality. In the second experiment, no mortality was found within the control groups. In the infection group of pale snails, two dead snails could be recorded on day 10 after infection whilst one dead snail occured in the infection group of dark snails on day 16 followed by a death of a further individual on day 17 after infection (Fig. 1). On day 19 after infection, a further individual died in the pale infection group (Fig. 1). The total mortality rate for pale snails at the end of the test was 30%, and 20% for dark snails (in relation to the respective morph groups). The overall mortality rate was 25%.

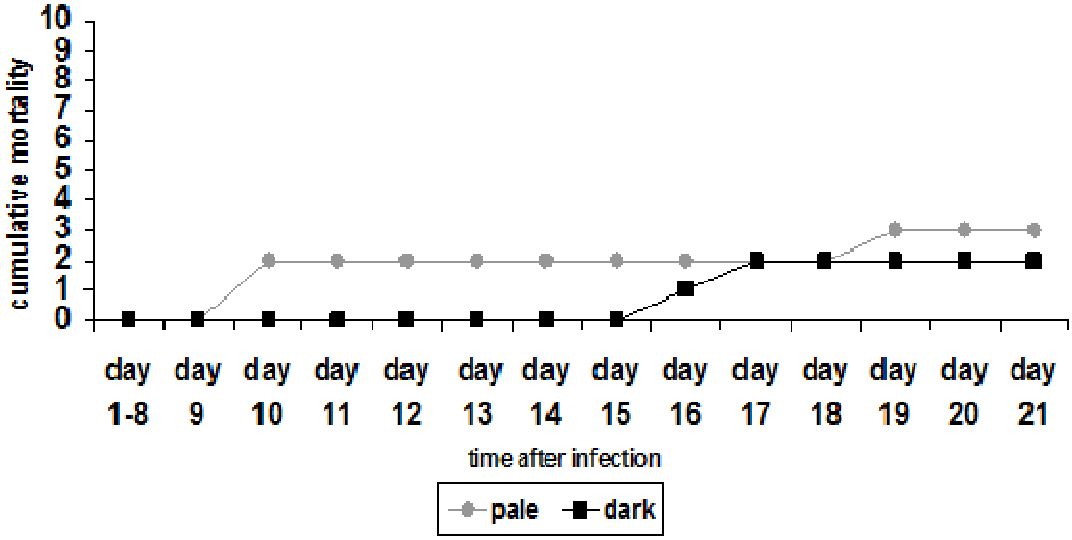


Figure 1. Mortality of *C. hortensis* in the second mortality experiment. Snails were exposed to approximately 30x the recommended application rate of Nemaslug® solution (*P. hermaphrodita*).

Mortality in C. virgata

In the controls of *C. virgata*, no mortality occurred in both morphs, pale and dark. In the infection group of pale morphs, cumulative mortality was recorded as follows: two snails on day four after, three animals on day seven, five animals on day eight, seven animals on day nine, eight animals on day 11 and nine animals on day 14 after infection (Fig. 2). For the infection group of dark morphs, cumulative mortality was recorded four days after infection (one snail), and six days (two snails), eight days (three snails) and 11 days (five snails) after infection (Fig. 2). In relation to the respective morph group the total mortality rate for pale snails was 90%, and 50% for the dark snails. The overall mortality rate was 70%.

