Ökophysiologische Konsequenzen und Bewältigung hoher Habitattemperaturen bei mediterranen Landschnecken

Dissertation
der Mathematisch-Naturwissenschaftlichen Fakultät
der Eberhard Karls Universität Tübingen
zur Erlangung des Grades eines
Doktors der Naturwissenschaften
(Dr. rer. nat.)

vorgelegt von

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Tübingen

2015

Gedruckt mit Genehmigung der Mathematisch-Naturwissenschaftlichen Fakultät der Eberhard Karls Universität Tübingen.

Tag der mündlichen Qualifikation:	26.06.2015
Dekan:	Prof. Dr. Wolfgang Rosenstiel
1. Berichterstatter:	Prof. Dr. Heinz – R. Köhler
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But wouldn´t you like to know the truth of what´s out there to have the proof…

(Steven Percy Harris 1988)

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Teil 1: Zusammenfassung

1.1 Titel der Promotion

Ökophysiologische Konsequenzen und Bewältigung hoher Habitattemperaturen bei mediterranen Landschnecken

Die vorliegende Dissertation wurde an der Universität Tübingen in Kooperation mit der HS Esslingen im Rahmen des *'Twinning projects'* „Wärmeflüsse, Thermodynamik und ökophysiologische Konsequenzen hoher Temperaturen bei mediterranen Landschnecken“ angefertigt.

1.2 Einleitung in das Thema

Trockene und warme Bedingungen, wie sie im Sommer in mediterranen Gebieten vorherrschen, stellen die dort lebenden Organismen vor schwere Herausforderungen. Um in einer solchen Umgebung zu überleben, ist eine vor Verdunstung schützende Oberfläche und ein der Witterung angepasstes Lokomotionsverhalten, das das Aufsuchen kühlerer Bereiche ermöglicht, von Vorteil. Auf den ersten Blick scheinen also Schnecken, die zu 75 % und mehr aus Wasser bestehen, deren Integument ständig feucht gehalten werden muss und die ihren Standort nicht innerhalb weniger Augenblicke ändern können, in heißen Gebieten ungeeignete Lebensbedingungen vorzufinden. Dennoch sind Schnecken im mediterranen Raum in großer Zahl und sehr oft auch an sonnenexponierten Standorten zu finden. Um in dieser auf den ersten Blick für Schnecken eher ungeeigneten Umgebung zu überleben, müssen diese Tiere morphologische und physiologische Anpassungen sowie Verhaltensweisen entwickelt haben, die ein Überleben unter den gegebenen Bedingungen ermöglichen.

Die zum Stamm der Mollusca (Weichtiere) zählende Klasse der Gastropoda (Schnecken) hat sich im Laufe ihrer Evolution an das Leben an Land angepasst. Die Fähigkeit, Sauerstoff aus der Luft zu beziehen, ermöglichte es einigen Vertretern dieser Klasse das Land zu besiedeln und damit einen völlig neuen Lebensraum einzunehmen. Dennoch besitzen Schnecken ein wasserdurchlässiges Integument, das an der Grenze zur Umwelt mit Schleim (Mukus) überzogen ist (Luchtel et al. 2001; Machin 1964). Um diese Mukusschicht aufrecht zu erhalten und um sich fortzubewegen müssen die Schnecken große Mengen Wasser abgeben und auch aufnehmen können. Gerade an Land droht Schnecken daher in warmen, trockenen

Gebieten der rasche Tod durch Austrocknung. Als erste Anpassung an ein trockeneres Habitat und als Schutz vor Prädatoren kann das Ausbilden einer kalzifizierten Schale gesehen werden. Bei Gefahr oder widrigen Bedingungen kann die Schnecke sich in die Schale zurückziehen. So wird die Fläche, über die Wasser verloren werden kann, auf den Eingang des Gehäuses reduziert. Um Wasserverlust in zurückgezogenem Zustand zu minimieren, können viele Schneckenarten die Öffnung mit einem Epiphragma verschließen (Withers et al. 1997). Das Epiphragma ist eine aus erstarrtem Mukus gebildete Absonderung des Mantelrandes, die mit Kalk verstärkt ist. Die Struktur des Epiphragmas gewährt Sauerstoffdiffusion und minimiert den Verlust weiteren Wassers (Barnhart 1983; Machin 1968). In einem Laborversuch haben Reuner et al. (2008) aktive und zurückgezogene Schnecken der Art *Cantareus apterus* [Born 1778] verglichen. Die in aktivem Zustand zu 75% aus Wasser bestehenden Schnecken waren in der Lage sich in ein Ruhestadium zurückzuziehen, in dem sie nach dem Versiegeln der Gehäuseöffnung im Laufe von sechs Monaten nur 14 % ihres Gesamtwassers verloren. In dieser immobilen Phase ist es den Schnecken jedoch unmöglich, der Aufheizung der Schale und damit auch dem Aufheizen des Körperinneren zu entgehen, da mit der Beschränkung der Wasserevaporation auch die damit einhergehende Kühlung des Körpers stark eingeschränkt wird. Bei Untersuchungen zur Anpassung von Schnecken an Ihren Lebensraum sollten daher Messungen, die Aufschlüsse über den Wasserverlust durch die Schale und die versiegelte Schalenöffnung geben, nicht fehlen.

Um dem Tod durch Austrocknung zu entgehen und um gleichermaßen einem übermäßigen Aufheizen des Körpers entgegen zu wirken haben die meisten Landschnecken ihre Aktivität auf die kühleren und feuchteren Tagesabschnitte, im mediterranen Sommer also auf den späten Abend bis zum frühen Morgen, verlegt (Pomeroy 1968; Yom-Tov 1971). Diese Anpassung der Aktivitätsphase ermöglicht es den Tieren, aktiv zu werden und sowohl Wasser als auch Nahrung aufzunehmen. Neben dem Verlegen der Aktivitätsphase in die Nacht und dem Zurückziehen des Körpers in die Schale haben einige Schneckenarten zusätzliche Strategien entwickelt um den heißen Monaten des Jahres oder den potentiell letalen Temperaturen des Tages zu entgehen. In der Arbeit von Yom-Tov (1971) konnte beispielsweise gezeigt werden, dass sich Schnecken der Art *Sphincterochila boissieri* [Charpentier 1847] zum Schutz vor Hitze und Austrocknung in den Boden eingraben, um Monate mit hoher Umgebungstemperatur und unzureichender Feuchte zu überstehen. Ähnliche Beobachtungen konnten von Staikou (1999) für die Art *Cepaea vindobonensis* [Férussac 1821] gemacht werden.

Eine weitere bemerkenswerte Verhaltensanpassung an die vor allem in Bodennähe schnell steigenden Temperaturen des mediterranen Sommers ist das Erklettern von höher gelegenen Orten. Wie bereits mehrfach nachgewiesen (Aubry et al. 2006; Di Lellis et al. 2012; Köhler et al. 2009; Schmidt-Nielsen et al. 1971; Yom-Tov 1971), erreichen die Bodentemperaturen im mediterranen Raum im Sommer schnell für Schnecken kritische Werte, während sie bereits einige Zentimeter über dem Boden deutlich abfallen. Um diesen potentiell letalen Bedingungen in Bodennähe zu entgehen, klettern Schneckenarten wie zum Beispiel *Xeropicta derbentina* [Krynicki 1836] oder *Theba pisana* [Müller 1774] in den Morgenstunden, wenn die Sonne das Habitat erwärmt und die Feuchte abnimmt, an der Vegetation nach oben. In anthropogen beeinflussten Gebieten werden auch Zäune, Telegraphenmasten, Autos oder gar andere, über Nacht ruhende Tiere erklettert (Aubry et al. 2006; Cowie 1985; Hazel & Johnson 1990; McQuaid et al. 1979). Besonders entlang von Straßen und Wegen, auf denen sich Menschen, Tieren und Fahrzeuge fortbewegen, konnte am Beispiel von *X. derbentina* gezeigt werden, dass dieses Kletterverhalten zur Verbreitung dieser Art beiträgt (Aubry et al. 2006), was sie in Südfrankreich zu einer invasiven Spezies werden ließ (Altena 1960).

Neben diesen Adaptationen im Verhalten der Tiere sind es vor allem stoffwechselphysiologische Anpassungen, die es den Tieren ermöglichen, in warmen Klimaten zu überleben.
Im Zuge dieser Arbeit sollen vor allem Anpassungen auf subzellulärer Ebene beleuchtet werden. Strukturelle Reaktionen von Geweben auf Hitzestress, wie sie bereits in früheren Studien dokumentiert wurden (Dittbrenner et al. 2009; Scheil et al. 2011; Troschinski et al. 2014), werden hier nicht berücksichtigt.
Insbesondere wenn sich Schnecken über einen längeren Zeitraum wegen ungünstiger Umweltbedingungen nicht bewegen können, müssen betroffene Individuen Vorkehrungen treffen, um diese schadlos zu überstehen. Im Falle von *Otala lactea* [Müller 1774] (Herreid II 1977) oder auch *T. pisana* (McQuaid et al. 1979) wird zum Beispiel der Sommer in einer Ruhephase verbracht. In dieser, auch Aestivationsphase genannten Ruhephase, wird der Metabolismus der Schnecke auf ein Minimum beschränkt.
Der Begriff der Aestivation wird in der Literatur, abhängig vom untersuchten Organismus, für unterschiedlich tiefgreifende Reduzierungen der Stoffwechselaktivität verwendet (Withers & Cooper 2010). In dieser Arbeit soll der Begriff als kurzzeitige bis mittelfristige Reduktion des Stoffwechsels der Schnecken verstanden werden, die dazu dienen temporär (wenige Stun-

den am Tag bis mehrere Tage) auftretende ungünstige Witterungen (vornehmlich heiße, trockene Perioden) zu überstehen.

Während der Aestivationsphasen werden die Herzschlagfrequenz (Wünnenberg 1991) und die Atmung deutlich reduziert (Ferreira-Cravo et al. 2010; Rees & Hand 1990). In dieser Phase kommt es bei Schnecken nur gelegentlich und unregelmäßig zum Gasaustausch mit der Umgebung, was als eine Art 'Apnoeatmung' interpretiert wird (Barnhart 1986; Schmidt-Nielsen et al. 1971; Storey & Storey 2010). Dies bildet zusammen mit dem bereits erwähnten Verschließen der Schalenöffnung einen effektiven Mechanismus, um den Wasserverlust während der Aestivationsphase zu minimieren (Withers et al. 1997). Bei diesen unkontrollierten Atemvorgängen ändert sich auch das innere Milieu der Schnecken (Barnhart 1986; Ferreira-Cravo et al. 2010; Withers et al. 1997). Mit fallendem Sauerstoffpartialdruck in der Hämolymphe konnte ein Anstieg der Natrium- und Kaliumkonzentration und damit ein Anstieg der osmotischen Konzentration in der Hämolymphe gemessen werden.

Da die Schnecken in der Aestivationsphase immobil sind, und sowohl Wasser als auch Nahrungsaufnahme damit unterbunden sind, muss die Schnecke Energiereserven nutzen und ihren Stoffwechsel anpassen. Um Energie einzusparen wird die im aktiven Zustand der Schnecke stattfindende Proteinbiosynthese und die Degradation von Proteinen unterbrochen (Ramnanan et al. 2009; Storey & Storey 2010).

Sowohl im aktiven Zustand als auch während der Aestivationsphase hat die Umgebungstemperatur einen großen Einfluss auf den Sauerstoffverbrauch und den Energieumsatz von Schnecken. Je nach Anpassung an ihr Habitat sind Schnecken in der Lage widrige Umstände und hohe Temperaturen bis zu einem physiologisch tolerierbaren Maximum zu ertragen. Wird dieses überschritten, kann die Schnecke in die Aestivationsphase übergehen. In aktivem Zustand erhöht sich, den Ergebnissen von Riddle (1977) zufolge, der Sauerstoffverbrauch von *Helix aspersa* (=*Cornu aspersum*) [Müller 1774] von 15 °C bis 25 °C deutlich. Überstieg die Temperatur 25°C, konnte in dieser Studie ein deutlicher Rückgang im Sauerstoffverbrauch der Tiere beobachtet werden und die Schnecken gingen in eine Aestivationsphase über. Eine vergleichbare physiologische Reaktion wurde für *Rabdotus schiedeanus* [Pfeiffer 1841] beschrieben, einer Schnecke, die an trockenere Verhältnisse angepasst ist. Im Vergleich konnte bei diese Art jedoch durchweg ein geringerer Sauerstoffverbrauch gemessen werden, was auf eine Adaptation an wärmere Umgebungen hindeutet (Riddle 1977). Im Vergleich zu aktiven Schnecken ist der Sauerstoffverbrauch von Schnecken

in der Aestivationsphase deutlich geringer (Rees & Hand 1990). Bei Studien zum Sauerstoffverbrauch von *Otala lactea* in der Aestivationsphase und beim Wiedererwachen aus selbiger konnte Herreid II (1977) einen Durchschnittsverbauch von 14 µl O_2/h bei Schnecken in der Aestivationsphase messen. Nach dem Wiedererwachen betrug der Durchschnittsverbrauch der Schnecken 50 µl O_2/h und erreichte Spitzenwerte von durchschnittlich 62-120 µl O_2/h in Phasen erhöhter Aktivität. Und auch während der Aestivationsphase konnte ein Einfluss der Temperatur auf den Sauerstoffverbrauch der Schnecken ermittelt werden. So konnten Schmidt-Nielsen et al. (1971) belegen, dass *Sphincterochila boissieri* bei 15 °C in der Aestivationsphase durchschnittlich 2,62 µl O_2/h verbrauchte, während es bei 35 °C 15,63 µl O_2/h waren.

Bei Untersuchungen zum Energiestoffwechsel von aktiven, wie auch in die Aestivationsphase übergehenden Individuen der Gattung *Oreohelix* [Pilsbry 1904] konnten Rees & Hand (1990) zeigen, dass verschieden hohe Stoffwechselniveaus bei diesen Schnecken unterschieden werden können. Der Stoffwechsel der Schnecken wurde in dieser Studie mit Hilfe einer kalorimetrischen Messmethode untersucht. Dabei wurden alle Stoffwechselvorgänge, bei denen durch aeroben Metabolismus Wärme entsteht, integriert und die daraus resultierende Wärmeleistung (in mW) als Maß für den Stoffwechsel der Schnecke herangezogen. Bei aktiven Schnecken konnten bereits zwei unterschiedliche Niveaus des Stoffwechsels gemessen werden, die um den Faktor 2-3 voneinander abwichen. Die Phasen niedriger Wärmeleistung (4–7 mW) wurden als Phasen angesehen in denen der Stoffwechsel normal abläuft, die Schnecke sich jedoch nicht bewegt. Die Phasen hoher Wärmeleistung (8-14 mW) hingegen wurden als Phasen direkter lokomotorischer Aktivität und starker Stoffwechselvorgänge interpretiert. Beim Übergang der Tiere in die Aestivationsphase wurde ein deutlicher Rückgang der Wärmeleistung (auf 0-2 mW) registriert, was als Herunterregeln des Stoffwechsels angesehen werden kann (Rees & Hand 1990).

Weitgehend unbekannt war indes zu Beginn der vorliegenden Arbeit, wie sich der Sauerstoffverbrauch der Schnecken in verschiedenen Phasen ihres Lebens bei verschiedenen Temperaturen aber ansonsten konstanten Umgebungsbedingungen verändert. Untersuchungen an unterschiedlich großen Schnecken einer Art könnten daher Aufschluss über den Sauerstoffverbrauch und damit einen ersten Hinweis über Anpassungen im Lebenszyklus an die vorherrschenden Temperaturen im Lebensraum geben. Ebenfalls fehlten Langzeitbeobachtungen zum Stoffwechsel von Schnecken. Ähnlich wie für den Sauerstoffverbrauch

wären Daten zum Stoffwechsel für verschiedene Größen von Schnecken hilfreich, um mögliche Anpassungen verschiedener Altersstadien der Schnecken an ihre Umgebung aufzudecken. Hinsichtlich beider Fragestellungen bestand daher Forschungsbedarf.

Neben den bisher beschriebenen Mechanismen der Energieeinsparung existieren zusätzlich zelluläre Schutzmechanismen, die es den Schnecken erlauben, auch längere Zeit an sonnenexponierten Standorten zu verweilen. Besonders beim Übergang in eine Aestivationsphase und beim Wiedererwachen aus einer Aestivationsphase, aber auch bei kürzeren Ruhephasen im Verlauf eine Tages sind intrazelluläre Schutzmechanismen nötig, um den Folgen einer Stresseinwirkung (wie zum Beispiel großer Hitze oder plötzlichen Schwankungen in der Verfügbarkeit von Sauerstoff) zu wiederstehen.
Eines der bekanntesten und phylogenetisch am stärksten konservierten Schutzsysteme gegen negative Auswirkungen hoher Temperaturen ist das der Hitzeschockproteine (Sørensen et al. 2003). Die Hitzeschockantwort wurde ursprünglich von Ritossa (1962) als Puffingmuster in den Chromosomen der Speicheldrüsen von *Drosophila busckii* [Coquillett 1901] Larven nach temporärer Erhöhung der Temperatur beobachtet. Tissiéres et al. (1974) konnten die bei Temperaturerhöhung gebildeten Proteine bei Larven von *Drosophila melanogaster* [Meigen 1830] identifizieren. Dem induzierenden Reiz nach wurden diese Proteine im Folgenden Hitzeschockproteine (*Heat shock proteins*, Hsp) genannt. Im Laufe der Zeit stellte sich heraus, dass nicht nur Hitze, sondern auch andere Stressoren, wie zum Beispiel organische Chemikalien und Schwermetalle ebenfalls zu einer verstärkten Bildung dieser Proteine führen können (Feder & Hofmann 1999; Kiang & Tsokos 1998; Köhler et al. 1992; Sørensen et al. 2003; Triebskorn et al. 1997). Heute ist eine Vielzahl verschiedener Hitzeschockproteine bekannt, die nach ihrem molekularen Gewicht unterschieden, bezeichnet und in Familien zusammengefasst werden. In ihrer Funktion als molekulare Chaperone sind Hitzeschockproteine auch in ungestresstem Zustand an vielen intrazellulären Stoffwechselwegen und Transportvorgängen, sowie bei der korrekten Faltung neu synthetisierter Proteine, der Stabilisierung von Proteinen, dem Verhindern der Aggregation denaturierter Proteine als auch bei der Degradation irreparabel beschädigter Proteine beteiligt (Feder & Hofmann 1999; Kiang & Tsokos 1998; Köhler 2009; Parsell & Lindquist 1993; Sørensen et al. 2003). Nach einem entsprechenden Impuls, wie zum Beispiel Hitzeexposition oder der Exposition gegenüber Chemikalien, kann eine Erhöhung des Hsp-Levels im Organismus festgestellt werden, der auf eine erhöhte Transkription von *hsp* Genen zurückzuführen ist (Köhler

2009; Lindquist & Craig 1988; Reuner et al. 2008; Sørensen et al. 2003). Diese induzierbaren Hsp-Formen stellen einen wichtigen Bestandteil der molekularen Reaktion bei der Anpassung von Organismen an ungünstige Bedingungen ihrer Umwelt dar.

Die bis heute am besten untersuchte Familie der Hitzeschockproteine ist die mit etwa 70 kDa Molekulargewicht charakterisierte Hsp70 Familie. Besondere Bedeutung im Zellstoffwechsel erlangt diese Proteinfamilie über ihre Fähigkeit neben der Chaperon-Funktion, die durch einen konstitutiven Basislevel an Hsp70 gewährleistet wird, auch nach oder während eines Stressimpulses teildenaturierte Proteine zu einem gewissen Prozentsatz in ihre ursprüngliche funktionale Form zurück führen zu können (Parsell & Lindquist 1993). Die durch einen Stressimpuls induzierbaren Vertreter der Hsp70 Familie können so den Auswirkungen proteotoxischen Stresses entgegenwirken (Jäättelä 1999; Mayer & Bukau 2005; Parsell & Lindquist 1993). Die Induktion von Hsp70 durch Umweltstressoren folgt dabei einer Kinetik, die von Eckwert et al. (1997) charakterisiert wurde. In ungestresstem Zustand ist ein konstitutiver Level an Hsp70 in der Zelle zu finden. Dieser ist abhängig von der derzeitigen Lebens- und Entwicklungssituation des Organismus und vom bewohnten Habitat (Mizrahi et al. 2010; Mizrahi et al. 2012), an das es sich anzupassen gilt. Mit steigender Stressintensität erhöht sich der induzierbare Hsp70-Level bis zu einem Maximum. In dieser Reaktionsphase können die Folgen des Stresseinflusses größtenteils kompensiert werden. Wird das Maximum überschritten, kommt es durch Überlastung des Schutzsystems, zu einem Abfall des Hsp70-Levels. In dieser Destruktionsphase ist der nötige Schutz der Zellen und der gebildeten Proteine nicht mehr vollständig gegeben und Schäden in Zellen, Geweben und Organen können sich manifestieren (Eckwert et al. 1997).

Speziell bei Schnecken konnte gezeigt werden, dass Hsp70 und andere Vertreter der Hitzeschockproteine eine wichtige Rolle im Leben von Landschnecken in heißen Habitaten spielen. Bei Untersuchungen an den nahe miteinander verwandten *Sphincterochila* [Ancey 1887] Arten *S. cariosa* [Olivier 1801] und *S. zonata* [Bourguignat 1853] konnte gezeigt werden, dass der Hsp70-Level in Folge von Hitzestress bei der im mediterranen Bereich vorkommenden *S. cariosa* schneller erhöht wurde als bei der in ariden Gebieten vorkommenden *S. zonata* (Mizrahi et al. 2012). Dies spricht für eine Anpassung an das jeweilig besiedelte Habitat. Im Hinblick auf ihre Fähigkeit, Austrocknung zu tolerieren konnte gezeigt werden, dass die an den ariden Lebensraum angepasste *S. zonata* in der Lage war, Wasser effektiver zurückzuhalten als die an den mediterranen Lebensraum adaptierte *S. cariosa* (Mizrahi et al.

2010). Im selben Experiment konnte belegt werden, dass *S. cariosa* bei beginnendem Trockenstress Hsp70 direkt hochregulierte, während *S. zonata* erst einige Tage nach Beginn des Trockenstresses Hsp70 verstärkt produzierte (Mizrahi et al. 2010). Bei Untersuchungen an aktiven und in eine Aestivationsphase übergegangenen Schnecken der Art *Cantareus apertus* konnte ein deutlicher Unterschied im Hsp70-Level festgestellt werden. Im Vergleich zu im Trockenschlaf (Aestivationsphase) befindlichen Schnecken wiesen hitzegestresste, aktive Individuen einen deutlich erhöhten Hsp70-Level auf, während sich der Hsp70-Level bei aktiven, jedoch ungestressten Schnecken und bei Schnecken im Trockenschlaf kaum unterschied (Reuner et al. 2008). Im Übergang von Trockenschlaf in erneute Aktivität konnten Arad et al. (2010) einen deutlich erhöhten Hsp70-Level in 'erwachenden' *S. cariosa* und *S. zonata* Individuen feststellen. Diese Experimente verdeutlichen, dass die Hsp70 Stressantwort nicht nur von der Intensität des Hitzeschocks, sondern auch von der Anpassung der Tiere an ihren Lebensraum und vom jeweiligen physiologischen Status abhängig ist. Doch nicht nur eine mögliche Aestivationsphase, sondern auch die Reproduktionsphase spielt eine wichtige Rolle bei der Betrachtung des Hsp70-Levels bei Landschnecken. So konnte gezeigt werden, dass *S. cariosa* und *S. zonata* während der Reproduktion auf einen erhöhten Hsp70-Level verzichten. Dafür konnte ein Anstieg des Hsp70 in den Eiweißdrüsen und in den Eiern der Schnecken festgestellt werden (Mizrahi et al. 2011). Dies deutet auf einen energetischen Trade-off zwischen Selbstschutz und Reproduktion hin. Ähnliche Befunde konnten in transgenen *Drosophila*-Stämmen, die Hsp70 überexprimieren, gefunden werden. Hier führte die künstliche Überproduktion an Hsp70 zu reduzierter Schlupfrate der Eier (Silbermann & Tatar 2000).

In natürlichen Populationen wirken zu verschiedenen Zeiten verschiedene Stressoren auf die Schnecken ein. Im Falle von *Xeropicta derbentina* [Krynicki 1836], einer in Südfrankreich invasiven Hygromiiden Art (Altena 1960; De Mattia 2007), konnte bereits gezeigt werden, dass Hsp70 ein wichtiger Teil eines Schutzmechanismus ist, der *X. derbentina* das Überleben in mediterranen Klimaten ermöglicht (Köhler et al. 2009; Scheil et al. 2011). Bei Untersuchungen an verschiedenen *X. derbentina* Populationen in Südfrankreich konnte festgestellt werden, dass diese Populationen in ihrer Intensität, auf Hitzestress zu reagieren, variieren (Troschinski et al. 2014).

Um den Einfluss des Umweltparameters Temperatur auf eine Population genauer zu untersuchen, ist es wichtig die Reaktionsmöglichkeiten der beteiligten Individuen im Verlauf ihrer Lebensabschnitte im Feld zu verfolgen. Weitgehend unbekannt war zu Beginn der vorliegen-

den Arbeit, inwieweit die Änderung der Umgebungstemperatur im Laufe des Tages den Hsp70-Level von freilebenden Individuen beeinflusst. Ebenfalls unklar war, ob die Tiere in den verschiedenen Phasen ihres Lebens unterschiedlich auf die Umgebungstemperatur reagieren. Die Tages- und Jahresrhythmik des Hsp70-Levels in natürlichen Populationen von *X. derbentina* sowie Untersuchungen zu deren Wachstum bedurften daher weiterer Untersuchungen. Ebenfalls unbekannt war, ob die Intensität der Hsp70 Stressproteininduktion bei diesen Schnecken durch die Länge der Hitzeexposition bei verschiedenen Temperaturen beeinflusst wird.

Neben dem Hsp Schutzsystem existieren jedoch noch weitere Systeme, die Organismen helfen, den Auswirkungen von Stress zu begegnen. Allen Sauerstoff verbrauchenden Organismen ist ein obligatorisch anfallender Stressor gemein: Bei der Zellatmung sowie bei der Biotransformation fallen, als Konsequenz der aeroben Lebensweise, reaktive Sauerstoffspezies (*reactive oxygen specises*, ROS) an (Ferreira-Cravo et al. 2010; Halliwell & Gutteridge 1989). Diese ROS (vornehmlich O_2^-, H_2O_2 und •OH) werden, soweit sie in physiologisch tolerierbarer Menge auftreten, zeitnah innerhalb derjenigen Zelle, in der sie gebildet werden, 'abgefangen' um ihre schädliche Wirkung umgehend zu neutralisieren (Storey 1996). Treten durch einen Stressor, wie zum Beispiel Hitze, ROS in übermäßiger Zahl auf, spricht man von oxidativem Stress (Sies 1997), und Schäden an Zellen können entstehen. Durch ihre höchst reaktive Natur können ROS sämtliche in der Zelle vorkommenden Makromoleküle angreifen und schädigen. Werden Lipide durch ROS geschädigt, kommt es zu einer oxidativen Degeneration derselben, der sogenannten Lipidperoxidation. Hierbei werden ungesättigte Fettsäuren durch Radikale angegriffen und reaktive Fettsäure-Radikale entstehen, welche wiederrum mit Sauerstoff reagieren und zu Lipidperoxyl-Radikale werden. Diese Lipidperoxyl-Radikale können eine Kettenreaktion in Gang setzten und weitere ungesättigte Fettsäuren angreifen. Durch Reaktion zweier Radikale zu einem Nicht-Radikal, durch kompletten Verbrauch der ungesättigten Fettsäuren oder durch ein Antioxidans kann die Kettenreaktion gestoppt werden kann. Das Endprodukt dieser oxidativen Degeneration sind Lipidperoxide. Durch Schäden an den Biomembranen von Zellen und Zellorganellen kann es zu Funktionsstörungen der Zellen kommen. Ein Maß für den Grad der Lipidperoxidation eines Organismus zu einem bestimmten Zeitpunkt und damit für den erfahrenen oxidativen Stress, kann über den *ferrous oxidation xylenol organge assay* (FOX assay) gegeben werden (Hermes-Lima et al. 1995; Monserrat et al. 2003).

Als Schutzsystem der Zellen stehen den ROS eine große Anzahl enzymatischer sowie auch nicht-enzymatischer antioxidativ wirkender Substanzen entgegen. Eine der wichtigsten Enzymgruppen ist die der Superoxiddismutasen (SOD). Die SOD katalysieren die Metabolisierung anfallender Superoxid-Anionen zu Wasserstoffperoxid (H_2O_2). Das entstandene Wasserstoffperoxid wird wiederrum durch andere Proteine, wie zum Beispiel der Katalase (CAT), einem weiteren obligatorisch in jeder Zelle vorliegenden Enzym, zu Sauerstoff und Wasser umgesetzt. Eine weitere zu erwähnende Enzymklasse sind die Glutathionperoxidasen (GPx), welche anfallende Peroxide (also auch Lipidperoxide und Wasserstoffperoxid) unter Anwesenheit von Glutathion entgiften.

Die drei erwähnten Enzymgruppen sind nur ein kleiner Teil einer komplexen Maschinerie zur Vermeidung von Zellschäden durch die Folgen oxidativen Stresses, bieten in der Forschung jedoch ein profundes Sortiment, um zusammen mit dem über den FOX-Assay messbaren Grad der Lipidperoxidation, Aussagen über die Konsequenzen und Bewältigung oxidativen Stresses machen zu können.

Betrachtet man eine natürliche Population von *Xeropicta derbentina* im Feld, so wird man großteils Individuen mit einer rein weißen Schalenfarbe beobachten. Jedoch wurden in den meisten bis dato untersuchten *X. derbentina* Populationen in Südfrankreich auch Individuen mit abweichendem Schalenfärbungsmuster gefunden und gemäß diesem Muster in verschiedenen Farbkategorien eingeteilt (Di Lellis et al. 2012; Köhler et al. 2009). Die Existenz von Schalenfärbungspolymorphismen bei Schnecken ist schon lange bekannt und Gegenstand der Forschung. Besonders bei verschiedenen *Cepaea* [Held 1837] Arten ist und war die Schalenfarbe immer wieder ein Ausgangspunk für ökophysiologische und evolutionsbiologische Forschung (Cain 1977; Goodhart 1987; Ozgo & Schilthuizen 2012; Richardson 1979). So konnte beispielsweise gezeigt werden, dass verschiedenen Farbmorphen von *Cepaea vindobonensis* [Férussac 1821] sich in ihrer Aktivität, ihrer Schalentemperatur und in ihrer Widerstandsfähigkeit gegenüber Trockenheit zum Teil deutlich unterschieden (Staikou 1999). Bei Untersuchungen an *Cepaea nemoralis* [Linné 1758] konnte des Weiteren gezeigt werden, dass sich verschiedene Farbmorphen dieser Art bezüglich der Innentemperatur und der durch Dehydration bedingten Mortalität nach Hitzeexposition unterschieden (Chang 1991; Heath 1975; Richardson 1974). Eine weitere Schneckenart, die einen Schalenpolymorphismus vorweist und häufig untersucht wurde, ist *Theba pisana* [Müller1774] (Cowie 1992; Köhler et al. 2013). Analog zu *Xeropicta derbentina* besiedelt auch *T. pisana* unter anderem

den mediterranen Lebensraum und tritt in Südfrankreich sympatrisch mit *X. derbentina* zusammen auf.

Die Färbung der Schale wurde in früheren Studien mit polymorphen Arten häufig im Kontext einer möglichen schnelleren Erwärmung dunklerer Morphen durch erhöhte Absorption von Strahlung untersucht (Hazel & Johnson 1990; Heath 1975). Diese Theorie wird jedoch durch aktuelle Forschungsergebnisse zunehmend in Frage gestellt. So konnten Scheil et al. (2012) keine Unterschiede in der Aufheizung und im Wärmeverlust unterschiedlich gefärbter *T. pisana* Morphen feststellen. Bei Untersuchungen zum Erscheinungsbild gefärbter Schneckenschalen bei verschiedenen Wellenlängen konnten Savazzi & Sasaki (2013) belegen, dass Schneckenschalen, die im sichtbaren Wellenlängenberich (VIS) dunkle Streifen auf hellem Hintergrund zeigten, im nahen Infrarotbereich (NIR) kaum oder keine Bänderung mehr aufwiesen. Sowohl Scheil et al. (2012) als auch Savazzi & Sasaki (2013) schließen bei solchen Schnecken, zu denen sowohl *T. pisana* als auch *X. derbentina* gehören darauf, dass die thermische Kapazität der Schneckenschale eher auf die Materialeigenschaften des Gehäuses als auf die im sichtbaren Wellenlängenbereich auftretenden Bänderungen zurückzuführen ist. Die Bänderung würde nach dieser Hypothese für die Erwärmung der Schale von *T. pisana* oder *X. derbentina* eine eher untergeordnete Rolle spielen. Vergleicht man jedoch die Schalen von *X. derbentina* oder *T. pisana* mit denen der erwähnten *Cepaea* Arten, so ist die Grundfarbe der Schale bei beiden Arten Weiß, während die Grundfarbe der *Cepaea* Arten von Gelb über Pink bis hin zu Braun variiert. Auch ist bei *Cepaea* die Bänderung deutlicher ausgeprägt als bei *X. derbentina* und *T. pisana*. Die bei *Cepaea* Arten gefundenen Unterschiede in der Erwärmung des Weichkörpers und der Schale durch Sonneneinstrahlung, könnten also durch unterschiedliche Materialeigenschaften und unterschiedlich hohe Absorption von Sonnenlicht der verschiedenfarbigen Schalen begründet sein, während die Großteils weißen Schalen von *X. derbentina* und auch *T. pisana* eine deutlichere Differenzierung zwischen den Morphen nicht zulassen. Jedoch gibt es auch bei diesen Arten Hinweise dafür, dass die Bänderung einen Einfluss auf die Erwärmung der Tiere hat. So konnte von Di Lellis et al. (2012) gezeigt werden, dass die Färbung der Tiere teilweise Einfluss auf den gemessenen Hsp70-Level hat, jedoch von den Faktoren 'Größe' und 'Gewicht' abhängig war.

Zu Beginn der vorliegenden Arbeit war nicht bekannt, wie sich verschiedene Morphen einer Schneckenpopulation bezüglich der Induzierbarkeit ihres Hsp70-Schutzsystems und ihrer Widerstandsfähigkeit gegen oxidativen Stress bei verschiedenen Temperaturen unterschei-

den. Gerade die Verbindung verschiedener biochemischer Parameter, wie zum Beispiel des Hsp70-Levels und des Grades der Lipidperoxidation bei verschieden gefärbten Morphen einer Art aus derselben Population, verspricht neue Aufschlüsse über deren Überlebensmöglichkeiten zu geben. Wie bereits eingangs erwähnt, spielen sowohl das Hsp-Schutzsystem als auch das Schutzsystem gegen oxidativen Stress eine bedeutende Rolle im Leben der Schnecken, besonders während und kurz nach einer Aestivationsphase. Bisher wurden viele Arbeiten über beide Schutzsysteme angefertigt, das Zusammenspiel dieser beiden Schutzsysteme verblieb bisher jedoch weitgehend unbeleuchtet.

1.3 Fragestellungen

In der vorliegenden Arbeit wurden hauptsächlich physiologische Aspekte der Anpassung von *Xeropicta derbentina* an ihren Lebensraum beleuchtet. Dabei lag der Fokus auf den subzellulären Schutzsystemen und Anpassungen des Stoffwechsels der Schnecken.

Ausgehend von der Aufnahme und Dokumentation des umweltrelevanten Stressors Temperatur im natürlichen Habitat einer exemplarisch ausgewählten Population in Südfrankreich wurde das Wachstum und die Färbung der Population im Verlauf des Jahres dokumentiert. Begleitend sollte sowohl die Tages- als auch die eventuell vorhandene Jahreskinetik im Hsp70-Level der Tiere durch mehrmalige Beprobung der Population über jeweils einen Tag zu vier verschiedenen Probezeiträumen ermittelt werden. Dabei wurden die folgenden Arbeitshypothesen adressiert:

- Die tageszeitlichen Änderungen im Temperaturverlauf des Habitats werden durch die Hsp70-Induktion in den Schnecken wiedergespiegelt.
- Neben den tageszeitlichen Schwankungen können auch jahreszeitliche Schwankungen der Umgebungstemperatur durch den Hsp70-Level der Schnecken abgebildet werden.
- Während des Wachstums der Individuen ist eine Änderung in der Morphenzusammensetzung der Population erkennbar.

Auf Basis der im ersten Kapitel erhobenen Ergebnisse wurde im zweiten Kapitel der Frage, ob der beobachtete Schalenpolymorphismus der Feldpopulation mit einer Präadaptation eines bestimmten Farbmorphs bezüglich seiner Hsp70-Stressproteininduktion einher geht, nachgegangen. Als ein weiterer Stressmarker sollte der Grad an Lipidperoxidation bei verschiedenen Temperaturen herangezogen werden um auf den erfahrenen oxidativen Stress rückzuschließen. Hierbei sollten die folgenden Arbeitshypothesen geprüft werden:

- Schnecken mit einer dunklen Schalenfarbe heizen sich stärker auf als hellere Morphen. Daher liegt eine Präadaptation dieser Morphe vor, die sich in höheren Hsp70-Leveln wiederspiegelt, um dem Einfluss von Hitze entgegen zu wirken.
- Dunkel gefärbte Schnecken erfahren durch stärkere Erwärmung auch einen höheren oxidativen Stress, was sich in erhöhter Lipidperoxidation niederschlägt.

Im dritten Kapitel wurden die Auswirkungen erhöhter Temperatur auf die Stressantwort gegenüber ROS mit Hilfe essentieller, antioxidativ wirkender Enzyme und deren Aktivität bei verschiedenen Temperaturen dargestellt. Durch die Kombination der bereits in Kapitel zwei etablierten Biomarker (Hsp70 und Lipidperoxidation) und der Analyse der Aktivität

antioxidativ wirkender Enzyme sollten diese protektiv wirkenden Mechanismen und ihr Zusammenspiel in der Anpassung von *Xeropicta derbentina* an ihren Lebensraum beleuchtet werden. Folgende Arbeitshypothesen lagen der Studie zu Grunde:

- Die antioxidativ wirkenden Enzyme Katalase und Glutathionperoxidase weisen eine temperaturabhängige Umsatzaktivität im Gewebe auf, die den Organismus bei hohen Temperaturen schützen.
- Es gibt einen Zusammenhang zwischen dem messbaren Hsp70-Level, dem Grad der Lipidperoxidation und der Aktivität der untersuchten Enzyme, welcher von der Temperatur abhängig ist.

In den Kapiteln vier, fünf und sechs wurde der Fokus weg von subzellulären Anpassungen und Schutzmechanismen hin zur Analyse metabolischer Abläufe verlegt. Die Gewinnung von Daten zum Energieumsatz der Schnecken und dem dabei verbrauchten Sauerstoff sowie Messungen zum Wasserverlust sollten Aufschluss darüber geben, wie sich der Stoffwechsel von *Xeropicta derbentina* bei verschiedenen Temperaturen und zu verschiedenen Aktivitätsphasen verhält.

Im vierten Kapitel wurde hierzu zunächst der Einfluss der Expositionszeit bei verschiedenen Temperaturen auf den Hsp70-Level der Schnecken beleuchtet. Zusätzlich wurden Messungen zur Evaporation von Wasser während dieser Exposition dargestellt. Des Weiteren sollten ausgedehnte Messungen über den Zeitraum von bis zu drei Tagen den Wasserverlust der Tiere exemplarisch aufzeigen um Approximationen über die Verdunstung über längere Zeit bei verschiedenen Temperaturen zu ermöglichen. Im Einzelnen wurden die folgenden Hypothesen dabei untersucht:

- Sowohl Temperatur als auch die Länge der Temperaturexposition haben einen Einfluss auf den Hsp70-Level der Schnecken.
- Die Verdunstung von Wasser durch die Schalenöffnung und die Schale der Schnecken ist abhängig von der Umgebungstemperatur, wobei höhere Temperaturen zu höherem Wasserverlust führen.

In Kapitel fünf wird dargestellt, wie hoch der Energieumsatz von *Xeropicta derbentina* bei zwei unterschiedlichen Temperaturen tatsächlich ist. Dieses Kapitel bezieht sich auf Untersuchungen, in denen Schnecken aus zwei zuvor definierten Größenklassen mit Hilfe eines Thermal Activity Monitor (TAM) kalorimetrisch untersucht wurden, wobei alle ablaufenden Stoffwechselvorgänge integrativ betrachtet wurden und die dabei freiwerdende Wärme als Leistung der Schnecke und somit als Maß für den Stoffwechsel des Tieres herangezogen

wurde. Insbesondere durch Langzeitbeobachtung sollten Energieniveaus herausgearbeitet werden, die eine Einordnung in Aktive Phasen, Ruhephasen und wenn möglich Aestivationsphasen zulassen. Folgende Arbeitshypothesen lagen diesem Kapitel zu Grunde:

- Der Energieumsatz der Schnecken ist abhängig von der Umgebungstemperatur. Bei niedriger Temperatur haben die Tiere einen niedrigeren Energieumsatz als bei hoher Temperatur.
- Schnecken geringerer Körpergröße haben absolut gesehen einen geringeren Stoffwechsel als größere Schnecken bei selber Temperatur.
- Schnecken geringerer Körpergröße reduzieren ihren Stoffwechsel bei unterbleibender Futteraufnahme schneller als große Schnecken.

In Kapitel sechs wurde der Frage nachgegangen, wie hoch der Sauerstoffverbrauch von *Xeropicta derbentina* bei umweltrelevanten Temperaturen zu verschiedenen Phasen ihres Lebens ist. Individuen aus definierten Größenklassen wurden hierbei bei drei verschiedenen Temperaturen auf ihren Sauerstoffverbrauch in Ruhe hin untersucht. Es standen folgende Hypothesen im Zentrum der Untersuchungen:

- Der Sauerstoffverbrauch kleiner Schnecken ist geringer als derjenige großer Individuen.
- Bei erhöhter Umgebungstemperatur ändert sich der Sauerstoffverbrauch der Schnecken.

Die Ergebnisse der kalorimetrischen Messungen, des Sauerstoffverbrauchs und des Wasserverlusts der Schnecken sollen zukünftig als Datenbasis für eine mögliche Computersimulation der Wärmeflüsse im Schneckenkörper genutzt werden und wurden in enger Absprache mit der Hochschule Esslingen und der dort involvierten Projektpartner ('*Twinning project*' „Wärmeflüsse, Thermodynamik und ökophysiologische Konsequenzen hoher Temperaturen bei mediterranen Landschnecken“) erhoben.

1.4 Material und Methoden

1.4.1 Der Testorganismus

In der vorliegenden Arbeit wurden Individuen von *Xeropicta derbentina* [Krynicki 1836] eingesetzt. Innerhalb der Klasse der Gastropoda ist diese Spezies der Ordnung der Pulmonata (Lungenschnecken) zugeordnet. Die Ordnung der Pulmonata ist derzeit in vier Unterordnungen, die Acteophila, die Basommatophora (Wasserlungenschnecken), die Systellommatophora und die Stylommatophora (Landlungenschnecken) unterteilt. *Xeropicta derbentina* gehört hierin zu den Stylommatophora (Landlungenschnecken). Innerhalb der Stylommatophora sind derzeit vierzehn Überfamilien gelistet. *Xeropicta derbentina* gehört hierin zur Überfamilie der Helicoidea und darin wiederrum zur Familie der Hygromiidae (Laubschnecken). Die Familie der Hygromiidae (Laubschnecken) umfasst fünf Unterfamilien (Ciliellinae, Geomitrinae, Hygromiinae, Monachinae und Ponentininae) und zwei Gattungen (*Plentuisa* sp. und *Trochulus* sp.). Hierbei gehört *Xeropicta derbentina* der Unterfamilie der Hygromiinae und dem darin enthaltenen Tribus der Trichiini an.

Ihr Verbreitungsgebiet ist ursprünglich die östliche Mittelmeerregion. Durch Verschleppung konnte sich die Art jedoch bereits bis Italien, Kroatien und Frankreich ausbreiten (Altena 1960; Aubry et al. 2005; De Mattia 2007). In ihrem Verbreitungsgebiet bewohnt *Xeropicta derbentina* meist offene, sonnenbeschienene, trockene Landschaften. Im Adultstadium wird diese Art 10-16 mm im Durchmesser groß. Die Schalenfarbe reicht von Weiß über leichte braune Bänderungen an der Unterseite der Schale bis hin zu braunen Bänderungen über die gesamte Schale hinweg, wobei jedoch in den meisten Fällen eine rein weiße Schale dominiert (Abbildung 1).

Abb.1: *Xeropicta derbentina* im natürlichen Habitat. Deutlich zu erkennen sind die unterschiedlichen Farbmorphen.

Die in Kapitel eins verwendeten Individuen wurden direkt im Feld beprobt. Die in den Kapiteln zwei bis sechs verwendeten Individuen von *Xeropicta derbentina* wurden nach dem Sammeln im Feld für zwei bis vier Wochen im Labor akklimatisiert. Die Tiere wurden in Plastikkontainern (20,5 x 30 x 19,5 cm) auf Terrarienerde (JBL Terra Basis, Neuhofen, Deutschland) gehalten, alle zwei Tage mit Fertignahrung für Kleinkinder (HIPP Gute Nacht Bio-Milchbrei, Hafer & Apfel, Pfaffenhofen, Deutschland) gefüttert und mit Wasser besprüht. Bis auf die in Kapitel zwei nach Farbmorphen getrennt gesammelten Schnecken erfolgte das Sammeln der für die Hälterung im Labor bestimmten Individuen im Feld nach zufälligem Muster. Alle in den Kapiteln der vorliegenden Arbeit verwendeten Tiere wurden auf einer Wiese bei Modène (Departement Vaucluse, Provence-Alpes-Côte d'Azur, Südfrankreich (N44° 6.055' E5° 7.937')) gesammelt. Durch die hohe Abundanz von *Xeropicta derbentina* im Probengebiet war die zufällige Entnahme der Versuchstiere für den Fortbestand der Population nicht von Bedeutung.