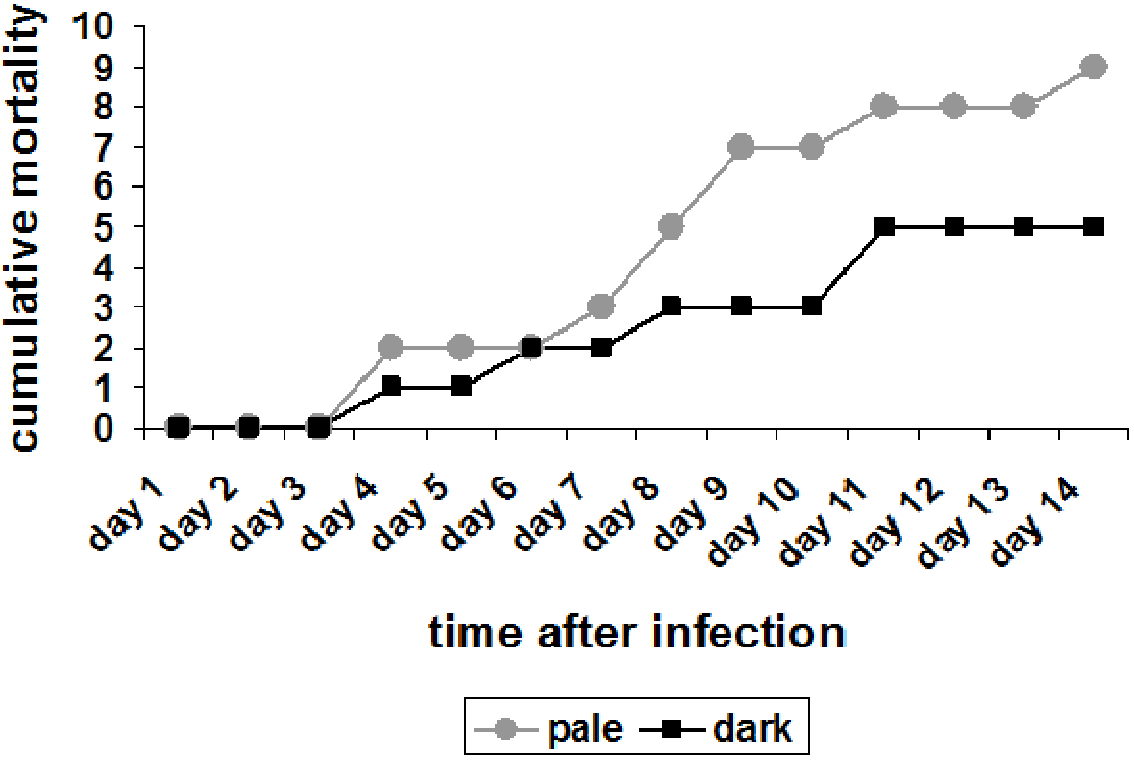


Figure 2. Mortality of *C. virgata*. Snails were exposed to approximately 10x the recommended application rate of Nemaslug® solution (*P. hermaphrodita*).

Phenoloxidase Assays

C. hortensis

In the first experiment with *C. hortensis*, the outcome of the ANOVA was significant ($p \leq 0.01$) in both the control (Tab. 1a) and the infection group (Tab. 1c). However, we did not observe any colour effects or colour*time interactions neither in the control nor in the infection group (Tab. 1b & d), but the corresponding effect tests revealed time as a significant ($p \leq 0.01$) influence in both cases (Tab. 1b & d).

Tab. 1 ANOVA results for control (a) and infected (c) *C. hortensis* in the first experiment, with corresponding effect tests (b: control, d: infected); p≤0.01; significant results are indicated by asterisks.

a. Analysis of Variance Control *C. hortensis*

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	5	574624,9	114925	3,8787
Error	77	2281477,5	29630	**Prob > F**
C. Total	82	2856102,4		0,0035*

b. Effect Tests Control *C. hortensis*

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Color	1	1	13221,79	0,4462	0,5061
Time	2	2	505414,96	8,5289	0,0005*
Color*Time	2	2	38441,16	0,6487	0,5256

c. Analysis of Variance Infection *C. hortensis*

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	5	1112784,8	222557	6,4822
Error	79	2712354,5	34334	**Prob > F**
C. Total	84	3825139,4		<,0001*

d. Effect Tests Infection *C. hortensis*

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Color	1	1	13221,79	0,3851	0,5367
Time	2	2	869157,59	12,6575	<,0001*
Color*Time	2	2	224073,79	3,2632	0,0435

The post- hoc Wilcoxon-tests showed the PO activity level in the control group to be elevated in a highly significant way seven days after onset of the experiment ($p \leq 0.00\overline{3}$) compared to both the base level (0h) as well as the level three days (3d) after start (Fig. 3a). The base level (0h) and the 3d-level did not differ significantly ($p \leq 0.01\overline{6}$) from each other (Fig. 3a). Concerning the infection group, it was found that three days (3d) after infection the PO activity level was significantly ($p \leq 0.01\overline{6}$) elevated compared to the base level (0h) (Fig. 3b). Seven days (7d) after infection the PO activity level was found to be most significantly ($p \leq 0.0003\overline{3}$) higher than the base level (0h) (Fig. 3b). No significant ($p \leq 0.01\overline{6}$) differences could be found between the 3d- and 7d-levels (Fig. 3b). The linear regression analysis (Fig. 4) revealed a slightly steeper increase of the PO activity level in the infection group (Fig. 4b) compared to the control (Fig. 4a).

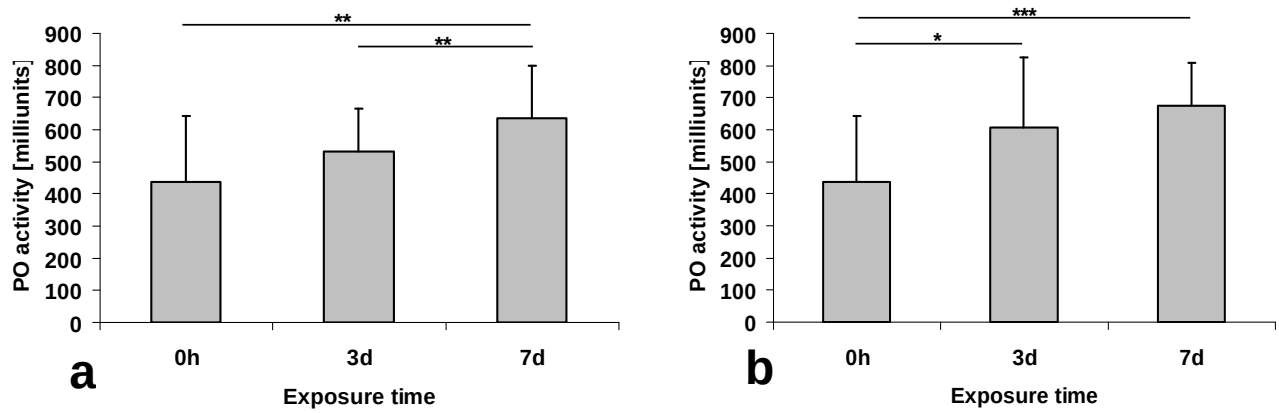
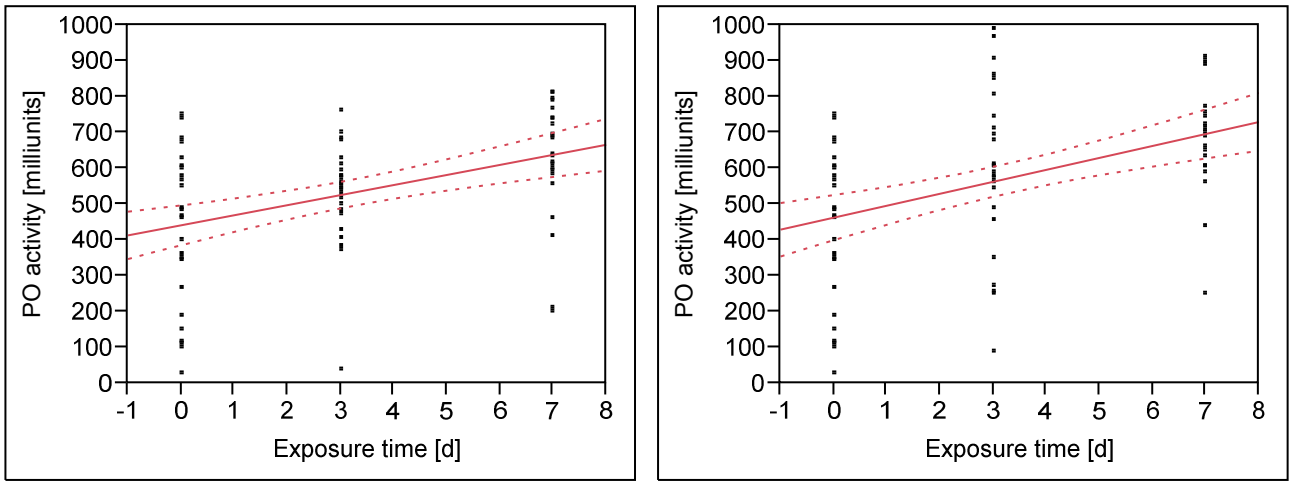


Figure 3. First experiment with *C. hortensis*: PO activity levels resulting in control (a) snails and snails infected (b) with *P. hermaphrodita* (Nemaslug®). Morphs were fused since no significant colour or colour*time effects were detected in the ANOVA. Means + sd; n=15; significant differences are indicated by asterisks (p≤0.01 :* significant; p≤0.00 :** highly significant; p≤0.0003 : *** most significant).