Systematik:

Phylum: Mollusca
Classis: Gastropoda
Subclassis: Orthogastropoda
Superordo: Heterobranchia
Ordo: Pulmonata
Subordo: Stylommatophora
Superfamilia: Helicoidea
Familia: Hygromiidae
Subfamilia: Hygromiinae
Tribus: Trichiini
Genus: *Xeropicta*

Nach Fauna Europaea (www.faunaeur.org am 05.11.2014)

1.4.2 Methodik zur Ermittlung von Temperaturverläufen in verschiedenen Höhen im Feld und Messung der Schalentemperatur

Im ersten Kapitel der vorliegenden Arbeit wurden in vier verschiedenen Monaten im Jahr 2011 jeweils in zehn verschiedenen Höhen Temperaturverläufe im natürlichen Habitat aufgezeichnet. Hierzu wurden im April 2011 mit Hilfe eines präzisen Thermometers (ELLAB Kopenhagen, Typ DM 825) je Stunde in den Distanzen 1, 2, 3, 5, 10, 15, 20, 25, 30 und 40 cm vom Boden entfernt die jeweils vorherrschende Temperatur ermittelt. Zur Erfassung der Bodentemperatur wurde der Messfühler direkt auf den Boden gesetzt. Die Protokollierung

der Messdaten wurde von Hand durchgeführt. In den weiteren Probemonaten wurde eine automatisierte Temperaturerfassung in den angegebenen Höhen durchgeführt. Hierzu wurde in Kooperation mit der HS-Esslingen eine Halterung für Sensoren entwickelt, die ein Platzieren der Sensoren in der entsprechenden Höhe ermöglichte. Auf einem Multiplexer wurden zehn Sensoren (Thermoelement Typ T Klasse1 nach IEC 584-3, Electronic Sensor, Heilbronn, Deutschland) zur Protokollierung der Lufttemperatur und zwei Sensoren (Thermoelement Typ T Klasse1 nach IEC 584-3, Electronic Sensor, Heilbronn, Deutschland) zur Erfassung der Bodentemperatur angebracht und mit Hilfe eines Agilent 34972A LXI Mehrkanal Datenloggers (Santa Clara, CA, USA) im Abstand von 15 Sekunden digital protokolliert. Die aus diesen Messungen gebildeten Mittelwerte wurden für die in Kapitel eins durchgeführten Analysen verwendet. Des Weiteren wurde die Schalentemperatur der in den Analysen des ersten Kapitels verwendeten Individuen erfasst. Hierzu wurden die Individuen, die für die Stressproteinanalyse im Feld gesammelt wurden, vorerst am jeweiligen Fundort belassen und die Schalentemperatur durch Auflegen des Messfühlers des Handthermometers ermittelt und protokolliert. Erst nach dieser Messung erfolgten das Absammeln der Probe und das Fixieren in flüssigem Stickstoff.

1.4.3 Erhebung des Wachstums und der Färbung einer Population im Feld

Die in Kapitel eins durchgeführte Erhebung zum Wachstum und zur Färbung der untersuchten *Xeropicta derbentina* Population im Jahr 2011 erfolgte in den vier Probemonaten April, Juni, August und Oktober begleitend zu weiteren durchgeführten Messungen. Um das Wachstum und die damit verbundene Färbung der Individuen in dieser Population zu ermitteln, wurde je Probemonat ein 1 x 3 m großes Stück Wiese zufällig ausgewählt und 250 der darin enthaltenen Individuen vermessen, deren Färbung gemäß der zuvor definierten Färbungsmuster (Dieterich et al. 2012; Dieterich et al. 2014; Köhler et al. 2009) ermittelt und die erhaltenen Daten von Hand notiert. Die Schalengröße wurde mit Hilfe einer digitalen Schieblehre ermittelt, gemessen wurde der maximale Schalendurchmesser.

1.4.4 Messung des Stressproteinlevels

Für die Messung des Stressproteinlevels der Schnecken (Kapitel 1-4) wurden die individuell in flüssigem Stickstoff fixierten Individuen mit je 2 µl Extraktionsgemisch (80 mM Kaliumacetat, 5 mM Magnesiumacetat, 20 mM Hepes und 2 % Proteasehemmer) pro 1 mg Gesamtge-

wicht der Schnecke mechanisch homogenisiert und bei 4 °C und 20000 rcf zentrifugiert. Aus dem erhaltenen Überstand wurden 5 µl zur Bestimmung des Gesamtproteingehalts der Probe nach Bradford (1976) verwendet, der Rest der Probe wurde mit SDS-Probenpuffer versetzt und zur weiteren Verarbeitung aufbewahrt. Die Trennung des mit SDS-Probenpuffer versetzten Proteingemisches erfolgte per SDS-PAGE (SDS-Polyacrylamidgelelektrophorese). Dabei wurden 40 µg Gesamtprotein der Probe aufgetrennt und im Anschluss mittels Elektrotransfer (Westernblot) auf eine Nitrocellulosemembran aufgebracht. Die mit Proteinen beladenen Nitrocellulosemembranen wurden daraufhin mit Pferdeserum geblockt, mit Antikörpern (1.Antikörper: mouse anti-human Hsp70, 2. Antikörper: goat anti-mouse IgG konjungiert an Peroxidase) inkubiert und die so markierten Hsp70 Proteine per Färbelösung (1 mM 4-Chloro(1)naphthol, 30 mM Tris, Methanol, H_2O_2) sichtbar gemacht. Die Auswertung erfolgte durch ein densitometrisches Messverfahren des optischen Volumens. Die erhaltenen Hsp70 Banden wurden zu einem aus *Theba pisana* [Müller 1774] hergestellten Proteinstandard, der je Gel doppelt aufgetragen wurde, ins Verhältnis gesetzt um Unterschiede in der Färbungsintensität der einzelnen Membranen zu berücksichtigen.

1.4.5 Hitzeexposition im Wärmeschrank

Die in den Kapiteln zwei, drei und vier untersuchten Schnecken wurden nach zwei bis vierwöchiger Akklimationszeit und Pflege im Labor in einem Wärmeschrank gegenüber verschiedenen Temperaturen exponiert. Die Exposition erfolgte in Plastikwannen (6,5 x 18 x 13 cm), die mit feuchtem Küchenpapier ausgekleidet waren. Um ein Entkommen der Schnecken währen der Exposition zu verhindern, wurden die Plastikwannen mit Klarsichtfolie verschlossen und neun kleine Löcher in diese gestochen. Durch diese bereits im Vorfeld praktizierte Vorgehensweise (Di Lellis et al. 2014; Dieterich et al. 2014; Dittbrenner et al. 2009; Köhler et al. 2009; Troschinski et al. 2014) konnte der Parameter Luftfeuchtigkeit konstant hoch gehalten werden und musste als Variable nicht berücksichtigt werden. Die Expositionszeit betrug in den Kapiteln zwei, drei und vier jeweils 8 Stunden, dabei wurden die Tiere in Kapitel zwei gegenüber 25, 33, 38, 40, 43, 45 und 48 °C und in Kapitel drei gegenüber 25, 38, 40, 43 und 45 °C exponiert. In Kapitel vier wurden die Schnecken für jeweils 1,5; 2,5; 4,5; 6,5 und 8,5 Stunden bei jeweils 20, 35, 40, 42 und 45 °C exponiert. Nach Ende der Experimente wurden die Schnecken einzeln in flüssigem Stickstoff schockgefroren und bis zur weiteren Analyse bei -20 °C im Gefrierschrank aufbewahrt. Individuen, die für die Messung des

Lipidperoxid-Levels in den Kapiteln zwei und drei, sowie für die Messung der Enzyme Katalase (CAT) und Glutathionperoxidase (GPx) in Kapitel drei verwendet wurden, wurden vor dem Schockgefrieren aus der Schale herauspräpariert. Hierzu wurde die Schale zwischen zwei Objektträgern geknackt und lediglich der Weichkörper der Schnecke für obig genannte Analysen schockgefroren.

1.4.6 Messung des Lipidperoxid-Levels

Die in den Kapiteln zwei und drei durchgeführte Messung des Lipidperoxid Levels fußt auf der von Hermes-Lima et al. (1995) entwickelten Methode des FOX (ferrous oxidation xylenolorange) Assays. Hierzu wurden die zuvor von der Schale befreiten und in flüssigem Stickstoff fixierten Schneckenweichkörper gewogen und mit Methanol (HPLC Qualität in der Verdünnung 1:2 zum Gewicht der Schneckenweichkörper, bei einer Dichte des Methanols von 0,791 mg/cm^3) auf Eis homogenisiert, bei 15000 rcf (=12638 rpm) und 4 °C zentrifugiert und die gewonnenen Überstände bei -80 °C zur weiteren Analyse zwischengelagert. Der FOX Assay wurde für die Analyse auf einer 96-kammrigen Mikrotiterplatte modifiziert. In jede Probenkammer (außer den Blanks) wurden je 50 µl 0,25 mM $FeSO_4$, 25 mM H_2SO_4, und 0,1 mM Xylenolorange gegeben. Zu diesem Gemisch wurden je 15 µl des zu untersuchenden Probenüberstandes gegeben und das Gesamtvolumen mit Aqua bidest. auf insgesamt 200 µl aufgefüllt. Die Master Blanks wurden mit je 200 µl Aqua bidest. gefüllt, die Proben Blanks statt mit 50 µl $FeSO_4$ mit je 50 µl Aqua bidest. Alle Proben wurden in dreifacher Ausführung pipettiert. Die Inkubationszeit betrug je 180 Minuten bei Raumtemperatur. Nach der Inkubation folgte die erste Absorptionsmessung bei 580 nm Wellenlänge ($A_{580\ nm\ (1.Messung)}$). Im Anschluss wurde je 1 µl einer 1 mM Cumolhydroperoxid (Chp) Lösung in die Probekammern gegeben und für weitere 30 Minuten inkubiert. Nach einer erneuten Absorptionsmessung bei 580 nm Wellenlänge ($A_{580\ nm\ +\ Chp\ (2.Messung)}$) konnte der Lipidperoxid Level der Proben berechnet werden. Hierzu wurde zuerst von allen Messwerten der Master Blank subtrahiert. Zur Berechnung der Probenwerte wurde vom Mittelwert der drei erhaltenen Probewerte der Proben der Proben Blank subtrahiert. Der Lipidperoxid-Level wird als Cumolhydroperoxid Äquivalente pro Gramm Nassgewicht der Probe (ChpE / g wet weight) dargestellt und berechnet sich nach der in Hermes-Lima et al. (1995) eingeführten Gleichung:

$$ChpE/g_{\ wet\ weight} = (A_{580\ nm\ (1.Messung)}/A_{580\ nm\ +\ Chp\ (2.Messung)})*1\ \mu l\ Chp_{1\ nmol} *V/V1 *DF$$

wobei V das Gesamtprobenvolumen (200 µl), V1 die zugegebene Menge an Probe (15 µl) und DF der Verdünnungsfaktor der Probe mit Methanol (hier: 2) ist.

1.4.7 Messung der Enzymaktivität für Katalase und Glutathionperoxidase

Im dritten Kapitel der vorliegenden Arbeit wurde die Aktivität der Enzyme Katalase und Gutathionperoxidase untersucht. Hierzu wurden die zuvor gegen Hitze exponierten Schnecken zunächst durch Knacken der Schale zwischen zwei Objektträgern aus der Schale herauspräpariert und der Weichkörper in flüssigem Stickstoff schockgefroren. Die Proben wurden bis zur weiteren Verarbeitung bei -80 °C zwischengelagert.

Zur Untersuchung der Katalase (CAT) Aktivität wurde das im Handel erhältliche Assay Kit von Cayman Chemicals (Item No. 707002, Cayman Chemicals Company, Michigan, USA) eingesetzt. Methodisch basiert dieses Kit auf einer Reaktion der in der Probe enthaltenen Katalase mit Methanol in der Anwesenheit von H_2O_2. Das bei der Reaktion gebildete Formaldehyd reagiert mit dem als Chromogen fungierenden und im Kit enthaltenen Purpald (4-amino-3-hydrazino-5-mecapto-1,2,4-triazol). Der dabei erfolgende Farbumschlag von transparent nach violett wurde mit einem Photometer bei 540 nm Wellenlänge gemessen. Der komplette Assay wurde auf einer 96-kammrigen Mikrotiterplatte durchgeführt. Zur Berechnung der Katalaseaktivität wurde folgende Formel verwendet:

CAT Aktivität [nmol/min/mg] = [(µM Formaldehyd der Probe/20 min)*Probenverdünnung]/1000

Zur Bestimmung der Glutathionperoxidase Aktivität wurde das im Handel erhältliche Assay Kit von Cayman Chemicals (Item No. 703102, Cayman Chemicals Company, Michigan, USA) eingesetzt. Der Aktivitätsnachweis der Glutathionperoxidase findet hierbei über einen indirekten Nachweis mittels gekoppelter Reaktion mit Glutathionreduktase (GR) statt. Oxidiertes Glutathion (GSSG), das bei der Reaktion von Hydroperoxiden mit der Glutathionperoxidase (GPx) anfällt, wird durch GR und NADPH in seine reduzierte Form umgewandelt (GSH). Die Oxidation von NADPH zu $NADP^+$ geht mit einer verminderten Absorption der Probe bei 340 nm einher. Die Minderung der Absorption bei 340 nm Wellenlänge im Photometer ist direkt proportional zur Aktivität der Glutathionperoxidase in der Probe. Die Änderung der Absorption wurde über einen Zeitraum von fünf Minuten gemessen. Diese Form des Assays erfasst alle von Glutathion abhängigen Peroxidasen der Probe. Der Assay wurde in 96-

kammrigen Mikrotiterplatten durchgeführt. Zur Errechnung der GPx Aktivität wurde folgende Formel verwendet:

GPx Aktivität [nmol/min/mg] = [((ΔA$_{340}$/min)/0,000373µM^{-1})*(0,19ml/0,02ml)*Probenverdünnung]/1000

1.4.8 Messungen zum Wasserverlust der Schnecken im Wärmeschrank

Die in Kapitel vier durchgeführten Messungen zum Wasserverlust der Schnecken bei verschiedenen Temperaturen wurden wie folgt durchgeführt:
Um eine Evaporation des Wasser von der Oberfläche und über das Epiphragma der Schnecken zu ermöglichen wurden verschließbare zylindrische Käfige aus Aluminiumgaze hergestellt. Je Versuchsansatz wurden zehn Schnecken gleicher Größe gewählt, in die Käfige eingebracht und der Käfig mit den Schnecken auf einer Feinwaage gewogen. Die Schnecken wurden anschließend in den vortemperierten Wärmeschrank für jeweils 1, 2, 4, 6 und 8 Stunden eingebracht und gegen die Testtemperaturen von 25, 30, 35, 40, 42 und 45 °C exponiert. Das der Schale anhaftende Wasser wurde durch eine 30-minütige Vorexposition entfernt und das Experiment danach bei t_0 als neuem Startzeitpunkt gestartet. Der Verlust an Gewicht nach der Testphase im Vergleich zum Gewicht davor wurde als durchschnittlicher Wasserverlust der zehn Schnecken angesehen. Das Experiment wurde je Temperatur und Expositionszeit dreimal wiederholt. Um die aus den Mittelwerten resultierenden linear angepassten Regressionen zu stützen, wurden zusätzlich zu obigen Messungen die für alle Temperaturen durchgeführt wurden, zusätzliche Langzeitmessungen von 24 h bei 20, 35 und 40 °C, 48 h bei 20 und 35 °C sowie 96 h bei 35 °C durchgeführt.

1.4.9 Stoffwechselmessungen mit Hilfe des Thermal Activity Monitor

Die in Kapitel fünf durchgeführte Messung des Stoffwechsels von *Xeropicta derbentina* wurde mit Hilfe eines Thermal Activity Monitor (TAM) 2277 (Thermometric AB, Jarfalla, Schweden) durchgeführt. Die kalorimetrische Messung basiert auf zwei identischen Messzellen, die auf einer konstanten Temperatur gehalten werden. Eine der Zellen dient dabei als Referenzzelle, während die andere Zelle als Messzelle dient. Alle in der Messzelle ablaufenden Reaktionen, bei denen Wärme als Produkt einer Umsatzreaktion (Wärmeleistung) entsteht oder Wärme aufgenommen wird, werden detektiert. Die dabei erzeugten Spannungen (Seebeck-Effekt) werden zur Referenzzelle in Bezug gesetzt, umgerechnet und mit Hilfe einer mitgelieferten Software in Computersignale übersetzt. Die Auflösungsgrenze des verwendeten Gerä-

tes lag bei ca. 0,4 µW. Das interne Hintergrundrauschen des Geräts war mit ca. 40 nW angegeben.

Für die Messung des Stoffwechsels der Schnecken wurde zunächst das Gerät auf die gewünschte Temperatur eingependelt und eine Baselinekorrektur durchgeführt. Die Schnecken wurden vor dem Experiment wie oben beschrieben gehältert und vor dem Experiment in eine feuchte Plastikbox mit nassem Zellstoff eingebracht. Die aktive Schnecke wurde vermessen und gewogen und eine korrespondierende Menge Wasser (errechnet aus der Wärmekapazität des Wassers und der Menge an Wasser im Weichkörper der Schnecke in Relation zu deren Größe und Gewicht; unveröffentlichte Daten U. Fischbach) in die Referenzzelle gegeben. Die Erfassung der Wärmeleistung erfolgte in Intervallen von 60 Sekunden. Es wurden zwei Größenkategorien an Schnecken vermessen. Bedingt durch den Innendurchmesser der Messzelle wurden Schnecken mit 5,3 – 5,8 mm Durchmesser als „Kleine Schnecken“ und Schnecken mit einem Durchmesser von 8,5 – 8,8 mm als „Große Schnecken“ eingestuft und untersucht. Drei Individuen jeder Größenkategorie wurden jeweils sieben Tage bei zwei unterschiedlichen Temperaturen (20 °C und 30 °C) eingesetzt und deren Wärmeleistung kontinuierlich erfasst. Zusätzlich wurde je Größenkategorie ein Langzeitversuch von je 24 Tagen durchgeführt. Bedingt durch die Konstruktion des TAM konnten die Tiere während der gesamten Messzeit nicht gefüttert oder überwacht werden.

1.4.10 Versuchsaufbau und Messung des Sauerstoffverbrauchs

Für die in Kapitel sechs durchgeführten Sauerstoffmessungen wurde eine hierfür geeignete Probenkammer eigens von der HS-Esslingen entwickelt. Die Notwendigkeit zur individuellen Messung des Sauerstoffverbrauchs einzelner Individuen war bei der Planung und Entwicklung dieses Mikrorespirometers wegweisend. Als Basis diente eine zylindrische Aluminiumkammer, die den Messfühler nach oben vor möglichem Kontakt mit dem Versuchsorganismus schützt. Der Sauerstoffsensor (ProChem Analytik, Xanten, Deutschland) wurde so gewählt, dass bereits Änderungen im Sauerstoffgehalt von 0,01 % gemessen werden konnten. Neben dem Sauerstoffgehalt wurden zusätzlich die Luftfeuchtigkeit (SHT21, Sensirion, Staefa, Schweiz), der Gasdruck (PAA-33X, Keller, Jestetten, Deutschland) und die Temperatur (miniature PT100, Elektronic Sensor, Heilbronn, Deutschland) im Innern der Kammer gemessen. Um zwischen dem Sauerstoffverbrauch bei Aktivität der Schnecke und dem Sauerstoffverbauch in Ruhe unterscheiden zu können wurde eine Webcam mit LED in den Deckel ein-

gebracht, um das Verhalten der Schnecke nachvollziehen zu können. In der hier durchgeführten Studie wurden jedoch nur inaktive Schnecken untersucht, um den Einfluss von Bewegungen auf den Sauerstoffverbrauch der Tiere zu vermeiden und zunächst den Sauerstoffverbrauch der Tiere in ihrer Ruhephase zu untersuchen. Die komplette Apparatur wurde so konzipiert, dass sie in ein Wasserbad eingebracht werden konnte, um eine konstante Temperatur zu gewährleisten. Im Laufe der Experimente konnte so die Temperatur als Parameter für unterschiedliche Messreihen angepasst werden. Es wurden Schnecken aus drei zuvor festgelegten Größenkategorien (Größe 1 = 0,65 – 0,85 cm, Größe 2 = 0,9 – 1,0 cm und Größe 3 = 1,0 – 1,25 cm) bei Testtemperaturen von 20, 30 und 38 °C untersucht. Um äußere Einflüsse wie Druckänderungen oder Erwärmung durch Sonneneinstrahlung zu minimieren, wurden die Prüfkammer und das Wasserbad in einen isolierenden Versuchsschrank eingebaut. Um eine Störung durch die LED der Webcam zu minimieren wurden je Stunde nur zehn Bilder aufgezeichnet, welche Rückschlüsse auf die Aktivität der Schnecke zuließen. Die Protokollierung der Messwerte des Sauerstoffsensors, des Thermosensors, des Feuchtigkeitssensors und des Drucksensors erfolgte mit Hilfe eines Agilent 34972A LXI Mehrkanal Datenloggers (Santa Clara, CA, USA) im Abstand von 10 Sekunden.

1.5 Ergebnisse und Diskussion

Kapitel 1:

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

Cell Stress and Chaperones, DOI: 10.1007/s12192-012-0393-8.

A. Dieterich, U. Fischbach, M. Ludwig, M.A. Di Lellis, S. Troschinski, U. Gärtner, R. Triebskorn, H. -R. Köhler (2012)

In dieser Studie konnte der Umweltfaktor Temperatur in Südfrankreich, dem natürlichen Habitat der zu untersuchenden *Xeropicta derbentina* Population, dokumentiert werden. Während der Probennahmen in vier unterschiedlichen Monaten (April, Juni, August, Oktober) konnte ein Temperatur-Höhenprofil erarbeitet werden, das abnehmende Temperaturen mit zunehmender Entfernung vom Boden aufzeigt. Die Beobachtungen dokumentierten ein stetiges Größenwachstum der Tiere bis hin zum Erreichen der Adultgröße im August. Mit zunehmender Größe konnte eine 'Entfärbung' der Individuen in dieser Population beobachtet werden. Die im Vorfeld definierte Farbkategorie 1, die komplett weiß gefärbte Individuen umfasste, dominierte mit zunehmender Größe, während die bei kleineren Schnecken im Frühjahr hauptsächlich vorgefundene Farbkategorie 3 – Tiere mit mehreren dunklen Streifen an der Schalenunterseite – im Adultstadium beinahe nicht mehr anzutreffen war. Die Farbkategorie 3 wurde folglich als Juvenilfärbung interpretiert. Die Beobachtungen zum Wachstum der Individuen legen einen einjährigen Lebenszyklus dieser Population nahe. Dies untermauert die bereits durch Kiss et al. (2005) und Staikou & Lazaridou-Dimitriadou (1991) dokumentierten Lebenszyklen dieser Art (in Staikou & Lazaridou-Dimitriadou (1991) als *Xeropicta arenosa* bezeichnet, einem veralteten Synonym).

Die Untersuchungen zum Hsp70-Schutzsystem der Schnecken ergaben eine deutlich positive Korrelation zwischen Hsp70 und der Schalentemperatur der Tiere in den Monaten April, Juni und August. Im Oktober konnte eine negative Korrelation der beiden Faktoren ermittelt werden. Dieser Befund wurde zum einen als energetischer *Trade-off* zwischen der Aufrechterhaltung des Hsp70-Schutzsystem und der einsetzenden Reproduktionsphase (Mayer & Bukau 2005; Mizrahi et al. 2011) im Herbst interpretiert; zum anderen wurde eine mögliche Überlastung des Hsp70-Schutzsystems während des Sommers und damit fortschreitende Zellschädigungen (Dittbrenner et al. 2009; Scheil et al. 2011) als Grund für diese negative

Korrelation diskutiert. Auch eine möglicherweise einsetzende Aestivationsphase und eine damit verbundenen Reduktion des kompletten Stoffwechsels (Reuner et al. 2008; Riddle 1981; Storey 2002) und somit auch eine Reduktion im Hsp70-Level der Schnecken wurde als mögliche Erklärung für die vergleichsweise schwache Hsp70-Induktion im Oktober berücksichtigt.

Neben dieser jahreszeitlichen Änderung im Hsp70-Level der Schnecken konnte auch eine tageszeitliche Änderung bei den Untersuchungen in dieser Studie festgestellt werden. In den Monaten April, Juni und August folgte der Hsp70-Level größtenteils der Umgebungstemperatur mit einer Erhöhung des Hsp70-Levels nach Sonnenaufgang bei steigender Temperatur. Im April, mit Höchsttemperaturen von 27,3 °C war der Hsp70-Level auf moderatem Niveau, während er in den heißen Monaten Juni und August, mit Höchsttemperaturen von 32,9 °C und 33,7 °C Umgebungstemperatur deutlich höher angesiedelt war. Während im Juni der Verlauf des Hsp70-Levels noch dem der Umgebungstemperatur entsprach, war er im August zunehmend unregelmäßiger und steigende Standardabweichungen – ein mögliches erstes Indiz einer einsetzenden Überlastung des Hsp70-Schutzsystems – wurden beobachtet. Im Oktober, mit Tageshöchsttemperaturen von 23 °C, konnte statt einer Erhöhung des Hsp70-Levels eher ein tendenzielles Einbrechen des Hsp70-Levels bei Tag beobachtet werden.

Kapitel 2:

Hsp70 and lipid peroxide level after heat stress in *Xeropicta derbentina* (Krynicki 1836) (Gastropoda, Pulmonata) with regard to different colour morphs.

Cell Stress and Chaperones, DOI: 10.1007/s12192-014-0534-3.

A. Dieterich*, S. Troschinski*, S. Schwarz, M.A. Di Lellis, A. Henneberg, U.Fischbach, M.Ludwig, U. Gärtner, R. Triebskorn, H.-R. Köhler (2014)

***Gleichwertige Erstautorenschaft**

In dieser Studie konnte gezeigt werden, dass *Xeropicta derbentina* Individuen mit der zuvor (bereits in Kapitel 1) definieren Farbkategorie 3 (hier definiert als Tiere mit einem breiten dunklen Streifen an der Schalenunterseite) im Bereich von 33 – 43 °C in der Lage sind, Hsp70 in stärkerem Umfang zu induzieren als Vertreter der anderen Farbkategorien (gestaffelt von rein weißen Tieren bis hin zu Individuen mit dunkler Bänderung über die komplette Schale hinweg). Diese Tiere scheinen damit in diesem Temperaturbereich effektiver an ihre Umwelt

angepasst zu sein als die anderen Farbmorphen. Wurde im Experiment diese Temperatur jedoch überschritten, fiel der allgemein beobachtete Abfall im Hsp70-Level bei 45 – 48°C bei der Farbkategorie 3 deutlich höher aus (der gemessene Hsp70-Level war damit deutlich erniedrigt) als bei allen anderen untersuchten Farbmorphen. Als möglichen Grund dafür wurde ein energetischer *Trade-off* zwischen der kostenintensiven Aufrechterhaltung eines überlegenen Hsp70-Schutzsystems auf der einen und der Abdeckung eines breiteren Temperaturbereiches auf der anderen Seite angeführt. Als Konsequenz dieser Befunde besteht die Möglichkeit, dass bei dieser Farbkategorie bei erhöhten Umwelttemperaturen (wie zum Beispiele einer ausgedehnten Hitzewelle in einem extrem heißen Sommer) - bedingt durch den überforderten Schutzmechanismus - Zellschäden schneller auftreten als bei anderen Farbmorphen (Dittbrenner et al. 2009; Scheil et al. 2011; Troschinski et al. 2014). Unbekannt bleibt jedoch, warum gerade die Farbkategorie 3 in diesem Versuch zu beschriebenem Verhalten neigt. Unklar bleibt auch, ob die untersuchte Population Schwankungen in der Zusammensetzung der Farbmorphen über Jahre hinweg aufweist oder nicht. Dieses für viele andere Heliciden beobachtete Phänomen (Cowie 1992; Johnson 2011; Ozgo & Schilthuizen 2012; Silvertown et al. 2011) wird oft als eine Anpassung an ein bestimmtes Mikroklima gedeutet. Die in dieser Arbeit erhobenen Daten zur Induzierbarkeit des Hsp70-Schutzsystems untermauern die bereits verfügbaren Daten zur Hsp70-Induktion bei *Xeropicta derbentina* in Südfrankreich (Di Lellis et al. 2014; Köhler et al. 2009; Troschinski et al. 2014).

Des Weiteren wurde in dieser Studie der Grad der Lipidperoxidation als Maß für den erfahrenen oxidativen Stress untersucht. Ausgehend von der Kontrolltemperatur bei 25 °C konnte ein Anstieg der Lipidperoxide bei 38 und 40 °C sowie bei 45 und 48 °C gemessen werden. Bei 43 °C fiel der Level an Lipidperoxiden auf nahezu Ausgangsniveau ab. Zur Erklärung dieses zweiphasigen Verlauf wurde ein temperaturabhängiges, protektiv wirkendes und mit dem Hsp70-Schutzsystem interagierendes System von antioxidativ wirkenden Enzymen und anderer kleine Moleküle (Aebi 1984; Gutteridge 1995; Gutteridge & Halliwell 1990; Halliwell & Gutteridge 1989) postuliert. Im Temperaturbereich von 25 – 38 °C stellte sich dieses antioxidative System bei Individuen der Farbkategorie 1 (rein weiße Tiere) als besonders effektiv heraus, während es besonders bei 45 und 48 °C bei Individuen der Farbkategorie 4 (dunkle Bänderung über die komplette Schale hinweg) nur schwach ausgeprägt war. Ein mögliches antagonistisches Verhalten des Hsp70-Schutzsystems und des antioxidativen Sys-

tems (hohe Lipidperoxidation bei hohem Hsp70-Level) wurde angenommen und mit einem energetischen *Trade-off* begründet.

Kapitel 3:

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

Journal of Experimental Biology DOI:10.1242/jeb.113167.

S. Troschinski, A. Dieterich, S. Krais, R. Triebskorn, H.-R. Köhler (2014)

Die in dieser Studie durchgeführten Versuche zielten darauf ab, die zwei essentiellen, antioxidativ wirkenden Enzyme Katalase (CAT) und Glutathionperoxidase (GPx) bezüglich ihrer Temperaturabhängigkeit und ihres Zusammenspiels mit dem Hsp70-Schutzsystem zu untersuchen. Generell war ein sehr hoher Level an Katalase in den Proben zu finden, der als unspezifischer protektiver und permanenter Schutz vor anfallenden ROS (*reactive oxygen species*), vor allem vor dem von ihr umgesetzten H_2O_2, gesehen werden kann. Zu ähnlichen Ergebnissen kamen auch Nowakowska et al. (2011) bei Untersuchungen zum Aestivationsverhalten von verschiedenen *Helix*-Arten. Zusätzlich konnte von Storey (1996) belegt werden, dass Tiere, die häufig hoher ROS Bildung ausgesetzt sind, ein erhöhtes Maß antioxidativ wirkender Enzyme in ihren Zellen aufrechterhalten. Bei Temperaturen von 43 und 45 °C wurde eine signifikante Erhöhung der Katalase Aktivität in den Proben festgestellt. Das Enzym Glutathionperoxidase, das ebenfalls den Abbau von H_2O_2 katalysiert, zeigte lediglich bei 40 °C eine deutliche Erhöhung in seiner Aktivität. Da beide Enzyme H_2O_2 entgiften, ist von einer Konkurrenzsituation um das Substrat auszugehen, was von Nowakowska et al. (2011) ebenfalls diskutiert wurde. Der parallel hierzu quantifizierte Grad an Lipidperoxidation zeigte einen erhöhten Lipidperoxidlevel bei 38 °C auf, jedoch nicht bei höheren Temperaturen. Dies wurde mit der erhöhten Aktivität der Katalase und der Glutathionperoxidase bei genau diesen höheren Temperaturen begründet. Zusätzlich wurde der Hsp70-Level der Schnecken gemessen. Hier zeigte sich eine generelle Reaktion auf steigende Temperaturen bis zu einem Maximum bei 38 bis 40 °C. Mit weiter steigender Temperatur kam es zu einem erneuten Rückgang des Hsp70-Levels. Diese Kinetik wurde bereits in früheren Studien beschrieben (Di Lellis et al. 2014; Dieterich et al. 2014; Köhler et al. 2009) und konnte somit bestätigt werden. Anhand der erhöhten Lipidperoxidation bei maximalem Hsp70-Level

(38 °C), wurde darauf geschlossen, dass ein energetischer *Trade-off* zwischen dem Hsp70-Schutzsystem und den antioxidativ wirkenden Enzymen vorliegen kann. Durch die Ergebnisse konnte gezeigt werden, dass neben dem Hsp70-Schutzsystem auch das der antioxidativ wirkenden Enzyme maßgeblich zum Überleben von *Xeropicta derbentina* in ihrem Habitat beiträgt.

Kapitel 4:

Einfluss verschiedener Temperaturen und Expositionszeiten auf die Wasserevaporation und die Hsp70-Induktion bei *Xeropicta derbentina* (Krynicki 1836).

(Unveröffentlicht)

A. Petschl und A. Dieterich (2013)

In dieser Studie wurde der Einfluss verschiedener Temperaturen (20, 35, 40, 42 und 45 °C) und Expositionszeiten (1,2, 4, 6 und 8 Stunden) auf den Hsp70-Level und die Wasserevaporation durch die Schalenöffnung und die Schale von *Xeropicta derbentina* untersucht. Dabei wurden je zehn Schnecken in eigens dafür konstruierten Zylindern aus Aluminiumgaze in einem Wärmeschrank exponiert. Nach einer halbstündigen Vorexposition um Irritationen der Messung durch Abdampfen von auf der Schale befindlicher Umgebungsfeuchte zu vermeiden, wurde das Gesamtgewicht der Schnecken bei Start der Messungen und das Gewicht der Schnecken nach Ende der Expositionszeit bestimmt. Die Differenz wurde als durchschnittlicher Wasserverlust der exponierten Schnecken gewertet. Es konnte dabei gezeigt werden, dass der Wasserverlust im untersuchten Zeitraum von einer bis acht Stunden linear verlief und auch bei längerer, exemplarisch durchgeführter, Exposition mit fortschreitender Zeit linear zunahm. Je höher die Expositionstemperatur war, desto höher war der Verlust an Wasser über die Zeit. Besonders bei mehr als 40 °C Expositionstemperatur konnte im Vergleich mit Temperaturen von 25 bis 40 °C ein deutlich erhöhter Wasserverlust gemessen werden. Durch die erzielten Ergebnisse konnte gezeigt werden, dass die Evaporation von Wasser, die auch zu Kühlung der Schnecke genutzt wird (Schmidt-Nielsen et al. 1971) und in inaktivem Zustand hauptsächlich über den Mantelrand bzw. das Epiphragma erfolgt (Machin 1966; Machin 1968), selbst bei längeren Expositionszeiten und Umgebungstemperaturen von bis zu 35 bis 40 °C noch keine schwerwiegenden Folgen für diese Schnecken zur Folge zu haben scheint. Der höchste gemessene Wasserverlust bei 35 °C betrug nach 96-stündiger

Exposition ca. 11,6 % des Gesamtgewichts der Schnecke. Nach Studien von Arad et al. (1998) waren Schnecken der Art *Lauria cylindracea* [Mendes da Costa 1778] in der Lage, einen Wasserverlust von 25 bis 40 % des Körpergewichts noch zu überleben, weshalb der hier beobachtete Wasserverlust als noch physiologisch tolerierbar eingestuft wurde. Bei Temperaturen von 45 °C hingegen wurde bereits nach acht Stunden ein Wasserverlust von 10,1 % des Körpergewichts ermittelt und somit die physiologisch tolerierbare Grenze überschritten. Diese Ergebnisse belegen die von Dittbrenner et al. (2009) postulierte Reaktionsgrenze von ca. 45 °C für diese Art und erklärt das beobachtete Bestreben der Schnecken (Kapitel 1), vor zu hohen Bodentemperaturen zu flüchten.

Begleitend zu den Messungen zum Wasserverlust wurden Untersuchungen zum Hsp70-Level durchgeführt. Durch die oben erwähnte halbstündige Vorexposition betrugen die Expositionszeiten bei diesem Teil der Studie 1,5; 2,5; 4,5; 6,5 und 8,5 Stunden. Bei 20 °C Expositionstemperatur konnte ein leichter Anstieg des Hsp70-Levels nach 6,5 Stunden beobachtet werden, der bei 8,5 Stunden wieder abgesunken war. Die gemessenen Hsp70-Werte für die Temperaturen 35 und 40 °C lagen bereits nach 1,5 Stunden deutlich über den bei 20 °C gemessenen Werte, stagnieren dann jedoch Großteils auf einem hohen Niveau (mit Ausnahme des Messwertes bei 4,5 Stunden Exposition bei 40 °C). Im Vergleich dazu und zu den erhaltenen Werten bei 20 °C konnte bei 45 °C bei allen Expositionszeiten ein deutlich geringerer Hsp70-Level gemessen werden. Dies lässt auf eine Überlastung des Hsp70-Schutzsystems schließen, sowie auf eine beginnende Denaturierung der Transkriptions-/Translationsmaschinerie und damit auf ein Zusammenbrechen des Systems. Ähnliche Verläufe des Hsp70-Levels in Abhängigkeit von der Intensität der Hitzebelastung wurden bereits in anderen Studien gefunden (Di Lellis et al. 2014; Köhler et al. 2009) und auch in den Kapitel zwei und drei registriert.

Kapitel 5:

Energy metabolism in the Mediterranean land snail *Xeropicta derbentina* measured by direct calorimetry.

(Unpublished manuscript)

U. Fischbach, A. Dieterich, F. Kolarov, D. Wharam, G. Gauglitz, U. Gärtner, H.-R. Köhler (2015)

In dieser Studie wurde die Abwärmeproduktion (Wärmeleistung) von *Xeropicta derbentina* als Spiegel ihres Stoffwechsels ermittelt. Hierzu wurden direkt-kalorimetrische Messungen in einem Thermal Activity Monitor (TAM) durchgeführt. Im Versuch wurden zwei zuvor definierte Größenkategorien von Schnecken bei jeweils 20 und 30 °C für jeweils 7 Tage untersucht. Zusätzlich zu diesen Messungen wurden Langzeitmessungen von 24 Tagen durchgeführt, um Messwerte für den Übergang in eine durch äußere Einflüsse und Nahrungsmangel induzierte Aestivationsphase zu gewinnen. Sowohl bei den einwöchigen, als auch bei den Langzeitmessungen konnten dabei zwei unterschiedliche Niveaus der Wärmeleistung eindeutig voneinander unterschieden werden. Diese zwei Niveaus wurden als 'aktiver' Stoffwechsel und 'inaktiver' Stoffwechsel gedeutet. Die Wärmeleistung des aktiven Stoffwechsel der größeren der beiden Größenkategorien lag bei 20 °C zwischen 120 und 145 µW, während sie bei kleineren Tieren bei 70 bis 85 µW lag. Die Wärmeleistung in der inaktiven Phase konnte mit 15 bis 75 µW für große, und 15 bis 25 µW für kleine Schnecken gemessen werden. Bei 30°C konnte in der aktiven Phase eine Wärmeleistung von 85 bis 110 µW für große und von ca. 80 µW für kleine Schnecken gemessen werden. Die inaktive Phase war durch eine Wärmeleistung von 30 bis 55 µW bei großen und 8 bis 15 µW bei kleinen Schnecken charakterisiert. Diese Ergebnisse spiegeln etwa die von Rees & Hand (1990) gefundenen Wärmeleistungen bei *Oreohelix* sp. wieder. Bei den Langzeitmessungen konnte ein Trend aufgezeigt werden, nach dem die mittlere Wärmeleistung in der inaktiven Phase bei beiden Temperaturexpositionen nach zuvor kontinuierlichem Absinken auf einem Minimalwert angekommen war. Dieser wurde dann meist über längere Zeiträume gehalten und wies regelmäßige Oszillationen zwischen 20 und 40 µW auf. Dieser Zeitpunkt wurde als Beginn einer Aestivationsphase gedeutet. In ihrer Arbeit über kalorimetrische Methoden konnten Lamprecht & Becker (1988) bereits ähnliche wiederkehrende Wärmeleistungsmuster bei Schnecken aufzeigen, was die hier aufgestellte Deutung untermauert. Im Vergleich zu den 7-Tages-Expositionen konnten die Langzeitexpositionen aus Kapazitätsgründen nur einmal

durchgeführt werden. Die starken Unterschiede in den Wärmeleistungen zwischen der Messung von kleinen Schnecken bei 30 °C über 7 Tage, die eine Wärmeleistung in der inaktiven Phase von nur 8 bis 15 µW ergaben, und der Langzeitbeobachtung einer kleinen Schnecke bei 30 °C wurden mit verschiedenen körperlichen Ausgangszuständen begründet. Die während der 7-Tages-Exposition untersuchten Schnecken wiesen dabei kaum Aktivitätsphasen auf und waren vermutlich schon beim Start des Versuchs in eine Aestivationsphase übergegangen. Vor diesem Hintergrund kann eine Wärmeleistung von 20 bis 40 µW auch als Stoffwechsel während einer Aestivationsphase interpretiert werden. Im Vergleich zu den Langzeitmessungen bei 20 °C konnte ein Absinken der Wärmeleistung auf das gemessene durchschnittliche Minimum bei den Messungen bei 30 °C früher aufgezeigt werden. Dies wurde als rascheren Übergang des Tieres in eine Aestivationsphase bei höheren Temperaturen gewertet.

Bei Berechnungen des Gesamtenergieumsatzes während der 7-tägigen Messungen konnte gezeigt werden, dass kleinere Tiere einen deutlich geringeren Energieumsatz aufwiesen als große. Bei 20 °C war der Energieumsatz der großen Schnecken um den Faktor 2,7 höher als der von kleinen Schnecken, bei 30 °C war er um den Faktor 4,9 höher. Auch die Häufigkeit, in der sich die Schnecken im aktiven Stoffwechsel befanden, änderte sich in Abhängigkeit von Temperatur und Schalengröße. Kleinere Individuen, die sich in den durchgeführten Experimenten generell seltener im Status eines aktiven Stoffwechsels befanden, reduzierten ihre Aktivitätszeit bei 30 °C um 50,4 % im Vergleich zu ihrer Aktivitätszeit bei 20 °C. Bei größeren Tieren konnte hierbei nur ein Rückgang von 13,6 % beobachtet werden.

Kapitel 6:

Measuring oxygen consumption and metabolic adaptation in the Mediterranean land snail *Xeropicta derbentina* using a novel camera-equipped microrespirometry system.

(Unpublished manuscript)

U. Fischbach, A. Dieterich, M. Ludwig, D. Wharam, U. Gärtner, H.-R. Köhler (2015)

In dieser Studie wurde der Sauerstoffverbrauch von drei vordefinierten Größenkategorien von *Xeropicta derbentina* bei drei unterschiedlichen, umweltrelevanten Temperaturen untersucht. Um dies bewerkstelligen zu können und um mögliche störende äußere Einflüsse zu unterbinden, war die *de novo* Konstruktion einer Messzelle zur Detektion des Sauerstoffver-

brauchs vonnöten. Mit dieser Messzelle wurde in dieser Studie der Sauerstoffverbrauch von ruhenden Schnecken gemessen. Um sicherzustellen, dass alle untersuchten Individuen dieselben Startkonditionen erhielten, wurde eine 24 stündige Vorexposition durchgeführt, bei der in einer separaten Klimakammer der Tagesverlauf der Temperatur und der relativen Luftfeuchte an einem durchschnittlichen Sommertag im August nachgestellt wurde. Die Ergebnisse zeigten eine signifikant positive Korrelation der Schalengröße mit dem Sauerstoffverbrauch über alle Temperaturen (25, 30 und 38 °C) hinweg. Bei der Betrachtung der Daten getrennt nach Schalengröße (Kategorie 1 = 0,74±0,06 cm, Kategorie 2 = 0,95±0,03 cm und Kategorie 3 = 1,17±0,06 cm) konnte bei Kategorie 1 kein signifikanter Effekt der Temperatur auf den Sauerstoffverbrauch ermittelt werden. Bei Kategorie 2 konnte ein signifikanter Rückgang des Sauerstoffverbrauchs bei 38 °C im Vergleich zu 25 und 30 °C gefunden werden. Bei den Untersuchungen zu Kategorie 3 konnte zwar ein deutlich sichtbarer, jedoch statistisch nicht signifikanter Rückgang des Sauerstoffverbrauchs bei 38 °C im Vergleich zu 25 und 30 °C ermittelt werden.

Da Kategorie 1 mehrheitlich noch juvenile Schnecken beinhaltet hatte, könnten diese Ergebnisse ein Anzeichen dafür sein, dass juvenile Schnecken noch keine Anpassungen ihres Stoffwechsels (hier ihres Sauerstoffverbrauchs) an erhöhte Temperaturen durchführen, während adulte Tiere ihren Stoffwechsel und somit ihren Sauerstoffverbrauch reduzieren können. Zumindest für die 'adulten' Kategorien 2 und 3 stehen die erzielten Ergebnisse im Einklang mit den von Riddle (1977) erhobenen Ergebnissen zur respiratorischen Regulation bei *Rabdotus schiedeanus* und *Helix aspersa* (heute: *Cornu aspersum*) bei 25°C und darüber hinaus. Ähnliche Ergebnisse konnten auch von Steigen (1979) bei Untersuchungen zur temperaturabhängigen Änderungen im Metabolismus (Sauerstoffverbrauch) von *Cepaea hortensis* [Müller 1774] belegt werden.