a. Linear Regression First experiment *C. hortensis*
Control
Linear Fit
Linear Fit
PO activity = 441,12943 + 28,05673*Exposure time

b. Linear Regression First experiment *C. hortensis*
Infection
Linear Fit
Linear Fit
PO activity = 462,12606 + 33,421322*Exposure time

Figure 4. Linear regression analysis results for the data resulting from the first PO assay experiment with *C. hortensis*; a: control snails, b: snails infected with *P. hermaphrodita* (Nemaslug®).

In the second experiment with *C. hortensis*, no significancies (p≤0.01) were found in the ANOVA (Tab. 2).

Tab. 2 ANOVA results for control (a) and infected (b) *C. hortensis* in the second experiment; p≤0.01.

a. Analysis of Variance Control *C. hortensis*

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	5	405922,5	81184,5	1,6172
Error	77	3865538,9	50201,8	**Prob > F**
C. Total	82	4271461,4		0,1655

b. Analysis of Variance Infection *C. hortensis*

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	5	744411,0	148882	3,0268
Error	82	4033390,5	49188	**Prob > F**
C. Total	87	4777801,4		0,0148

C. virgata

In the control group of *C. virgata*, the outcome of the ANOVA was significant (p≤0.01) (Tab. 3a) and the corresponding effect tests revealed time as the only significant (p≤0.01) factor whereas no influence of colour or colour*time interaction could be detected (Tab. 3b).

Tab. 3 ANOVA results for control (a) and infected (c) *C. virgata*, with corresponding effect tests (b: control, d: infected); p≤0.01; significant results are indicated by asterisks.

a. Analysis of Variance Control *C. virgata*

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	7	950,6130	135,802	6,4984
Error	70	1462,8460	20,898	**Prob > F**
C. Total	77	2413,4590		<,0001*

b. Effect Tests Control *C. virgata*

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Color	1	1	99,68113	4,7699	0,0323
Time	3	3	822,68845	13,1224	<,0001*
Color*Time	3	3	72,60855	1,1582	0,3320

c. Analysis of Variance Infection *C. virgata*

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	7	1056,1341	150,876	6,1223
Error	72	1774,3551	24,644	**Prob > F**
C. Total	79	2830,4891		<,0001*

d. Effect Tests Infection *C. virgata*

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Color	1	1	99,68113	4,0449	0,0481
Time	3	3	667,43753	9,0278	<,0001*
Color*Time	3	3	345,23318	4,6696	0,0049*

The post-hoc Wilcoxon test showed that compared to the base level (0h), all PO activity levels decreased within the control, with the 24h- and 5d-levels being highly significantly ($p\leq0.001\overline{6}$) and the 3d-level being significantly ($p\leq0.008\overline{3}$) lower (Fig. 5a). The 3d-level was highly significantly ($p\leq0.001\overline{6}$) higher than the 24h-level, and significantly ($p\leq0.008\overline{3}$) higher than the 5d-level (Fig. 5a).

For the infection group, the ANOVA revealed significancies (p≤0.01) (Tab. 3c). In the corresponding effect tests a significant (p≤0.01) influence of time was detected, as well as a

significant (p≤0.01) colour*time interaction. There was no significant (p≤0.01) influence of colour only, although it should be noted here that this "lack" of significance is due to the rigid p-value of 0.01 (instead of the 'usually-applied' 0.05) (Tab. 3d). The post-hoc Wilcoxon test revealed no significant (p≤0.003125) differences concerning PO activity levels between the two colour morphs, pale and dark, at any time point tested (Fig. 5b). However, it was found that the PO activity level in dark morphs had highly significantly (p≤0.000625) decreased 24h after infection compared to the base level (0h), yet 3d after infection the PO activity level was highly significantly (p≤0.000625) elevated again compared to the 24h level (Fig. 5b). Also, in the dark morphs the 5d-level was found to be significantly (p≤0.003125) increased compared to the 24h-level, whereas no significant (p≤0.003125) differences could be detected between the base level and the 3d-level, the base level (0h) and the 5d-level, or the 3d-level and the 5d-level (Fig. 5b). Despite the fact that the difference was not significant (p≤0.003125), in the pale morphs the PO activity level appeared to be lower by trend five days (5d) after infection than at the beginning of the experiment (0h, base level) (Fig. 5b).

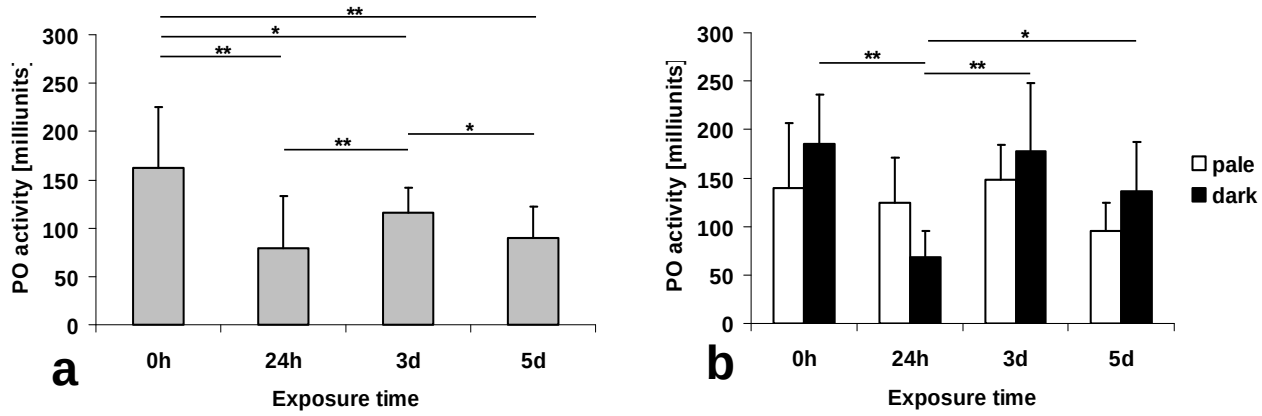


Figure 5. *C. virgata*: PO activity levels resulting in control (a) snails and snails infected (b) with *P. hermaphrodita* (Nemaslug®). As neither colour nor colour*time effects were detected in the ANOVA of the control group, morphs were fused for the post-hoc Wilcoxon test. Morphs remained split in the infection group as a significant colour*time interaction was found in the ANOVA of this data. Means + sd; n=10; significant differences are indicated by asterisks, for control (a) snails p≤0.008 :* significant and p≤0.001 :** highly significant; for infected (b) snails p≤0.003125:* significant and p≤0.000625:** highly significant.

Discussion

The mortality experiments in this study revealed differences between the tested snail species, *C. hortensis* and *C. virgata*, in respect to their sensitivity to infection with *Phasmarhabditis hermaphrodita* nematodes. This became evident in pre-tests already, since for *C. virgata* the single recommended Nemaslug® application rate proved to be lethal whereas for *C. hortensis* a five-fold increased application rate as used by Wilson et al. (2000) had no lethal effects in

the first mortality experiment with this species. Although the number of nematodes applied to each *C. hortensis* individual was further increased to 30x the supplier-recommended application rate in the second mortality experiment, the mortality rate remained low with a total number of five dead snails (25%) three weeks after infection. This is in contrast to a total number of 14 dead snails (70%) two weeks after infection in the mortality experiment with *C. virgata*, where only 10x the recommended application rate was applied to each snail. Yet, these results corroborate the findings of Wilson et al. (2000) that most snail species are less susceptible to infection by *P. hermaphrodita* than its target-species, *Deroceras reticulatum*, and that higher parasite numbers and longer exposure times are required to obtain mortalities. However, concerning the mortality experiments with *C. hortensis*, we received a lower mortality rate (25% at 30x the recommended application rate) than Wilson et al. (2000) (>70% at 5x the recommended application rate). These differences are possibly due to differences in the test set-ups: whereas Wilson et al. (2000) had lined the walls of the experimental boxes with woven copper mesh in order to confine snails to the soil, our test organisms were not restricted in this manner but manually placed back onto the ground coverings daily, if necessary. Therefore, it is possible that snails in our test set-up had less contact with nematodes in the ground coverings. However, contrary to Wilson et al. (2000), our test organisms in the infection groups were not only exposed to nematode-treated soil but exposed to individual direct applications of nematode solution as well. Such individual infections are known to be more successful than mass infections (Sauerländer, 1979), and can therefore be regarded as a good measure to compensate for possibly less contact with nematode-treated soil. It is possible that the higher mortality rates in *C. hortensis* observed by Wilson et al. (2000) are also due to the snails' contact with the copper mesh linings. Copper is known for its high toxicity to gastropods (Ravera, 1977; El-Gendy et al., 2009; Sawasdee et al., 2011), and uptake does not only occur by ingestion but via the foot as well (Ryder & Bowen, 1977). Consequently, the comparatively higher mortality rate for *C. hortensis* in the experiment of Wilson et al. (2000) could be caused by a combination of parasitic stress and copper toxicity. Another plausible explanation for the differing mortality rates might arise from variations in virulence of different Nemaslug® packages which can occur through, for example, deficient storage conditions. For our experiments, however, we can rule out this possibility as storage was conducted precisely according to the manufacturer's instructions, and each package was positively pre-checked for mobility of nematodes before the experiments.