1.6 Abschließende Betrachtung

In der vorliegenden Arbeit konnten neue Erkenntnisse über physiologische Grenzen und Anpassungen an hohe Habitattemperaturen im Leben von *Xeropicta derbentina* erzielt werden. Mit dem im Frühjahr stattfindenden Schlupf der Tiere im Feld beginnt für *X. derbentina* ein Leben unter zum Teil schwersten Bedingungen. Die in der ersten Lebensphase wichtige rasche Zunahme an Größe und Gewicht wird von zunehmend steigenden Umgebungstemperaturen begleitet. Die im Frühsommer bis Spätsommer beinahe täglich auftretenden hohen Umgebungstemperaturen von mehr als 45 °C in Bodennähe zwingen die Schnecken dazu, den aufgeheizten Boden zu verlassen und vertikale Objekte zu erklimmen. Doch selbst in einigem Abstand zum Boden kommt es zu einer starken Erwärmung der zu dieser Tageszeit ruhenden Tiere. Im Herbst, wenn die Schnecken bereits ihre endgültige Körpergröße erreicht haben und die Temperaturen mit denen des Frühjahrs vergleichbar sind, ist es vor allem die einsetzende Reproduktionsphase, die den Schnecken energetische Reserven abverlangt.

Die untersuchte Population von *X. derbentina* in Südfrankreich weist, wie schon durch Kiss et al. (2005) vermutet, einen einjährigen Lebenszyklus auf, an dessen Ende im Herbst und frühen Winter der Tod der Mehrheit der Individuen steht. Nur ein kleiner Teil der Tiere überlebt den Winter und reproduziert im Frühjahr.

Besonders im Sommer stoßen die Schnecken immer wieder an physiologische Grenzen, die Anpassungen der Schnecken an die vorherrschenden Habitattemperaturen notwendig machen.

Die Untersuchung des Hsp70-Levels verdeutlichte die Wichtigkeit dieses Schutzsystems für *X. derbentina*. Im Frühjahr konnte ein konstanter Basislevel, der den heranwachsenden Jungtieren bei intrazellulären Prozessen, bei der Neusynthese von Proteinen und beim puffern von Temperaturmaxima half, aufgezeigt werden. Bei Individuen, die den heißen Tageshöchsttemperaturen (32,9 °C im Juni und 33,7 °C im August, gemessen 5 cm über dem Grund) während des Sommers täglich ausgesetzt waren, war ein deutlich erhöhter, den Temperaturverlauf des Tages abbildender Hsp70-Level zu finden, der die Folgen der Hitzeeinwirkung am Tage pufferte und in den kühleren Abendstunden Reparaturvorgänge und Neusynthese wichtiger Proteine unterstützte. Im Herbst hingegen, wenn die Temperaturen wieder auf das Niveau des Frühjahrs absanken (Maximaltemperatur war im Oktober 23,0 °C und im April 27,3 °C), war kein erhöhter Hsp70-Level mehr zu messen. Mit steigender Temperatur war hier sogar eine Reduktion im Hsp70-Level zu beobachten. Vor dem Hintergrund

der Reproduktion ist ein energetischer *Trade-off* zwischen Schutz auf der einen und dem Investieren von Energie zur Produktion von Nachwuchs auf der anderen Seite ein plausibler Grund für den beobachteten Einbruch der Hsp70 Induktion (Mizrahi et al. 2011; Sørensen & Loeschcke 2002).

Mit zunehmendem Größenwachstum der Individuen geht – populationsweit betrachtet – eine zunehmende Entfärbung einher. Bestand die untersuchte Population zu Beginn des Jahres noch aus Individuen aller Farbkategorien (wobei zumindest eine dieser als Juvenilfärbung interpretiert wurde), konnte sie nach Abschluss des Wachstums im Hochsommer als beinahe rein weiß bezeichnet werden. Trotz dieser Tatsache waren immer wieder Individuen mit gebänderter Schale zu beobachten. Bei der gezielten Untersuchung dieser gefärbten Individuen, die nach Auffassung bisheriger Studien, bedingt durch eine höhere Absorption an Sonnenlicht, einen Nachteil in sonnenexponierten Habitaten haben sollten (Hazel & Johnson 1990; Heath 1975), waren es vor allem die mittelstark gefärbten Individuen, die einen erhöhten Hsp70-Level bei manchen Temperaturen zeigten. Waren sie im Temperaturbereich unter 43 °C noch in der Lage, signifikant mehr Hsp70 zu produzieren als die anderen Farbmorphen, so waren es bei Temperaturen über 43 °C ebenfalls diese Schnecken, deren Hsp70-Level stärker zurückging als der ihrer anders gefärbten Artgenossen.

Eine physiologische Grenze für länger wirkende Temperaturen scheint hierbei zwischen 40 und 45 °C zu liegen, da bei weiteren Untersuchungen stets ein maximaler Hsp70-Level zwischen 38 und 40 °C, gefolgt von einem deutlichen Einbrechen des Hsp70-Levels zwischen 43 und 45 °C gefunden werden konnte. Dieses auch von Di Lellis et al. (2014) beobachtete Einbrechen des Hsp70-Levels weist damit vermutlich auf die von diesem Schutzsystem maximal tolerierbare Last hin. Dabei ist zu bemerken, dass der Hsp70-Level bei *X. derbenina* nicht nur durch die Intensität, sondern auch durch die Länge des Temperatureinflusses beeinflusst wird. Je höher die einwirkende Temperatur, umso schneller wird der Hsp70-Level auf den Maximalwert erhöht, während ein milderer Temperatureinfluss meist zu einer stetigen Erhöhung des Hsp70-Levels bis zum Maximum führt.

Der mit zunehmender Temperatur und dem damit bedingten erhöhten Auftreten reaktiver Sauerstoffspezies (ROS) einhergehende oxidative Stress ist eine weitere Bedrohung für die der Sonne ausgesetzten Schnecken. Viele dieser während der Zellatmung anfallenden Sauerstoffspezies (besonders H_2O_2) werden bereits bei Entstehung in der Zelle durch antioxidativ wirkende Enzyme und andere Antioxidantien 'entgiftet'. Es zeigte sich, dass der Level der

Lipidperoxidation, also der Grad der durch oxidativen Stress bereits angegriffenen Lipide der Zellen, besonders dann sehr hoch war, wenn auch der Hsp70-Level ein Maximum zeigte. Hingegen konnte nur ein geringer Level an Lipidperoxiden gemessen werden, wenn der Hsp70-Level nach Überschreiten von 40°C bereits wieder geringer wurde. Dies lässt auf eine Konkurrenz um die zu Verfügung stehende Energie schließen. Da sowohl die Aufrechterhaltung des Hsp70-Schutzsystems als auch die Etablierung erhöhter Level antioxidativ wirkender Stoffe in der Zelle kostenintensiv sind, ist hier von einem energetischen *Trade-off* zwischen diesen beiden Schutzmechanismen auszugehen. Während bei niedrigen Temperaturen die konstitutiv in der Zelle vorliegenden Level an Hsp70 und antioxidativ wirkenden Stoffen ausreichen, um schwache Temperatureinflüsse abzupuffern, greifen mit zunehmender Temperaturerhöhung diese beiden Schutzsysteme ein, um Schäden in den Zellen zu minimieren. Das anfänglich induzierte Hsp70-Schutzsystem wird mit zunehmender Temperatur offenbar zu "kostenintensiv" und wird nach dem Erreichen des Maximalwertes bei 38-40 °C nach sechs bis acht Stunden Exposition wieder herunterreguliert. Zu dieser Phase kommt es jedoch zu verstärkter Aktivität von Enzymen, die dem antioxidativ wirkenden System zugeordnet sind (hier anhand der Aktivitätsmaxima der Glutathionperoxidase (GPx) bei 40 °C und der Katalase (CAT) ab 43 °C dargestellt). Dieses Ineinandergreifen der beiden Systeme ist in den Phasen maximaler Hsp70-Induktion, in denen erhöhte Lipidperoxid Level festgestellt werden konnten, deutlich zu sehen.

Mit zunehmender Erhöhung der Umgebungstemperatur geht eine erhöhte Evaporation von Wasser durch die Schale und die per Epiphragma versiegelte Schalenöffnung einher. Während die Temperaturen im Frühjahr nur geringe Mengen Wasser verdunsten lassen, sind es vor allem die hohen Temperaturen des Sommers, die den Verlust an Wasser zu einem ernst zu nehmenden Problem werden lassen. Überschreitet die Temperatur längerfristig 40 °C, können binnen weniger Stunden über 5 % des Körperwassers verdunsten, während solche Verlustraten bei niedrigeren Temperaturen erst nach mehr als einem Tag gemessen werden konnten. Die beobachteten *Trade-off* Situationen besonders an der Grenze der tolerierten Temperatur von ca. 40 °C legen nahe, dass die Schnecken weitere Anpassungen vornehmen müssen, um hohe Temperaturen zu überleben.

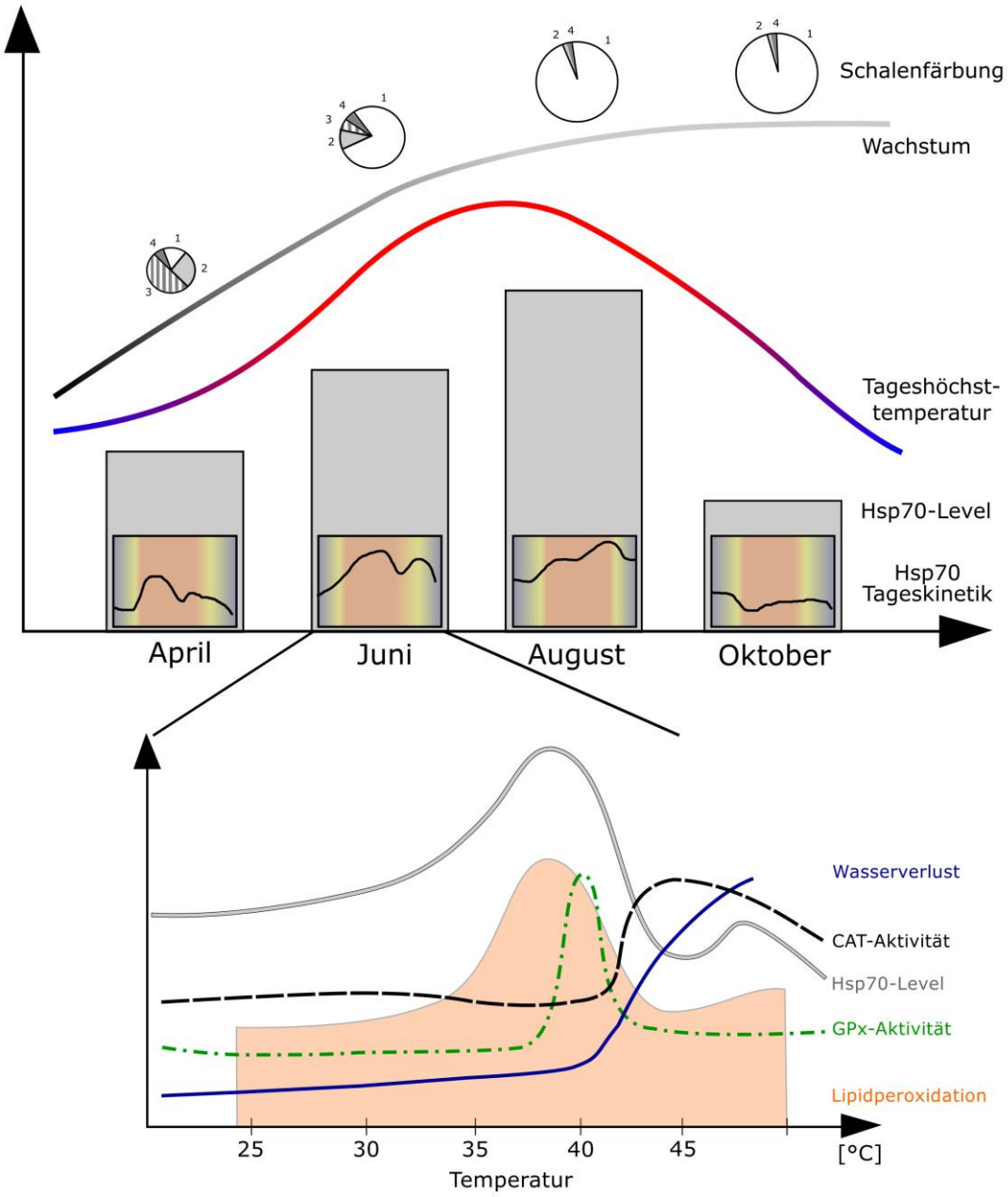
Um wertvolle Energie einzusparen, ist es den Schnecken möglich, ihre Stoffwechselintensität und ihren Sauerstoffverbrauch anzupassen.

Große Schnecken weisen generell einen höheren Energieumsatz auf als kleinere Individuen. Bei Änderungen der Temperatur und in Abwesenheit von Futter waren die Schnecken in der Lage, ihren Stoffwechsel deutlich zu reduzieren und somit Energie einzusparen.
Der Sauerstoffverbrauch – und somit eine Quelle der reaktiver Sauerstoffspezies – wird, vornehmlich bei größeren Individuen, mit zunehmender Temperatur reduziert. Die Reduktion des Sauerstoffverbrauchs bei größeren Tieren, die damit den Wasserverlust durch Atmung und die erhöhte Bildung von ROS vermeiden, ist ebenfalls als Anpassung an die zu ihrer Lebensphase vorherrschenden Habitattemperaturen zu sehen.

Vergleicht man die im Frühjahr bis Frühsommer lebenden kleineren Schnecken mit den im Sommer und Spätsommer lebenden Adulten, sind die Anpassungen an ihr Habitat deutlich zu sehen:
Kleinere Schnecken müssen moderate Umgebungstemperaturen mit nur kurzen Temperaturmaxima überstehen. Der in den Zellen konstitutiv vorliegende Level an Hsp70 und antioxidativ wirkenden Enzymen reicht wohl im Allgemeinen aus, um den Temperaturen der Umgebung zu widerstehen und sowohl den Wasserverlust als auch mögliche Zellschädigung zu kompensieren. Bei Erhöhung der Temperatur sind die Tiere in der Lage den, Hsp70-Level anzuheben und den Energieverbrauch durch Regulation des Stoffwechsels zu reduzieren. Der Sauerstoffverbrauch muss dabei nicht reduziert werden. Die übrige Energie kann für das Wachstum verwendet werden. Die Flucht vor den Temperaturen in Bodennähe ist noch nicht allzu relevant, weswegen diese Tiere eher in Bodennähe zu finden sind.
Im Sommer müssen jedoch die nunmehr größeren Individuen oftmals längere Phasen hoher Hitzeeinwirkung überstehen. Die rasche Verdunstung körpereigenen Wassers und die zunehmende Denaturierung von Proteinen, sowie Schäden durch Lipidperoxidation bedingen einen erhöhten Hsp70-Level, mit dem Ziel, Proteotoxizität zu limitieren. Bei drohender Überlastung greifen andere Schutzsysteme ein, die ein Überleben sichern. Die Reduktion des Stoffwechsels kann nur insoweit realisiert werden, dass noch genug Energie zur Aufrechterhaltung der energieintensiven Schutzsysteme zur Verfügung steht. Um die Generierung reaktiver Sauerstoffspezies zu unterbinden und um Wasserverlust zu minimieren, wird der Sauerstoffverbrauch und damit die Atmung reduziert. Um weitere Energie einzusparen, ist ein Übergang in die Aestivationsphase denkbar. Die eingesparte Energie kann dann für die Aufrechterhaltung der Schutzsysteme und zur Rückhaltung von Wasserreserven genutzt wer-

den. Die Tiere sind dazu gezwungen die heißeren Luftschichten in Bodennähe zu verlassen, das typische Kletterverhalten ist zu beobachten.

Letztlich zeigt jedoch die hohe Abundanz der Art in Südfrankreich und ihre fortschreitende Verbreitung, dass die in der vorliegenden Arbeit skizzierten Anpassungen an heiße Klimaten *Xeropicta derbentina* dabei helfen, in diesen Habitaten erfolgreich zu überleben.



Graphische Zusammenfassung: Mit zunehmendem Wachstum der Population ist eine Entfärbung zu beobachten. Die Zusammensetzung der Population ändert sich hin zu einer starken Dominanz des weißen Farbmorphs (1). Die höchsten Tagestemperaturen wurden im Hochsommer (Juli und August) gemessen. Der durchschnittliche Hsp70-Level der beprobten Individuen war im Sommer höher als im Frühjahr und Herbst. Die Tageskinetik des Hsp70-Levels weist im April, Juni und August positive Korrelationen mit der umgebenden Temperatur auf. Im Oktober ist ein geringerer durchschnittlicher Hsp70-Level zu finden als in den anderen untersuchten Monaten. Die Tageskinetik des Hsp70-Levels ist im Oktober negativ korreliert.
Bei der Exposition gegenüber unterschiedlich hoher Temperaturen kann ein Hsp70-Maximum zwischen 38 und 40°C nachgewiesen werden. Beim Überschreiten dieser Temperaturen ist ein Einbrechen des Hsp70-Levels zu beobachten. Die Lipidperoxidation ist in Phasen hoher Hsp70-Induktion ebenfalls hoch, was auf einen energetischen *Trade-off* zwischen Hsp70-Schutzsystem und antioxidativ wirkenden Schutzmechanismen hindeutet. Um der Lipidperoxidation entgegenzuwirken kommt es kurz vor dem Einbrechen des Hsp70-Levels zu einer verstärkten Aktivität der antioxidativ wirkenden Enzyme Glutathionperoxidase (GPx) und Katalase (CAT), durch deren Aktivität geringere Lipidperoxidation nachgewiesen wurde. Der Wasserverlust der Schnecke steigt mit zunehmender Temperatur an. Überschreitet die Temperatur 40°C ist ein deutlich höherer Wasserverlust zu verzeichnen.

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Teil 2: Eigenanteil an den durchgeführten Arbeiten

Kapitel 1:

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

Cell Stress and Chaperones, DOI: 10.1007/s12192-012-0393-8.

A. Dieterich, U. Fischbach, M. Ludwig, M.A. Di Lellis, S. Troschinski, U. Gärtner, R. Triebskorn, H. -R. Köhler (2012)

Die Probennahmen für die in diesem Kapitel analysierten Individuen erfolgte in Kooperation mit M.A. Di Lellis, S. Troschinski und S. Krais (Universität Tübingen) sowie mit U. Fischbach und M. Ludwig (Hochschule Esslingen) auf einem Privatgrundstück, zu dem der Zugang über C. Mazzia (Université d'Avignon et des Pays de Vaucluse) gestattet war. Kompletter Eigenanteil an der Aufarbeitung der Proben, sowie an der statistischen Auswertung und dem Erstellen des Manuskripts. Beiträge zur Komplettierung des Manuskripts und bei der automatisierten Erhebung von Umweltparametern im Rahmen des *Twinning Projects* durch U. Fischbach, M. Ludwig und U. Gärtner (Hochschule Esslingen). Unterstützung bei der Erhebung von Messwerten war durch M.A. Di Lellis, S. Krais und S. Troschinski gegeben. Die Korrektur des Manuskripts erfolgte durch H.-R. Köhler (Universität Tübingen). Die fachliche Betreuung wurde von H.-R. Köhler und R. Triebskorn (Universität Tübingen) übernommen.

Kapitel 2:

Hsp70 and lipid peroxide level after heat stress in *Xeropicta derbentina* (Krynicki 1836) (Gastropoda, Pulmonata) with regard to different colour morphs.

Cell Stress and Chaperones, DOI: 10.1007/s12192-014-0534-3.

A. Dieterich*, S. Troschinski*, S. Schwarz, M.A. Di Lellis, A. Henneberg, U.Fischbach, M.Ludwig, U. Gärtner, R. Triebskorn, H.-R. Köhler (2014)

***Gleichwertige Erstautorenschaft**

Die Probennahme zu den in Kapitel 2 durchgeführten Experimenten erfolgte in Kooperation mit M. A. Di Lellis, A. Henneberg (Universität Tübingen), U. Fischbach und M. Ludwig (Hochschule Esslingen) auf einem Privatgrundstück, zu dem der Zugang über C. Mazzia (Université

d'Avignon et des Pays de Vaucluse) gestattet war. Die experimentelle Durchführung erfolgte gemeinsam mit S. Troschinski (Universität Tübingen). Die Aufarbeitung und Auswertung der Hsp70-Proben wurde in komplettem Eigenanteil geleistet. Die Aufarbeitung und Auswertung der FOX-Proben wurde durch S. Troschinski bewerkstelligt. Die statistische Aufarbeitung aller Proben erfolgte in Kooperation mit S. Troschinski und S. Schwarz (Universität Tübingen). Das Manuskript wurde in Absprache mit S. Troschinski angefertigt, die Korrektur des Manuskripts erfolgte durch H.-R. Köhler (Universität Tübingen). Beiträge zur Vervollständigung des Manuskripts erfolgten durch U. Fischbach, M. Ludwig und U. Gärtner (Hochschule Esslingen) im Rahmen des *Twinning Projects*. Fachliche Betreuung durch H.-R. Köhler und R. Triebskorn (Universität Tübingen).

Kapitel 3:

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

Journal of Experimental Biology DOI:10.1242/jeb.113167.

S. Troschinski, A. Dieterich, S. Krais, R. Triebskorn, H.-R. Köhler (2014)

Die Probennahme zu den in Kapitel 3 durchgeführten Experimenten erfolgte in Kooperation mit S. Troschinski und M.A. Di Lellis (Universität Tübingen) auf einem Privatgrundstück, zu dem der Zugang über C. Mazzia (Université d'Avignon et des Pays de Vaucluse) gestattet war. Die experimentelle Durchführung erfolgte in Kooperation mit S. Troschinski. Die Aufarbeitung und Auswertung der Hsp70-Proben erfolgte in komplettem Eigenanteil, die Durchführung und Aufarbeitung der Enzymassays erfolgte durch S. Troschinski in Kooperation mit S. Krais (Universität Tübingen) und A. Dieterich. Die statistische Auswertung erfolgte in Kooperation mit S. Schwarz (Universität Tübingen). Das Manuskript wurde von S. Troschinski in Kooperation mit A. Dieterich erstellt und von H.-R. Köhler (Universität Tübingen) korrigiert. Fachliche Betreuung durch H.-R. Köhler und R. Triebskorn (Universität Tübingen).

Kapitel 4:

Einfluss verschiedener Temperaturen und Expositionszeiten auf die Wasserevaporation und die Hsp70-Induktion bei *Xeropicta derbentina* (Krynicki 1836).

(Unveröffentlicht)

A. Petschl und A. Dieterich (2013)

Dieses Kapitel beruht auf einer Studie, in der auch eine Zulassungsarbeit für das höhere Lehramt angefertigt wurde. Die Planung der in dieser Zulassungsarbeit durchgeführten Versuche erfolgte durch die Staatsexamenskandidatin A. Petschl unter der Betreuung von A. Dieterich und H.-R. Köhler (Universität Tübingen). Einarbeitung und Anleitung der Staatsexamenskandidatin sowie Hilfestellung bei der Durchführung der Experimente und deren Auswertung erfolgten durch A. Dieterich und H.-R. Köhler. Die Ausarbeitung der Arbeit erfolgte durch A. Petschl. Fachliche Betreuung durch H.-R. Köhler.

Kapitel 5:

Energy metabolism in the Mediterranean land snail *Xeropicta derbentina* measured by direct calorimetry.

(Unpublished manuscript)

U. Fischbach, A. Dieterich, F. Kolarov, D. Wharam, G. Gauglitz, U. Gärtner, H.-R. Köhler (2015)

Die Planung des Experiments erfolgte in Zusammenarbeit mit U. Fischbach (Hochschule Esslingen) und F. Kolarov (Universität Tübingen). Die Messungen am TAM 2277 (Thermometric AB, Jarfalla, Sweden) des Instituts für physikalische und theoretische Chemie der Universität Tübingen wurden durch A. Dieterich und F. Kolarov durchgeführt. Die Versuchstiere wurden durch A. Dieterich in Südfrankreich auf einem Privatgrundstück, zu dem der Zugang über C. Mazzia (Université d'Avignon et des Pays de Vaucluse) gestattet war, gesammelt. Auswertung und Ausarbeitung des Manuskripts erfolgte durch U. Fischbach. Fachliche Betreuung durch U. Gärtner (Hochschule Esslingen), D. Wharam, G. Gauglitz und H.-R. Köhler (alle Universität Tübingen).

Kapitel 6:

Measuring oxygen consumption and metabolic adaptation in the Mediterranean land snail *Xeropicta derbentina* using a novel camera-equipped microrespirometry system.

(Unpublished manuscript)

U. Fischbach, A. Dieterich, M. Ludwig, D. Wharam, U. Gärtner, H.-R. Köhler (2015)

Eigener Beitrag zur Konzeption und Entwicklung der neu gebauten Sauerstoff-Messapparatur und bei der Versuchsplanung. Die Probennahme der für das Experiment benötigten Versuchstiere erfolgte auf einem Privatgrundstück, zu dem der Zugang über C. Mazzia (Université d'Avignon et des Pays de Vaucluse) gestattet war, durch A. Dieterich (Universität Tübingen), U. Fischbach und M. Ludwig (Hochschule Esslingen). Bau, Kalibration und Verbesserung der Messapparatur sowie Durchführung und Auswertung des Experiments durch U. Fischbach. Inhaltliche Beiträge durch M. Ludwig. Fachliche Betreuung durch U. Gärtner (Hochschule Esslingen), D. Wharam und H.-R. Köhler (beide Universität Tübingen).

Teil 3: Wissenschaftliche Arbeiten

Kapitel 1: **Daily and seasonal changes in heat exposure and the Hsp70 level of individuals from a field population of *Xeropicta derbentina* (Krynicki 1836) (Pulmonata, Hygromiidae) in Southern France.**

Cell Stress and Chaperones, DOI: 10.1007/s12192-012-0393-8.

A. Dieterich, U. Fischbach, M. Ludwig, M.A. Di Lellis, S. Troschinski, U. Gärtner, R. Triebskorn, H. -R. Köhler (2012)

Abstract:

The Mediterranean land snail *Xeropicta derbentina* forms huge populations in Southern France. In order to characterize heat exposure and the induction of the 70kD heat shock protein (hsp70) response system during the life cycle of this snail, a selected population from the Vaucluse area, Provence, was investigated encompassing the issues of morphological life cycle parameters (shell size and colouration), the daily courses of heat exposure at different heights above the ground, of shell temperature, and that of the individual Hsp70 levels. The study covered all four seasons of the year 2011. Snails were found to be annual, reaching their final size in August. The shell colouration pattern showed high variation in juveniles (spring) with a strong tendency towards becoming uniformly white at old age in autumn. In all seasons, ambient air temperature decreased with increasing distance from the ground surface during daytime while remaining constantly low in the night. Overall, the Hsp70 level of individuals followed the ambient temperature during diurnal and seasonal variations. Correlation analysis revealed a positive association of individual shell temperature and Hsp70 level for the most part of the life cycle of the snails until late summer, whereas a negative correlation was found for aged animals indicating senescence effects on the capacity of the stress response system.

1. Introduction:

Climbing vertical structures to avoid lethal ground temperatures is a common and frequently recognized adaptive behaviour of land snails to their environment (Aubry et al. 2006; Kiss et al. 2005; Storey 2002). Besides other behavioural adaptations like burrowing in the soil dur-

ing the day or hiding beneath fallen leaves, climbing is one of the most obvious responses of snails to adverse conditions in the field during daytime. Measurements of the ground temperature and several centimetres above show a dramatic decrease of the air temperature even a few centimetres above the ground (Köhler et al. 2009). Shifting the activity to the cooler and moister night hours is another common behaviour of land snails in their response to hot environments (Abdel-Rehim 1983; Di Lellis et al. 2012). In the climate of Southern France with hot and dry summers, ground temperatures frequently reach 50□C and more. For snails that consist of roughly 75% water (Reuner et al. 2008), such temperatures are lethal (Dittbrenner et al. 2009).

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

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

As demonstrated for helicoid land snails (Dittbrenner et al. 2009; Scheil et al. 2011), Hsp70 clearly increases when the animals heat up. Apart from the intensity of stress affecting the increase of the Hsp70 level in the organism, the exposed life stage also influences the degree of Hsp70 induction. Young or larval stages are especially known to be able to induce Hsp70 to a higher degree than older or senescent organisms (Mayer and Bukau 2005; Köhler 2009). Furthermore, it was shown that Hsp70 levels varied on a seasonal basis, monthly, or even on a daily scale (Nakano and Iwama 2002; Tomanek and Sanford 2003; Schill et al. 2002; Köhler et al. 2001). The induction of Hsp70 was found to vary depending on the environmental conditions the species or a specific population encountered. For example, two closely related *Sphincterochila* species from two different habitats (Mediterranean vs. desert) ex-

pressed different levels of Hsp70 when they were exposed to adverse conditions, reflecting a pre-adaptation to their environment (Arad et al. 2010; Mizrahi et al. 2010, 2012).

To date, little is known about the diurnal changes in the Hsp70 level under field conditions in different seasons of a year, particularly for animals living in non-aquatic systems. We investigated a selected population of *X.derbentina* in respect to the daily course of their Hsp70 level in four different months of 2011. Furthermore, we continuously recorded the ambient temperature at different heights over ground during all samplings. According to the known heat-inducibility of stress proteins, we expected the Hsp70 level of the snails to correspond to the external temperature profile recorded in the field. Investigations covered different months and, consequently, different life stages of this annual species. Our aim was to provide a solid data basis to estimate the severity of heat stress and the capacities of the Hsp70 system to counteract this stress during the life-cycle of this annual land snail species.

2. Material and Methods:

2.1 Test organism:

In this study *Xeropicta derbentina* (Krynicki 1836), a hygromiid land snail, was investigated. All samples of *X. derbentina* were collected from a meadow in the vicinity of Modène, department Vaucluse, Southern France (N44°4.034' E5°11.041'). Samples were taken randomly from this population. The sampling site was not used agriculturally and no pesticides were applied by the owner. Sampling took place during four different months in 2011 to make sure that different climatic conditions were present during sampling and different life stages of *X. derbentina* could be collected. Samples were taken on April 18th, June 13th, August 30th and October 17th 2011. All samples were taken on sunny days with none to only little cloudiness. In April, ten snails were collected hourly and individually submerged in liquid nitrogen after recording the following parameters: (1) the heights at which individuals were resting, measured with a yard stick, (2) the temperatures at the surface of their shells, in the middle of the first whorl, that was exposed to the sun, using a medical precision thermometer (EL-LAB Copenhagen, type DM 825), (3) the shell diameter using a digital calliper, and (4) the patterns of shell colouration as introduced by Köhler et al. (2009). For *X. derbentina,* colour category 1 consisted of white shells only, while in category 2 animals with a single small

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

2.2 Hsp70 analysis:

For Hsp70 analysis, only individuals which have been resting between 5 and 20 cm above the ground were taken. The individually frozen samples were homogenized with appropriate volumes of extraction buffer (80 mM potassium acetate, 5 mM magnesium acetate, 20 mM Hepes and 2% protease inhibitor at pH 7,5) according to their body weight including the shells. All homogenization steps were performed on crushed ice to prevent degradation of proteins. The samples were centrifuged for ten minutes at 13722 rpm (=20000 rcf) using an Eppendorf Centrifuge 5804R at 4°C. The protein content of the supernatants was determined according to Bradford (1976) using 96-well plates and a plate reader (Bio-Tek Instruments, Winooski, VT, USA). Total protein (10-40µg / sample, depending on the intensity of resulting Hsp70 bands in preliminary analyses) was analysed using minigel SDS-PAGE (12% acrylamide, 0.12% bisacrylamide, 30’ at 80 V plus 90’ at 120 V). The proteins were transferred to nitrocellulose membranes by semi-dry electrotransfer. After incubation in blocking solution (50% horse serum in TBS) for two hours, the nitrocellulose membranes were incubated with the first monoclonal α-Hsp70 antibody (mouse anti-human Hsp70, Dianova, Hamburg, Germany, dilution 1:5000 in 10% horse serum / TBS) on a lab shaker at room temperature overnight. This antibody cross-reacts with all isoforms of the Hsp70 family. To remove surplus Hsp70 antibodies, the nitrocellulose membranes were rinsed in TBS for five minutes. After two hours of incubation with the secondary antibody (goat anti-mouse IgG

conjugated to peroxidase, Jackson Immunoresearch, West Grove, PA, dilution 1:1000 in 10% horse serum/TBS) the nitrocellulose membranes were rinsed again for five minutes in TBS. Subsequently, the membranes were stained in a solution containing 1 mM 4-chloro(1)naphthol, 0.015% H_2O_2, 30 mM Tris pH 8.5 and 6% methanol. Digitalization of the nitrocellulose membranes was carried out using an Epson Perfection V350 Photo scanner. For each band, the optical volume (= band area x average grey scale value) was calculated with E.A.S.Y. Win 32 (Herolab, Wiesloch, Germany). The optical volumes of the bands were related to a standard sample containing supernatant of full body extract of *Theba pisana* (Müller 1774) snails. In each minigel SDS-PAGE, this standard sample was run in duplicate. All data (means ± standard deviations) were calculated by ten individuals.

2.3 Additional sampling for field distribution and colouring:

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

2.4 Recording of temperature at different heights:

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

2.5 Statistics:

All data were checked for normal distribution using the Shapiro-Wilk W-Test in JMP 9.0.0. (SAS Institute Inc.). Since the data were not normally distributed, non-parametric tests had to be applied. To compare sample sets describing the change of shell diameter during the

year, individual Wilcoxon tests were performed between all examined months. Correction for multiple testing was accomplished by adjusting the significance level according to Bonferroni. The resulting α-level was 0.0083. Correlation between the parameters Hsp70 level, shell temperature, shell diameter and climbing height were performed using SAS JMP 9.0.0. A Spearman's ρ test was performed to check for significance and α-levels were also corrected according to Bonferroni as mentioned above.

3. Results:

3.1 Shell growth and colouration:

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

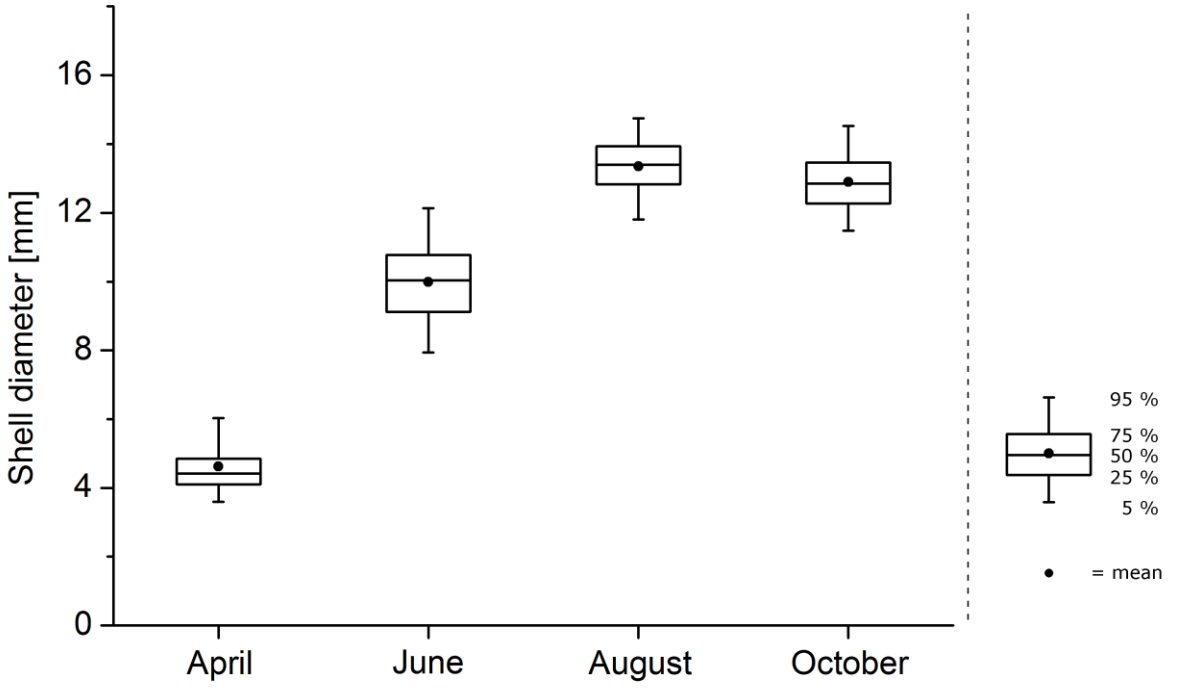

Fig. 1: Increase in shell diameter of samples taken in 2011. Boxes indicate 25%, 50% and 75% percentiles of all samples taken during the corresponding sampling day. Black dots = mean shell diameter, whiskers: 5% and 95% percentiles

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

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

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

3.2 Hsp70 induction and ambient parameters:

In June, the lowest measured temperature 5 cm above the soil surface was 13.2°C measured at 4am. The maximum temperature at the same height was 32.9°C at 2pm. In August the temperature 5 cm above ground ranged from 10.4°C (6am) to 33.7°C (3pm). In these two months the temperature exceeded 30°C during the day which made conditions different from those in April and October. In April the lowest temperature of all samplings was measured. At a height of 5 cm above the soil surface it was found to be 4.8°C (4am). The maximum temperature at this height in April was 27.3°C (5pm). In October the temperature in 5 cm above ground varied from 7.6°C (7am) to 23.0°C (3pm). In all months an increase of air temperature after sunrise was observed as well as a decrease after sunset. By comparing the

temperature at different heights, a gradient with decreasing temperatures at increasing heights above the ground was found to be established during the day. At night and during sunrise and sunset, only little temperature differences were recorded at different heights. In April, sunrise was roughly at 6:30am and sunset roughly at 8:30pm. In June, sunrise took place around 6am and sunset around 9:30pm. In August, sunrise took place at approximately 7am and sunset at 8:30pm. In October, sunrise took place at roughly 8am and sunset at 7pm. On June 13th a sudden decrease in ambient temperature was recorded at all heights at 4pm. At this time, clouds temporarily covered the sky and ambient temperature decreased transiently. Five centimetres above the ground the overall mean temperature of the sampling day in April was calculated to be 14.1°C, in June 22.3°C, in August 22.9°C and in October 14.7°C. Temperatures at heights between 1 cm and 30 cm above ground are presented in Figure 2 for each sampling.

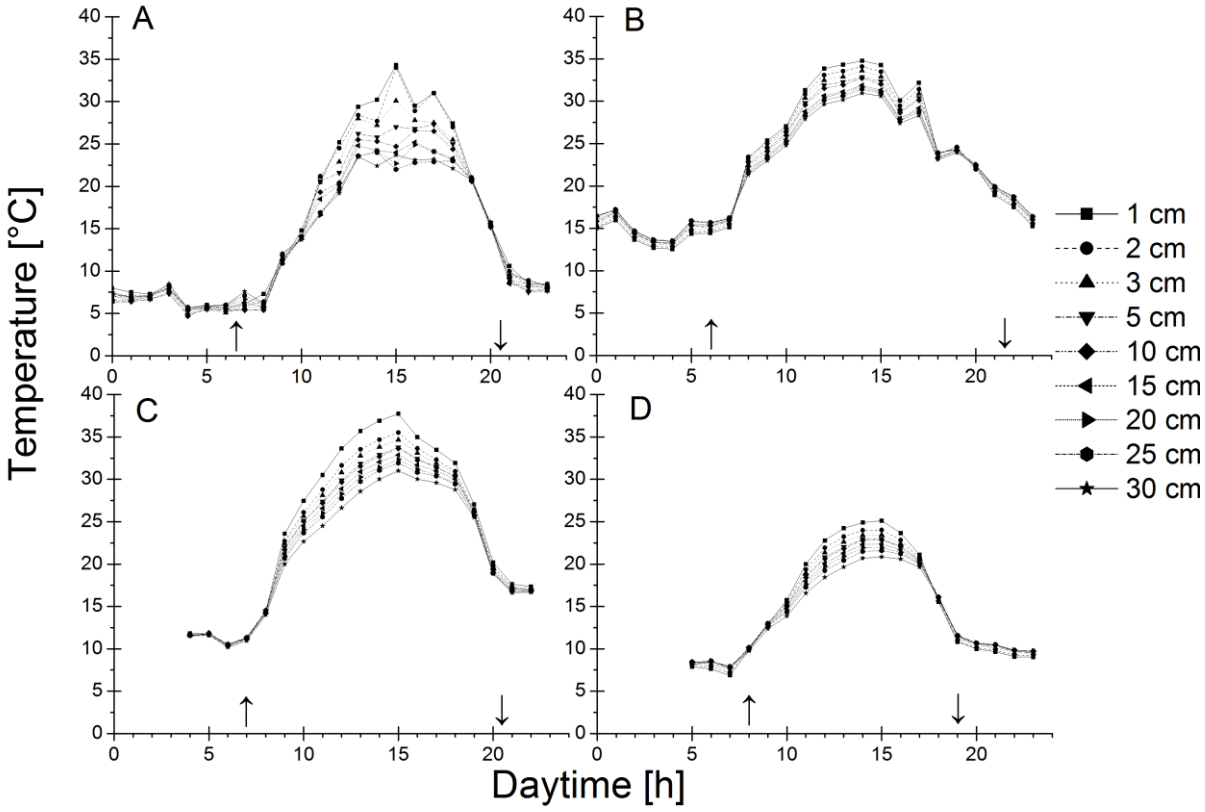

Fig. 2: Daily course of the air temperatures at different heights above the ground in Modène, France, during samplings in 2011. A: April 18th, B: June 13th, C: August 30th, D: October 17th. Sunrise is indicated by ↑ and sunset by ↓

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

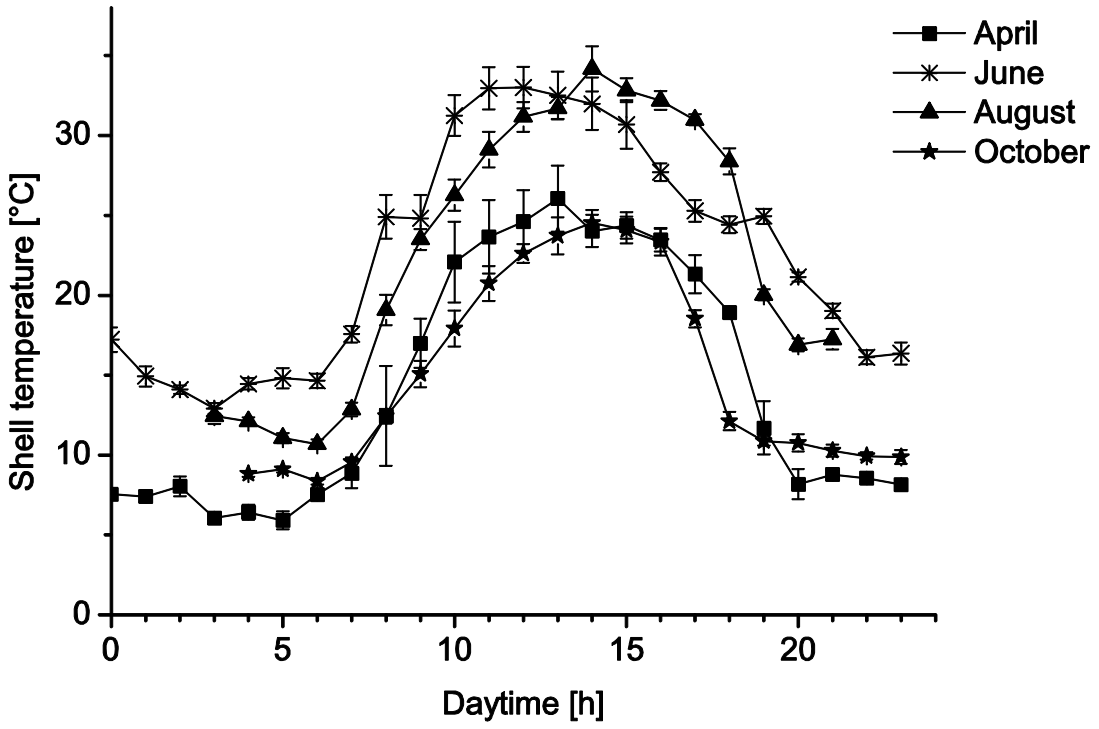

Fig. 3: Daily course of the shell temperature in four different months in 2011. Error bars indicate the standard deviation of ten samples taken per hour. Each data point represents the mean value of ten individuals

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

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

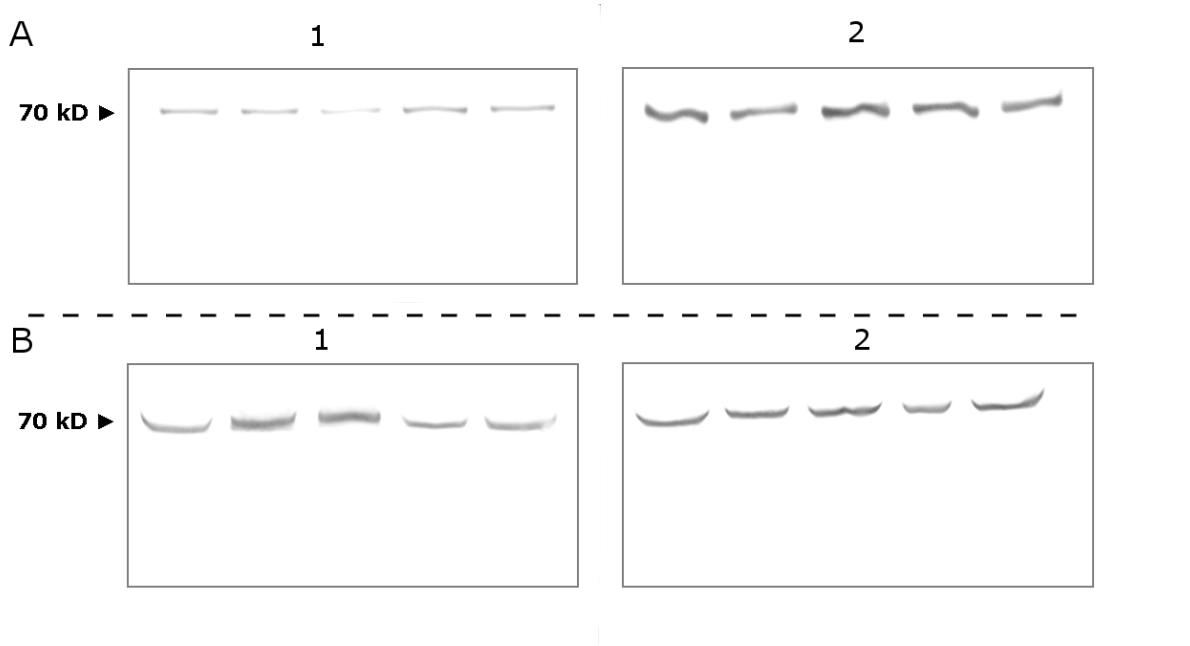

Fig. 4: Western blots for two different seasons and two different sampling times. A: Samples from June 2011; 1=0h, 2=15h daytime. 10µg of total protein were separated per lane. B: Samples from October 2011; 1=0h, 2=15h daytime. 40µg of total protein per lane were separated. Each band represents a single individual.

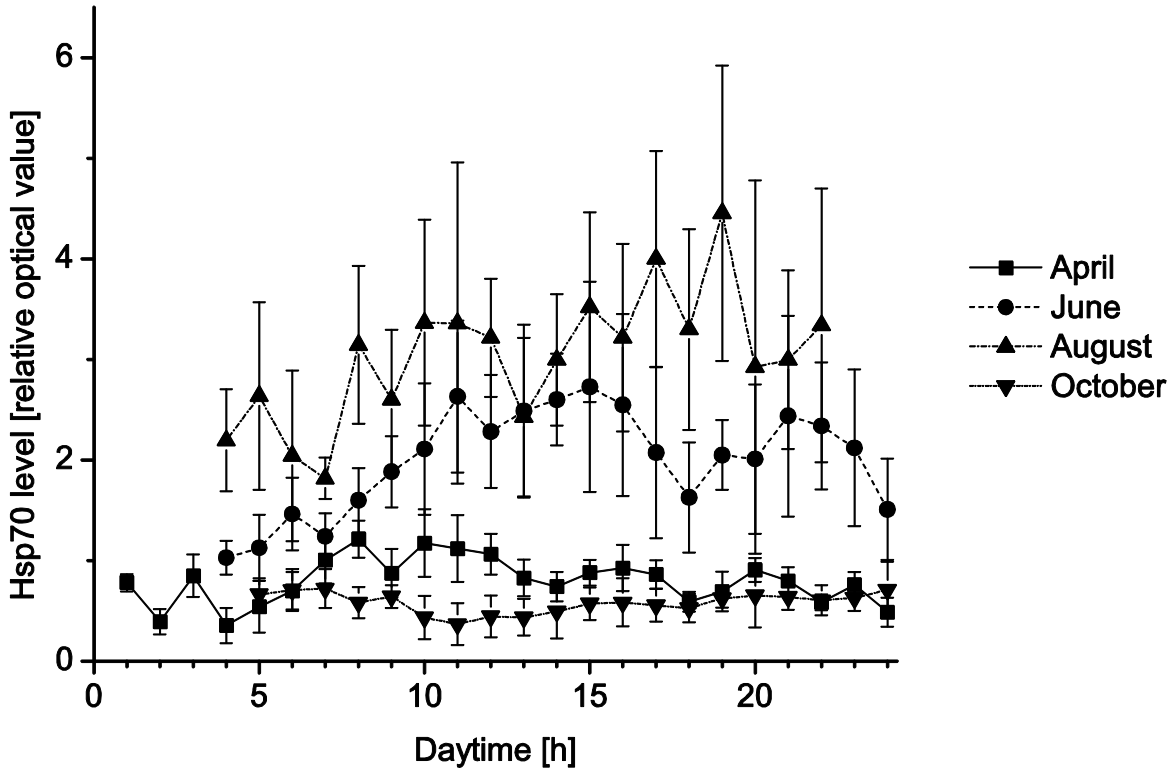

Fig. 5: Daily course of mean Hsp70 levels (n=10) obtained from samples taken in 2011. Error bars indicate the standard deviation of ten samples taken per hour.

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

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

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

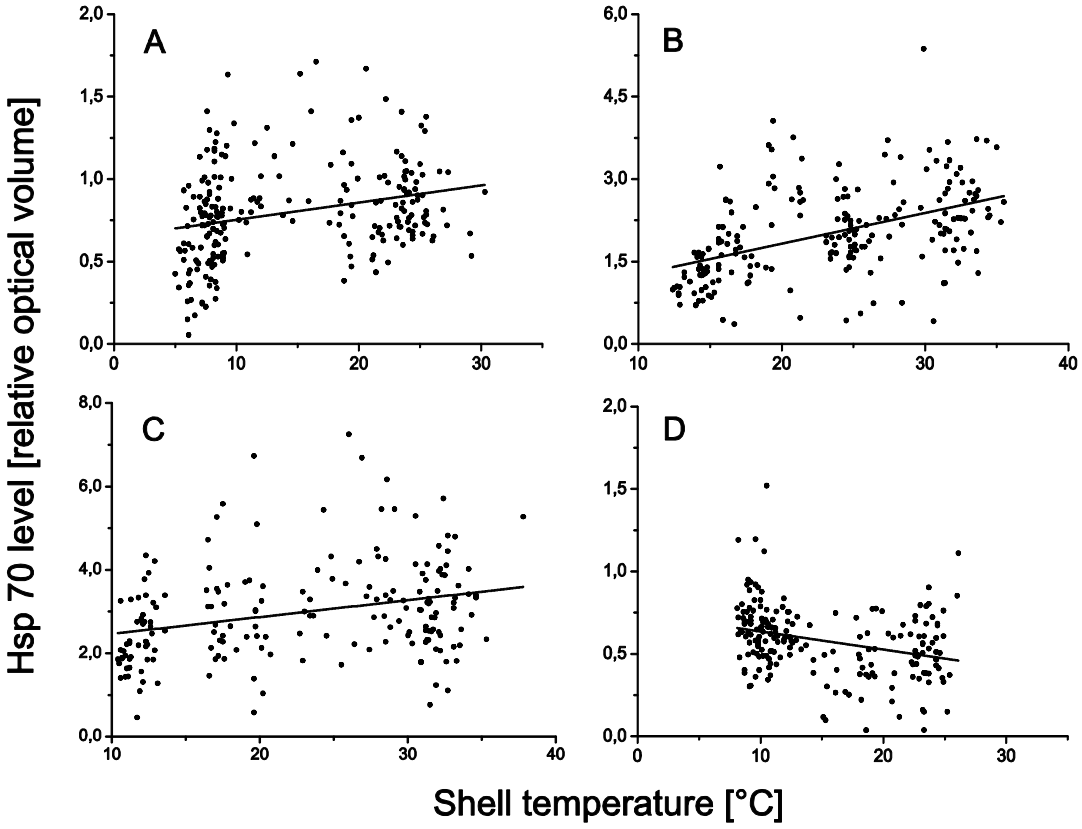

Fig. 6: Correlation between Hsp70 level and external shell temperature in the four different months of sampling. A: April, B: June, C: August, D: October. In April, June, and August, a significant positive correlation between Hsp70 level and shell temperature was present. In October a significant negative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

4. Discussion:

In the present study, a field population of *X. derbentina* from Southern France (Modène, Vaucluse) was used to investigate the molecular stress response to ambient temperature. This was accomplished during four different snapshots of a single day, each of these in four

different months of one year. In addition to the Hsp70 analysis we notice the development of colouration and growth in individuals of this population.

4.1 Snail growth and colouring:

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

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

studies, samplings in May revealed partly phenotypic 'mixed' populations of *X.derbentina* as well.

4.2 Hsp70 induction:

Since another study (Di Lellis et al. 2012) has revealed influence of the factor "climbing height" on the Hsp 70 level, we have only used snails that were taken from a pre-defined range of height for stress protein analysis. Within this range no significant effect of the climbing height or correlation between this factor and another parameter was found. This enabled us to relate the stress protein response to the factors "temperature" and "season".

In Southern France, *X. derbentina* snails that consist of 78% water [including shell, measured as a mean of 15 fully hydrated snails dried to the nearest 0,01g body weight, measurements performed by A.D. and U.F. in July 2011; similar results were found in *Cantareus apterus* (Born 1778) by Reuner et al. (2008)], have to face comparatively hot conditions during the day. Due to their inactivity during the day, they are not able to take up water from food or from their environment to cool down or to prevent desiccation. During all samplings, activity of snails was found to take place in the cooler night until the early hours of the morning when the sun has not yet heated up the ground. No activity was observed during the day, thus escaping higher temperatures by moving into shaded regions is not an option for *X. derbentina*. Rising ambient temperature results in higher temperature on the surface of the shell and, consequently, also in higher temperature inside the body (Di Lellis et al. 2012). To prevent misfolding of proteins and to counteract consequences of heat stress and desiccation, Hsp70 is usually up-regulated (Sørensen et al. 2003; Mayer and Bukau 2005; Köhler 2009; Kiang and Tsokos 1998; Feder and Hofmann 1999). In our study, a positive correlation between the Hsp 70 level and the temperature at the shell surface could be observed for April, June, and August only. In the samples taken in October, a negative correlation for these two variables was found. When comparing the temperature – stress response relationships from April and October, it became obvious that snails lost their ability to react properly to heat stress in October, even though ambient temperature in these two months was almost the same. These findings may have occurred for the following reasons.

It is known that older, senescent individuals have reduced Hsp70 levels compared to younger ones (Sørensen and Loeschcke 2002; Mayer and Bukau 2005; Köhler 2009). This

may be due to an energetic trade-off between the maintenance of the stress response system and reproduction (Mizrahi et al. 2011). Furthermore, continuously repeated exposure to high temperature during summer, accompanied by a shortage in energy supply may have reduced the ability of the snails' cells to fully express the energy-costly Hsp system. Moreover, the overwhelming of this stress response machinery in turn could have resulted in cellular pathology as shown by Dittbrenner et al. (2009), Scheil et al. (2011), and S. Troschinski, University of Tübingen (unpublished data) for Mediterranean land snails. The limitation of the stress protein system by environmental parameters resulting in a reduced capacity of organisms to overcome environmental stressors has already been postulated by Nakano & Iwama (2002) and Tomanek (2002). In cases of "overwhelmed" stress physiology, additional stressor action will not result in an induction but rather in a decrease of Hsp 70 levels (Eckwert et al. 1997; Tomanek 2002). It is likely that the present results obtained for the October snails should be seen as a consequence stemming from an exhaustion of the stress response system as it was shown before by Scheil et al. (2011) for *X. derbentina*.

Another assumption that could explain the absence of Hsp70 induction in the October snails is, as reported in many studies, that snails, especially in the Mediterranean area, are often able to enter an aestivating phase when conditions turn unfavourable. During this phase metabolism is reduced and the internal milieu of the snails changes (Herreid II 1977; Riddle 1981; Umezurike and Iheanacho 1983; Storey 2002). In our French field population snails did not enter the aestivation phase in April, June, and August as they were foraging on the ground in the night hours during sampling. In October, snails were almost exclusively found resting on the vegetation and only very few snails were active during the night. If snails had entered a temporal aestivation phase due to physiological exhaustion, the low level and limited induction of Hsp 70 in snails collected in October could be explained according to the findings of Reuner et al. (2008) who found dormant snails not to express much Hsp70 compared to heat-shocked active ones. Kiss et al. (2005) have shown that populations of *X.derbentina* may be able to change their survival strategy and shift from an annual to a biennial life-cycle, and some of these populations were found to aestivate. In our population it is more likely that snails entered a short-term aestivation-like phase to temporarily cope with a prolonged phase of dry conditions during autumn 2011. Equivalent to the findings in 2011, predominately small snails were found in spring 2012 on the same sampling ground (personal communication C. Mazzia, University of Avignon). Therefore, it is highly unlikely

that large parts of the population had entered a prolonged aestivation phase. In this case, snails with intermediate shell sizes would have been found in spring 2012. As no aestivation was observed, apart from some periods in autumn, we conclude that aestivation is not part of the survival strategy of the investigated population.

Our results reveal not only a seasonal change of Hsp70 level as reported in several other studies (Nakano and Iwama 2002; Tomanek 2002; Tomanek and Sanford 2003; Arad et al. 2010), but, for one of the few times (Ulmasov et al. 1999), also a daily change in Hsp70 expression in the field. Regarding this daily course it is obvious that Hsp70 levels follow the increase in ambient temperature. In April, where temperatures were lower than in June or August, only a slight increase in the Hsp70 level could be shown during the day. This slight increase indicates that ambient temperatures at that time seemed to generally be below the threshold temperature at which *X. derbentina* starts to up-regulate Hsp70 for their survival. According to Köhler et al. (2009) this threshold temperature should be estimated to be around 30°C. In experiments where *X. derbentina* was exposed to different temperatures, 24 - 25°C were used as a control (Dittbrenner et al. 2009; Köhler et al. 2009; Scheil et al. 2011). On the day of data collection in April, temperatures >25°C occurred for five hours only with a measured maximum of 27.3°C. In June the Hsp70 levels followed the rise of ambient temperatures till early afternoon and decreased again with sunset in the evening. Additionally at night a slight elevation of the Hsp70 level was found. This additional Hsp 70 peak most likely corresponds to the activity period of the snails that typically starts a few hours after sunset, when temperatures had decreased. During this period snails were often found on the ground, eating, moving around, and probably being in a phase where the snails have to deal with balancing their internal milieu and producing new proteins (Herreid II 1977; Riddle 1981; Umezurike and Iheanacho 1983; Storey 2002). This happens at a time of the year when snails have not yet reached their final body size, as shown by our results on shell size. Hence, the induction of Hsp70 during the night could be seen as a consequence of the need to chaperone newly synthesized proteins necessary for the animals' growth (Köhler 2009; Mayer and Bukau 2005). In August the daily course of Hsp70 was shown to remain at a high level but with high standard deviations. A possible reason for this effect could be the interaction of high temperature at that time of the year and the energy-costly proceeding maturation of reproductive organs. This may have led to a beginning collapse of the Hsp70 protection system in some individuals. Particularly those snails that are still growing and have not

entered maturation seemed to produce high levels of Hsp70 to counteract heat stress; others, which have grown to their final size, may have started with egg production which poses an additional stress on them, overcharging the capacity of the molecular stress response and resulting in a sub-optimal Hsp70 level. In October temperatures did not reach 25°C. The recorded maximum in October was 23.0°C. Given the fact that such a temperature is not high enough to induce Hsp70, only a 'base level' of constitutional Hsp70 should remain which was the case in our investigation.

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

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

5. Acknowledgements:

The authors would like to thank Wolfgang Rähle, Edmund Gittenberger, Sergej Sereda and Thomas Wilke for their contribution in snail species determination and discussion. Furthermore, we would like to thank Stefanie Krais and Katharina Graf for assistance in field work, Simon Schwarz for his help with the statistics and Nishrin Schacherbauer for proofreading. The study was financed by the German Research Council (DFG Ko 1978/5-3) and the University of Tübingens's twinning project with the University of Applied Sciences Esslingen under the supervision of H.-R.K., U.G., Stefan Rösler and David Wharam.

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Kapitel 2: **Hsp70 and lipid peroxide level after heat stress in *Xeropicta derbentina* (Krynicki 1836) (Gastropoda, Pulmonata) with regard to different colour morphs.**

Cell Stress and Chaperones, DOI: 10.1007/s12192-014-0534-3.

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Abstract:

Terrestrial snails which live under dry and hot conditions need efficient mechanisms of adaptation to counteract the problems of desiccation and over-heating. A profoundly heat tolerant snail species is the Mediterranean *Xeropicta derbentina*, exhibiting different shell colour morphs ranging from pale white to darkly banded. Considering that dark-pigmented snails are believed to have a disadvantage due to faster heating, we investigated possible differences in the stress markers Hsp70 and lipid peroxideation between four pre-defined colour morphs which were exposed to different temperatures for eight hours. The highest Hsp70 levels were observed in response to 38-40°C. Levels decreased when this temperature was exceeded. Snails of a pre-defined colour category 3 (with a large black band at the umbilicus side of the shell) showed the most prominent Hsp70 response. Lipid peroxideation levels also showed a maximum at 38°C but displayed a second peak at rather high temperatures at which the Hsp70 level already had decreased (45-48°C). Particularly pure white snails (category 1) and the most pigmented ones (category 4) were found to have different levels of lipid peroxidation at 38°C and 45°C compared to the other morphs. A hypothesis involving a combined two-phase defence mechanism, to which both, the Hsp70 protection system and the antioxidant defence system, may contribute, is discussed.

1. Introduction:

Hot and dry conditions, as being common during summer in Southern France, constitute hostile conditions for terrestrial animals with high water content, like snails. Embodying of more than 75% water (Reuner et al. 2008), snails per se are vulnerable against desiccation

and overheating. Nevertheless, *Xeropicta derbentina* (Krynicki 1836), a hygromiid land snail species, occurs in high abundance in Southern France, where it was first recorded in 1949 (Altena 1960) and, in the following, has successfully spread over this area. Its origin lies in the Eastern Mediterranean from where it was presumably introduced during the Second World War. In its adult state, *X. derbentina* reaches shell sizes up to 16 mm in diameter. This annual species (Dieterich et al. 2012; Kiss et al. 2005) can often be found in areas with scarce vegetation, at the borders of agricultural areas and along roads. The ability to climb vertical objects can not only be seen as a way to protect these snails from overheating in consequence of high ground temperatures, as postulated by different authors (Cowie 1985; Pomeroy 1968). Furthermore it can be seen as a way of dispersal (Aubry et al. 2006), as snails are frequently found to be attached on mobile devices like cars.

Most of the yet investigated populations of *X. derbentina* in Southern France were mainly characterized by individuals that carried a pure white shell (Dieterich et al. 2012; Köhler et al. 2009) when they have reached their final size. Among these pale individuals lower percentages of individuals were observed which were characterized by a darkly pigmented banding of the shell and which can be categorized according to the banding pattern described in previous studies (Di Lellis et al. 2012; Dieterich et al. 2012; Dittbrenner et al. 2009; Köhler et al. 2009).

Polymorphism in shell colouration is a well-known phenomenon in a number of land snail species. Different morphs of one of the best-studied genus, *Cepaea* sp., were found to differ in their activity, their resistance against desiccation and their shell temperature, partly depending on the natural habitat in which they were collected (Staikou 1999). Moreover, numerous studies on *Cepaea nemoralis* (Linnaeus 1758), one of the most polymorphic land snail species in Europe (Cain 1977; Goodhart 1987), revealed differences between shell colour morphs: this was the case for the reflectance of the shell, the internal temperature after solar radiation, and the extent of dehydration and mortality after severe heat exposure (Chang 1991; Heath 1975; Richardson 1974). Another frequently studied snail species is *Theba pisana* (Müller 1774), which also shows a highly polymorphic shell banding (Cowie 1984; Köhler et al. 2013) and is found in Southern France as well as in coastal plains of the Mediterranean Sea. The northernmost boundary of its distribution is Southern England and Wales; furthermore *T. pisana* can be found in Northern Africa and in Australia. *T. pisana* was,

analogous to *X. derbentina*, observed to climb vertical objects, thereby preventing overheating (McQuaid et al. 1979). Quite frequently, the shell pigmentation of snails has been linked to higher shell temperatures, higher internal temperatures, and a quicker heating of dark banded morphs caused by solar radiation (Hazel and Johnson 1990; Heath 1975). Especially in habitats with high temperatures this should be a great disadvantage for darker individuals. Nevertheless, banded morphs – even though in smaller amounts – are abundant in hot and dry habitats. Hence, some kind of pre-adaptation can be assumed in banded or darker morphs. On the other hand, it has been reported that shells of differently coloured morphs of *T. pisana* did neither differ in heating nor in heat loss when being illuminated by light with a natural spectrum (Scheil et al. 2012a).

As mentioned above, confrontation with elevated habitat temperatures leads, like in most animals, to behavioural adaptations. In land snails the most prominent adaptations are climbing (Arad et al. 1993; Aubry et al. 2006; Cowie 1985; Pomeroy 1968) and shifting their activity to the night hours. Besides these, also physiological responses like the up-regulation of protective biochemical systems are common defence mechanisms to cope with the consequences of heat (Jäättelä 1999; Kregel 2002). One of the best known and frequently investigated mechanisms in dealing with elevated temperatures is the heat shock protein (Hsp) protection system (Feder and Hofmann 1999). Hsps are proteins which, beside other functions, assist newly synthesised proteins in their folding. This chaperoning function allows organisms to cope with elevated temperatures and to reduce protein malfolding. Hsps are categorized according to their molecular weight, and best investigated is the 70kDa family – Hsp70. As a marker of effect, Hsp70 has been frequently used in characterizing the molecular stress response of different organisms to heat and other stressors (Daugaard et al. 2007; Dieterich et al. 2012; Feder and Hofmann 1999; Köhler et al. 2001; Mayer and Bukau 2005). However, some Hsp70 isoforms are also expressed under non-stress conditions. These constitutively expressed stress proteins have chaperone function in protein folding processes, stabilize proteins in intracellular trafficking, and play an essential role in the assembly, degradation, and intracellular localization of proteins (Fink 1999; Hendrick and Hartl 1993; Mayer and Bukau 2005). It is known that different populations of a species can differ in their Hsp70 content and in their ability to induce Hsp70 as a response to heat, depending on their natural habitat and on the organisms' general ability of Hsp70 induction as, for example, shown in whole body homogenates of *Drosophila* sp flies (Bahrndorff et al. 2006; Krebs and

Feder 1997; Sørensen et al. 2001). In case of *X. derbentina*, analyses of whole body homogenates have shown that the Hsp70 level of individuals depends on the population (Di Lellis et al. 2014), the life stage, the season, and the intensity of heat exposure (Dieterich et al. 2012) as well as on the total load of heat stress over a given period of time (Köhler et al. 2009; Scheil et al. 2011). Furthermore, was been shown that several populations of *X. derbentina* deriving from the same area have developed different heat response strategies characterized by different levels of Hsp70 (Troschinski et al. 2014).

Elevated temperatures not only lead to higher amounts of Hsp70, but can also lead to oxidative stress, as higher temperatures are known to generate reactive oxygen species (ROS) that include the superoxide anion radical ($^{\cdot}O_2^-$), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($^{\cdot}OH$). These ROS have deleterious effects on DNA, proteins, and lipids - the latter affected by peroxidation leading to the formation of lipid peroxides and a disturbance of biomembranes (Gutteridge and Halliwell 1990). In aerobic organisms, ROS are continuously formed as by-products of metabolism and are scavenged or detoxified by antioxidant defence systems (Halliwell and Gutteridge 1989; Sies 1997; Storey 1996). Whenever these systems are overwhelmed by a sudden burst of generated ROS, oxidative damage rapidly manifests in cells (Abele et al. 1998; Pannunzio and Storey 1998).

Measuring the amount of oxidative waste products such as lipid peroxides is a common method to assess an organism's ability to cope with oxidative stress which has been applied to marine (Jena et al. 2009) and terrestrial molluscs (Scheil et al. 2012b) before. Lipid peroxides can be quantified by the ferrous oxidation xylenol orange method (FOX-assay) (Hermes-Lima et al. 1995; Monserrat et al. 2003).

To date, only little is known about the influence of a snail's shell colour on the Hsp70 level or the extent of oxidative damage, reflected by lipid peroxidation, at different temperatures. In this study we will therefore address the question of pre-adaptation of different shell colour morphs of *X. derbentina* to passive heating by analysing their Hsp70 level and their level of lipid peroxidation after exposure to elevated temperature for a fixed period of time in an artificial scenario.

2. Material and Methods:

2.1 Test organism and sampling setup:

Equal sample sizes from a single, annual field population of *Xeropicta derbentina* (Krynicki 1836) were taken. To avoid any negative influence of aging, the investigated specimens were all collected in early summer, where the growth of the snails is almost finished but production of eggs has not yet taken place. In former studies, June revealed to be the best time in the year to perform such studies (Dieterich et al. 2012).

Samples were collected in the vicinity of Modène (Vaucluse, Provence, Southern France, N44°6.055' E5°7.937') in June 2012. The sampling site was not used for agricultural purpose, thus, no pesticides were applied. Individual snails were sorted according to their colour category as predefined in other studies (Di Lellis et al. 2012; Dieterich et al. 2012; Köhler et al. 2009). Colour category 1 snails were defined as snails which carry a uniformly white shell. In colour category 2 snails with only a narrow light pigmented single band on the umbilicus side of the shell or with a light brownish shell colour on the umbilicus side of the shell were grouped. Category 3 snails bore a dark pigmented thick band on the umbilicus side of the shell or more than one light pigmented band. In colour category 4 snails with multiple bands on the umbilicus side of the shell and pigmentation on the apical side of the shell were grouped (Figure 1). The snails were allowed to acclimatise to laboratory conditions (25°C) for three weeks until further processing. They were kept in plastic containers (20.5 x 30 x 19.5 cm) filled with a layer of ground cover material (JBL, Terra Basis, Neuhofen, Germany). Snails were fed organic milk mash (Hipp, Pfaffenhofen, Germany) *ad libitum* twice a week. Every other day boxes were cleaned and sprayed with water to keep humidity.

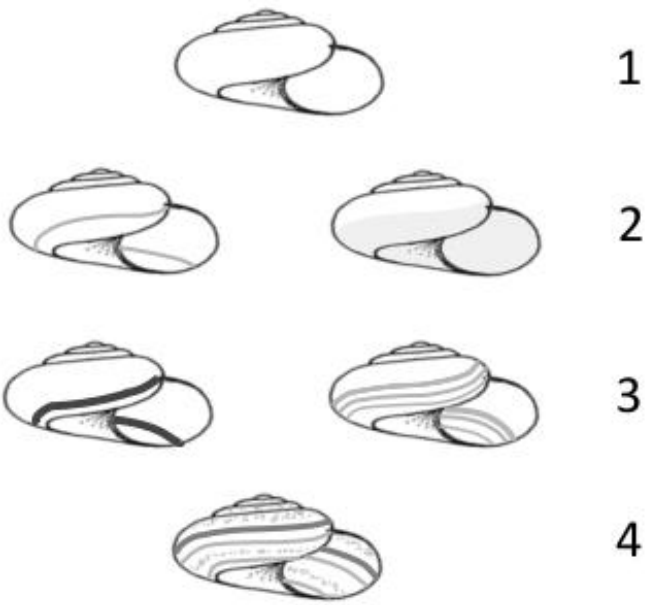

Fig. 1: Illustration of the four different colour morphs of X. derbentina, modified version based on Köhler et al. 2009

For experimental purpose 22 snails of each colour morph were randomly chosen and transferred into separate plastic boxes (18 x 13 x 6.5 cm) with a moist paper towel used as ground cover. The boxes were sealed with plastic foil to prevent the escape of snails during the experiment and to ensure a water saturated atmosphere. This was done to prevent fluctuations in the results that might have appeared as there was no possibility to control the humidity during the experiment. To ensure air circulation, the foil was perforated with nine small holes with 2 mm in diameter. Subsequently, the snails were exposed for eight hours in heating cabinets to temperatures of 25, 33, 38, 40, 43, 45, and 48°C. After heat exposure, the snails were immediately and individually frozen in liquid nitrogen for further analyses. The shell of the specimens taken for the FOX assay was cracked between two glass slides and removed prior to freezing. Samples were stored at -25°C until further analyses. To ensure comparability of biochemical data, snails of similar size were chosen for analysis. To avoid effects that might be addressed to senescent animals, only snails between 0.7 and 1.1 cm representing late juveniles or young adults were used for the experiment. Only individuals that survived the exposure phase were used in the experiments. To check for the snails´ survival, individuals were tabbed with a blunt needle. Retraction movement of the foot was seen as a sign of survival. As no mortality was detected during the experiments, all treated snails were used.

2.2 Hsp70 Analysis:

For Hsp70 analysis twelve out of the above-mentioned twenty-two individuals from each experimental setup were taken. While two individuals of them were kept as a backup and stored at -20°C, ten individuals were subsequently analysed as follows: The individually frozen snails were homogenized as a whole on crushed ice in appropriate volumes of extraction buffer (80 mM potassium acetate, 5 mM magnesium acetate, 20 mM Hepes and 2% protease inhibitor at pH 7.5) according to their body mass including the shell (2 µl buffer each mg snail weight). After ten minutes of centrifugation at 13722 rpm (= 20000 rcf) in an Eppendorf Centrifuge 5804R at 4°C, the resulting supernatant was divided into two portions. The first portion was used to calculate the total protein content using a standard procedure (Bradford 1976) in 96-well plates and a plate reader (Bio-Tek Instruments, Winooski, VT, USA). The second portion (40µg of total protein) was processed for the minigel SDS-PAGE (12% acrylamide, 0.12% bisacrylamide, 30' at 80 V plus 90' at 120 V). The proteins were transferred to a nitrocellulose membrane by semi-dry electro blotting. Subsequently, the membranes were transferred into blocking solution (50% horse serum in TBS) for two hours. After blocking, the membranes were incubated with a monoclonal α-Hsp70 antibody, cross reacting with all isoforms of the Hsp70-family, (mouse anti-human Hsp70, Dianova, Hamburg, Germany, dilution 1:5000 in 10% horse serum/TBS) on a lab shaker at room temperature overnight. The following day, the membranes were rinsed in TBS for five minutes to remove surplus antiboy. After that step, the second antibody (goat anti-mouse IgG conjugated to peroxidase, Jackson Immunoresearch, West Grove, PA, dilution 1:1000 in 10% horse serum/TBS) was applied for two hours. Following another five minutes of rinsing in TBS, the membranes were stained in staining solution (1 mM 4-chloro(1)naphthol, 0.015% H_2O_2, 30 mM Tris pH 8.5 and 6% methanol). Digitalisation was done using an Epson Perfection V350 Photo scanner. For each band, the optical volume (= band area x average grey scale value) was calculated with E.A.S.Y. Win 32 (Herolab, Wiesloch, Germany). The achieved optical volumes of the samples were related to a standard (full body extracts of *Theba pisana* (Müller 1774)) which was run in duplicate on every single gel. All stained membranes showed a single band of Hsp70 protein for each sample separated in the minigel SDS-PAGE. No broken bands were observed during the whole experiment (exemplarily shown in Figure 2). All given data were calculated as a mean of ten individuals.

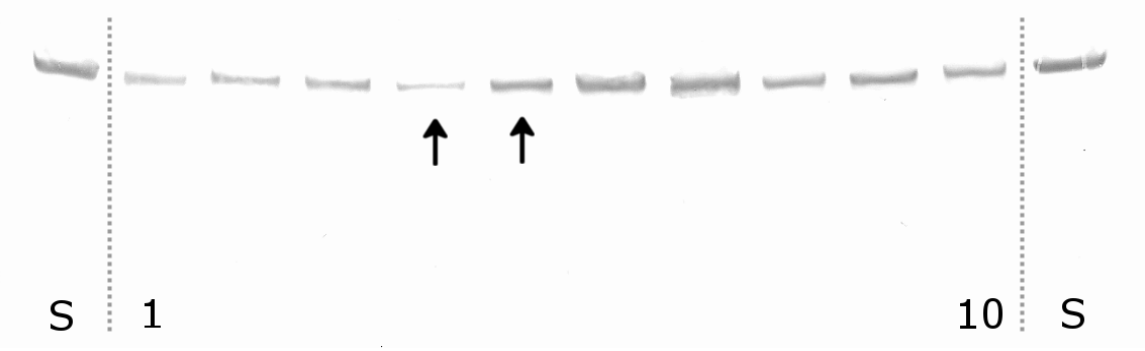

Fig. 2: Representative picture of a stained nitrocellulose membrane. The standard (S) was run in duplicate. Ten randomly chosen samples are shown. The amount of total protein used for analysis was 40 µg for each sample. Arrows indicate two representative samples with a high (lane 5) and a low (lane 4)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

2.3 FOX Assay:

In this study we conducted a modified FOX assay according to the method described by Hermes-Lima et al. (1995). Ten of the stored samples per exposure group (without shell) were used for this assay. The individuals were weighed and homogenized in ice-cold HPLC grade methanol (dilution 1:2; the required amount of methanol is calculated by: wet weight of the individual / density of methanol (0.791 g/cm^3)), centrifuged at 15.000 rpm (= 21130 rcf) at 4°C for 5 min in an Eppendorf Centrifuge 5804R. Supernatants were stored at -80°C. The assay was conducted using 96-well plates and a plate reader (Bio-Tek Instruments, Winooski, VT, USA). In each well (except for the blank) 50µl of each reagent was added following this order: 0.25 mM $FeSO_4$, 25 mM H_2SO_4 and 0.1 mM xylenol orange. Then, 15 µl of sample supernatant was added and the final sample volume adjusted to 200 µl with aqua bidest. For each sample, three wells were prepared (3 replicates) and a mean value was calculated. Master blanks contained 200 µl of aqua bidest.

Samples were incubated at room temperature for 180 min and absorbance was read at 580 nm (A_{580nm}). Subsequently, 1 µl of 1 mM cumenehydroperoxide (Chp) solution was added to the samples, incubated for 30 min at room temperature and again read at 580 nm ($A_{580nm+CHP}$).

The content of lipid hydroperoxides in the samples is expressed as cumenehydroperoxide-equivalents per gram wet weight (ChpE / g wet weight) and was calculated according to the equation by Hermes-Lima et al. (1995):

$$ChpE/g_{\text{wet weight}} = (A_{580nm}/A_{580nm+CHP}) * CHP_{1nmol} * V/V1 * DF$$

where V= total sample volume (200 µl), V1=added sample supernatant volume (15 µl) and DF= dilution factor with methanol (2).

2.4 Statistics:

All data were checked for normality using the Pearson-D´Agostino Omnibus Test. The Levene´s test was used to check for homogeneity of variance. In both sample sets normal distribution and homogeneity of variance was present, therefore, parametric test statistics could be applied. Because of a highly significant ($p < 0.001$) interaction between the factors 'temperature' and 'colour category' in both sample sets, the interpretation of a two way ANOVA was avoided. Instead, we performed one way ANOVAs on our data, sorted by temperature, followed by Tukey-Kramer-HSD tests to reveal the differences in Hsp70 and lipid peroxidation levels among the colour categories for each temperature tested and between the tested temperatures ignoring the shell colouration. For statistics we used SAS Jmp10 (SAS Institute Inc. 2012). The Pearson-D´Agostino Omnibus Test was carried out using the SolverStat Plugin (Comuzzi et al. 2003) for Excel. Levels of significance were set to: $0.01 < p \leq 0.05$: * (slightly significant); $0.001 < p \leq 0.01$: ** (significant); $p \leq 0.001$: *** (highly significant).

3. Results:

3.1 Hsp70 Analyses:

Generally, snails showed a distinct response in their Hsp70 levels after exposure to different temperatures. As shown in figure 3, the overall Hsp70 level (ignoring the shell colouration) was found to be slightly significantly ($p = 0.0117$) elevated in those snails exposed to 38°C and highly significantly ($p < 0.001$) reduced in snails exposed to 45°C compared to laboratory conditions at 25°C. A maximum Hsp70 level was observed in snails exposed to 38°C. The

measured Hsp70 level of these snails was found to be highly significantly ($p < 0.001$) elevated compared to those of individuals exposed to 45°C and 48°C and significantly ($p = 0.002$) elevated compared to individuals exposed to 43°C. The lowest Hsp70 level was found in snails exposed to 45°C. The observed Hsp70 level of these snails was found to be slightly significantly ($p = 0.0179$) lower compared to snails exposed to 48°C, significantly lower compared to the measured Hsp70 level in snails exposed to 43°C and highly significantly ($p < 0.001$) lower compared to the Hsp70 levels of snails exposed to the other temperatures tested.

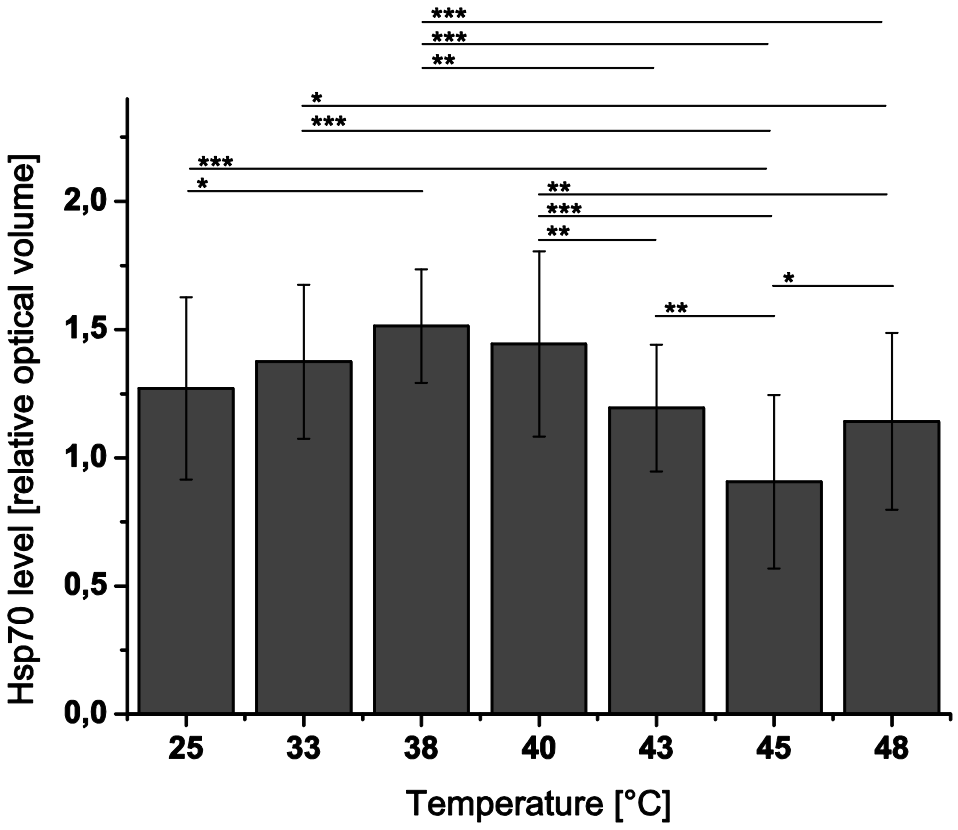

Fig. 3: Hsp70 levels of X. derbentina after exposure to different temperatures for 8 hours, irrespective of shell colouration (means ± SD; n = 40). Asterisks show significant differences between the different exposure temperatures: $0.01 < p \leq 0.05$ (*), $0.001 < p \leq 0.01$ (**); $p \leq 0.001$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

While the Hsp70 levels were found to be almost identical at 25°C among the four colour categories, the individuals of the different categories responded differently to elevated temperatures. Particularly the heat response of individuals of colour category 3 diverged from the other morphotypes (Figure 4). At 33°C, snails from category 3 started to express a tentatively higher Hsp70 level than the other morphs. After exposure to 38°C and 40°C, the resulting Hsp70 levels of category 3 snails were significantly higher than those of the other colour

categories (at 38°C : category 3 differed from category 1 with p = 0.0382, from category 2 with p < 0.001 and from category 4 with p = 0.002. At 40°C: category 3 differed from category 1 with p = 0.0050, from category 2 with p < 0.001 and from category 4 with p < 0.001). Exposure to 43°C led to a remarkable breakdown of the Hsp70 level in category 3 snails. At 45°C, not even half the Hsp70 level was measurable compared to the findings at 40°C. In the exposure groups of 45°C and 48°C, the category 3 snails were found to express the lowest measured Hsp70 level, compared to the other colour categories. Comparing the Hsp70 level of category 3 with category 1 snails at 48°C, a slightly significant (p = 0.0412) lower Hsp70 level was found (Figure 4).

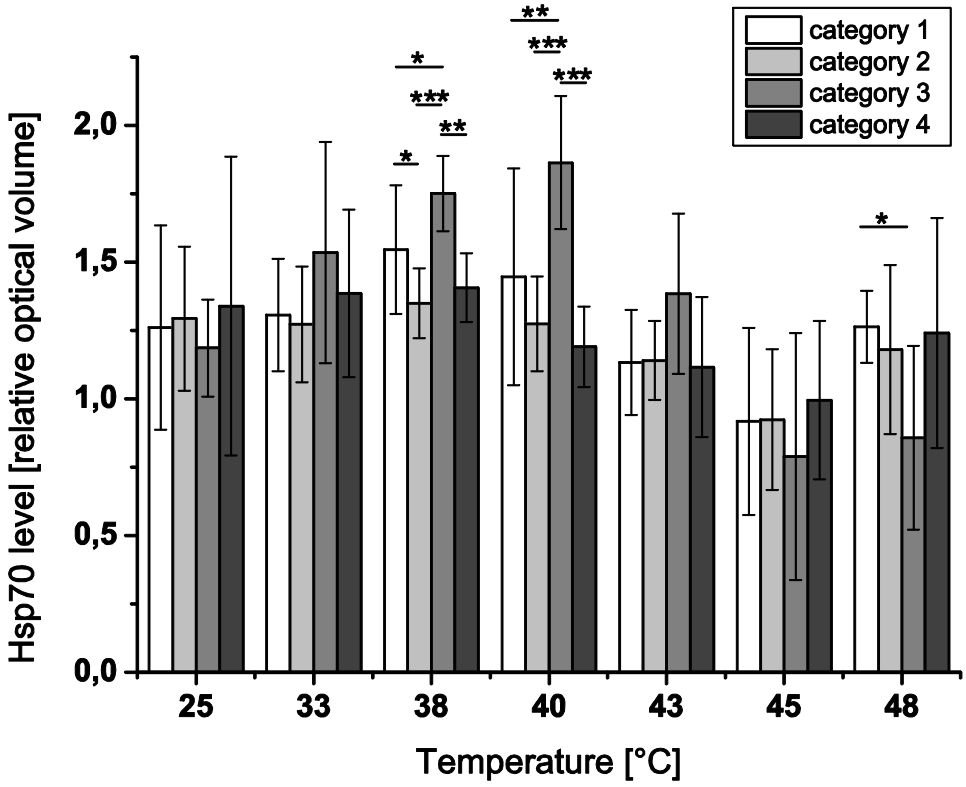

Fig. 4: Hsp70 levels of each morph category after exposure to elevated temperatures (means ± SD; n = 10). Asterisks show significant differences between the categories within an exposure group: 0.01 < p ≤ 0.05 (*), 0.001 < p ≤ 0.01 (**); p ≤ 0.001 (***)

3.2 FOX Assay:

Generally, the amount of ChpE / g wet weight increased after exposure to 38 and 40°C (compared to control condition at 25°C) followed by a decrease after exposure to 43°C with

a level even lower as the control level (25°C). A second increase of ChpE / g wet weight was observed after exposure to very high temperatures (45 and 48°C).

The observed differences between the colour morphs are displayed in Figure 5. After exposure to 33, 38, and 45°C significant differences were found to be present. At 33°C, a slightly significant difference was observed between categories 2 and 3 ($p = 0.0485$) whereby category 2 snails showed a lower level of ChpE / g wet weight. A highly significant difference between category 1 and all other colour categories was observed after exposure to 38°C (all comparisons with $p < 0.001$). Here, snails of category 1 had the lowest level of lipid peroxides measured at this temperature. This level almost mirrored the control level, and thus showed a ˈdelayedˈ reaction to increasing temperatures compared to the other categories.