Comparing the mortality rates and times of snail deaths, there were, in case of *C. virgata*, striking differences between the two colour morphs, pale and dark, tested in our experiment. In the pale morphs, overall mortality was distinctly higher than in the dark morphs. Also, the mortality rate started to increase to a greater extent at earlier time points in the pale morph group than in the dark morph group. These differences can be caused by a higher parasitic load following higher susceptibility to nematode infection in pale morphs as it was found by Cabaret (1983) for *Theba pisana* and *C. virgata* infected with *Muellerius capillaris*. Similar results are known for *Eobania vermiculata* (Lahmar et al., 1990). Since we did not quantify the actual parasitic load in our test snails, we cannot draw a final conclusion on whether differences in the capacity of the morphs' defense system to prevent infection or differences in their capacity to respond to infection are responsible for the variation between morphs concerning mortality. In *C. hortensis*, we did not find morph-specific differences in mortality to the same extent as in *C. virgata*, as both morphs showed a similarly low overall mortality rate at the end of the experiment with only a slightly higher rate for the pale morphs. Yet, it should be noted that mortality in pale morphs of this species started to occur five days earlier than in dark morphs. This, again, is possibly due to a lower susceptibility to nematodes in the dark morphs, as stated above for *C. virgata*. However, on the basis of the generally low mortality in *C. hortensis*, this should possibly be considered with some caution.

Regarding *C. hortensis*, we did not find any differences concerning PO activity between the two morphs tested since no colour or colour*time effects were detectable in the ANOVAs. This holds true for both the control as well as the infection groups, meaning that there were no differences in constitutive as well as inducible PO activity levels between the morphs at the time points and under the treatments tested in this study. This is partly contrary to what has been previously found in studies on other invertebrates, namely insects (Armitage & Siva-Jothy, 2005; Wilson et al., 2001), where higher constitutive PO activity levels and a resulting higher pathogen resistance could be attributed to darker morphs. However, it is in accordance with our findings on constitutive PO activity levels in *Theba pisana* and *Cornu aspersum maximum* snails and results of previous tests with *C. hortensis* conducted in our laboratory, where no morph-specific differences in constitutive PO activity levels were found as well (Scheil, A.E., unpublished data). Since a high constitutive or prophylactic PO activity level is not exclusively beneficial to an individual but also bears nutritional costs (Siva-Jothy & Thompson, 2002) as well as a high risk of oxidative stress (Kumar et al., 2003) and the occurrence of cytotoxic effects (Ballarin et al., 1998), it is reasonable that high constitutive PO levels are avoided for these reasons. The results of the first experiment with *C. hortensis*

show a clear increase of PO activity levels over test time both in the control as well as in the infection group. However, the linear regression analysis revealed a slightly steeper increase of PO activity in the infection group, which can be interpreted as an effect of the nematode infection. This is in concordance with results from experiments with other molluscs, namely oysters, where it was shown that parasitic challenge increases PO activity in the hemolymph (Butt & Raftos, 2008). Yet, it should be noted that the differences in PO activity levels between the control and the infection group of *C. hortensis* in our study are not overly prominent, which relates to the low mortality found for this species as discussed above. The second experiment with *C. hortensis* revealed that within the short time span between the beginning of the infection and 24h after infection, no effects of parasite stress concerning PO activity levels are observable. Again, this probably also relates to the only minor mortality observed in this snail species.