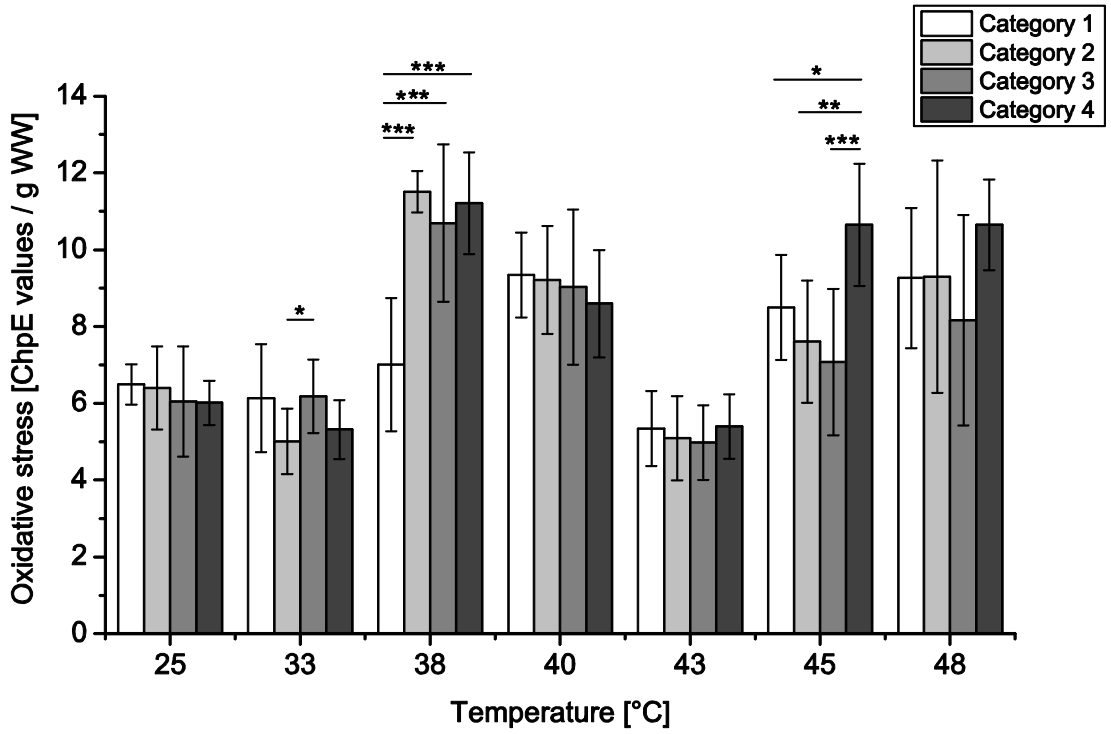

Fig. 5: Lipid peroxide levels (ChpE per gram wet weight) of each colour category after exposure to elevated temperatures (means ± SD; n = 10). Asterisks show significant differences between the colour categories within an exposure group: $0.01 < p \leq 0.05$ (*), $0.001 < p \leq 0.01$ (**); $p \leq 0.001$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

After exposure to 45°C, snails of category 4 showed an increase in ChpE / g wet weight differing from the response of the other colour categories: the level of lipid peroxides in colour category 4 was slightly significantly elevated compared to category 1 ($p = 0.0270$), signifi-

cantly elevated compared to category 2 ($p = 0.0010$), and highly significantly elevated compared to category 3 ($p < 0.001$). Furthermore, category 3 snails tended to exhibit a lower level of ChpE / g wet weight in response to extreme temperature exposures at 45°C and 48°C, compared to the other categories (Figure 5).

4. Discussion:

4.1 Hsp70 Analysis:

As reported in other studies related to adaptations of land snails from Southern France to elevated temperatures (Di Lellis et al. 2012; Dieterich et al. 2012; Köhler et al. 2009; Scheil et al. 2011; Troschinski et al. 2014), *X. derbentina* reacts to heat stress with a clear Hsp70 induction when being confronted with increasing temperatures. A maximum induction of Hsp70, the intensity of which depended on the identity of the population, was regularly observed between 38° and 40°C in different populations (Di Lellis et al. 2014; Troschinski et al. 2014). Exceeding these temperatures, a decrease in Hsp70 level was found, with the lowest measured values at 45°C, followed by a second, minor increase in Hsp70 at 48°C (Di Lellis et al. 2014; Troschinski et al. 2014). Both, qualitative and quantitative aspects of these heat stress response kinetics were also recorded for the *X. derbentina* specimens investigated in the present study. Therefore it can be considered as a general response of the snails to artificially induced heat stress.

In this study we focused on possible differences among the four colour categories found in the field and their ability to induce Hsp70 as a response to passive heating. As revealed by our data, category 3 snails showed a higher capacity to induce Hsp70 compared to snails of any other category. In the study of Dieterich et al. (2012) category 3 snails were mainly found during spring. Thus, it is possible that category 3 shell colouration may be regarded predominantly as a 'juvenile' colouration pattern that disappears when the next shell whorl is formed with proceeding growth. Particularly shells with more than two small brown bands could mainly be observed in young snails with a size of 3 to 5 mm. Snails with a single large black or brown band, as those used in this study, mainly correspond to larger size and could be observed during the entire year. To prevent effects that may arise from working with very young or senescent snails, our experiments only used individuals collected in June. Never-

theless, differences in the induction of Hsp70 were found. Category 3 snails, for a reason we do not yet understand, were able to induce higher levels of Hsp70 than the other categories when heated up to a maximum of 43°C, indicating that a particular heat response strategy may be associated with a distinct phenotype. In the temperature range between 33°C and 43°C category 3 snails were found to be pre-adapted to elevated temperatures in a better way, as indicated by higher Hsp70 levels. In contrast, category 3 snails were shown to exhibit the lowest Hsp70 content of all categories when the temperature exceeds 43°C. Apparently, the Hsp70 protection system of the category 3 snails seems to be more effective below 43°C, compared to the other categories, and seems to get easily overwhelmed when temperatures exceed 43°C. It is likely that the maintenance of a superior protection system is very cost-intensive. Therefore, it seems that snails of the colour category 3 are less able to cope with temperatures higher than 43°C, probably as they are no longer able to invest these high energy costs in this protection system. Consequently, at very high temperatures, category 3 snails may show lesions on the cellular level, as described in different studies (Dittbrenner et al. 2009; Scheil et al. 2011; Troschinski et al. 2014), earlier in comparison to the other morphs. In years with very hot summers, this may be a disadvantage for category 3 snails. It is not yet known if *X. derbentina* populations change their composition of colour morphs during the years and how different local temperatures may influence the morph frequencies within a population of these snails. However, the phenomenon of morph frequency fluctuations throughout the years has been reported for other helicoid land snails before (Cowie 1992; Johnson 2011; Silvertown et al. 2011). As summarized by Ozgo and Schilthuizen (2012), the shell colour of *Cepaea nemoralis* was found to be associated with a gene locus coding for the different background shell colours in this species, while the banding was found to be associated with another locus, linked to the colour coding one. *Cepaea nemoralis*, as reviewed in Goodhart (1987), was often found to adapt its shell banding and colouration to the habitat. Particularly in warmer regions and in more sun-exposed habitats, yellow unbanded or at least 'effectively unbanded' (snails with at least the top two bands missing and appearing unbanded in the most views) specimens were found to be more abundant than specimens with all five bands expressed on the shell or with a darker background colour. This indicates a natural selection of morphotypes by climate. On the other hand, other examples are given which rather point to a local area effect, as, in some studies cited in this review, snails from one predominant colour morph were found to inhabit differently struc-

tured habitats and no change in morph frequencies was found. Further, a change in the colouration frequency of *Cepaea nemoralis* over more than 40 years was reported in the study of Ozgo and Schilthuizen (2012). The authors speculated on anthropogenic change of environment and the increase in temperature in the sampling region to be possible reasons for these morph frequency changes. For the investigated population of *X. derbentina* in this study, as well as for other populations of this species in the vicinity investigated so far (Di Lellis et al. 2014; Köhler et al. 2009; Troschinski et al. 2014), no historic data about the change in morph frequencies are available. Without long term studies like the above mentioned ones, a possible area effect that may explain the predominant white coloured category 1 snails, remains speculative. In our study not the predominant pale category 1 did express the highest Hsp70 level and, therefore, may be best protected against the consequences of heat. Compared to *Cepaea nemoralis,* the 'effectively unbanded' category 3 snails were found to have an increased Hsp70 level when being exposed to 38°C and 40°C. A possible explanation for this may lie in a varying adaptation to the climatic conditions in the different colour categories over the years. As mentioned in Sørensen et al. (1999), adaptation to heat over several generations can lead to a decreased Hsp70 level in *Drosophila buzzatii* lines. If this would also be the case in the investigated *X. derbentina* population, the better adapted categories 1 and 2 would show reduced Hsp70 inducibility as a matter of an energetic trade-off with the possible advantage of a more successful reproduction. The significantly higher Hsp70 levels in category 3 snails may point to a weaker adaptation to the local climate. To date it is not know if *X. derbentina* shows a similar genetically controlled mechanism of shell colouration and shell banding as found in *Cepaea nemoralis* before. The change in morph frequency distribution over a long period of time as well as the genetics of this species needs further investigations to clarify these aspects. In contrast to other publications dealing with Hsp70 induction or shell colouration, we excluded solar radiation as a heat source by heating the snails in heating cabinets. Different heating of shells caused by different shell colouration intesity, as it has often been proclaimed (Heath 1975; Moreno-Rueda 2008; Richardson 1979), cannot be taken to explain the differences in Hsp70 levels in this experiment. Therefore, some kind of pre-adaptation may have been evolved for the different colour categories of the investigated *X. derbentina* population.

To be consistent with the methodology applied in earlier studies on Mediterranean land snails (Dittbrenner et al. 2009; Köhler et al. 2009; Scheil et al. 2011; Troschinski et al. 2014)

we exposed the snails for eight hours in a heating cabinet. The principles of Hsp70 induction in *X. derbentina* have been studied in relation to the heat load (Köhler et al. 2009), and also the daily Hsp70 level kinetics in different seasons was reported before (Dieterich et al. 2012). However, data on the temporal kinetics of the Hsp70 system in *X. derbentina* in response to different temperatures are still lacking. Scheil et al. (2011) found that exposure of *X. derbentina* to a very high but still sub-lethal temperature of 45°C led to a maximum induction of Hsp70 after two hours, followed by a subsequent decline. After exposure to temperatures of about 25°C, a maximum Hsp70 level was observed after four hours in the same snail species. These results indicate an interrelation of exposure time and temperature, two parameters which are likely not linked in a linear way. No such data are yet available for temperatures in between these two extremes and it is not known whether different colour morphs of these snails induce their maximum Hsp70 level after the same period of experienced heat stress or, possibly, differ in this respect. The latter may explain the divergent '8h snapshot' Hsp70 data recorded in the present study.

4.2 FOX Assay:

An assumed time dependency of maximum levels, as it was discussed for Hsp70, does not have to be considered for the measurement of lipid peroxides: Scheil et al. (2012) exposed snails of *Theba pisana* for 8h at 43°C, took samples at four time points (0, 2, 4 and 8h), and observed a significant increase of lipid peroxides after 4h which stayed constantly high until the end of the experiment. It became evident that long heat exposure elevated the lipid peroxide level, but without any subsequent decline.

Our results on temperature-induced oxidative damage, as reflected by the relative amount of lipid peroxides, in general revealed a clear increase of lipid peroxidation after 8h, primarily after exposure to 38°C and 40°C and, secondly after exposure to 45°C and 48°C. The elevated levels of lipid peroxides indicate cellular damage as a consequence of oxidative stress which was caused by heat exposure. This 'two-peak' pattern is particularly remarkable in view of the low lipid peroxide level at 43°C. A possible explanation may lie in the activity of the antioxidant defence system which includes both a number of enzymes and also small molecules. Enzymes of this defence system that directly degrade ROS include: superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) (Aebi 1984; Gutteridge 1995; Halliwell and Gutteridge 1989). Aside of these enzymes, there are much more antioxidants

acting as free radical scavengers or substrates. For example, most importantly reduced glutathione or other enzymes like glutathione reductase (GSR) or glutathione-S-transferase (GST), which additionally need reduced glutathione as a cofactor for their activity (Meister 1988; Pannunzio and Storey 1998; Radwan et al. 2010). In the context of our results, we assume that one or several enzymes of the antioxidant defence system may have an activity optimum or are expressed in a higher amount at low temperatures (25 and 33°C), in the following called 'defence mechanism 1'. Whereas another enzyme (or a complex of enzymes), in the following called 'defence mechanism 2', displays either its optimum or at least a high level at 43°C, thus limiting lipid peroxidation at this temperature. In addition to these enzymes, also a higher amount of scavenger molecules of the non-enzymatic defence system could cause this effect.

It has been shown in aquatic invertebrates (Zhou et al. 2010) that antioxidant protection systems, as well as the stress proteins Hsp60 and Hsp70, are induced by heat. It is likely that both, the heat shock proteins and the antioxidant protection system, are responsible for the snails´ survival in the heat. With respect to the interrelation of these two protection systems, one may speculate as follows: The 'defence mechanism 1' may counteract slight oxidative stress and may be expressed in a rather constitutive way with a slight induction potential above 38°C. Whenever a more severe stress factor (in this case heat) challenges one of the sub-systems of this 'defence mechanism 1' (e.g. the Hsp70 system) to react, the other subsystem may be silenced because of an energetic trade-off. When the first defence mechanism starts getting overwhelmed with increasing stress intensity (e.g. at temperatures above 43°C), the 'defence mechanism 2' may take over to ensure further survival. When we apply this hypothesis to our results, the high level of Hsp70 observed at 38°C and 40°C was probably indicative of Hsp70 being a sub-system of 'defence mechanism 1', associated with an increased extent of oxidative damage, reflected by increased levels of lipid peroxides, because of an energetic trade-off. Thus, the antioxidant defence was probably at a minimum at these temperatures. The 'defence mechanism 2' protection system may become of importance when the energy-intense Hsp70 protection system is getting overwhelmed between 40°C and 43°C. Such a protective system may lead to lower lipid peroxide levels at 43°C and to the observed reduction in Hsp70 level at 43°C and 45°C. Such an interpretation, however, remains speculative in respect to the biochemical antioxidant components involved in 'de-

fence mechanism 1' and 'defence mechanism 2', even though it helps to explain the different patterns observed for Hsp70 and lipid peroxidation following increasing heat stress.

Regarding the different morphs of *X. derbentina*, 'defence mechanism 1' seems to be more efficient in colour category 1, whereas 'defence mechanism 2' is poorly pronounced in colour category 4, respectively compared to the other morphs.

This hypothesis should be addressed by further studies in which the focus should lie on different component enzymes of the antioxidant defence system. Nevertheless in our present study the suitability of the measurement of lipid peroxides via the FOX assay as a biomarker for heat-induced oxidative stress in terrestrial snails could be demonstrated. In combination with the well established marker for proteotoxicity, Hsp70, a more detailed statement about the health conditions of *X. derbentina* individuals in Southern France and their possibility to react to harsh environmental conditions can be given.

5. Acknowledgements:

The authors would like to thank Christophe Mazzia, University of Avignon, for the provision of our sampling site in Modène. The study was funded by the German Research Council (DFG, Ko 1978/5-3) and the Twinning Projects Grant of Tübingen University. The authors would like to thank two unknown reviewers for their useful comments on an earlier version of this manuscript.

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Kapitel 3: **Antioxidant defense and stress protein induction following heat stress in the Mediterranean snail *Xeropicta derbentina* [Pulmonata, Hygromiidae].**

Journal of Experimental Biology. DOI:10.1242/jeb.113167.

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Abstract:

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

1. Introduction:

In the Mediterranean climate, which is characterized by dry and hot summers, animals need particular adaptations to ensure survival under extreme environmental conditions. Especially terrestrial snails with their water-permeable skin (Machin 1964) and their external shell easily face the risk of desiccation and over-heating. One example is the pulmonate land snail

Xeropicta derbentina (Krynicki, 1836), which occurs in high numbers in southern France. These snails possess special behavioral and physiological adaptations to their habitat: climbing on vegetation to escape from hot ground temperatures or shifting activity phases to favorable time periods (Pomeroy 1968; Yom-Tov 1971) can be seen as behavioral adaptations whereas aestivation attended by metabolic depression (Guppy and Withers 1999; Bishop and Brand 2000; Storey 2002) during periods of extreme dry conditions is an example for a physiological mechanism of adaptation. Furthermore, there are different mechanisms acting on the biochemical level, which are known to play an important role in the thermotolerance of animals.

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

Aerobic organisms must deal with the continuous generation of ROS as byproducts of metabolism (Halliwell and Gutteridge 1989). These products are molecules derived from molecular oxygen and include the superoxide anion radical ($^{\cdot}O_2^-$), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($^{\cdot}OH$), the latter being highly reactive and most destructive (Pannunzio and Storey 1998). To minimize their destructive action, ROS should be rapidly eliminated. All cells possess constitutive antioxidant defences, which include enzymes and small molecules that detoxify or scavenge ROS (Halliwell and Gutteridge 1989). Enzymes that directly degrade ROS include: (i) superoxide dismutase (SOD), which catalyses the dismutation of superoxide into hydrogen peroxide and oxygen, (ii) catalase (CAT), which degrades hydrogen peroxide, and (iii) glutathione peroxidase (GPx), which degrades hydrogen peroxide and also lipid peroxides generated by lipid peroxidation (Aebi 1984; Halliwell and Gutteridge 1989; Gutteridge

1995). Beside these enzymes there are much more antioxidants acting as free radical scavengers or substrates (e.g. the most important one is reduced glutathione (GSH)) involved in the detoxification of hydroperoxides, or other enzymes like glutathione reductase (GR) or glutathione-S-transferase (GST), which additionally need reduced glutathione as a cofactor for their activity (Meister 1988; Pannunzio and Storey 1998; Radwan et al. 2010).

The activation of antioxidant defenses is an essential factor in protecting an organism from cellular damage when environmental conditions become deleterious. Changes in the activities of antioxidant enzymes have been found in many organisms in response to anoxia (Hermes-Lima and Storey 1996, 1993; Pannunzio and Storey 1998; Lushchak et al. 2001), freezing (Hermes-Lima and Storey 1993; Joanisse and Storey 1996), and also heat stress (Lushchak and Bagnyukova 2006b; Heise et al. 2006; Verlecar et al. 2007). An increase in antioxidants during aestivation in snails (Hermes-Lima et al. 1998; Ramos-Vasconcelos and Hermes-Lima 2003; Nowakowska et al. 2009) has also been shown. Furthermore, the application of oxidative stress indices can be used as biomarker of environmental pollution (Jena et al. 2009; Luna-Acosta et al. 2010; Radwan et al. 2010).

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

bentina, elevated Hsp70 levels in response to heat exposure have been found in a number of recent studies (Köhler et al. 2009; Scheil et al. 2011; Dieterich et al. 2013; Di Lellis et al. 2014; Troschinski et al. 2014).

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

In an earlier study (Dieterich et al. 2014), we conducted heat exposure experiments with the result of a clear decrease of lipid peroxides at a distinct temperature (43°C) in *X. derbentina*, which brought us to the hypothesis that this effect might be due to an activation of the antioxidant defense machinery. Here, we investigate the effects of different temperatures on the activity of the two enzymes catalase and glutathione peroxidase as representatives of the antioxidant defense system, by exposing snails of the species *X. derbentina* to different heat exposure regimes (25, 38, 40, 43, and 45°C) in the laboratory. In addition, we also determined lipid peroxidation levels (as a marker for oxidative stress) and the 70kDa heat-shock protein, Hsp70. Furthermore, we aimed at assessing the role of the antioxidant defense mechanism in this snail's ability to counteract high temperatures. Thus, we investigated, for the first time, the interplay between the antioxidant defense system and the Hsp70 response in this context.

2. Material and Methods:

2.1 Test organism and sampling:

Individuals from a single population of the terrestrial snail, *Xeropicta derbentina,* were collected in the last week of May 2013 in Modène, Provence, Southern France. The sampling site was dry, open, and sun-exposed.

Snails were collected and kept in plastic containers (20.5 × 30 × 19.5 cm) in a density of approximately 200 individuals per box.

2.2 Experimental setup:

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

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

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

2.3 Catalase assay:

To measure the catalase activity in the samples, we used Cayman´s Catalase Assay Kit (Item No. 707002, Cayman Chemical Company, Michigan, USA). The method is based on the reaction of catalase with methanol in presence of H_2O_2. Produced formaldehyde is measured calorimetrically with purpald (4-amino-3-hydrazino-5-mercapto-1,2,4-triazole) as the chromogen, which forms a bicyclic heterocycle with aldehydes and changes from colorless to a purple color upon oxidation.

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

Formaldehyde standard wells were prepared containing 100µl of assay buffer (100mM potassium phosphate, pH 7.0), 30µl of methanol, and 20µl of standard (concentrations 0, 5, 15, 30, 45, 60, and 75µM formaldehyde) per well. Two positive control wells were filled with 100µl of assay buffer, 30µl of methanol, and 20µl of catalase (control: bovine liver CAT).

Sample wells were prepared in duplicates containing 100µl of assay buffer, 30µl of methanol, and 20µl of sample. Because the amount of catalase added to the well should result in an activity between 2-35nmol/min/ml, it was necessary to dilute the samples with sample buffer (1:2000).

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

Catalase activity was calculated using the following equation:

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

2.4 Glutathion peroxidase assay:

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

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

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

For each sample, the change in absorbance (ΔA_{340}) per minute was determined and GPX activity was calculated by the following equation:

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

2.5 FOX-assay (quantification of lipid peroxides):

In this study we conducted a modified FOX assay deriving from the method described by Hermes-Lima et al. (1995). The individuals were weighed and homogenized in ice-cold HPLC grade methanol (dilution 1:2; the required amount of methanol is calculated by: wet weight of the individual / density of methanol (0.791 g/cm^3)), centrifuged at 15.000 g and 4°C for 5 minutes. Supernatants were stored at -80°C until further analysis. The assay was conducted using 96-well plates. In each well (except for the blank) 50µL of each reagent was added following this order: 0.25mM $FeSO_4$, 25mM H_2SO_4, and 0.1mM xylenol orange. Then, 15µL of sample supernatant was added and the final sample volume adjusted to 200µL with aqua bidest. For each sample, three wells were prepared (3 replicates) and a mean value was calculated. Master blanks contained 200µL of aqua bidest.

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

The content of lipid hydroperoxides in the samples is expressed as cumenehydroperoxide-equivalents per gram wet weight (CHPE / g wet weight) and was calculated according to the equation by Hermes-Lima et al. (1995):

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

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

2.6 Hsp70 analysis:

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

2.7 Statistics:

All data were checked for normality and homogeneity of variance using the D'Agostino Omnibus Test and Levene's test. Data from catalase and FOX assay were transformed (square root; log) to guarantee a normal distribution of the data. To detect significant differences within the treatments, we used ANOVA followed by the Tukey-Kramer HSD post-hoc test.
Data were analyzed using JMP 9 (SAS Institute Inc., Cary, NC) and Microsoft Excel 2007 (Microsoft Corporation, Redmond, USA).

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

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

3. Results:

3.1 Catalase:

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

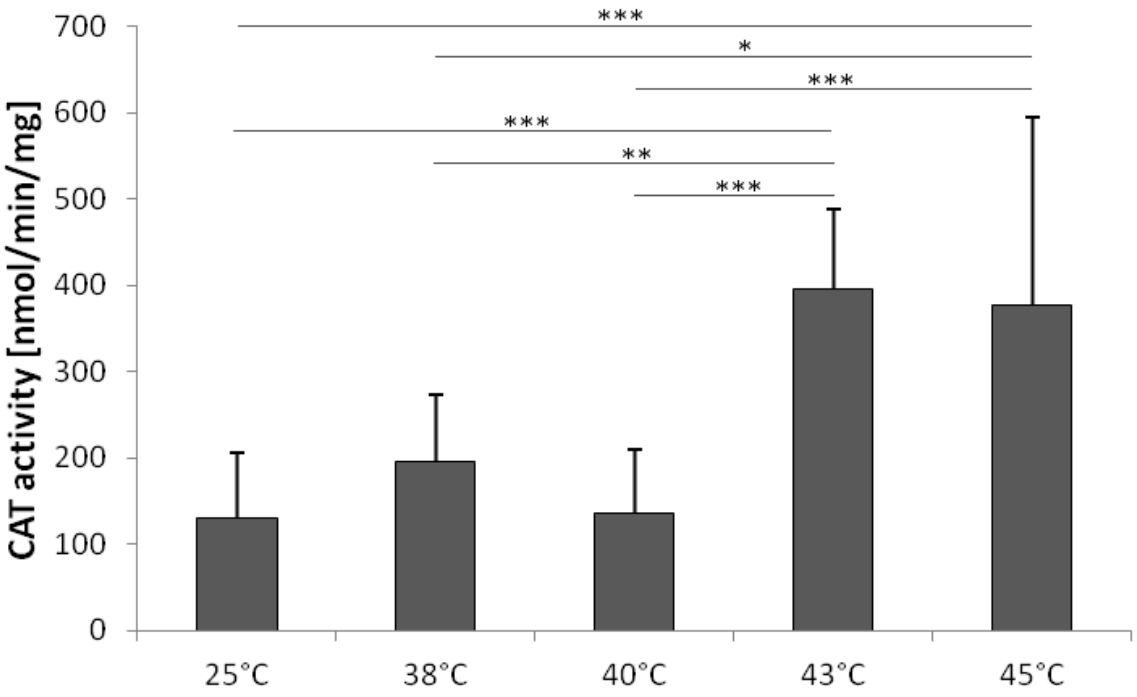

Fig. 1: Catalase activity in *X. derbentina* after different temperature treatments (means + s.d., n=10). Asterisks indicate significant differences between the groups: 0.01 < *p* ≤ 0.05 (*), 0.001 < *p* ≤ 0.01 (**); *p* ≤ 0.001 (***).

3.2 Glutathione peroxidase:

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

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

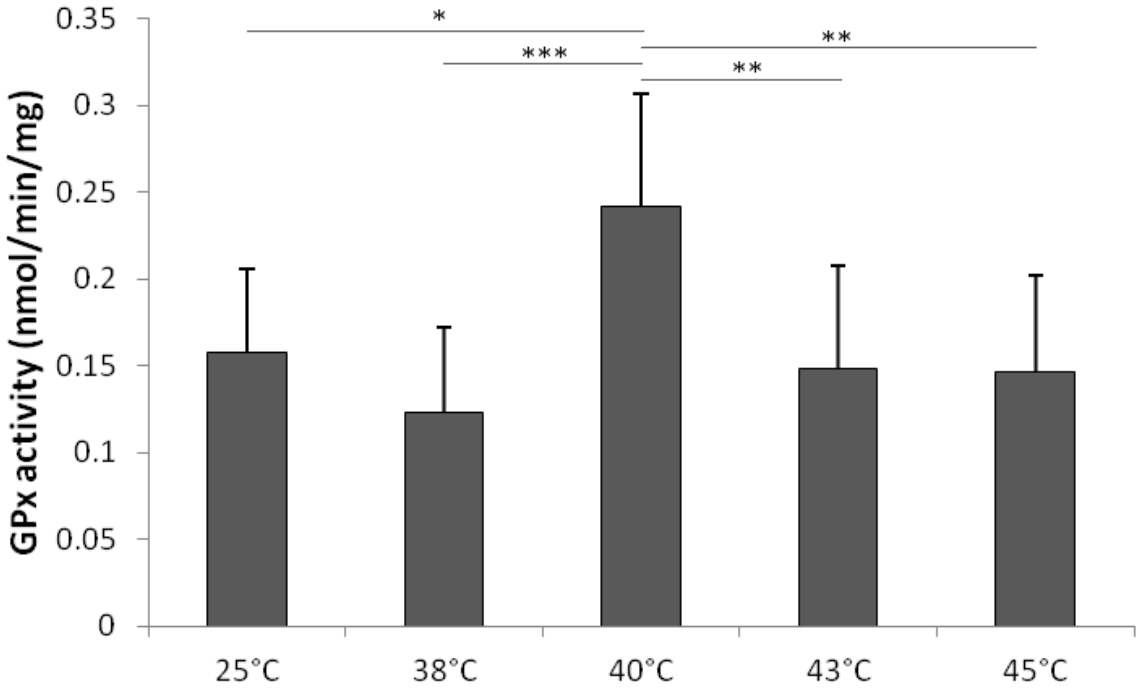

Fig. 2: Glutathione peroxidase activity in *X. derbentina* after different temperature treatments (means + s.d., n=10). Asterisks indicate significant differences between the groups: $0.01 < p \leq 0.05$ (*), $0.001 < p \leq 0.01$ (**); $p \leq 0.001$ (***).

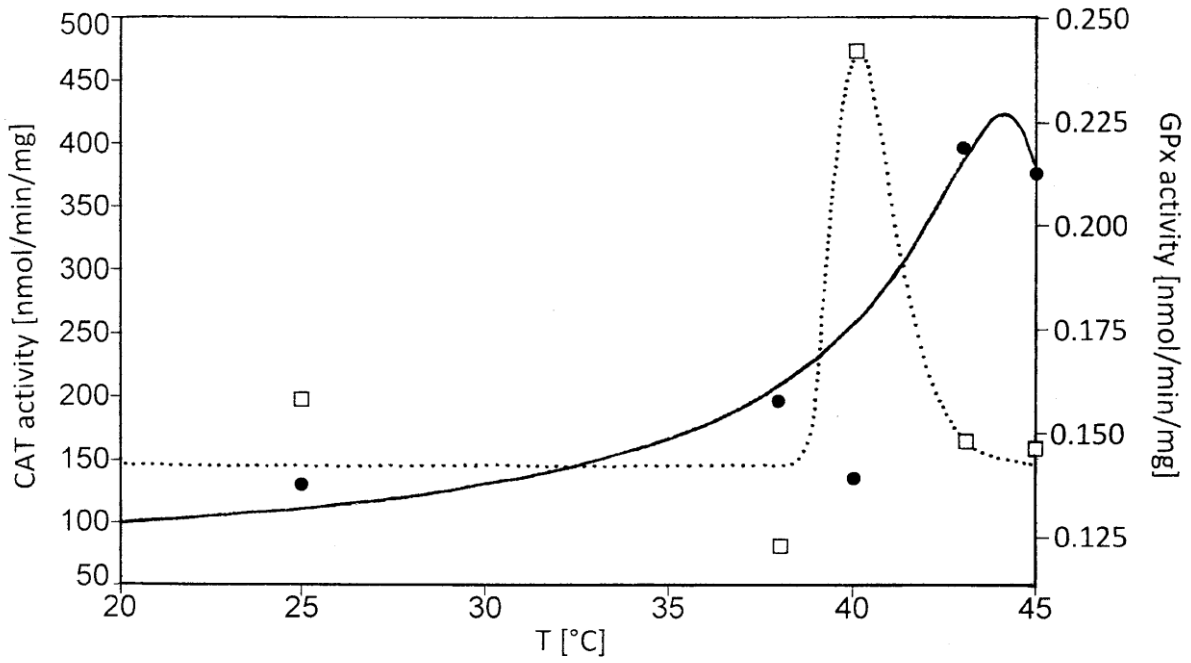

Fig. 3: Non-linear regression analysis of catalase (black dots, solid line, left scale, CAT) and glutathione peroxidase (squares, dotted line, right scale, GPx) activities *vs.* temperature (T).
$(CAT)^{-1} = 0.011 - (7.369 \cdot 10^{-7}) T^{2.5} + 3.757\, e^{T}$ with $r^2 = 0.773$
$GPx = 0.142 \exp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

3.3 Lipid peroxidation:

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

3.4 Hsp70:

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

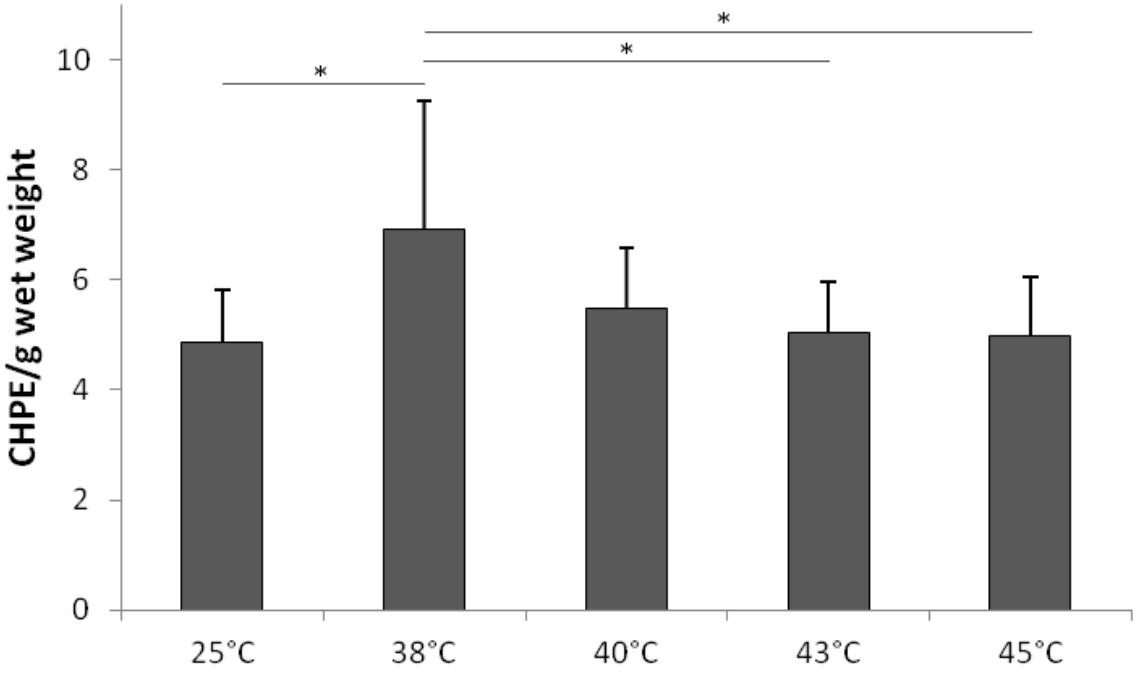

Fig. 4: Levels of lipid peroxides (expressed as CHPE per gram wet weight) in *X. derbentina* after different temperature treatments (means + s.d., n=10). Asterisks indicate significant differences between the groups: $0.01 < p \leq 0.05$ (*).

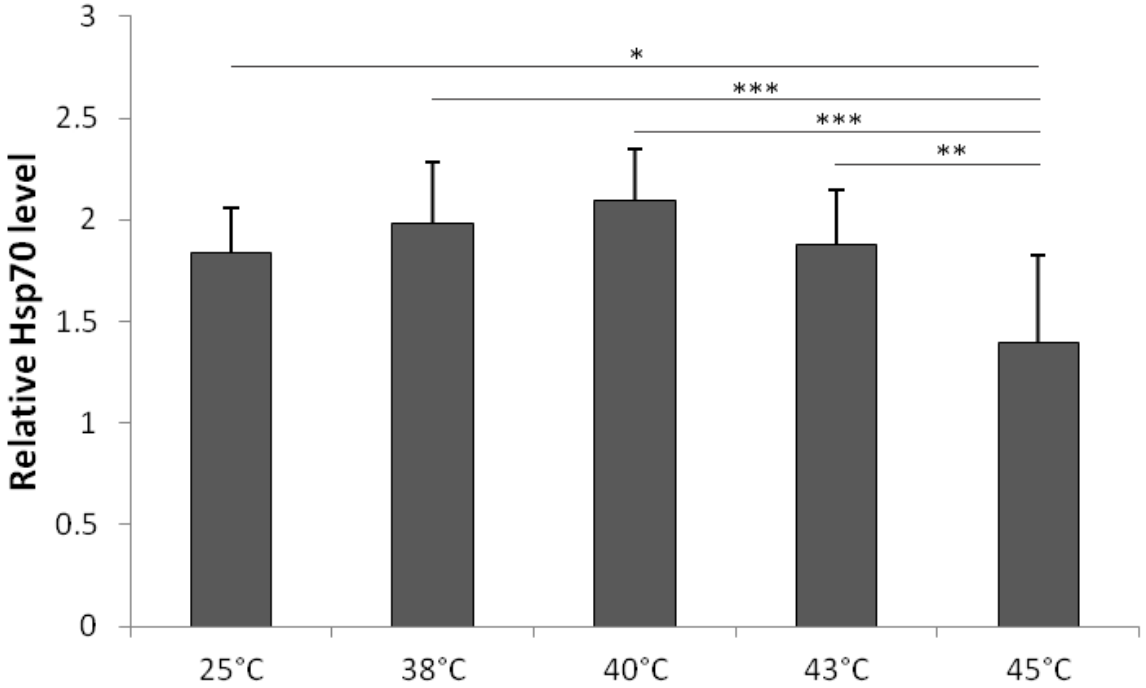

Fig.5: Relative Hsp70 levels in *X. derbentina* after different temperature treatments (means + s.d., n=10). Asterisks indicate significant differences between the groups: $0.01 < p \leq 0.05$ (*), $0.001 < p \leq 0.01$ (**); $p \leq 0.001$ (***).

4. Discussion:

Terrestrial snails inhabiting dry and hot habitats experience daily periods of high temperatures due to the absorbance of solar radiation. The pulmonate *Xeropicta derbentina* is a well adapted organism to such unfavorable conditions. Thus, this snail species had become a common object for the investigation of physiological heat stress responses in the last years (Dittbrenner et al. 2009; Köhler et al. 2009; Dieterich et al. 2013; Scheil et al. 2011; Di Lellis et al. 2012; Di Lellis et al. 2014; Troschinski et al. 2014). In contrast to the well-documented induction of heat-shock proteins in response to heat exposure, the activation of the antioxidant defense system is poorly understood in this context in this terrestrial snail species.

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

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

We used the determination of lipid peroxides via FOX assay as index for oxidative stress. When we compare the levels of lipid peroxides with the observed levels of antioxidant en-

zyme activity in the different temperature treatments, a clear physiological response is obvious: after exposure to 38°C, we detected an increase in lipid peroxides which was followed by an increased activity of GPx in the 40°C treatment. In consequence to this elevated enzyme activity, the level of lipid peroxides decreased. After exposure to 43 and 45°C, we measured a significant increase of CAT activity associated with low lipid peroxide levels which is indicative for the highly effective work of this enzyme against the reactive oxygen species H_2O_2. Furthermore, the increase of CAT activity was also associated with a decrease in activity of GPx. Our data suggest that, here in our artificial heat exposure experiment, GPx has its activity optimum at 40°C, whereby CAT activity remains unaffected staying on its 'base level'. But when exceeding this temperature, reaching 43 and 45°C, a boost in CAT activity, associated with a decrease in GPx activity, lead to a reduction of damaging effects of H_2O_2 (mirrored by low lipid peroxide levels). This phenomenon reflects a competition between CAT and GPx for the same reactive oxygen species (ROS), since both enzymes degrade H_2O_2. In a study by Nowakowska et al. (2011), this competing action between CAT and GPx could also be demonstrated in two molluscan species (*Helix aspersa* and *Helix pomatia*): here, extremely low levels of CAT activity were usually associated with extremely high activities of GPx.

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

In the last years, several studies demonstrated that antioxidants, i.e. catalase and GPx, play an important role during aestivation as a mechanism of preparation for the oxidative stress that accompanies arousal in snails (Hermes-Lima and Storey 1995; Storey 1996; Hermes-Lima et al. 1998; Storey 2002; Ramos-Vasconcelos and Hermes-Lima 2003; Nowakowska et al. 2009; Nowakowska et al. 2010; Nowakowska et al. 2011). Beside this well-documented phenomenon and the role of the antioxidant defense system in this context, it is generally known that heat can induce oxidative stress. An increase in temperature stimulates all metabolic processes, for example it elevates oxygen consumption which can result in oxidative

stress due to an increase in ROS as by-products during intensified metabolism (Storey 1996; Lushchak 2011). The induction of oxidative stress due to elevated environmental temperature was shown in several organisms (Heise et al. 2006; Bagnyukova et al. 2007a; Lushchak and Bagnyukova 2006a, b; Verlecar et al. 2007; Bocchetti et al. 2008) and was associated with an increase in antioxidants (Lushchak and Bagnyukova 2006b; Bagnyukova et al. 2006; Bagnyukova et al. 2007b). For example, in the mussel *Perna virdis*, increased activities of CAT and GPx (beside other antioxidants) were recorded (Verlecar et al. 2007). In the present study, we could demonstrate that terrestrial snails undergo oxidative stress as a result of elevated temperature which suggests the activation of physiological mechanisms to scavenge produced ROS. We could show that CAT and GPx activities were increased as enzymatic antioxidant defenses in a temperature-dependent, serial way of induction, indicating an essential role of antioxidants in the thermotolerance of *X. derbentina*.

Also the Hsp70 induction kinetics recorded here were in accordance with previous findings (Köhler et al. 2009; Troschinski et al. 2014; Di Lellis et al. 2014). In these studies the maximum Hsp70 level was observed at temperatures around 38 and 40°C applied for 8h, followed by a rapid Hsp70 decline when ambient temperature exceeded 40°C. Our data support these results, since we found a maximum heat shock protein induction at 40°C. The significant decrease of the Hsp70 level, especially in the 45°C exposure group, is assumed to be due to an overwhelmed stress protein machinery (destruction phase), which is in accordance with the kinetics of stress protein induction described by Eckwert et al. (1997).
Molecular chaperones as the heat shock proteins are primary sensors of misfolded proteins and assist in refolding processes. Some isoforms of Hsp70 are stress-inducible proteins that repair damaged proteins and prevent protein aggregation. The regulation of the expression of Hsp70 in gastropods has been linked to different factors of the developmental or ecological level (Tomanek and Somero 2002; Arad et al. 2010; Mizrahi et al. 2010). Furthermore, in *X. derbentina*, seasonal and intraspecific variations in the Hsp70 induction could be found leading to different survival strategies in *X. derbentina* populations (Dieterich et al. 2013; Troschinski et al. 2014). Generally, it is known that Hsp induction is used as an important survival strategy in land snails living under extreme environmental conditions (Mizrahi et al. 2010, 2012). In this context, heat shock proteins are essential for ‘repairing’ partly malfolded

proteins due to damaging effects of ROS, so an up-regulation of these proteins may be important for an organisms' cellular fitness (De Oliveira et al. 2005).

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

It has to be mentioned that we just investigated a "snap-shot" of the biochemical heat response after 8 hours of exposure. A previous study showed *X. derbentina* to exhibit a maximum level of Hsp70 after two hours of exposure to 45°C, whereas, in a 25°C treatment, the maximum stress protein induction was reached after four hours of exposure (Scheil et al. 2011). Furthermore, the activity of antioxidant enzymes (CAT and SOD) and levels of glutathione in *Helix aspersa* were measured at different time points during awakening process after aestivation. Results indicated differences in the glutathione levels but none in enzyme activities (Ramos-Vasconcelos and Hermes-Lima 2003). For further studies, it might be interesting to investigate different time points during heat exposure to get a more detailed pic-

ture of the physiological processes, especially of the antioxidant defense system, involved in the thermotolerance of terrestrial snails.

4.1 Conclusions:

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

List of symbols and abbreviations

CAT	catalase
DNA	deoxyribonucleic acid
FOX	ferrous oxidation xylenol orange method
GPx	glutathione peroxidase
GR	glutathione reductase
GSH	reduced glutathione
GST	glutathione S-transferase
H_2O_2	hydrogen peroxide
Hsp70	72 kDa heat shock protein
Hsps	heat shock proteins
$^{\cdot}O_2^-$	superoxide anion radical
$^{\cdot}OH$	hydroxyl radical
ROS	reactive oxygen species
SOD	superoxide dismutase

5. Acknowledgements:

Thanks go to Bärbel Baum for technical support and Simon Schwarz for discussion and help with the statistics.

Funding:

This research received no specific grant from any funding agency in the public, commercial or not-for-profit sectors.

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Kapitel 4: Einfluss verschiedener Temperaturen und Expositionszeiten auf die Wasserevaporation und die Hsp70-Induktion bei *Xeropicta derbentina* (Krynicki 1836).

A. Petschl, und A. Dieterich (2013): (Unveröffentlicht)

1. Einleitung:

Das im Sommer in Südfrankreich vorherrschende trockene und heiße Klima konfrontiert die dort lebenden Schnecken, wie zum Beispiel *Xeropicta derbentina* [Krynicki 1836], mit für Schnecken ungünstigen Bedingungen. Die zu mehr als 75 % aus Wasser bestehenden Schnecken (Machin 1964; Reuner et al. 2008) sehen sich durch die äußeren Bedingungen ständig der Gefahr des Austrocknens (Arad et al. 1998; Machin 1966) und der Gefahr der Überhitzung ausgesetzt. Besonders durch das Fehlen einer potenten Verdunstungsbarriere in der Haut und durch die eingeschränkte Mobilität der Tiere sind Anpassungen der Tiere an ihren Lebensraum vonnöten, die ein Überleben in diesem Habitat sicherstellen. Als primärer Schutz gegen Prädatoren und Verdunstung kann bei Landschnecken das Ausbilden einer Schale gesehen werden. *Xeropicta derbentina*, eine aus dem östlichen Mittelmeerraum eingeschleppte (Altena 1960; Aubry et al. 2005; Kiss et al. 2005) Art aus der Familie der Hygromiidae, ist bekannt dafür, auch unter trockenen, heißen Bedingungen zu überleben (Köhler et al. 2009; Troschinski et al. 2014). Sieht sich die Schnecke ungünstigen äußeren Bedingungen, wie zum Beispiel zu hohen Bodentemperaturen, ausgesetzt, versucht *X. derbentina*, diesen durch Erklettern vertikaler Objekte wie Pflanzen oder anderen Strukturen zu entgehen (Aubry et al. 2006). *X. derbentina* kann daher oft in größeren Aggregationen an der Vegetation gefunden werden und verweilt dort den Rest des Tages in sonnenexponierter Lage (Di Lellis et al. 2012). Als Schutz vor Verdunstung ist *X. derbentina* in der Lage, die Schalenöffnung und den Raum dahinter mit einem Epiphragma zu verschließen. Für das Überleben der Schnecken ist das Rückhalten von Wasser essentiell (Arad et al. 1998), da nach dem Verlust von zu viel körpereigenem Wasser rasch der Tod eintritt. Unklar ist indes, wie hoch der Wasserverlust bei *X. derbentina* durch ihre Schale und durch ein Epiphragma bei unterschiedlicher Expositionszeit gegen verschieden hohe Temperaturen ist.

Neben einer Schale ist es vor allem das unter Hitzestress eingreifende Hsp70-Schutzsystem, das *X. derbentina* dazu befähigt, selbst in sonnenexponierten, heißen Gebieten zu überleben. Das Hsp70-Schutzsystem kann bis zu einem gewissen Grad den durch Hitzeeinwirkung

anfallenden Schäden in der Zelle entgegenwirken. Die bereits untersuchte Reaktionskinetik des Hsp70-Schutzsystems (Kapitel 1 (Di Lellis et al. 2014; Eckwert et al. 1997; Feder & Hofmann 1999)) konnte bereits die Temperaturabhängigkeit des messbaren Hsp70-Levels belegen und Reaktionsgrenzen auch für *X. derbentina* aufzeigen (Köhler et al. 2009). Unklar bleibt indes, ob und wie sich der messbare HSp70-Level bei verschieden langer Expositionszeit gegenüber verschieden hohen Temperaturen ändert.

In dieser Studie lag der Fokus darauf, die Verdunstungsrate durch die versiegelte Schalenöffnung und die Schale bei verschieden lange einwirkenden Temperaturen zu bestimmen. Die Änderung im Gewicht der Schnecke kann dabei als Näherung für das verdunstete Wasser gesehen werden. Begleitend sollte der zeitliche Verlauf der Hsp70 Stressproteininduktion bei verscheiden hohen Temperaturen erfasst werden.

2. Material und Methoden:

2.1 Versuchstiere:

In der vorliegenden Arbeit wurde *Xeropicta derbentina* (Gastropoda, Hygromiidae) aus Südfrankreich (Modène, Departement Vaucluse) als Testorganismus verwendet. Die Tiere wurden im Juni 2012 auf einer Wiese, die vom Besitzer nicht kommerziell genutzt wird und nicht mit Pestiziden oder Düngemitteln belastet wurde, gesammelt. Die Hälterung erfolgte nach gängigen Hälterungsmethoden (Dieterich et al. 2012; Köhler et al. 2009; Scheil et al. 2012; Scheil et al. 2011).

2.2 Exposition im Wärmeschrank und Wasserverlustmessung:

Für die Exposition im Wärmeschrank wurden aus feinmaschiger Aluminiumgaze zylindrische Käfige gebaut (d=4 cm, h=6 cm), die verschlossen werden konnten. Je zehn Schnecken wurden gegenüber 20, 35, 40, 42 und 45 °C für jeweils 1,5; 2,5; 4,5; 6,5 und 8,5 Stunden exponiert. Wegen hoher Schwankungen bei Wasserverlustmessungen in einem Vorversuch (A. Petschl, Daten nicht dargestellt), wurde der Start der Wasserverlustmessungen im Hauptexperiment um 30 Minuten verzögert. Nach dieser Zeit stabilisierten sich die gemessenen Werte und die Expositionszeit wurde gestartet. Sie betrug dadurch entsprechend 1, 2, 4, 6 und 8 Stunden. Zur Ermittlung des Wasserverlustes wurden je zehn Schnecken pro Exposition in

jeweils einen Gazezylinder eingebracht und für 30 Minuten in den vortemperierten Wärmeschrank gestellt. Nach 30 Minuten wurde das Gewicht des Zylinders inklusive der Schnecken gewogen. Nach Ende der Exposition wurde erneut gewogen und die Differenz der Gewichte protokolliert. Jeder Versuch wurde dreimal wiederholt. Der Verlust an Gesamtgewicht wurde als mittlerer Wasserverlust von zehn Schnecken aufgefasst.
Zusätzlich wurden exemplarisch je zehn Tiere über längere Zeiträume gegen 20, 35 und 40 °C exponiert. Bei 20 °C wurden die Schnecken erneut nach 24 und 48 Stunden gewogen, bei 35 °C nach 24, 76 und 96 Stunden und bei 40 °C nach 24 Stunden. Diese Langzeitexpositionen unterstützten die linearen Regressionsfunktionen, die zur Darstellung des mittleren Wasserverlustes über die Zeit berechnet wurden, mit weiteren Daten.

2.3 Hsp70 Analyse:

Nach Ende der Gewichtsmessung wurden je Exposition zehn Schnecken individuell in flüssigem Stickstoff fixiert und zur späteren Analyse bei -20 °C aufbewahrt (Für Tiere, die gegenüber 42°C exponiert wurden, wurde keine Hsp70 Analyse vorgenommen). Die Probenaufarbeitung für die Hsp70 Analyse erfolgte gemäß Köhler et al. (2009) und der in Kapitel 1 dargelegten Methodik mittels SDS-PAGE und anschließendem Immunoblot. Die gefärbten Nitrozellulosemembranen wurden digitalisiert und mit Hilfe von E.A.S.Y Win 32 (Herolab, Heidelberg, Deutschland) ausgewertet. Zur Vergleichbarkeit der Daten wurden je Gel zwei Standards (*Theba pisana* [Müller 1774] Ganzkörperhomogenat) verwendet, mit deren Hilfe der relative Hsp70 Gehalt je Probe bestimmt wurde. Für Details der Methodik siehe Köhler et al. (2009) und Dieterich et al. (2012).

2.4 Statistische Auswertungen:

Die gewonnenen Werte wurden mit Hilfe von JMP 10.0 (SAS System, USA) auf statistische Unterschiede hin untersucht. Auf Normalverteilung wurde mittels Shapiro-Wilk-Test getestet. Da die Daten weitgehend nicht normalverteilt waren wurde im Anschluss ein Kruskall-Wallis-Test mit anschließendem Steel-Dwass post-hoc Test, der die Datensätze miteinander verglich durchgeführt. Die Signifikanzniveaus wurden mit $0{,}05 \geq p > 0{,}01$ = schwach signifikant (*), $0{,}01 \geq p > 0{,}001$ = signifikant (**) und $0{,}001 \geq p$ = hoch signifikant (***) festgelegt.

3. Ergebnisse:

3.1 Wasserverlustmessungen:

Betrachtet man die Gesamtheit der Daten, so ist eine Zunahme des mittleren Wasserverlusts mit steigender Expositionstemperatur zu beobachten.

Bei einer Temperatur von 20 °C war nach einer Stunde ein Gewichtsverlust von 0,64±0,195 % des Ausgangsgewichts zu verzeichnen. Nach acht Stunden konnte ein Gewichtsverlust von 0,909±0,429 % gemessen werden. Im Langzeitversuch mit 24 Stunde verloren die Schnecken gemittelt 1,626±0,742 % an Gewicht, bei 48 Stunden waren es 2,308±0,841 %.

Bei 35°C konnte nach acht Stunden ein Gewichtsverlust von 1,267±0,711 % und bei 24 Stunden bereits ein Gewichtsverlust von 3,598±2,555 % gemessen werden. Nach 96 Stunden betrug der Wasserverlust bereits 11,756±1,967 % des Ausgangsgewichtes.

Bei 40 °C konnte nach acht Stunden ein Gewichtsverlust von 1,376±0,698 % gemessen werden. Nach der 24 Stunden Exposition betrug er 3,747±0,430 %.

Wurde die Expositionstemperatur auf 42 bzw. 45 °C erhöht, wurden, verglichen mit den erzielten Messwerten bei 20 bis 40 °C, deutlich höhere Gewichtsverluste gemessen.

Bei 42 °C betrug der Wasserverlust nach einer Stunde bereits 1,826±064 %, während er nach acht Stunden 4,017±0,587 % betrug.

Bei 45 °C konnte in der ersten Stunde ein Gewichtverlust von 3,597±0,417 % gemessen werden. Nach acht Stunden belief sich dieser auf 10,118±1,06 % des Ausgangswertes (Abbildung 1).

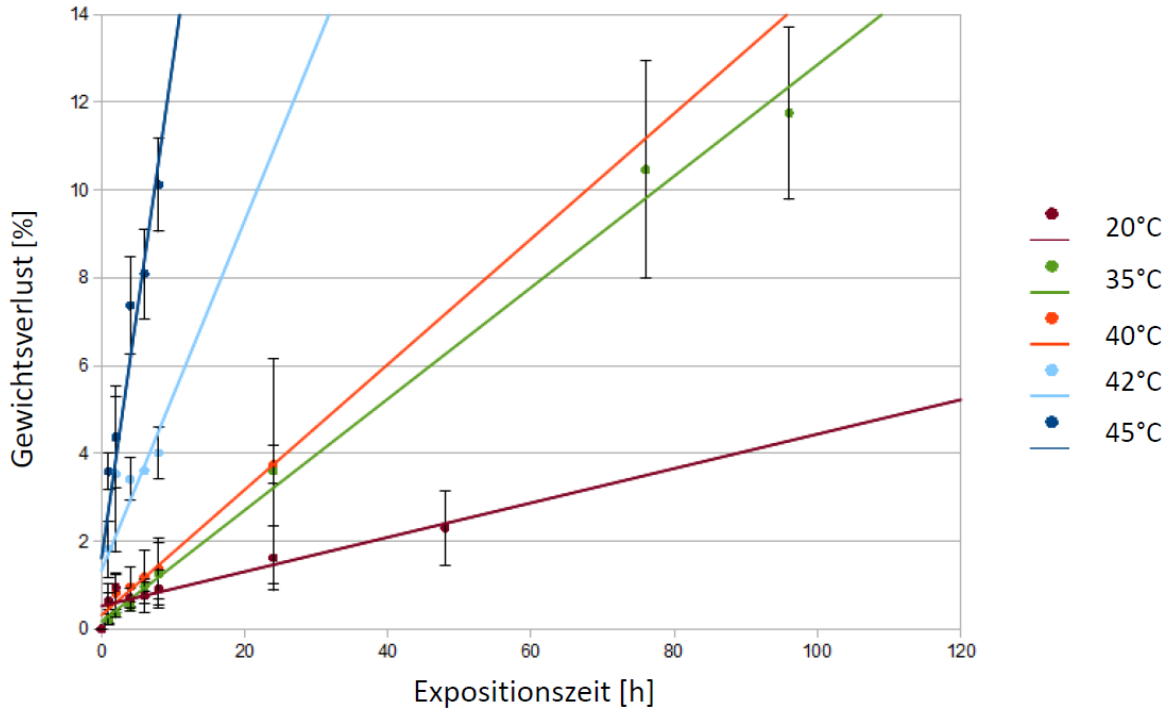

Abb. 1: Prozentualer Gewichtsverlust der Versuchstiere im zeitlichen Verlauf. Jeder Datenpunkt entspricht dem Mittelwert aus drei unabhängigen Messungen mit je 10 Versuchstieren mit zugehöriger Standardabweichung. Verändert nach Petschl (2013).

Die in Abbildung 1 dargestellten linearen Regressionsfunktionen geben für die betrachteten Expositionszeiten eine gute Näherung an. Bei längerer Exposition der Schnecken ist allerdings ein Übergang in eine Sättigungskurve anzunehmen. Ausgehend von der Steigung der linearen Fit Funktionen ist ein Erreichen eines Plateaus (Zustand in dem kein Wasser mehr verloren werden kann) bei 42 und 45 °C schneller zu erwarten als bei 20 °C.

In Abbildung 2 ist zur Veranschaulichung dieser Tatsache der prozentuale Gewichtsverlust pro Zeiteinheit für die gewählten Expositionstemperaturen aufgetragen.

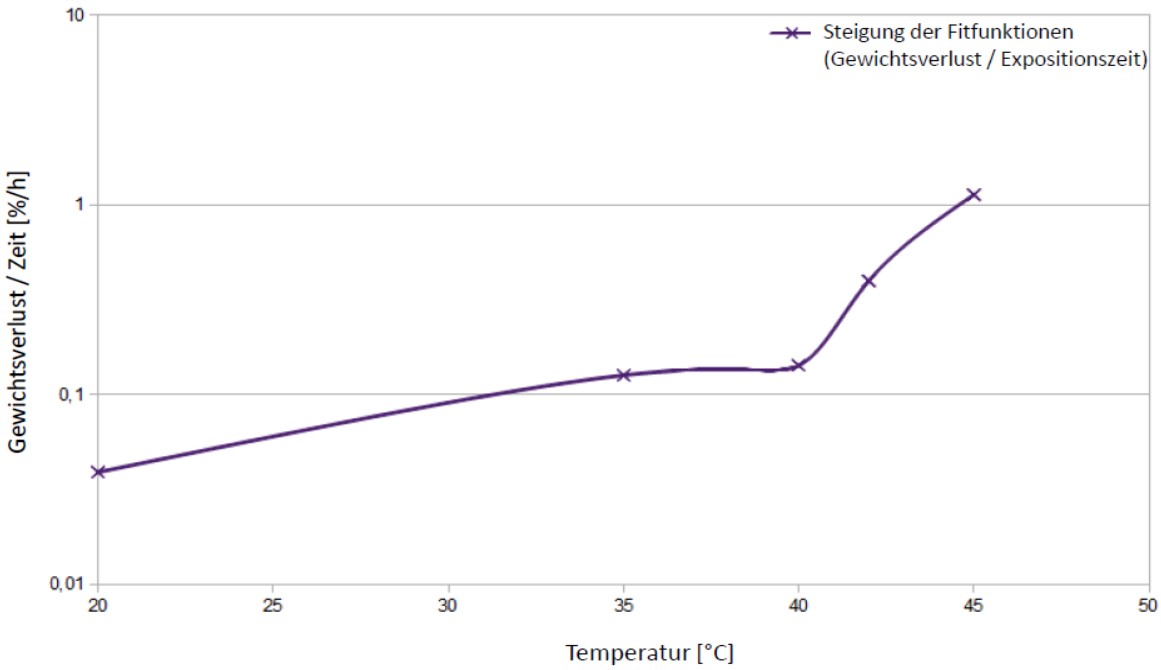

Abb. 2: Gewichtsverlust pro Zeit über die einzelnen Versuchstemperaturen. Verändert nach Petschl (2013).

3.2 *Hsp70 Analyse:*

Bei 20 °C Expositionstemperatur konnte ein leichter Anstieg der Hsp70-Werte mit zunehmender Expositionszeit bis 6,5 Stunden gemessen werden. Nach 8,5 Stunden fiel dieser wieder geringfügig ab. Bei den Expositionstemperaturen 35 und 40 °C konnten (bis auf 4,5 Stunden Exposition bei 40 °C) durchweg höhere Hsp70-Level gemessen werden als dies bei 20 °C der Fall war. Der höchste gemessene Hsp70-Level lag bei 35 °C bei einer Expositionszeit von 2,5 Stunden (0,84±0,22). Bei einer Expositionstemperatur von 40 °C konnte bereits nach 1,5 Stunden mit 0,73±0,24 der höchste gemessene Hsp70-Level für diese Temperatur ermittelt werden. Sowohl bei 35 °C als auch bei 40 °C konnten, nach einem Rückgang von Hsp70 bei einer Expositionszeit von 4,5 Stunden, ein erneuter Anstieg im Hsp70-Level beobachtet werden. Bei einer Expositionstemperatur von 45 °C konnten bei sämtlichen Expositionszeiten (mit Ausnahme von 4,5-stündiger Exposition bei 40 °C) die niedrigsten Hsp70-Level gemessen werden. Diese lagen meist schwach signifikant bis signifikant unter denen der anderen Expositionstemperaturen (Abbildung 3).

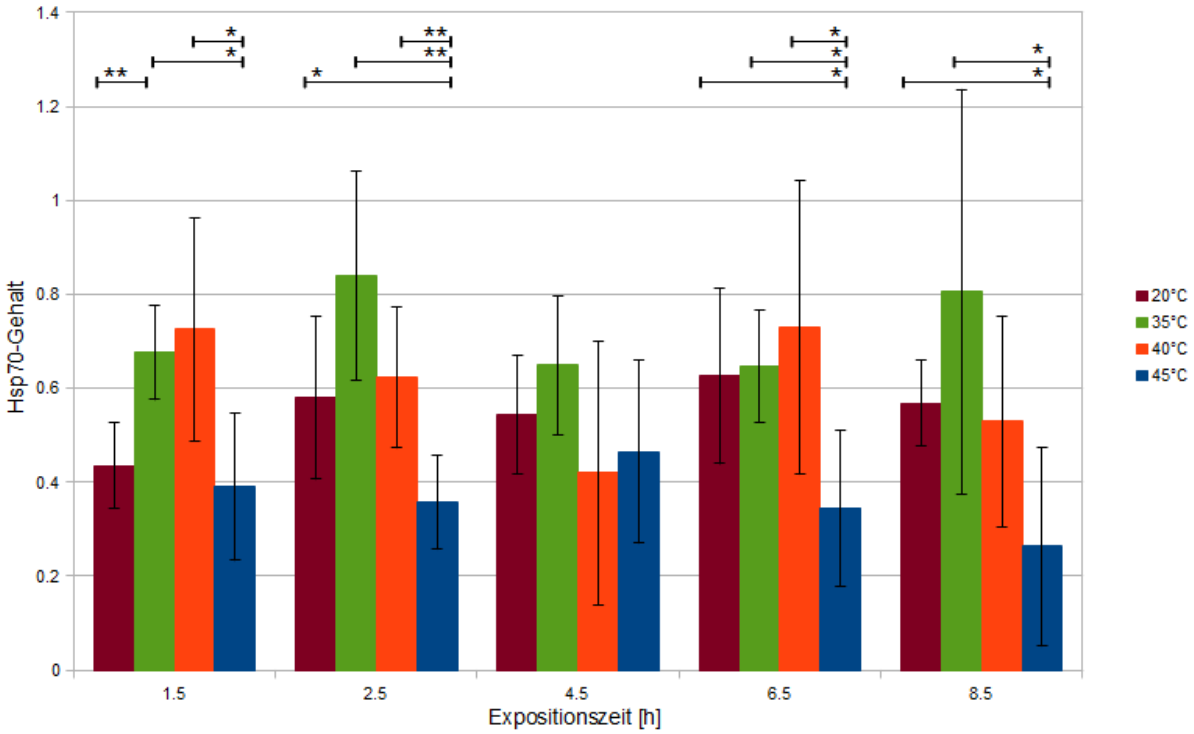

Abb. 3: Hsp70-Level in Abhängigkeit von der Expositionszeit bei verschiedenen Expositionstemperaturen.

4. Diskussion:

In der vorliegenden Studie konnte klar der Einfluss der Temperatur und der Expositionszeit auf den Wasserverlust von *X. derbentina* belegt werden. Dabei konnte ein allmählicher Wasserverlust bei 20 °C Umgebungstemperatur gemessen werden, der bis zur maximalen Expositionszeit näherungsweise linear verlief. Bei höheren Expositionstemperaturen von 35 und 40 °C erfolgte ein deutlich höherer Wasserverlust, der jedoch verglichen mit Literaturangaben (Arad et al. 1998) noch in einem physiologisch tolerierbaren Rahmen war. Bei Erhöhung der Expositionstemperatur auf 42 und 45 °C konnten bereits nach wenigen Stunden der Exposition Wasserverluste protokolliert werden, die diejenigen von 35 und 40 °C bei gleicher Expositionszeit um ein Mehrfaches übertrafen (8 h bei 35 °C = 1,267±0,711 % Wasserverlust, 8 h bei 40 °C = 1,376±0,698 % Wasserverlust, 8 h bei 42 °C = 4,017±0,587 % Wasserverlust und 8 h bei 45 °C = 10,118±1,060 % Wasserverlust). Dies deutet auf ein Überschreiten der über diesen Zeitraum maximal tolerierbaren Umgebungstemperatur hin. Wie bereits von Dittbrenner et al. (2009) beschrieben lag die Immobilität von *X. derbentina* bei einer Exposi-

tion gegen 48 °C zwischen 0 und 30 % (abhängig von der untersuchten Population). Da in diesem Experiment in beiden Teilversuchen nur überlebende Tiere verwendet wurden (eine Rückziehbewegung durch Berühren mit einer Sonde wurde als Lebenszeichen gewertet), kann die Reaktionsobergrenze von *X. derbentina* bei etwa 45 bis 48 °C bei einer Expositionszeit von bis zu acht Stunden angesiedelt werden. Dies spiegelt sich auch in den Ergebnissen der Hsp70-Analyse wieder. Bei Umgebungstemperaturen von 20 °C ist ein mittlerer Hsp70-Level zu finden, welcher der Schnecke bei physiologischen Zellabläufen hilft, wie zum Beispiel bei der Neusynthese und korrekten Faltung von Proteinen. Er kann als konstitutiv angesehen werden. Bei Erhöhung der Umgebungstemperatur und, damit bedingt, mit der Erhöhung von falsch gefalteten Proteinen in der Zelle, wird der Hsp70-Level durch Neusynthese weiterer Hsp70 Proteine erhöht. (Befunde für 35 und 40 °C). Wird die physiologisch tolerierbare Obergrenze erreicht, kommt es zu einem Einbrechen des Hsp70-Levels (bei 45 °C), was als Überlastung des Systems angesehen werden kann. Die tendenziell niedrigeren Hsp70-Level nach 4,5 Stunden Expositionszeit könnten darauf hinweisen, dass an einem normalen Tag im natürlichen Habitat die Maximaltemperaturen meist nur für kurze Zeit erreicht werden und die Schnecke somit einen energetischen *Trade-off* eingeht, um nicht zu viel Energie in die Produktion von Hsp70 zu investieren. Da dies durch die vorliegenden Daten jedoch nicht geklärt werden kann, bleibt diese Erklärung spekulativ.

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Kapitel 5: Energy metabolism in the Mediterranean land snail *Xeropicta derbentina* measured by direct calorimetry.

U. Fischbach, A. Dieterich, F. Kolarov, D. Wharam, G. Gauglitz, U. Gärtner, H.-R. Köhler (2015): (Unpublished manuscript)

Abstract:

Heat dissipation of *Xeropicta derbentina*, a Mediterranean helicoid snail, was measured in individuals of two different size groups and at temperatures of 20°C and 30°C for 7 days. Two distinct levels of thermal output for all specimens were recorded during non-estivating phases, corresponding to temporally 'active' and 'inactive' states. The integral consumption of energy was found to decrease for small individuals at the higher test temperature. For both size groups the integral fraction of expended energy associated with activity decreased at 30°C, for large specimens, however, an increased time of activity was recorded. The observation of regular thermal fluctuations in inactive snails demonstrated remarkable ranges of heat production for *X. derbentina*. A frequency analysis of these inactive phases highlighted the existence of increased frequency components for the higher test temperature. Additionally, an approach has been made towards an integrative physiological model of the investigated species by utilizing the results of direct calorimetry, oxygen consumption, and geometrical data of *X. derbentina* to combine these methods to a more comprehensive model.

1. Introduction:

Xeropicta derbentina (Krynicki 1836) (Gastropoda, Hygromiidae), a terrestrial, pulmonate snail, is abundant in the southern part of France, where it is often found in areas with only scarce vegetation. Originating from the Eastern Mediterranean, *X. derbentina* has first been recorded there in 1949 (Kiss, Labaune, Magnin, & Aubry, 2005; Van Regteren Altena, 1960) presumably because being introduced in southern France during the Second World War. Although this species has to cope with hot and dry conditions in its habitat it has been spreading northwards ever since. This annual species reaches adult shell sizes up to 15 mm. Its shell morphology is mainly dominated by a white colour (Dieterich et al., 2013) but a

smaller percentage of individuals also shows a darkly pigmented banding (Dieterich et al., 2014). In July and August southern France air temperatures few centimetres above the ground reach values up to 45°C and values over 55°C on the soil surface, which constitutes hostile conditions for snails. As their water content is higher than 75% (Reuner, Brümmer, & Schill, 2008), desiccation and overheating poses a major threat to them. The frequently observed behaviour of climbing vertical objects, like blades of grass but also cars, is not only believed to be a mechanism of protection from unfavourable conditions on the soil ground but also the reason for the rapid spread of this snail species in southern France (Aubry, Labaune, Magnin, Roche, & Kiss, 2006). At above-ground positions snails experience cooler conditions and form clusters of many temporally 'inactive' individuals that quickly build up an epiphragm to reduce water loss through the aperture. Thus, the overall energy balance of *X. derbentina* is characterized by the trade-off between evaporative water loss needed for convective cooling and the demand to minimize the total water loss.

From a previous study it is known that elevated temperatures lead to a significant decrease in oxygen consumption for mid-level sized individuals of *X. derbentina* (Fischbach et al., submitted) with intact capability to physiologically counteract thermal stress. Therefore, an approach has been made to further investigate the effects of metabolic adaptations at higher temperatures by direct calorimetry. Complementary to this information a physiological model will be presented that effectively combines direct calorimetry and respirometry and, thereby, reveals further physiological parameters of this species.

The metabolic rate (energy demand) of living organisms can be captured indirectly by measuring the rate of oxygen consumption or directly from the rate of heat production. All processes that are involved with energy turnover produce heat and, therefore, direct calorimetry gives an integral view of all effects taking place in the sample, including side effects, resulting in an integral thermic signal. Only in cases of complete aerobic metabolism data on oxygen consumption can directly be transformed into metabolic rates by using a fixed oxy-calorific equivalent (also called 'calorimetric equivalent') depending on the composition of food, i.e. carbohydrates, lipids, and protein (Gnaiger, Shick, & Widdows, 1989). The complex relationship between energetics and oxygen consumption has been described by Gnaiger (1980).Thus, the use of an oxy-calorific equivalent requires 'a detailed thermochemical analysis of the biochemical pathways' to allow for an interpretation in terms of ATP-turnover and depends on whether the organism is in aerobic or anaerobic state. In the latter the ca-

loric equivalent of ATP-turnover is reduced by up to 30% for the fermentation of glucose. In the anaerobic state of an animal the measurement of metabolic activity in terms of oxygen consumption would result in a significant discrepancy to the true metabolic activity since it only captures aerobic rates of energy turnover. The use of direct calorimetry is, therefore, essential for the study of anaerobic metabolism of animals that are frequently exposed to low oxygen conditions. Peakin (1973) additionally stated that for hygrophilous animals direct calorimetry has the advantage of avoiding physiological stress due to desiccation by providing more natural conditions.

Direct calorimetry has been applied on a wide range of biological systems such as microbial cultures but also facilitates the monitoring of complete ecological entities like forest, sediments, and lakes, and their interaction with adjacent units due to toxicological interference. An overview of investigated fields of calorimetric experiments as well as the different calorimetric equipment is given in Drong and Lamprecht (1993). Direct calorimetry measurements of poikilothermic animals have been reported among others for honeybee workers (Fahrenholz, Lamprecht, & Schricker, 1989) and some reptiles (Lamprecht & Matuschka, 1985).

Among the few researchers that investigated snails with direct calorimetry are Becker and Lamprecht (1977). They identified oxygen consumption and heat production in the snail *Biomphalaria glabrata* to be significantly higher if the animals were infected with the parasite *Schistosoma mansoni*. Additionally, the comparison of transformed values of oxygen consumption with values of heat production showed that the metabolism of infected and uninfected individuals was always in the aerobic state.

In a further study Becker (1980) demonstrated that uninfected animals (1.02 µW/mg wet weight) did not show a higher heat production than infected individuals (0.96 µW/mg). But when locomotion was specifically blocked the thermal output for infected snails (0.3 µW/mg wet weight) was significantly higher than for uninfected specimens (0.22 µW/mg). Becker (1980) concluded that “infected snails restrict their movements to compensate for the higher basal metabolism”. The comparison of uninfected individuals revealed that the activity of *B. glabrata* requires approximately 80% of the total energy expenditure. A similar impact of movement on the calorimetric output has been reported by Pamatmat (1983) in the mussel *Ischadium demissum demissum*, which revealed extreme fluctuations of metabolic rates with time and between individuals as well. The author showed that these variations

declined if the movement of the mussels' shell valves was blocked. Furthermore, the orientation of the animal resulted in a measureable difference in heat output. Long-term measurements indicated that metabolic rates became cyclic after a certain period of adaptation inside the chamber of the calorimeter. Interruptions of this state immediately resulted in unpredictable fluctuations again.

Lamprecht and Becker (1988) presented calorimetric measurements in combination with endoscopy observations of a snail (species not mentioned). They further raised the discussion whether temporally observed phases of high calorimetric output corresponded to high metabolic activity or if this was just the result of the snail's locomotion. The authors clearly demonstrated that small ripples in the calorimetric signal indicate movements of the snail, whereas phases of a quick increase and a subsequent slow and almost linear decrease are the result of metabolic responses that happen periodically. Contradictory to the findings of Becker (1980) locomotion only resulted in these small ripples and activity was not responsible for 80% of overall energy turnover.

Pamatmat (1978) reported measurements of heat production and simultaneous oxygen consumption for the sea snail *Littorina irrorata* and the fiddler crab *Uca pugnax*. During 16 h of measurements an unproportional decrease of oxygen uptake and heat production of both species was observed resulting in an increased oxy-calorific coefficient, which has been used as an indicator for the onset of anaerobic metabolism. Above a given level of oxygen consumption, however, the commonly used coefficient was obtained.

A similar disproportion between heat production and oxygen consumption has been reported for some marine bivalve molluscs showing too little oxygen consumption to represent total metabolism, whereas others revealed ratios that were in close accordance to the theoretical values proposed for the turnover of common food substances (Hammen, 1979). The author additionally showed that for at least four species of bivalves the rate of heat production exceeded the equivalent one for oxygen consumption even at periods of maximum respiration (Hammen, 1980). This leads to an additional question: which fraction of total metabolism is aerobic and which is anaerobic at a certain time of anoxia since phases of maximal respiration are no longer supposed to be periods of peak metabolism.

The involvement of possible anaerobic metabolic pathways before and during a short period of estivation of the land snail *Oreohelix* has been investigated by Rees and Hand (1990). Heat production during the non-estivating phase revealed two distinct metabolic states, the

'standard' and 'active' metabolism, which differed by a factor of 2-3 from one another. By simultaneous measurements of gas exchange *Oreohelix* was found to have a fully aerobic metabolism in both states which depends primary on the turnover of carbohydrate. Rapid transition into estivation took place within 4 days resulting in six fold reduced respiration rates, which is believed to be an 'adaptation to survival in desiccating environments'.

In order to establish a model that enables narrowing down additional physiological parameters needed for a comprehensive description of *X. derbentina*'s metabolism the current study is investigating the metabolic rate of *X. derbentina* at different ambient temperatures. The resulting information is used in combination with previous measurements of oxygen consumption and geometrical data of *X. derbentina*. Therefore, our data on energy metabolism is indispensable to draw up an integrative model on the complex physiology taking place in this snail species.

A particular challenge for this analysis of metabolic rates is the rather small size of the animal, which hampered the use of standard calorimeters and, therefore, required a very precise measurement system. This, in turn, facilitated the analysis of recurring and stable frequency patterns that were observed for individuals in their 'metabolically' inactive state. Our high precision system could be used to analyse these fluctuations as well as alternating phases of activity and inactivity of this species in a very detailed way. Additionally, long-term measurements were performed to examine the physiological reactions of the animals when their metabolism passes into estivation.

Thus, using individuals of different sizes exposed to different temperatures, this study aims at answering the following questions:

- How large are the differences between standard/inactive and active metabolic states and what is the factor between them?
- To what extent is the total metabolic energy turnover as well as its division into active and inactive phases influenced by the animals' size and by ambient temperature?
- How much time do individuals spend in standard and active metabolic states and does this alternation follow a regular cycle?
- How is the frequency of thermal output for individuals at different metabolic states influenced by the animals' size and by ambient temperature?

- Does the combination of calorimetry, oxygen consumption, and geometrical data deliver reliable information on additional physiological parameters, such as the animals' breathing frequency?

2. Material and Methods:

2.1 Test organism:

In the present study *X. derbentina* (Krynicki 1836), a hygromiid land snail, was investigated. All specimens were collected from a meadow close to Modène, department Vaucluse, southern France (N44°6.055' E5°7.937'). The owner neither applied any pesticides to the landsite nor used it as farming land. All collected snails were transferred to Germany and kept well hydrated and fed at ambient temperature and humidity in a terrarium. In this study only white shelled individuals were used (in accordance with the coloration classification patterns used by (Köhler et al., 2009)). Due to geometrical limitations of the calorimeter chamber snails larger than 10 mm could not be analysed. Therefore, two size groups were tested, called 'small' and 'large' snails. In the two groups the size ranged from 5.3-5.8 mm and 8.5-8.8 mm of diameter, respectively, measured at their largest cross-section. Additionally, the wet weight of all specimens was determined at the beginning of each measurement. From both size groups n=3 distinct individuals were tested for a period of 7 days at 20°C and 30°C, respectively. To allow for the observation of complete estivation of *X. derbentina* two additional long-term measurements of n=1 large individual for each temperature were conducted that each lasted for 24 days. The accomplishment of all measurements was, therefore, rather time-consuming and resulted in approximately 5 continuous months of measurements.

2.2. Calorimetry:

Measurements of heat dissipation due to metabolic energy turnover were accomplished with a "Thermal Activity Monitor" (TAM) 2277 manufactured by Thermometric AB (Jarfalla, Sweden), a micro calorimeter based on the principle of isothermal titration calorimetry. The apparatus consisted of two identical, highly conductive and adiabatically coated cells. The first cell served as the sample cell whereas the second one was used as a reference cell to compensate outer thermal disturbances and thereby increasing the accuracy of the device.

For calorimetry measurements it was important use a reference medium with a heat capacity of maximum similarity to the measured animals. In the present study water has been used as reference medium. For the calculation of the required water mass the specific heat capacities of the shell and the soft tissue of *X. derbentina* at different temperatures has been used, all of which was obtained in preceding studies (data unpublished). Additionally, previous measurements of the water content and mass distribution of shell und soft tissue of the snails (data unpublished) delivered a proportion of approximately 2.3 for the mass of soft tissue to the mass of the shell. These results have been used to account for the different heat capacities of shell and soft tissue material. Therefore, each individual snail was weighted and the masses of shell and soft tissue were calculated subsequently. Based on this ratio and the heat capacity of each component a capacity of the mixture was calculated and the equivalent amount of water with the same capacity was used as a reference for each measurement.

The amount of diffusive water loss for the snails during the time of each measurement has not been considered since diffusion out of the measurement cell was negligible and, therefore, a saturated atmosphere can be assumed. Furthermore, the evaporation and condensation processes were taking place in both the measurement cell and the reference cell, resulting in effectively no influence on the overall heat signal of the measurement due to water loss. The resolution of the calorimeter was approximately 0.4 µW, and noise was in the range of about 40 nW.

2.3 Separation of active and inactive energy turnover:

To distinguish between ‘active’ and ‘inactive’ states of metabolism thresholds have been defined for each measurement separating these two states. Since most of the recorded data followed an asymptotical course in the first days, the definition of a constant threshold was found inappropriate. Therefore, active phases/peaks were identified and separated block by block and the corresponding starting and end points were connected by a straight line. The integral below this resulting ‘baseline’ energy course was defined to be ‘constitutive’ and thus part of the inactive state. Subsequently, active and basal metabolic energy turnover were related to the overall energy turnover using the software package MATLAB R2013b (Mathworks, Natick, MA, U.S.A.).

2.4 Statistics:

Due to the long time of measurements required for each individual only a small sample size could be realized. Therefore, statistical tests are not applicable to evaluate significances. Whenever statistical data are presented for a particular time series of a measurement, the values are indicated as boxplots showing 25%, medians and 75% quartiles.

3. Results:

3.1 Seven-days measurements:

3.1.1 Time course of measurements:

Figure 1 exemplarily shows the results of the 7-days-measurements for one specimen of each size group and temperature. All results displayed in Figure 1 show two characteristic levels of thermal output that will be referred to as 'active' and 'inactive' states of snails. During measurements A, C and D the snails were active from the beginning of each measurement and show a much higher initial output than in measurement B, where the snail was inactive at most of the time.

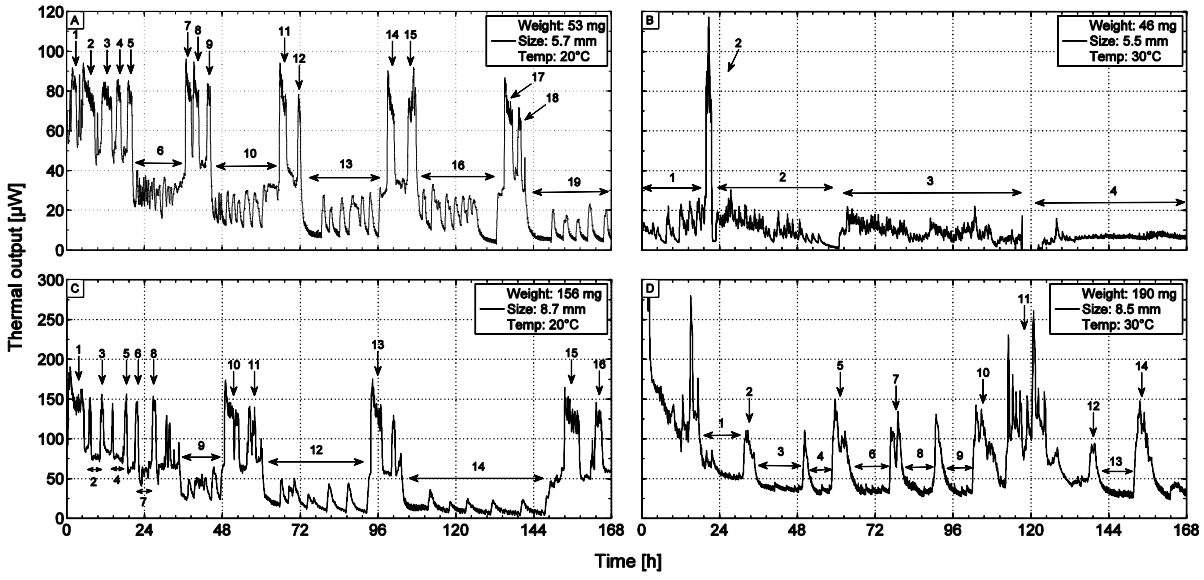

Fig. 1: Thermal output of an exemplary selection of 7-days-measurements for one specimen of each size group and temperature. Presented measurements are 'small2' at 20°C (**A**), 'small1' at 30°C (**B**), 'large2' at 20°C (**C**), and 'large3' at 30°C (**D**).

The median values and the distribution of the selected active and inactive time intervals from Figure 1 (numbered arrows) are given in Figure 2. The time intervals were chosen in a way that they accurately represent the levels of active and inactive thermal output. Intervals that were too short or rather noisy were excluded. For small snails (A, B) the active state results in approximately 4-5 times the thermal output compared to the inactive state regardless of the temperature. For larger specimens (C, D), however, the ratio between active and inactive thermal output differs for 20°C and 30°C, showing maximum ratios of 6-7 at the lower temperature but only a maximum ratio of 5 at the higher temperature.

At 20°C mean values for the active metabolic state vary between 70-85 µW and 120-145 µW for small and large animals, respectively, and 15-25 µW and 15-75 µW at the inactive state for small and large animals, respectively. At 30°C the mean values for the active state of the large animals range from 85-110 µW. A comparison to the small specimen is inappropriate since measurement B revealed only a single active phase with a mean value of 80 µW. For the inactive state values between 8-15 µW and 30-55 µW were reached for small and large animals, respectively.

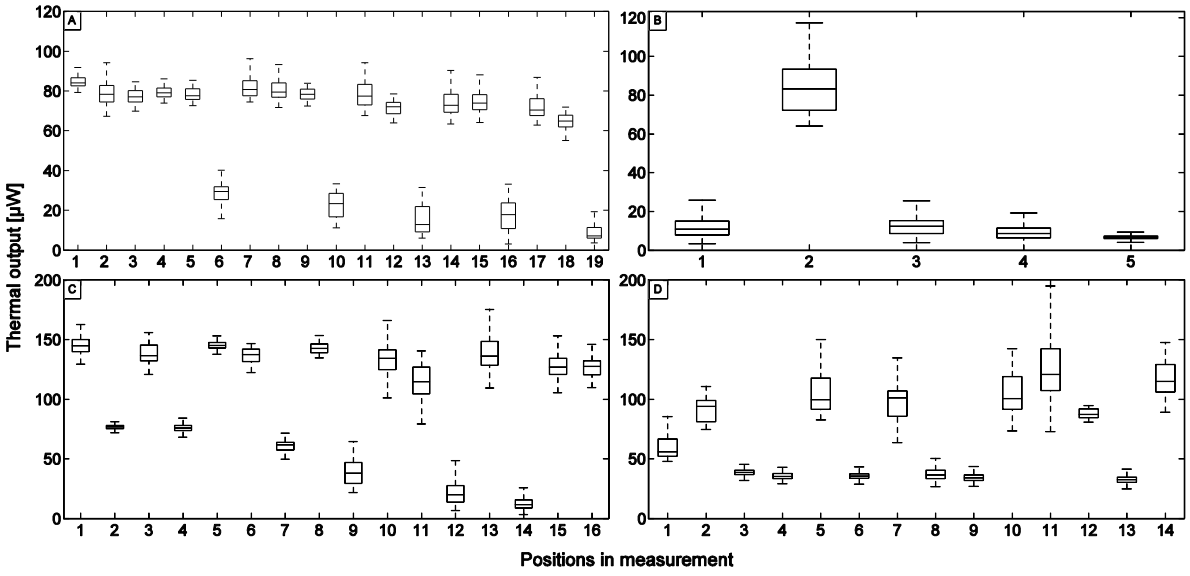

Fig. 2: Boxplots of the highlighted active and inactive phases in Figure 1.

Figure 1 and 2 additionally show that the median values of the inactive states are asymptotically decreasing over time. For measurements A and C a similar, yet smaller, decline can be seen for the active phases. It was generally observed that the measurements at 20°C clearly

show this kind of behaviour, whereas it could not be seen that clearly for measurements at 30°C.

Particularly for the larger specimens (Figure 1, C and D) the overall time courses of all measurements generally show lower 'active' levels at higher temperatures. In contrast to this, after 2-3 days, when the asymptotical decrease has settled, the 'inactive' metabolic level was higher at the higher temperature.

3.1.2 Total energy turnover and the division into active and inactive states:

The power output of the calorimeter was integrated numerically by the trapezoid-rule with Excel 2010 (Microsoft Inc., Redmond, WA, U.S.A.) to obtain data for energy turnover of *X. derbentina* measured by its heat production.

The overall energy turnover for all tested snails is given in Figure 3 for both temperatures. In measurement 'small3' at 20°C the snail had only three active phases, which is one reason for the lower total energy consumption. Furthermore the calorimeter needed to get readjusted by +10 µW, which is another small source of uncertainty in this measurement. Measurements 'small1' and 'small2' at 30°C also showed only very little phases of activity, resulting in the likewise low total energy turnover. Additionally, measurement 'small3' at 30°C produced an asymptotical decrease in thermal output during the first day, which was not present at the other measurements for small specimens. Hence, the smaller specimens show a rather high variance in the data.

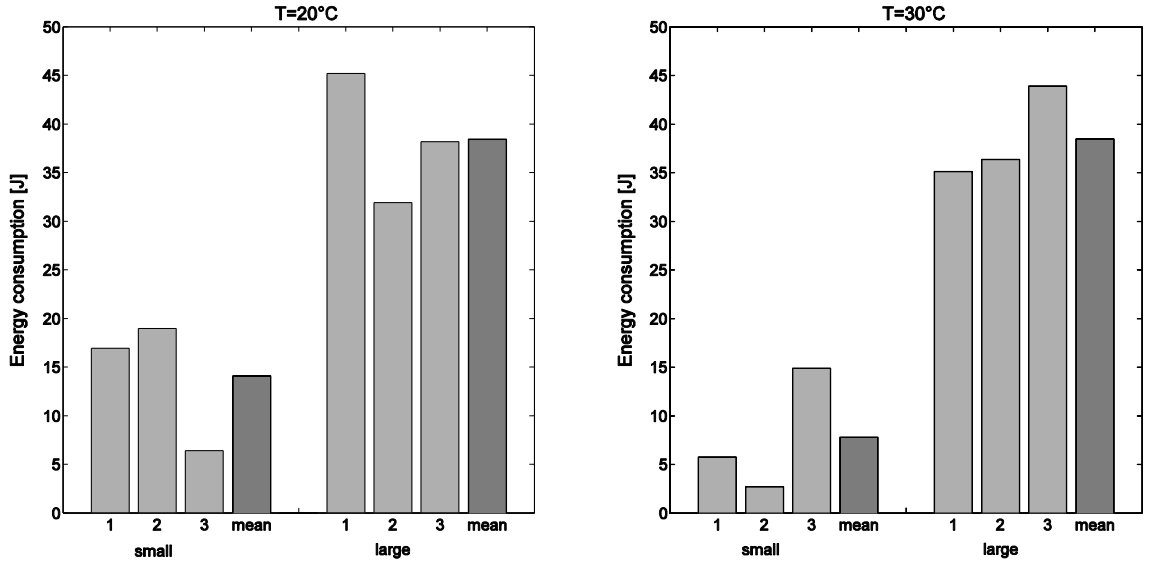

Fig. 3: Total energy turnover for all tested snails calculated by integration for the temperatures 25°C and 30°C. The darkened bar represents the mean value of each block.

The mean values at both temperatures for each size group show that energy consumption is similar for large snails but seem to decrease for small snails when temperature is raised from 20°C to 30°C. The total energy consumed was 2.7 and 4.9 times higher in large animals compared to small ones at 20°C and 30°C, respectively.

To account for the mentioned asymptotical decrease in thermal output within the first day of measurement we calculated integrals for data recorded at t>1d. The resulting means, however, were very similar to the ones displayed in Figure 3.

The separation of 'active' from 'inactive' energy turnover is given in Figure 4. From the mean values, on top of every group, it can be seen that the energy consumption to maintain the active state generally decreases for both small and large animals when temperature is increased from 20°C to 30°C. The extent of this decrease is more distinctive for the small specimen.

Small snails show a higher fraction of active state energy than large snails at 20°C, whereas at 30°C large snails have a little higher active energy demand than the small snails.