In *C. virgata*, no effects of colour were observed in the control group at any time, meaning that there are no differences in constitutive (base) levels of PO activity between the morphs, and that the morphs do not react differently to control maintenance conditions. Interestingly, there were effects of time in the control group, as the PO activity level had decreased as early as 24h after start of the experiment and remained comparatively low throughout the rest of the experiment. A possible explanation for this phenomenon comes from the fact that *C. virgata* is known to form clusters (Stugren & Coman, 1990; personal observations), resulting in high density spots in certain areas in the wild as well as in laboratory maintenance boxes. For insects, it is known that immune prophylaxis is density-dependent (Wilson & Reeson, 1998; Barnes & Siva-Jothy, 2000; Wilson et al., 2002), and it is also known that higher PO activity levels are expressed under high density conditions (Wilson et al., 2001). When the *C. virgata* snails were transferred to individual maintenance boxes at the beginning of the test, high density conditions did no longer apply to the snails and density-dependent prophylactic PO activity levels were possibly down-regulated. Although, to our knowledge, density-dependent immune prophylaxis has not been shown for molluscs so far, it is possible that similar density-dependent mechanisms are present in molluscs as well, and that our results give a first hint to this. It is also noteworthy in this context, that *C. hortensis*, which usually is not observed forming clusters (personal observation), in comparison did not show a down-regulation of PO activity under test conditions.

For the infection group of *C. virgata*, effects of time as well as effects of a colour*time interaction on PO activity were observed, however, no effects of colour only could be detected. Interaction effects render interpretation of the data more complex (Quinn & Keough,

2003), and since there were no colour effects but an interaction effect of colour*time in our case, interpretation should be carried out with some caution. Yet, one should keep in mind that colour effects in this case were not classified significant because of the rigid p-value of 0.01 chosen to account for some of the data being either not normally distributed or showing unequal variances. In case of a p-value of 0.05 colour effects would have been significant. Consequently, the examination of differences in PO activity levels between the two morphs at a given time point and of differences between PO activity levels of a given morph at different time points was mandatory. Although there was a tendency for higher PO activity levels in the dark infected morphs at most time points (except for 24h), the post-hoc tests revealed no significant differences concerning PO activity levels between the two morphs, pale and dark, at any given time point of the experiment. Hence we conclude that there are no differences in PO-based immune response following *P. hermaphrodita* infection between pale and dark *C. virgata* snails. This conclusion is further strengthened by the fact that there were no differences detectable within the pale infection group over the whole time course of the experiment. Additionally, in the dark infection group, even though there was a significant decrease of PO activity at 24h (which corresponds to the decrease found in the control group), the PO activity re-increased within the experimental time to the base level again.

In summary, our study shows that the differential sensitivity of pale and dark *C. virgata* morphs to *P. hermaphrodita* and its lethal effects, and the obvious general tolerance of *C. hortensis* to infection with this nematode which we observed, cannot be explained by the PO activity data. Nevertheless, we could demonstrate that in *C. virgata* there is a morph-specific tolerance to *P. hermaphrodita* infection with the dark shell morphs being less affected by this nematode.

Acknowledgements

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Tagungsbeiträge

SETAC Europe 18th Annual Meeting, Warschau (2008)
Poster: Zürn, A, Mazzia, C, Capowiez, Y, Köhler, H-R, Triebskorn, R:
Effects of increased temperatures on hepatopancreatic cells and stress protein levels in mediterranean land snails

ESF Workshop Genetics of thermal adaptation in ectotherms, Wageningen (2008)
Vortrag: Zürn, A, Dittbrenner, N, Mazzia, C, Capowiez, Y, Köhler, H-R, Triebskorn, R: Cellular adaptation and recovery in response to heat stress in Mediterranean snails (*Xeropicta derbentina, Theba pisana* and *Cernuella virgata*)

StEvE-Meeting (2009), Eberhard Karls Universität Tübingen, Tübingen Vortrag: Scheil, A, Triebskorn, R, Köhler, H-R: Heat tolerance in Mediterranean land snails – what are the underlying mechanisms?

IOBC-WPRS Meeting Slug and Snail Control in the 21st Century, Cardiff (2010)
Poster: Scheil, A, Köhler, H-R, Triebskorn, R: Antioxidant defense capacity in *Theba pisana*
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