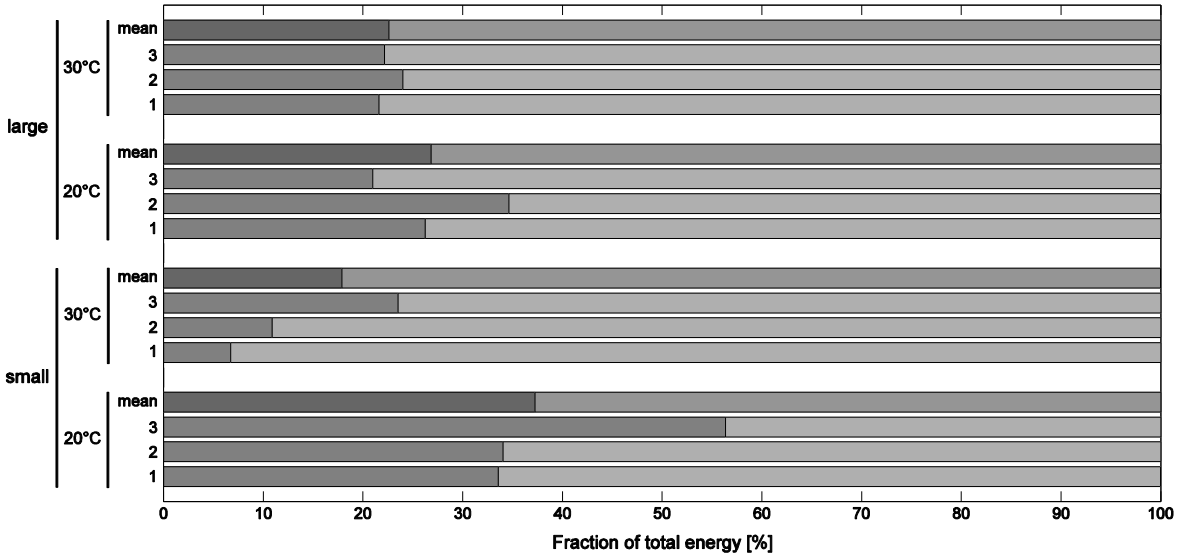

Fig. 4: Division of active and inactive energy turnover (standard metabolism) for both size groups and temperatures. Dark bars represent the active parts, pale bars represent the inactive parts. The upper stacked bar shows the mean value of each block.

3.1.3 Phases of active and inactive metabolism over time:

On the basis of the aforementioned definition the calculation of the time intervals corresponding to 'activity' and 'inactivity' was performed. The results on time spent in 'activity' and 'inactivity' are given in table 1.

Table 1: Time fractions of activity and inactivity for all measurements.

[% of total time]		**20°C**			**30°C**		
		1	**2**	**3**	**1**	**2**	**3**
small	active	22.4	34.4	26.5	1.2	1.0	39.1
	inactive	77.6	65.6	73.5	98.8	99.0	60.9
large	active	34.4	36.9	27.7	30.7	23.1	31.7
	inactive	65.6	63.1	72.3	69.3	76.9	68.3

With reference to total measurement time larger specimens showed longer times of activity at 20°C. From an energetic point of view the aforementioned results followed a contrary pattern, namely a lower 'active metabolic' fraction of total energy demand for larger animals at 20°C. There can be two reasons for this: a baseline shift towards a higher metabolic level and, secondly, a real decrease of the active metabolic energy level fraction.

The 'active time' fractions for 30°C basically show the same relation but, due to measurements 1 and 2 of the small animals, in which almost no activity at all could be observed, no comparison is drawn. Comparing the time of activity for the large specimens at 20°C to 30°C it is obvious that this trend declined again for higher temperatures.

A comparison of the means of each parameter combination and the corresponding relative change of the time spent in 'activity' and 'inactivity' is given in table 2. The overview shows that small animals approximately halve their time spent in 'activity' and, therefore, react more sensitive to an increasing temperature. The large individuals are only reducing their 'activity' by roughly 14%.

Table 2: Means of each parameter combination and relative change of the time spent in 'activity' and 'inactivity'.

[% of total time]		**20°C**	**30°C**	**change**
		average	**average**	**%**
small	active	27.8	13.8	-50.4
	inactive	72.2	86.2	+19.4
large	active	33.0	28.5	-13.6
	inactive	67.0	71.5	+6.7

3.2 Long-term measurements:

Figure 5 shows the results of the long-term measurements for a single large snail at 20°C and 30°, respectively. At 20°C (A) the individual showed a very regular pattern of inactive phases followed by shorter active metabolic peaks that were 2.5-5.5 times higher than standard metabolism. The investigated snail at 30°C (B) showed a very distinct activity pattern in the first two days. After day 2, even in these very inactive phases, a distinct oscillating behaviour in heat output can be seen with an amplitude of approximately 20 µW ranging from 20 to 40 µW. As in Figure 1 (A,C,D) both long-term data sets displayed a very slow decrease of thermal output with time, converging asymptotically towards a minimum that was reached at approximately 8-9 days for A and at about 2-4 days for B when the snails may enter estivation.

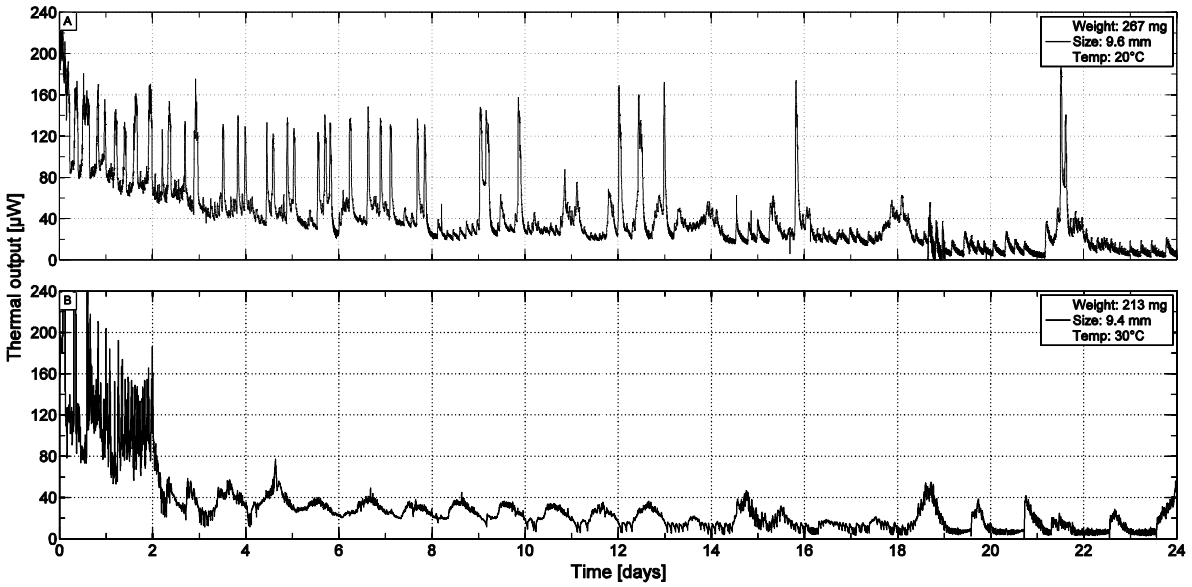

Fig. 5: Thermal output of the two long-term measurements of 24 days for one large specimen at both temperatures 25°C and 30°C.

3.2.1 Frequency patterns:

In order to closer investigate the observed frequency patterns of all measurements the signals were transformed and *decomposed*(??) into its frequency components (discrete Fourier transformation, DFT) by a fast Fourier transformation (FFT) algorithm using the software package MATLAB R2013b (Mathworks, Natick, MA, U.S.A.). The analysis concentrated on one measurement for each size and temperature. The course of metabolic heat output for every measurement was largely characterized by two patterns: a regular oscillation in the inactive state and a much lower frequency of recurring active phases. Therefore, the frequency analysis had to be carried out separately for these two regions (Figure 6 and 7).

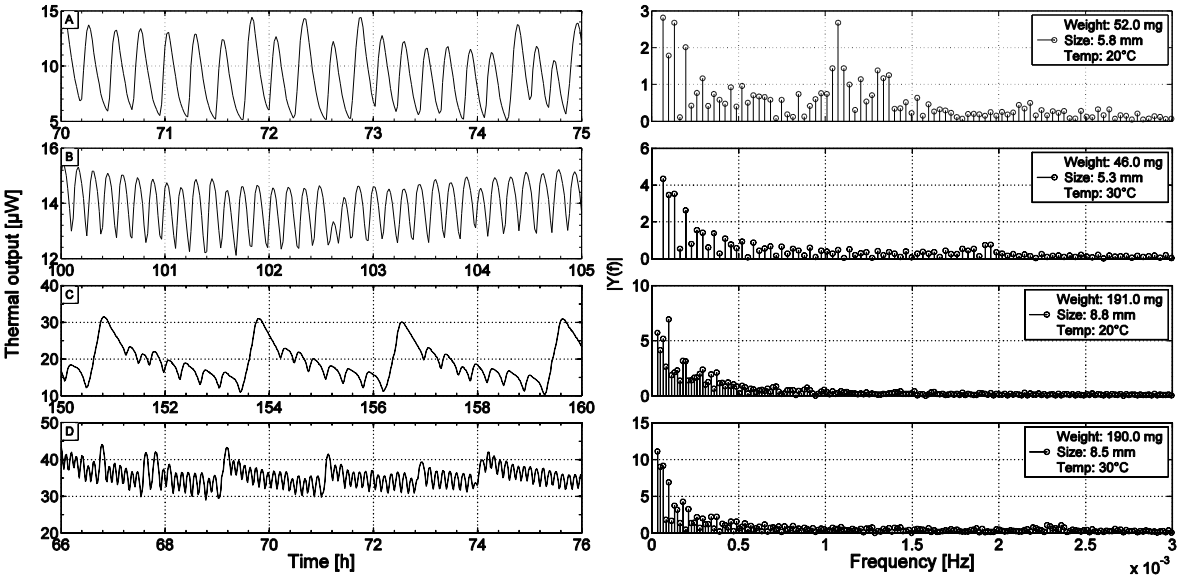

Fig. 6: Frequency analysis of an inactive phase for one specimen of each size group and temperature. Corresponding to the naming in Figure 1 and 3, presented measurements are 'small1' at 20°C (A), 'small3' at 30°C (B), 'large1' at 20°C (C), and 'large3' at 30°C (D).

Figure 6 shows the thermal output of a different selection of four individuals in time sections corresponding to 'inactivity'. The number of peaks for the inactive state are 22 (A), 35 (B), 25 (C), and 63 (D) ranging time-wise between 0.17-0.28 h (A), 0.1-0.17 h (B), 0.23-0.65 h (C), and 0.1-0.23 h (D). The mean distance between the peaks was 0.235 h, 0.145 h, 0.394 h, and 0.127 h for measurements A, B, C, and D, respectively. The frequency analysis showed the existence of larger frequency components for higher temperatures. For specimen A (small, 20°C) dominant frequency components at about 1.1-1.4E-3 Hz with peak-to-peak amplitudes of 2.5-5.3 µW could be detected, whereas for specimen B (small, 30°C) the major frequency increased to about 1.9E-3 Hz with lower amplitudes of 1.5 µW. Measurement C (large, 20°C) shows the superposition of two frequencies, a lower one with 1E-4 Hz and a higher component of 7.2-8.9E-4 Hz. A similar overlay is found for specimen D (large, 30°C), with frequencies of 1.3-1.7E-4 Hz and 2.2-2.4E-3 Hz, both being higher than the ones found for the equally sized animal at the lower temperature.

Figure 7 shows the thermal output of the previous examples for a longer time span of 24-120 h (day 2 to end of day 5) with their corresponding frequencies. The repeating phases of high metabolic activity are clearly visualized occurring with frequencies in the range of 1-1.2E-5 Hz, 1.8-7.7E-5 Hz, 1.6-4.7E-5 Hz, and 1.6-3.1E-5 Hz for measurement A, B, C, and D,

respectively. There were even lower components, which were, however, related to a very slow oscillation over time and not related to recurring active phases. Measurement B was corrected in a way to straighten negative values that were probably linked to fast condensation processes inside the chamber. No clear pattern in the arrangement of active phases could be found with regard to different temperatures and snail sizes.

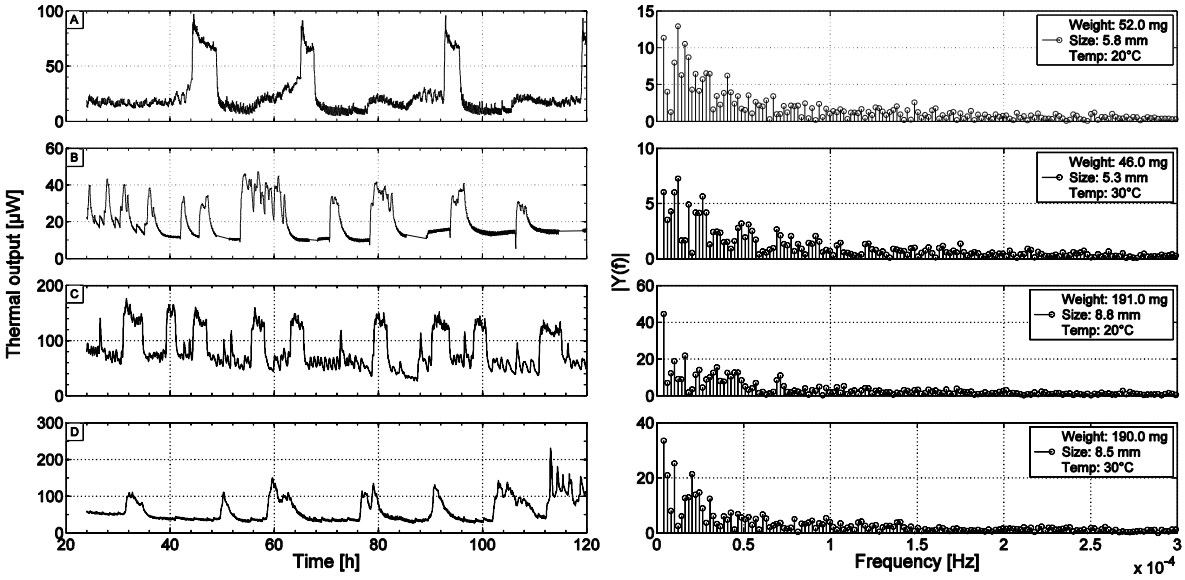

Fig. 7: Frequency analysis of recurring active phases for one specimen of each size group and temperature. Presented measurements are the same as in Figure 6.

4. Discussion:

All measurements presented in Figure 1 clearly showed two distinct levels of metabolism, an inactive and an active state, corresponding to the ones mentioned by Rees and Hand (1990). The active state is probably also related to locomotion (Lamprecht & Becker, 1988; Rees & Hand, 1990), whereas in inactive states the snail remains withdrawn in its shell.

The work of Blazka (1955) showed that oxygen consumption only decreases very slowly for starving individuals kept under high humidity conditions. This goes along with our findings of a very slow decrease of thermal output in the long-term measurements (Figure 5) where snails were most probably entering estivation. The large difference in the onset of this low level of metabolism between these two individuals is probably owed to their different

physiological fitness, which is related to the current food and reproduction status as well as humidity level. Beyond these unknown factors *X. derbentina* tends to approach estivation earlier when the ambient temperature rises, which may represent a basic strategy to avoid costly energy expenses when ambient conditions are unfavourable.

Concerning the occurrence of regular patterns the results of Figure 1 A (small, 20°C) showed a slightly shifted daily rhythm of one pronounced activity phase that the snail is still following despite the lack of any light source in the chamber specifying daily phases of brightness and darkness. Phases of inactivity lasted about 15-20h and were constantly followed by active phases. The shift results from increasing phases of inactivity of specimen A, which indicates that the animal saves more and more energy the longer the period of food and water deprivation takes during the measurement. A similar course was found in measurement A (small, 20°C) and D (large, 30°C) of Figure 7, whereas specimens B (small, 30°C) and C (large, 20°C) clearly showed more times of activity.

Comparing the larger individual C with the small individual A at 20°C in Figure 1 clearly shows that the prolongation of inactive phases occurs even earlier for the larger specimen, which may be due to its older age and the higher necessity of saving its resources. Dieterich et al. (2013) have shown older individuals of *X. derbentina* to save resources when down regulating stress protein expression. At the upper temperature of 30°C only the larger individual (D) showed a daily rhythm with approximately two phases of activity per day.

With reference to the total energy consumption (Figure 3) it became obvious that for small individuals a regulation is taking place resulting in lower values as the temperature transitions from 20°C to 30°C. Smaller individuals, therefore, seem to be more ‘aware’ of the higher temperature, which makes it necessary for them to adjust their metabolism towards a lower level. The reason for this observation may be the higher specific surface (surface to volume ratio) of smaller individuals. Since the heat transfer rate is depending on the corresponding surface the heat flux for smaller specific surfaces is higher and, therefore, smaller snails are more affected by an increasing temperature that approaches a critical limit. Larger individuals, however, have shown a notable reduction in active metabolic level (Figure 1, C and D) at higher temperatures, which indicates the presence of adaptation processes for larger specimens as well.

The division of active and inactive energy fractions (Figure 4) showed that ‘active’ energy fractions of both size groups decrease for a transition towards a higher ambient tempera-

ture, which may be indicative of the animal's basic mechanism of preserving metabolic energy at more difficult thermal conditions. Additionally, at 20°C a significant decrease of active energy fraction was observed for larger specimen compared to the small individuals. This trend could not be found for the higher test temperature, where the active fraction only non-significantly differed for larger individuals. The reason for this may be the existence of a lower limit of activity phases. If temperature rises, active metabolic energy will be saved. But this decrease may, however, have a lower limit before *X. derbentina* passes into estivation, which could be connected to fixed activity cycles, such as feeding times and liquid intake that individuals are still trying to follow inside the measurement chamber. This limit may have, therefore, already been reached for both size groups at the higher test temperature in a way that no further reduction was possible. The findings of Becker (1980) stating that activity is responsible for 80% of total energy turnover cannot be confirmed.

The results from the fractions of time spent in the 'active' metabolic state (Table 1) revealed an increasing time fraction for larger individuals at 20°C, which at first glance seemed to misfit the findings from Figure 4. However, this is still possible as larger individuals reduce their active metabolic energy consumption and, thus, are able to spend longer periods in the active state. Unfortunately this result was not reproduced by the specimen kept at 30°C. As the energy fractions showed a largely constant active demand for both size groups it can be assumed that both size groups spend an approximately equal time in activity. Poikilothermic animals usually become more active at high temperatures and less active at low temperatures (Mellanby, 1939). In this respect, *X. derbentina* shows an anomalous response as the time share of activity for large individuals subsequently decreases again as temperature rises. However, this is not unknown to happen (Kerkut & Taylor, 1958) but found to be transient until the 'normal' behaviour adjusts again. Therefore, the reduced time of activity as well as the observed lower active thermal output level during activity (Figure 1, C and D) can be interpreted as a deliberate action to counteract difficult thermal conditions.

For both size categories a characteristic difference exists between their surfaces, which can be approached by a sphere. The ratio of their mean surfaces is, therefore:

$$\left(\frac{large}{small}\right)_{surface} = \left(\frac{8.65^2}{5.55^2}\right) = 2.4 \quad (1)$$

According to table 2 the ratio of the change of time spent in 'activity' is:

$$\left(\frac{large}{small}\right)_{activity\ \ change} = \left(\frac{13.6}{50.4}\right) = 0.27 \quad (2)$$

Despite the fact that the data for small individuals is rather critical it seems that the ratio of change in 'activity' for the transition from 20°C to 30°C is inversely proportional to the ratio of their surfaces for large and small individuals:

$$0.27 \approx 2.4^{-1} = 0.41 \quad (3)$$

This highlights the fact that the behaviour of *X. derbentina* is directly influenced by the aforementioned heat flux, and, therefore, its exposition to ambient temperature.

The frequency analysis for the 'inactive' state presented in Figure 6 revealed a clear dependency on the investigated temperature for both sizes of *X. derbentina*, namely the existence of increased frequency components for the higher test temperature. In a broader sense this result resembles the findings of Kerkut and Ridge (1962), who reported a proportionality of ambient temperature and the frequency of neuronal activity in *Helix aspersa*. Although the thermal output of *X. derbentina* is only remotely linked to the activity of its brain, this accordance is rather unexpected. The calorimetric system monitors the thermal output of the complete organism and, therefore, the identified oscillations may most probably represent muscular activity of *X. derbentina* in phases of inactivity since these energetic processes give the largest contribution to thermal output. Crozier and Stier (1925) as well as Bailey and Lazaridou-Dimitriadou (1991) found an increasing heart rate for higher temperatures in *Limax maximus*, *Helix aspersa*, and *Helix lucorum*. With increased heart rate the metabolic expenditure rises, which explains the higher 'inactive' levels found at higher temperatures for the large specimens (Figure 1, C and D). A reasonable heart rate in land snails, however, is in the range of 5E-1 Hz (Schwartzkopff, 1954) and, therefore, our results on energetic frequencies do not directly reflect the heartbeat. The consequences of this increased effort at higher temperatures, however, may likely be expressed by the observed frequency increase and may represent indices for thermal stress.

Comparing the courses of thermal output in Figures 6 A/C and 6 B/D one can see that the pattern changes from a single major component to a superposition of two frequencies for larger animals. The reason for this change remains unclear but this effect is most probably

related to their older age and corresponding physiological processes, which demand energy and take place at lower frequencies.

Therefore, the 'features with a relatively quick increase and a rather slow and nearly-linear decrease' mentioned by Lamprecht and Becker (1988) that were clearly connected to inactivity can be confirmed by our finding of regular oscillations in the inactive state. For *X. derbentina*, however, activity usually resulted in a much higher thermal output level compared to Lamprecht and Becker's unmentioned snail species. After all, the regular and distinct oscillations found in the present study show that *X. derbentina* can undergo remarkable changes in heat production although being a poikilothermic animal without a distinct temperature regulation system. These changes happen within few minutes. The observed phases of metabolic change could not be associated with phases of locomotion since the latter are most probably connected to the much higher active levels found, similar to the results from Rees and Hand (1990). In contrast, our observed fluctuations in heat output likely represent internal activity changes.

4.1 Approach to an integrative physiological model:

With the help of physical modelling reasonable statements concerning the physiological state of *X. derbentina* can be made despite the lack of a broad data basis in our study.

From the basics of physiology it is clear that oxygen consumption, blood flow and metabolism are directly linked to each other and depending on the current needs of an organism. Air gets inhaled and the oxygen is passed over to the hemolymph, which is transported by the beating heart. Despite the snail having an open cardiovascular system that is very different to the human circulation, basic principles must still be applicable (Müller, 2001).

By the combination of the obtained data with measurements of the oxygen consumption (Fischbach et al., submitted) and the size of *X. derbentina*'s lung additional metabolic parameters of the snail, like breathing frequency or the reduction of oxygen concentration per breath of air can be determined to further characterize the physiological state of the animal. The size of the snail's lung was gained from detailed nuclear magnetic resonance imaging (NMRI) measurements (unpublished) that were performed at the Institute of Physics, University of Würzburg, corresponding to the 'non-movement state' of the snail, i.e. when the snail is withdrawn in its shell.

The oxygen consumption can be calculated by the following equation:

$$oxygen\ consumption\ \left[\frac{mol}{s}\right] = \frac{breat\,hing\ \ volume\ \ per\ \ minute\ \left[\frac{l}{min}\right] \times O_2\ reduction\ \ per\ \ breat\,h\ [\%]}{100 \times 22{,}41\left[\frac{l}{mol}\right] \times 60} \qquad (4)$$

The breathing volume is:

$$breathing\ volume\ [mm^3] = \frac{breat\,hing\ \ volume\ \ per\ \ minute\ \left[\frac{l}{min}\right]}{breat\,hing\ \ frequency\ \left[\frac{1}{min}\right]} \times 10^6 \qquad (5)$$

The lung volume is:

$$lung\ volume\ [mm^3] = breathing\ volume\ [mm^3] \times\ lung\ volume\ to\ breathing\ volume\ ratio\ [-] \qquad (6)$$

Based on a solely consumption of glucose, the measured thermal output is:

$$thermal\ output\ [W] = \frac{caloric\ \ value\ \ of\ \ glucose\ \left[\frac{kJ}{mol\ \ glucose}\right] \times oxygen\ \ consumption\ \ \left[\frac{mol\ \ O_2}{s}\right]}{amount\ \ of\ mol\ O_2\ to\ turn\ \ over\ 1\ mol\ of\ \ glucose\ \left[\frac{mol\ \ O_2}{mol\ \ glucose}\right]} \times 10^3 \qquad (7)$$

The stoichiometry for the oxidation of glucose reveals 6 mol of oxygen to be needed per mol glucose. Together with the caloric value of glucose of 2826 kJ, equations (1) to (4) can be combined to express the unknown 'O_2 reduction per breath' by the other two unknown variables 'breathing frequency' and 'lung volume (LV) to breathing volume (BV) ratio':

$$O_2\ reduction\ [\%] = \frac{thermal\ \ output\ \ [W] \times 6\left[\frac{mol\ \ O2}{mol\ \ glucose}\right] \times \frac{LV}{BV}\ [-] \times 22{,}41\left[\frac{l}{mol}\right]}{lung\ \ volume\ \ [mm^3] \times breat\,hing\ \ frequency\ \ \left[\frac{1}{min}\right] \times 2826\left[\frac{kJ}{mol\ \ glucose}\right]} \times 6E6 \qquad (8)$$

With this equation of three unknowns a surface plot was created that shows 'O_2 reduction per breath' as a function of 'breathing frequency' and the 'LV/BV ratio'. With the measured values of 'thermal output' and 'lung volume' this surface shows possible combinations of the three unknown properties that would all satisfy the results of the comprised methods, direct calorimetry (present paper), oxygen consumption (Fischbach et al., submitted) and geometrical data of the lung by NMRI (unpublished).

This set of equations only holds true if the metabolism of *X. derbentina* is aerobic and, therefore, this was checked before. Fischbach et al. (submitted) presented data of oxygen consumption in *X. derbentina* for three different size groups in their inactive state for the temperatures 25°C, 30°C, and 38°C. In the present study, the size of the measurement chamber inside the calorimeter limited the size range of the measurements. Fischbach's et al. size

group 2 with an average diameter of 0.9-1.0 cm closely matched the investigated 'large snails' in the present study and, therefore, oxygen consumption values at 30°C of this group will be used to check for aerobic metabolism. The division of 'caloric value of glucose / amount of mol O_2 to turn over 1 mol of glucose' in equation 4 is the calorimetric equivalent [kJ / l O_2] and varies between 19.63-21.13 kJ/ l O_2 (neglecting the influence of temperature) depending on the composition of the nutrients (Lusk, 1924). Calculating the theoretical thermal output for *X. derebentina* individuals with a diameter of 0.9-1.0 cm with their average oxygen consumption of 1.56E-10 mol/s at 30°C (Fischbach et al., submitted) results in a heat output of 73.7 µW, 68.5 µW, and 70.3 µW, for a solely consumption of carbohydrates, lipids, and proteins, respectively. This is in close accordance to the data recorded for the inactive state of *X. derbentina* in the present study (compare Figure 7) considering that the individuals in this case were slightly smaller than the ones from the Fischbach et al. (submitted) study and, therefore, values of approximately 70 µW fit very well to slightly upwards corrected values from Figure 7. This shows that the 'inactive' metabolic state of *X. derbentina* is aerobic. The NMRI measurement to obtain the geometrical size of the lung (35.13 mm^3, unpublished) has been performed with an individual of approximately 0.95 cm size and, therefore, fits the 'large' group of snails in the present study and 'group 2' of Fischbach et al. (submitted). Thus, the value of 70 µW was used for the calculation of the response surface at 30°C for inactive individuals sized between 0.9-1.0 cm. Assuming that *X. derbentina* is also aerobic at a slightly lower temperature, an additional dataset was calculated for 25°C using the oxygen consumption 1.04E-10 mol/s (Fischbach et al., submitted) measured for equally sized snails at 25°C and the caloric equivalent.

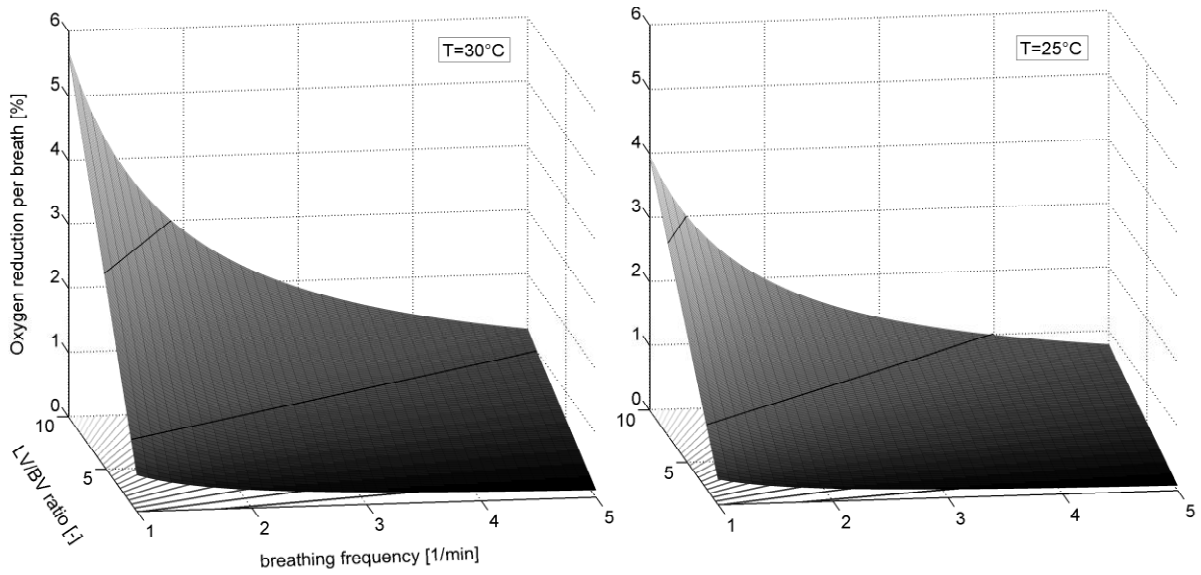

Fig. 8: Response surface calculated for oxygen reduction per breath vs. breathing frequency and factor of lung volume to breathing volume for a medium sized snail of 0.9-1 cm in aerobic state, a corresponding heat output of approximately 70 µW and a lung volume of 35.1 mm^3 at a temperature of 30°C.

Figure 8 shows the resulting surfaces of 'oxygen reduction per breath' vs. 'LV/BV ratio' and 'breathing frequency' with additional contour lines for constant values of 'oxygen reduction' for both temperatures. It can be seen that 'oxygen reduction per breath' needs to be increased in order to maintain the metabolic state when either 'breathing frequency' gets lower or when 'LV/BV ratio' gets higher, which is a plausible relationship. The range of 'breathing frequency' is very reasonable compared to values of about 3.5 breath cycles per minute found for *Helix pomatia* (Maas, 1939).

It is known that an average human being reduces the oxygen concentration by approximately 5%, from an oxygen concentration of 21% to 16% in air, per breath (Schmidt, Lang, & Heckmann, 2005). In this regard the highly efficient alveolar design of mammal lungs is considered to be much more efficient than the rather basic respiratory system of pulmonate snails and, therefore, an upper reasonable limit of 'O_2 reduction' may be defined by a value of 3%. In combination with the assumption of a lower limit of 1% (black lines in Figure 8) the delimited remaining area in the 3D plot describes all possible physiological states of *X. derbentina* based on the measurements and on the taken assumptions. Due to the lower level of oxygen consumption at 25°C this area is smaller than the one calculated for 30°C.

The data generated in the frame of the present study and the resulting insights may provide

the basis for a future, more sophisticated, integrative model of *X. derbentina* physiology and energetics.

5. Acknowledgments:

The authors would like to thank Christophe Mazzia for the delivery of snails and the provision of his land site to collect the animals.

The study was financed by the University of Tübingen's Twinning Project with the University of Applied Science Esslingen under the supervision of H.-R. K., U. G., D. W. and Stefan Rösler.

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Kapitel 6: Measuring oxygen consumption and metabolic adaptation in the Mediterranean land snail *Xeropicta derbentina* using a novel camera-equipped micro-respirometry system.

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Abstract:

The oxygen consumption of *Xeropicta derbentina*, a Mediterranean helicoid snail was measured in individuals of varying sizes and at a range of temperatures. In comparison with control conditions at 25°C oxygen consumption in individuals of medium size decreased significantly at the highest tested temperature (38°C). A similar but non-significant trend was observed for the largest and, therefore, oldest snails but no metabolic regulation was found for specimens belonging to the smallest size category. The linear correlation between the logarithm of oxygen consumption and the logarithm of shell-free weight reveals that oxygen consumption is proportional to a power function of shell-free weight. The exponent α for the power function was found to depend inversely on test temperature and, thus increased for decreasing temperatures.

1. Introduction:

Xeropicta derbentina (Krynicki 1836) (Gastropoda, Hygromiidae) is a terrestrial, pulmonate snail abundant in southern France, whence it was introduced from the eastern Mediterranean. In southern France, *X. derbentina* was first reported in 1949 (Aubry, Labaune, Magnin, Roche, & Kiss, 2006; Kiss, Labaune, Magnin, & Aubry, 2005; Van Regteren Altena, 1960) and, since then, has been spreading northwards. Its shell diameter reaches up to approximately 15 mm and its outer shell morphology is dominated by a uniformly white color, but also dark-banded specimens can be found. In July and August, when air temperatures in their habitat reach up to 45°C a few centimeters above the ground and even exceed 55°C at the soil surface, the snails climb vertical objects, like blades of grass or other vegetation. The observed climbing behaviour is believed to be a part of the snails' survival strategy and is one of the reasons for *X. derbentina*'s spread throughout southern France. Once individuals have reached cooler conditions at positions high above the ground they become inactive to

avoid desiccation. During hot and dry summer days, *X. derbentina* remains inactive for long periods of the time, develops an epiphragm to seal its aperture and thus minimize water loss by evaporation, and may form clusters of hundreds of individuals at positions distant from the soil surface. Detailed measurements of temperature, air velocity, and humidity have shown that this snail species withstands ambient temperatures of over 40°C for a whole day. In this context it has been shown that one of the mechanisms to survive these conditions is the production of heat shock proteins that serve as a primary defence to protect cells from proteotoxic damage (Dieterich et al., 2013). Nevertheless, not only the molecular stress response but rather the entire metabolism needs to be adapted to high ambient temperature in these snails in order to withstand severe climatic conditions.

The present study thus aimed at providing further information to understand the highly developed physiological survival mechanisms of *X. derbentina*. Here, we used oxygen consumption as a proxy for metabolic activity to investigate possible metabolic regulation processes that take place at high temperatures.

The influence of temperature on oxygen consumption in snails has been investigated by Mason (1971), who recorded respiration rates of twelve species at 5°C, 10°C, and 15°C. His findings suggest a general rise in the metabolic rate with increasing temperature with only a single species displaying stagnant oxygen consumption at the highest test temperature. Mason confirmed these results for the species *Hygromia striolata* and *Discus rotundatus*. This tendency has also been confirmed by Rising and Armitage (1969) for two different species of slugs, *Limax maximus* and *Philomycus carolinianus*.

Schmidt-Nielsen, Taylor, and Shkolnik (1971) investigated the influence of temperature on *Sphincterochila boissieri*, a snail inhabiting the deserts of the Middle East and thus facing a very dry and hot climate. The authors showed that higher temperatures resulted in higher oxygen consumption but that the extent of this relationship mirrored the data recorded for non-desert snails. They concluded that no metabolic adaptation to temperatures up to 35°C was present, but, as the lethal temperature for *S. boissieri* is known to be 50-55°C, higher experimental temperatures up to this limit should be investigated. Furthermore, they noticed remarkable variations in consumption (“oxygen bursts”) which appeared periodically and were believed to be intrinsic since they occurred under constant external conditions and may, therefore, be characteristic for the metabolism of *S. boissieri*. Herreid (1977) has also

observed this behaviour for *Otala lactea* obtained from Morocco. His results showed highly variable oxygen consumption rates of six individuals after arousal from dormancy which was triggered by increased humidity.

Riddle (1977) compared oxygen consumption between the desert snail *Rabdotus schiedeanus* and the garden snail *Helix aspersa* and found a general rise in oxygen consumption for temperatures up to 25°C. Beyond 25°C a metabolic regulation became visible for both species, and oxygen consumption declined, contrary to the results of Schmidt-Nielsen et al. (1971). Riddle concluded that the "depressed metabolism (…) of high temperatures is adaptive in conserving metabolizable energy". This was especially evident for the desert snail *R. schiedeanus* that in general showed a lower oxygen demand. These studies were confirmed by Dallas, Curtis, and Ward (1991) showing that individuals of *Trigonephrus sp.* had a significantly higher oxygen demand at 15°C than at 25°C, corresponding to an active and an inactive state of these Namibian desert snails. In comparison with the similarly sized snails *Helix aspersa* and *Otala lactea*, oxygen consumption was still considerably lower showing the metabolic adaptation of *Trigonephrus* to much warmer climatic conditions.

Riddle (1975) demonstrated that atmospheric humidity has a strong influence on the oxygen consumption of the desert snail *R. schiedeanus*, resulting in significantly higher consumption with increasing relative humidity. The comparison with *Helix aspersa* additionally revealed the lower metabolic rates for the desert snail at high temperatures when humidity was very low (Riddle, 1977).

Comparative oxygen measurements by Steigen (1979) for banded and unbanded morphs of *Cepaea hortensis* revealed the oxygen consumption to generally increase between 5-15°C and to stagnate for the unbanded morph at 25°C.

Nopp (1965) reported the difference in oxygen consumption between starving, dry-sleeping, and active pulmonates. Starving *Arianta arbustorum* individuals showed significantly higher oxygen consumption rates than fresh-fed conspecifics. Furthermore, dry-sleeping individuals further reduced their oxygen consumption to approximately half of the 'hungry-level'. Nopp (1965) mentioned that this dry-sleep level is only reached after 2-4 days of inactivity. Blazka (1955) and Nopp (1965) also report that oxygen consumption in pulmonate land snails drops drastically at the beginning of estivation and subsequently remains almost constant from the second week of estivation onwards.

The absolute body size of an organism and therefore its weight is a very important factor in determining the rate of metabolic processes. This correlation is a classic topic of physiology. At the end of the 19th century Rubner (1883) noticed that the weight-specific metabolic rate of homoeothermic animals decreases with increasing body size. If, however, metabolic rate is calculated per unit body surface almost constant values can be obtained. This is known as the 'surface rule' stating that the surface of two geometrically similar bodies can be expressed by the 2/3 power of weight multiplied by a constant (the cubic root of the volume is a linear value and its square, therefore, has the dimension of a surface).

A general mathematical expression for the dependency between metabolic rate and weight, the metabolic scaling, is given by the well-known allometry formula:

$$M = bW^{\alpha} \qquad (1)$$

where M is the metabolic rate, expressed by oxygen consumption, W is the body weight, α is the scaling exponent, and b is a specific constant. Equation (1) can also be written as:

$$\log M = \log b + \alpha \log W \qquad (2)$$

which results in a linear relationship between logM and logW with the slope α. If α=1 equation (2) represents an isometric relationship, which results in a doubling of the metabolic rate as the weight doubles. If α=2/3 one directly obtains the relationship corresponding to the surface rule. Kleiber (1947) reviewed applications of the surface law in zoophysiology and showed that, for a certain group of mammals, the metabolic rate was proportional to the 0.756th power of body weight. According to Kleiber (1947) the surface rule cannot be the sole explanation for the metabolic activity of animals but the behavior of a species has to be considered as well. Another general review of metabolism and body size is given by Bertalanffy (1957). The author supposed that three different 'metabolic types' exist and that each species essentially belongs to one of these types. In the first type metabolism and growth is described in such a way that metabolic rate is proportional to the surface or the 2/3 power of weight following the assumptions of the surface rule. In the second type the rate is proportional to weight itself and, in the third type, an intermediate proportionality between surface and weight is found with 1>α>2/3. In recent years the generality of the 2/3 or the 3/4 power of weight metabolic scaling has been questioned (Chown et al., 2007; Kozłowski, Konarzewski, & Gawelczyk, 2003). Glazier (2005) reviewed published data and

showed that significant deviation exists from the 2/3 or 3/4 scaling model among mammals (0.38-1.11), squamate reptiles (0.27-1.26) and invertebrates (-1.2-2.05) (α became negative when juveniles were included). These deviations could not be explained by theoretical models, such as the resource-transport-network model (West & Brown, 2005; West, Brown, & Enquist, 1997), which predicts an exponent α of 0.75, from molecules to whole organisms, and describes the way "materials are transported through space-filling fractal networks of branching tubes". An alternative model based on cell size describes the change in metabolic scaling as a result of the way body size changes. Growth driven by an increasing cell number results in an isometric scaling of metabolism, whereas growth via an increasing cell size results in an exponent α=0.67 (Kozłowski et al., 2003). The results of Chown et al. (2007) showed a broad variation of α (0.67-1) for an interspecific analysis of eight ant species, which also corresponded to the cell growth types of Kozłowski et al. (2003). Furthermore, ontogenic factors were investigated, such as phases of fast growth and reproduction that increase metabolic scaling, resulting in the finding that the exponent α may change during the course of ontogeny (Glazier, 2006). Recently Glazier (2009) reviewed data of 19 ectothermic species and showed that the mean value of the metabolic exponent α was significantly higher for active animals (0.918) compared to inactive ones (0.768).

For poikilothermic invertebrates contradictory results concerning the exponent α have been published. Liebsch (1929) investigated three species of Helicidae showing a direct proportionality to weight and hence α=1, and the measurements carried out with *Cepaea vindobonensis* by Bertalanffy and Müller (1943) support this relationship. This was confirmed by the findings of Kienle and Ludwig (1956) who measured oxygen consumption in H*elicella candicans*. In contrast to these results Wesemeier (1960) found the average slope of the curve in a log-log plot for four different species of land snails to be α=0.76. Wesemeier also criticized the results of Liebsch (1929) for not taking the state of locomotion into account. By comparing *Helix pomatia* at different levels of activity he showed that phases of resting and movement have an influence on the slope α resulting in values of 0.8 for moving snails and 0.71 for resting individuals. Furthermore he noted that Liebsch (1929) had calculated the regression coefficient as an average value for different species without accounting for interspecific variation. Wesemeier's (1960) results for α in pulmonate land snails varied between 0.71 and 0.85 showing that there might not be an exact correlation between weight and metabolism, such as stated by the surface law, but rather a species-specific relationship. In addition

the author confirmed the result of Kienle (1957) where the lung surface of *Helix pomatia* was found to be proportional to $W^{0,74}$. However, it is still unclear whether this is an intrinsic and therefore species-specific genetically fixed feature or if metabolism itself is limiting the growth of the lung surface. Steigen (1979) presented results of *Cepaea hortensis* for energy metabolism at temperatures from 5°C-25°C showing a continuous decrease of α for increasing temperatures for banded (except for 20°C) and unbanded morphs ranging from 1.06-1.23 and 0.96-1.60, respectively.

Mason (1971) found a linear relationship on a double log plot between weight and oxygen consumption for an interspecific comparison between twenty terrestrial snail species that grew to a large size with regression coefficients of 0.74, 0.65, and 0.71 for their slope at 5°C, 10°C, and 15°C, respectively.

Czarnołęski et al. (2008) showed that phases of slow and fast growth in ontogeny affect the relationship between metabolism and size in *H. aspersa* resulting in an almost isometric relation (α=1) for the initial fast-growing ontogenic phase and a lower value of α for the slow-growing phase. Similar variations in α due to different developmental stages of 421 individuals of *Cornu aspersum* were documented by Gaitán-Espitia, Bruning, Mondaca, and Nespolo (2013).

Air-breathing water snails (Basommatophora) have been investigated by Berg and Ockelmann (1959) who found variations in α ranging from 0.45 to 1.0. They concluded that seasonal changes were the reason for this and also pointed out that “the relation, oxygen consumption to body size, is not a fixed, unchangeable quantity characteristic for all species ...”. Additionally, Duerr (1967) presented measurements for *Lymnaea stagnalis appressa*, another air-breathing water snail, and found a direct proportionality of respiration to its weight.

Acclimation to different temperatures within an appropriate time span is known to pose a physiological problem for some species that may result in different oxygen consumption rates, as reported by a number of authors (Herreid, 1977; Mason, 1971; Rising & Armitage, 1969; Schmidt-Nielsen et al., 1971; Segal, 1961; Steigen, 1979). This effect, however, is not addressed in the current study.

Clearly, the discussion of the interrelationship of temperature, body size, and metabolism in terrestrial snails has a long-standing tradition. In this context, our study is aimed at answering the following questions:

(1) Does a change in temperature affect the oxygen consumption of *X. derbentina*?
(2) If so, does the metabolism of individuals of different shell diameter react to the same extent?
(3) If so, does the metabolism of active and inactive individuals react in the same way when temperature is changed?
(4) What is the value of the exponent α for *X. derbentina* and how does it depend upon the ambient temperature?

2. Methods:

2.1 Test organism:

The present study uses *X. derbentina* (Krynicki 1836), a hygromiid land snail. Specimens were collected from an untreated meadow on private property and close to Modène, department Vaucluse, southern France (N44°6.055' E5°7.937') between August and early November 2013. The owner neither applied any pesticides to the landsite nor used it as farm land. All collected snails were carried to the laboratory, kept hydrated and fed lettuce and baby porridge (*Hipp Gute Nacht BIO* oat & apple, Pfaffenhofen, Germany).

To assure constant starting conditions for all measurements snails were preconditioned in a separate climate chamber that reproduced the 24h humidity and temperature cycle characteristic for an average day in August in southern France. Immediately before the measurements, the individuals' mass and shell diameter were measured. In this study only 'uniformly white' morphs were used in accordance with the shell coloration pattern classification used by Köhler et al. (2009).

Measurements were carried out for 20°C, 30°C, and 38° C for three different size-groups of snails, group 1 with 0.65-0.85 cm shell diameter (n=23), group 2 with 0.9-1.0 cm (n=22) and group 3 with 1.0-1.25 cm (n=25). For each size-group and for each temperature at least n=7 snails were analyzed to assure a minimum of required statistical reliability. Due to the highly time-consuming procedure a larger number of individuals could not be analyzed.

2.2 Oxygen consumption:

The necessity to both observe the snails and measure oxygen consumption in parallel as well as the need for a high accuracy required a *de novo*-construction of a purpose-built microrespirometer. For the measurement of oxygen consumption a cylindrical aluminum chamber containing a defined air volume was constructed. To capture both inactive and active phases independently, snails were measured individually. The top of the chamber was equipped with an oxygen sensor *O_2 Tracer* (*ProChem Analytik*, Xanten, Germany) which is based on an electrochemical cell with a sensitivity of 0.01% O_2. The principle of this sensor is similar to a battery or a fuel cell, where chemical energy is transformed into a continuous electric current. A varying concentration of the reactant oxygen results in a variable corresponding current. For a constant total pressure, which is essential for the measurements, a defined rate of oxygen, according to the partial pressure of oxygen p_{O2}, diffuses through a membrane and participates in the reaction.

The top of the chamber was sealed with acrylic glass that allows the observation of the snails' activity. A custom plastic cover was designed, in which a small webcam and an LED were integrated for the observation of the animal's activity. This permitted the separate determination of oxygen consumption rates both in active and inactive phases. The entire respirometer chamber was then placed in a water bath to adjust the environment for the different test temperatures. To minimize the thermal impact of the observation webcam and, therefore, to allow stable oxygen signals to be recorded, only 10 pictures per hour with a flashing LED were taken, otherwise LED and webcam were turned off. Furthermore, gas pressure (*PAA-33X, Keller*, Jestetten, Germany), temperature (miniature *PT100*, Electronic Sensor, Heilbronn, Germany) and humidity (*SHT21, Sensirion*, Staefa, Switzerland) inside the chamber were monitored. On the basis of the measured relative humidity the dry gas pressure was calculated by subtracting the water vapor pressure from the total pressure. The saturated vapor pressure (SVP) was calculated according to the *Magnus*-formula with the temperature T given in degree Celcius:

$$SVP\ [Pa] = 6{,}112\ \times\ e^{\frac{17{,}62\ \times T}{243{,}12\ + T}}\ \times\ 100 \qquad (3)$$

Total dry gas pressure, temperature and air volume finally revealed the total molar amounts of the substances n_{total} inside the chamber, as stated by the ideal gas equation:

$$p_{total\,,dry} \times V_{chamber} = n_{total} \times R \times T_{chamber} \quad (4)$$

with *R* being the universal gas constant.

Using *Dalton's* law and the definition of the spatial fraction of oxygen r_{O2} in the mixture, one obtains for the amount of oxygen:

$$n_{O2} = \frac{p_{O2} \times V_{chamber}}{R \times T_{chamber}} = \frac{r_{O2} \times p_{total\,,dry} \times V_{chamber}}{R \times T_{chamber}} \quad (5)$$

The time evolution of each measurement revealed the variation of the amount of oxygen [mol/s], which is equal to the oxygen consumption of each specimen.

The automatic LED and webcam power switch was realized by using a separate *LabView* program that controlled a *National Instruments* (Austin, TX, USA) NI-9472 Card connected to the LED and to a relay that linked the USB-connection from the webcam to the computer. Pictures were taken automatically by the open-source software *iSpy*. Signals for oxygen, gas pressure, and temperatures were acquired every ten seconds by an *Agilent* (Santa Clara, CA, USA.) 34972A LXI Data Acquisition / Data Logger Switch Unit and saved as *.*csv*-files for further analysis. Humidity signals were taken with the software from *Sensirion* that was included with the *Evaluation Kit*. Relative humidity was not controlled during the experiments but was always in the range of 25-50% depending on ambient conditions and the measured snail.

The validity of the measurements was confirmed by a baseline record of a constant level of oxygen for the empty chamber at each temperature.

The oxygen sensor, which was directly screwed into the acrylic glass at the chamber´s top, was provided with a hardware implementation for temperature correction, integrated in its outer casing, and thus located outside the measurement chamber. To minimize environmental temperature gradients on this hardware that would immediately influence the oxygen signal, the measurement took place in an insulated *Rittal* (Herborn, Germany) cabinet. The measurement chamber was then placed in an additional plastic case that was used as a water bath and the water circulation was realized via an external setup. This arrangement resulted in a very stable microclimate inside the cabinet since the measurement chamber was

conditioned by the temperature of the circulating water and was properly insulated from external influences (Figure 1).

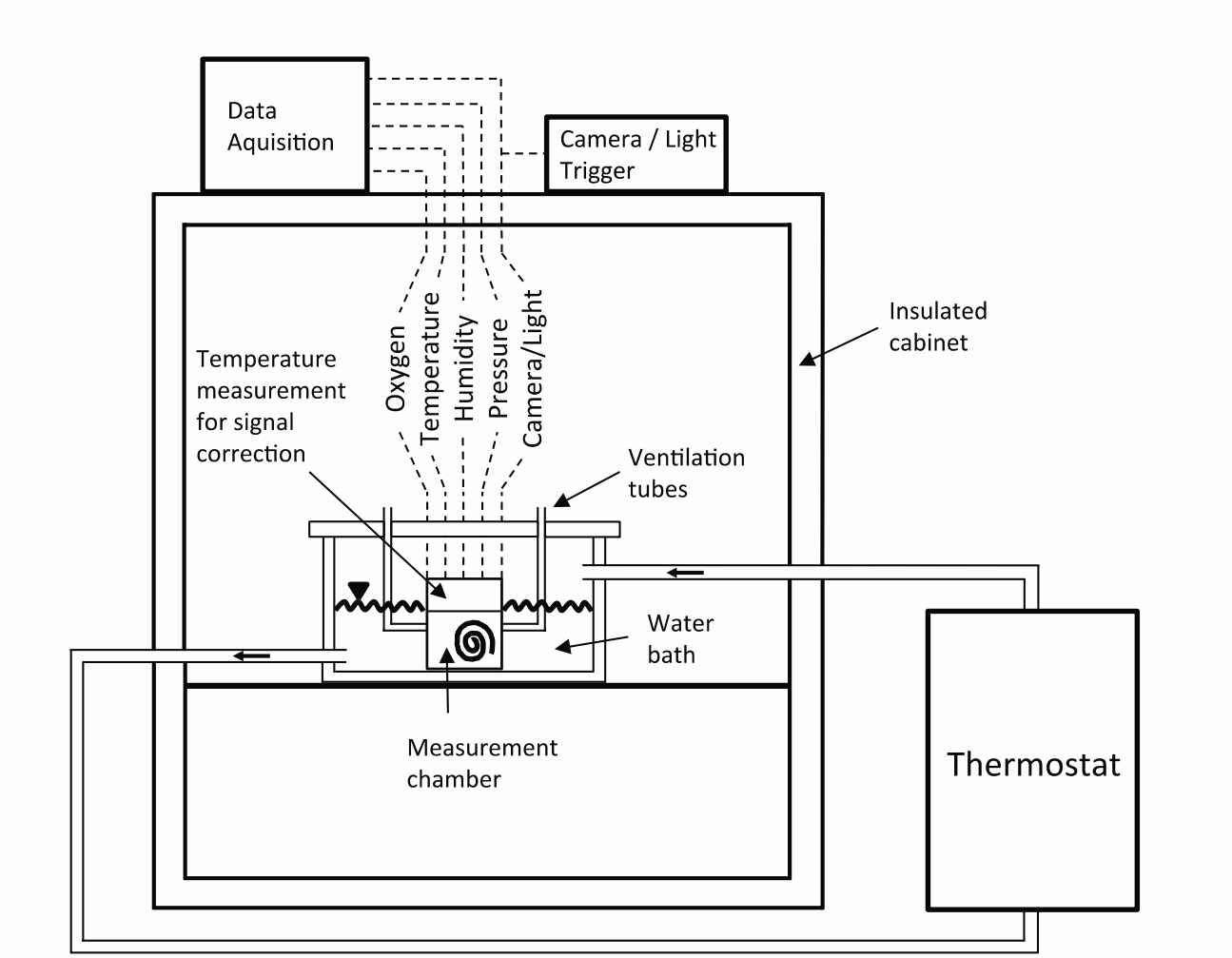

Fig. 1: Schematic depiction of the developed camera-equipped microrespirometry system used in this study.

Additionally, baseline measurements were taken with an empty chamber and a linear correlation (r=-0.856, Pearson) was obtained between the oxygen signal and the temperature that was measured at the position of the outer casing of the oxygen sensor (cp. Figure 1). This was subsequently used to correct the measured oxygen signal for unavoidable temperature drifts. With this correction the oxygen signals for baseline measurements were shown to vary in a range of ±0,01% O_2 level, representing the maximum accuracy of the oxygen sensor and demonstrating the high accuracy of the measurements.

The gas pressure sensor was placed directly above the water level to reduce the hose length outside of the water bath, and thus minimize the influence of slight temperature gradients.

In order to avoid the impact of different movement activity on the measurements, only data recorded for individuals in their inactive phases were used for this study.

2.3 Statistics:

Data were checked for normal distribution using the Shapiro-Wilk W-Test in JMP 11.0 (SAS Institute Inc.).In order to obtain a normal distribution, data for oxygen consumption had to be transformed by calculation of the 4^{th} root. In the following, the original data will always be presented together with the transformed data on two vertical axes but statistical analyses were exclusively applied to the transformed data. Additionally, homogeneity of variances was guaranteed by non-significance in Levene's tests. Significance was then checked by ANOVA with a post-hoc Tukey-Kramer test.

If not specified otherwise $p \leq 0.05$ was defined significant (*), $p \leq 0.01$ was highly significant (**), and $p \leq 0.001$ was conclusively (***) (very highly) significant.

On the basis of fitted power series a two-parameter function has been determined with Excel 2010 (Microsoft Inc., Redmond, WA, USA) representing oxygen consumption depending on shell free weight and temperature to unify both dependencies.

3. Results:

3.1 Influence of shell size on the oxygen consumption:

Shell size was significantly and positively associated with oxygen consumption, independent of the investigated temperatures (ANOVA, Tukey-Kramer HSD test between all groups, figure 2). Mean values for each size group were 0.44E-10±0.27E-10 mol/s (group 1, small), 1.06E-10±0.59E-10 mol/s (group 2, medium) and 2.0E-10±1.18E-10 mol/s (group 3, large) for the non-transformed data. The mean shell sizes were 0.74±0.06 cm, 0.95±0.03 cm and 1.17±0.06 cm for shell size categories 1, 2, and 3, respectively.

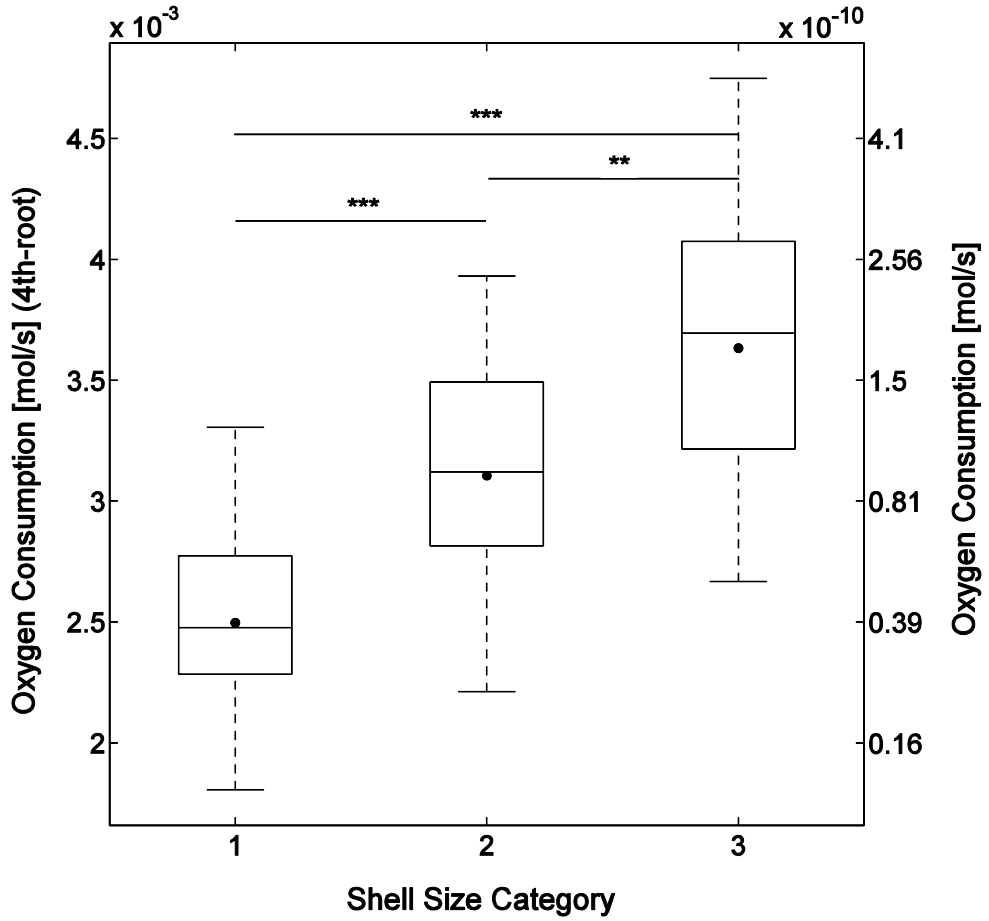

Fig. 2: Oxygen consumption for shell size groups 1, 2, and 3 (left: original data, right: transformed data). Black dots represent means. Box plots represent 25%, medians and 75% quartiles.

3.2 Influence of temperature on the oxygen consumption:

To account for the different investigated temperatures a two-way ANOVA was applied with the two factors 'shell size category' and 'temperature'. This analysis, however, showed the interaction between shell size category and temperature to be close to the limit of significance ($p=0.053$). Hence, a one-way ANOVA with subsequent Tukey-Kramer HSD test was applied for each shell size category. The results for shell size category 1 (the 'small' individuals) did not show any significant differences between the three different test temperatures 25°C, 30°C, and 38°C (Tukey-Kramer HSD, $p>0.99$ for all temperatures, Figure 3). Means are 3.99E-11±1.48E-11 mol/s, 4.74E-11±3.67E-11 mol/s, and 4.51E-11±2.91E-11 mol/s for 25°C, 30°C, and 38 °C, respectively.

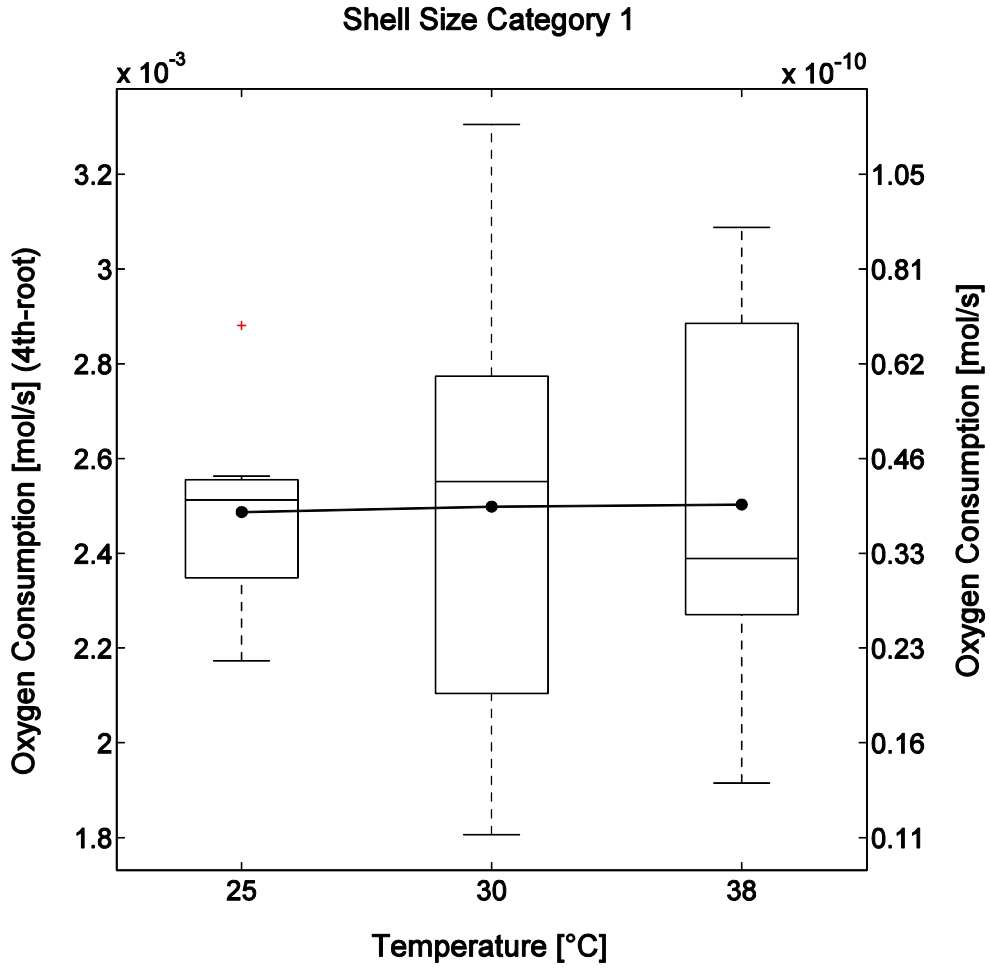

Fig. 3: Oxygen consumptions for the three tested temperatures for snails belonging to shell size category 1 (left: original data, right: transformed data).

The data obtained for shell size category 2 (Figure 4), however, revealed a very highly significant decrease (Tukey-Kramer HSD, $p<0.001$) in oxygen consumption at 38°C (0.51E-10±0.33E-10 mol/s) vs. 30°C (1.56E-10±0.55E-10 mol/s). A highly significant (Tukey-Kramer HSD, $p<0.01$) decrease in oxygen consumption at 38°C could also be found vs. 25°C (1.04E-10±0.25E-10 mol/s).

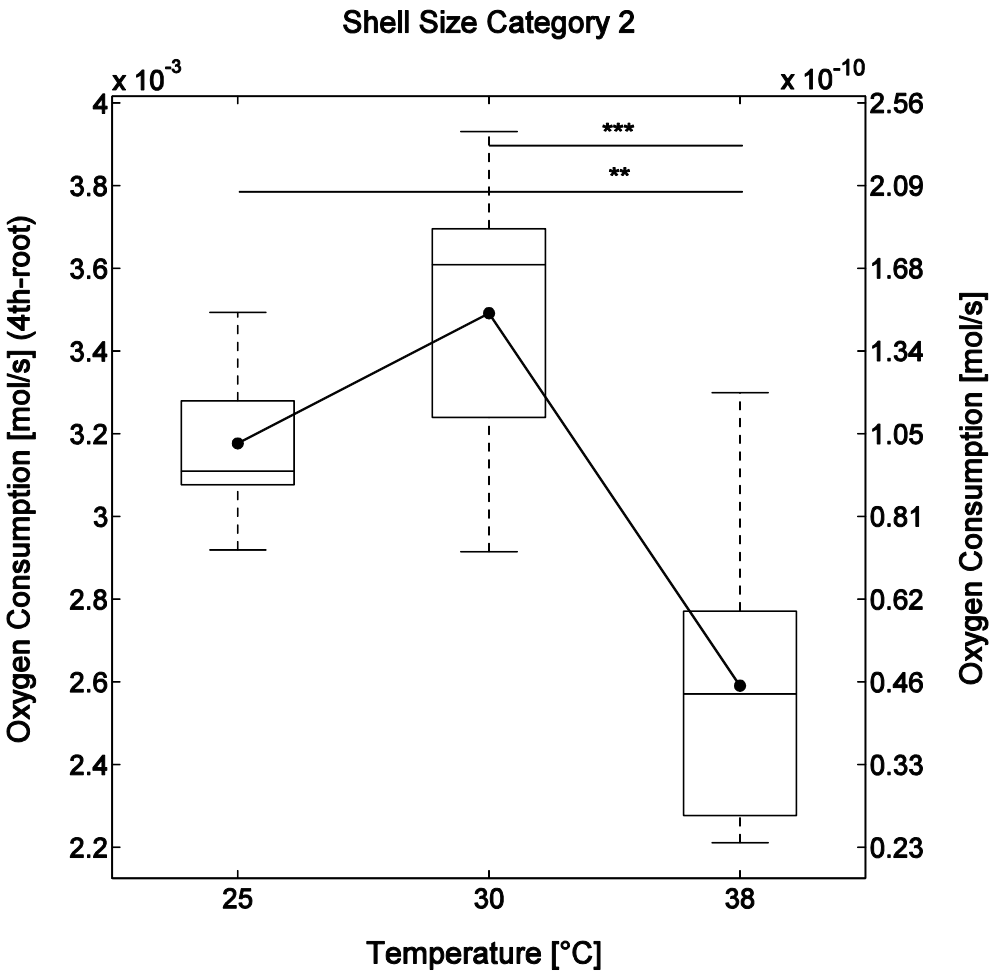

Fig. 4: Oxygen consumptions for the three tested temperatures for snails belonging to shell size category 2 (left: original data, right: transformed data).

Data recorded for the 'large' individuals, those of shell size category 3 (Figure 5) showed a similar drop in oxygen consumption at the highest temperature, 38°C. Between 25°C and 30°C no decline in metabolism was visible. Despite lacking significance, the oxygen consumption rates at 38°C revealed a trend towards reduced metabolism at that temperature (Tukey-Kramer HSD, p=0.07). Means were 2.27E-10±1.11E-10 mol/s, 2.48E-10±1.33E-10 mol/s, and 1.30E-10±0.78E-10 mol/s for 25°C, 30°C, and 38°C, respectively.

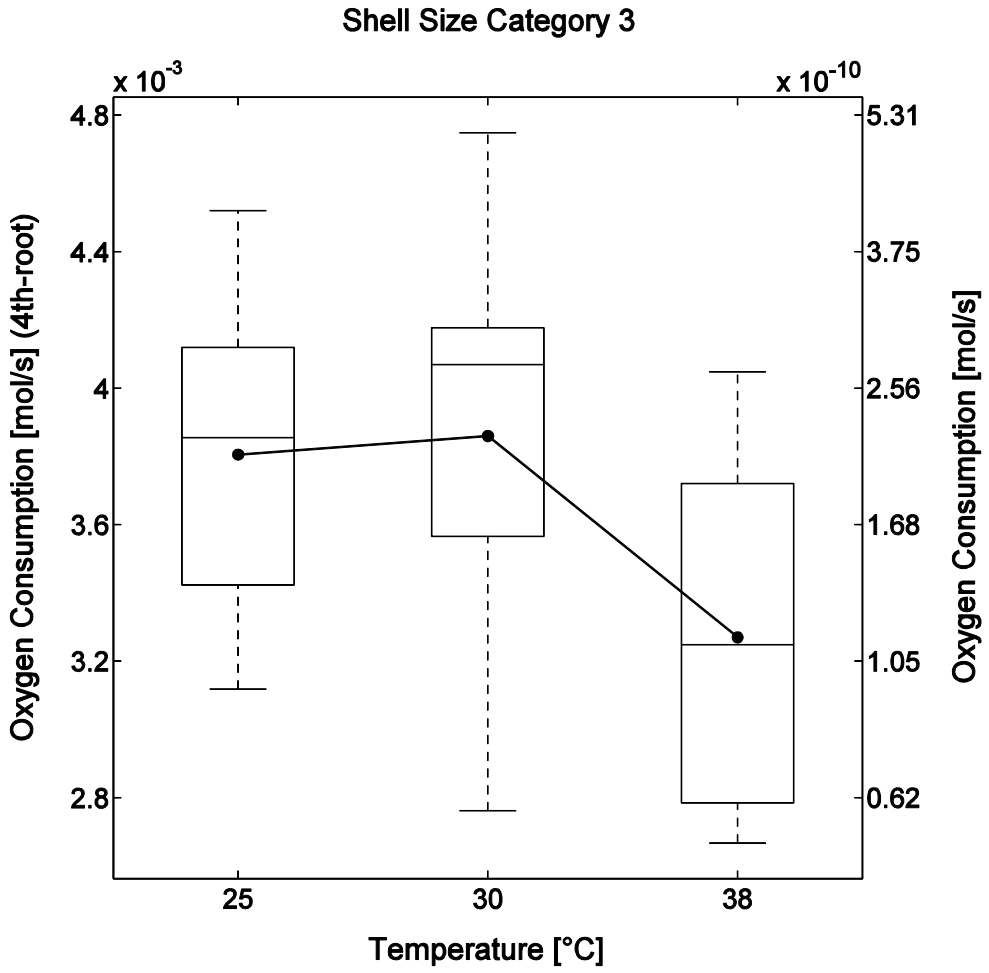

Fig. 5: Oxygen consumptions for the three tested temperatures for snails belonging to shell size category 3 (left: original data, right: transformed data).

3.3 Influence of weight on the metabolism at different temperatures:

This potential interrelationship was investigated using the shell-free weight of each animal. Therefore, empty shells of *X. derbentina* were weighed and correlated to their outer shell diameter yielding an approximately cubic fit (n=26, R^2=0.981) that was used to subtract the shell weight from each specimen.

The analysis of intraspecific weight influence on oxygen consumption required appropriate fitting of the data points, which was achieved with the software package MATLAB R2013b (Mathworks, Natick, MA, USA).

For 25°C the fitting procedure resulted in α=1.221 and b=2.835E-13 with R^2=0.875 (figure 6). The uncertainty in coefficients is given by the 95% confidence intervals of 1.000-1.442 and 9.856E-14-8.157E-13 for α and b, respectively.

The fit for 30°C resulted in R^2=0.566 for α=1.033 and b=8.216E-13 (figure 7). The uncertainties for α and b were higher due to the lower R^2 value compared to the measurements at 25°C given by the intervals of 0.633-1.432 and 1.203E-13-5.611E-12 for α and b, respectively. The power fit function for 38°C revealed its maximum of R^2=0.362 for α=0.689 and b=2.18E-12 (figure 8). As mentioned before uncertainty in both coefficients is high as R^2 is even lower than calculated for the data at 30°C (0.294-1.083 and 3.291e-13-1.444e-11 for α and b, respectively).

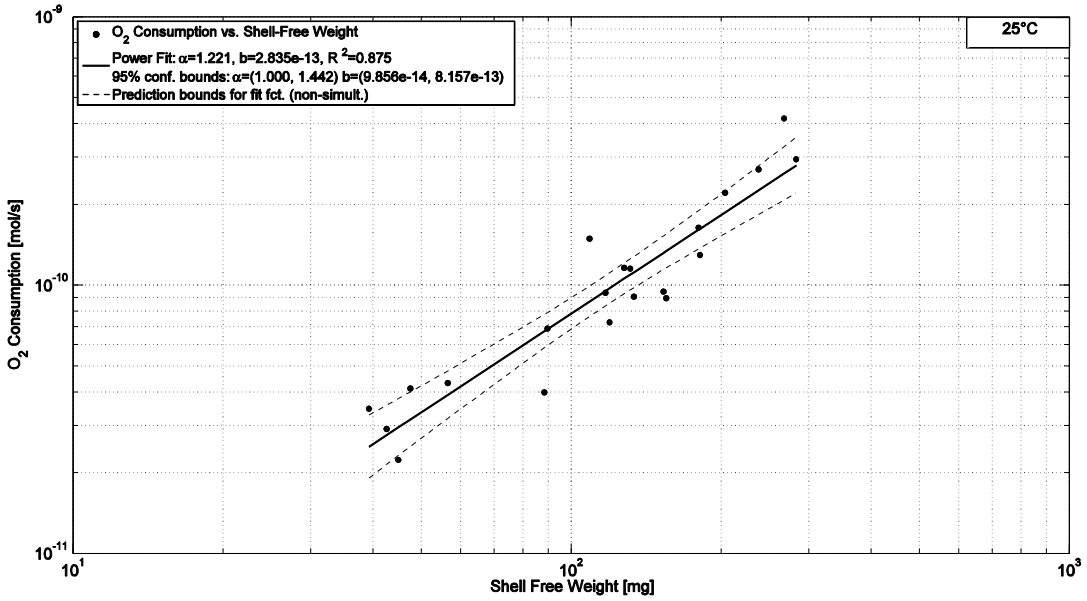

Fig. 6: Oxygen consumption vs. shell free weight for 25°C, logarithmic scaling. To minimize the uncertainty of the resulting fit coefficients, the basic dataset, which can be approached by a non-linear power function $M=b*W^{\alpha}$, was transformed by plotting the data in a double logarithmic diagram. By doing so the power function is transformed into a straight line with the slope α. MATLAB performed the fit with a linear least-squares minimization technique to calculate the coefficients b and α. The resulting uncertainty for the fitted coefficients is also calculated by MATLAB. Additionally, the upper and lower prediction bounds (95% confidence) for the fitted curve were calculated. Calculation has been applied with the original data since a transformation by the 4^{th} root would result in different fit coefficients that are not comparable to the existing literature.

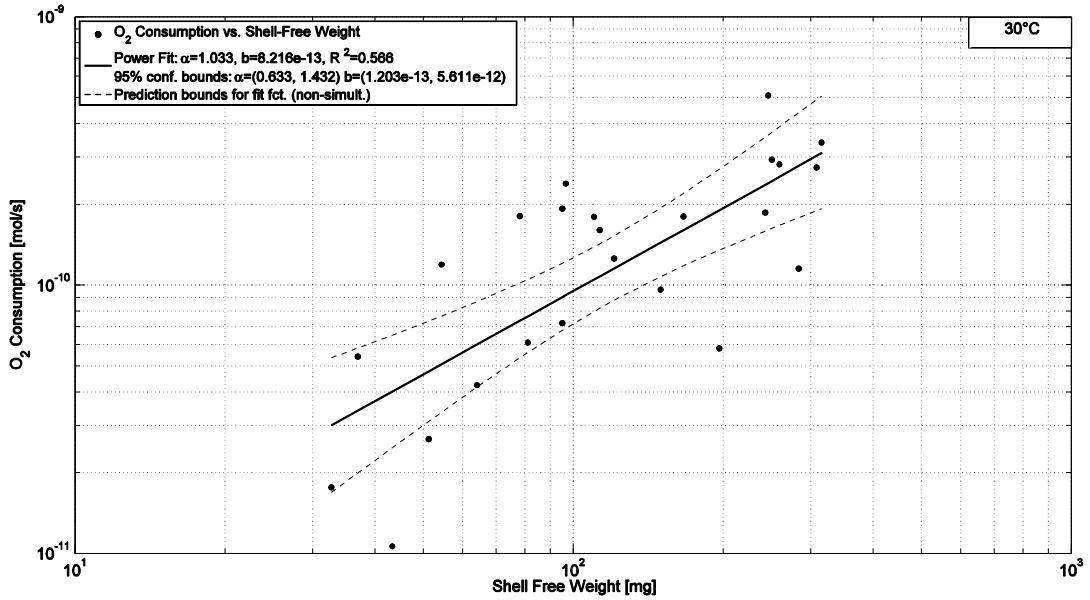

Fig. 7: Labels as in Figure 6. Oxygen consumption vs. shell free weight for 30°C, logarithmic scaling.

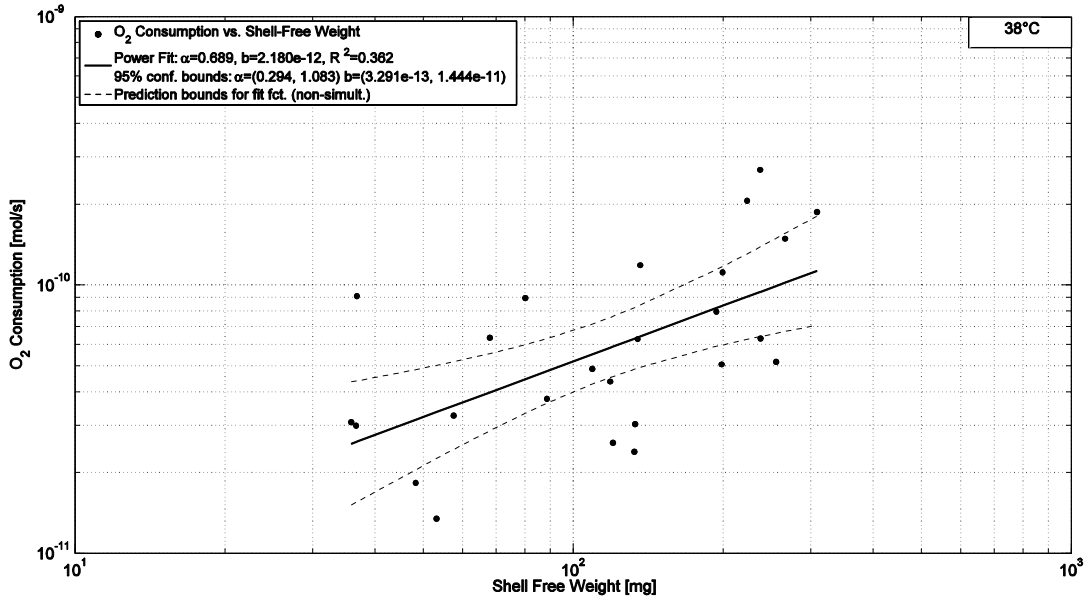

Fig. 8: Labels as in Figure 6. Oxygen consumption vs. shell free weight for 38°C, logarithmic scaling.

4. Discussion:

In the present study a population of *Xeropicta derbentina* from southern France (Modène, Vaucluse) was used to investigate the oxygen consumption at three different ambient temperatures. Additionally three shell size groups were differentiated and the influence of temperature was studied separately for each group.
Finally, the effect of mass on metabolic rate was analyzed, which is expressed by the characteristic curve of oxygen consumption over shell-free weight for each temperature.

4.1 Effect of shell size:

Independent of the temperature, an increase in oxygen consumption with increasing shell size was found. This relation is plausible since larger snails have greater energy demands and therefore higher metabolic rates. The development of the mean values for each shell size category shows a more or less constant trend suggesting a linear relation for the data when the temperature influence is neglected. However, no statistical evidence can be derived from this relation as the data for 'shell size category' contains non-uniformly distributed values of the shell diameter.

4.2 Effect of temperature for each shell size group:

In southern France *X. derbentina* has to face xeric and hot conditions during the day. As reported by Dieterich et al. (2013) snails are almost entirely inactive during the day, which limits their food and water uptake to times of activity on the ground at night and in the early morning when cooler temperatures prevail. By climbing vertical objects the snails escape from lethal ground temperatures during daytime and quickly become inactive to avoid desiccation. Schmidt-Nielsen et al. (1971) argued that in very harsh habitats the major physiological threats for snails are thermal death, desiccation and starvation. The solution to these problems basically includes metabolic regulation at high temperatures, control of water loss during inactivity, and low metabolic rates during dormancy (Riddle, 1975). A metabolic adaption of snails at higher temperatures has been reported by a number of researchers (Dallas et al., 1991; Riddle, 1977; Steigen, 1979).
The results for shell size category 1 indicate that no thermal regulation is present for small individuals of *X. derbentina* mirrored by the lack of any significant differences for all investigated temperatures (Figure 3). Apparently, this effect is related to the animals' young age

and their ongoing adolescence. Young individuals may tolerate higher temperatures before regulation of metabolism takes place. This argument, however, has still to be confirmed. Effects of increased oxygen consumption due to locomotion can be precluded since every recorded individual was inactive during the measurements.

The results for shell size category 2 (Figure 4), however, are in good agreement with the results of Riddle (1977) who detected strong regulations for both of his test species, *Rabdotus schiedeanus* and the garden snail *Helix aspersa*, beyond 25°C. A similar result was presented by Steigen (1979) who showed temperature compensation of *C. hortensis* in a temperature range of 15°C-25°C.

Snails belonging to shell size category 3 (Figure 5) showed a non-significant trend similar to that of category 2 snails. The reasons for the differences between distinct sizes – and respectively ages – of *X. derbentina* may be explained by their general capacity to adjust physiological processes to external temperatures at different life stages. Given that the induction of stress proteins reflects a snail's ability to modify metabolic processes, it should be taken into account that 70-kD stress protein (Hsp70) levels are reduced in older individuals and that it is more difficult to induce Hsp70 in old individuals (Köhler, 2009; Mayer & Bukau, 2005). This reduced capability to physiologically counteract heat stress is probably based upon accumulated stress effects in individuals of old age at the end of summer (Dieterich et al., 2013) and the energetic trade-off between maintaining the molecular stress response system and reproduction (Mizrahi, Heller, Goldenberg, & Arad, 2011). It is plausible that this physiological limitation is not restricted to stress protein induction but extends to other basic metabolic processes, such as the O_2 consumption. Consequently, 'older' individuals of *X. derbentina* may not be able to maintain high metabolic activity in the heat, due to a general 'weakening', while younger individuals can. Yengkokpam et al. (2008) showed that, in particular, a connection between oxygen consumption and Hsp70 production exists as he reported for the fish *Labeo rohita*.

Variation in oxygen consumption, resulting in a rather high standard deviation in all test groups, may be due to irregular pneumostome opening and periodical release of CO_2 during measurements as it was described by Barnhart and McMahon (1987).

We conclude that a change in ambient temperature affects the oxygen consumption of *X. derbentina*. The degree of this impact, however, depends upon their life history, i.e. individuals in phases of strong growth and ongoing adolescence do not show any metabolic down-

regulation at higher temperatures, whereas individuals of medium and large size, and hence of older age, show a clear metabolic shift in response to high temperatures. However, the question as to what extent active individuals also showed metabolic adaptations and how far size/age affected activity in general cannot be answered, since all measurements were performed on individuals in their inactive phases.

4.3 Weight-/Metabolism relationship:

The relation between metabolism and body size, respectively body weight, of living organisms has been under intense discussion for almost 175 years. With respect to homeotherms, particularly mammals, the results according to Kleiber (1947) suggest that their metabolic rate grows with approximately 0.75 the power of weight. Bertalanffy (1957) distinguishes between three different growth types, those obeying the surface rule ($\alpha=2/3$), those growing proportionally to their weight ($\alpha=1$) and an intermediate type ($2/3<\alpha<1$). In his detailed review he states that snails of the family of *Helicidae* belong to the first group with a metabolic rate directly proportional to weight, hence $\alpha=1$.

Our results demonstrate a clear relationship with temperature, similar to the one observed by Steigen (1979), namely a decrease in α for increasing temperatures. For 25°C the value of α was 1.22, which is higher than 1.0 and outside the range postulated by Bertalanffy. For 30°C the value of α is approximately 1 indicating a direct proportionality to weight consistent with the results of Liebsch (1929) and also Kienle and Ludwig (1956). At the highest temperature of 38°C, α declines even further to the value of approximately 0.69 that is close to the value for animals obeying the surface rule.

Wesemeier (1960) presented results for both active and inactive specimens of *Helix pomatia* and reported an increased value of α for the active individuals that he could not explain. For *Xeropicta derbentina* it is well known that these snails become more and more inactive as temperatures increase. Even though no active snail was measured in the present study, it may be that snails at the lower test temperatures have a higher state of 'internal' activity than the ones measured at the highest temperature, which are trying to lower their metabolism as much as possible. It is therefore conceivable that the state of activity, indirectly triggered by temperature, has an influence on α.

It has to be mentioned that measurements become less accurate at high temperatures due to unavoidable methodological reasons and therefore, an evaluation of the goodness of fit is

difficult to interpret. The first reason for this is due to the lower oxygen consumptions at higher temperatures and the increasing sensitivity of the respirometric system at higher temperature differences between measurement chamber and the outer environment. The second reason may be due to increased thermal stress on *X. derbentina* that arises at higher temperatures. Under the assumption that this effect starts primarily in only a few individuals and not in the entire group of animals at the same time, the overall variance increases and therefore R^2 declines.

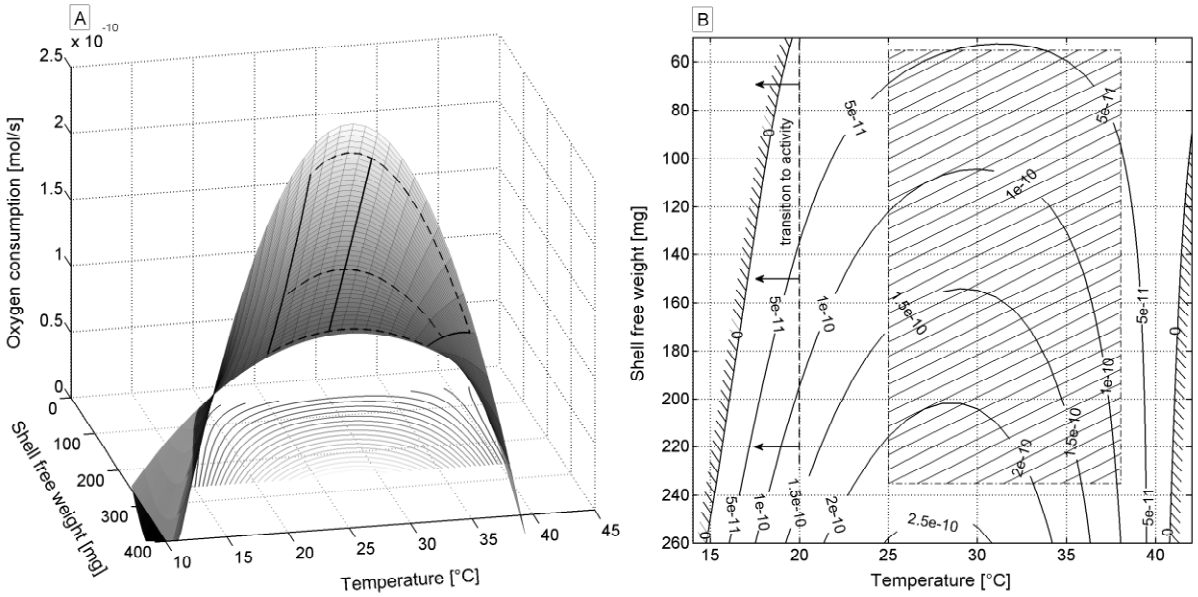

Fig. 9: A: Response surface calculated for oxygen consumption vs. temperature and shell free weight. The resulting equations of the power fits from Figures 6-8 have been used to calculate oxygen consumptions for each temperature using the mean shell free weights of each shell size category (ref. Figure 2) resulting in 3x3 datapoints. For the representation of oxygen consumption of each size category over temperature a quadratic fit was generated. The three coefficients for the absolute (a_1, a_2, a_3), linear (b_1, b_2, b_3), and quadratic term (c_1, c_2, c_3) of each size category were subsequently plotted vs. the mean shell-free weights and interpolated again with a quadratic fit yielding $a_{(m)}$, $b_{(m)}$, and $c_{(m)}$. The surface for oxygen consumption can then be represented by a function of the form O_2 consumption = $a_{(m)} + b_{(m)}*T + c_{(m)}*T^2$.
B: Two-dimensional plot of constant O_2 consumptions (isoboles) in a temperature vs. shell free weight diagram. Zero isoboles represent the theoretical limits (zero O_2 consumption) deduced by extrapolation. The hatched area shows the region of interpolation.

A complete view of the dependence of oxygen consumption upon temperature and body size is given in Figure 9A. The changing curvature of the surface at elevated temperatures is related to the trend towards lower exponents α (solid lines for T=25, 30 and 38°C corresponding to our measured data). The exponent α represents the curvature of the solid line at

each section and clearly shows a value <1 (negative curvature for increasing body weight) for T=38°C and increases further up to a value >1 for the lowest temperature.

For given shell free weights (dashed lines) the surface includes the results from Figures 3, 4 and 5 showing the decline in metabolism with increasing temperatures, particularly for high body weight. Here, the response surface has been extrapolated to temperatures beyond the upper and lower limits chosen in our study (14°C – 42°C). The resulting plot reveals conditions under which the oxygen consumptions theoretically would yield negative values, representing possible limits of physiology. Additionally, isobolic curves of constant oxygen consumptions are depicted in Figure 9B displaying the 'regions' of predicted physiological limits (zero or negative oxygen consumptions for both very low and very high ambient temperatures). Even though these 'limits' derive from extrapolations and are, thus, purely theoretical they fit rather well to behavioural and biochemical adaptations of *X. derbentina* to environmental temperatures. The lower temperature 'limit' (15-20°C) corresponds to conditions triggering arousal from inactivity resulting in the transition into active movement and feeding activity. However this region is associated with a high degree of uncertainty due to its rather distant extrapolation from measured data as indicated by the hatched surface. The upper temperature 'limit' (slightly above 40°C) corresponds astonishingly well to the temperature that induces the maximum Hsp70 response after 8h exposure (Troschinski et al., 2014). For even higher temperatures, the Hsp70 response in *X. derbentina* starts to be overwhelmed, indicating the biochemical limits of this species, as also extrapolated for the O_2 consumption in our model.

In conclusion, using a highly sophisticated microrespirometer constructed especially for the investigation of inactive snails we have been able to show that the relation between oxygen consumption and size of *X. derbentina* is not quantitatively fixed but is directly dependent upon ambient temperatures. This may well be associated with the observed metabolic depression at higher temperatures for medium-sized and large snails. The data generated within the frame of the present study and the resulting insights may provide the basis for a further and more general analysis of the physiological properties and constraints of xerophilic land snails.

5. Acknowledgments:

The authors would like to thank Christophe Mazzia for the delivery of snails and the provision of his meadow to collect the animals. Additional thanks are given to IPG Laser GmbH for their provision of the Rittal Cabinet. Furthermore we would like to thank Simon Schwarz for his help with the statistical analysis. The study was financed by the University of Tübingen's Twinning Project with the University of Applied Science Esslingen under the supervision of H.-R. K., U. G., D. W. and Stefan Rösler.

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Danksagung

Die vorliegende Arbeit wurde an der Eberhard Karls Universität in Tübingen, in der Arbeitsgruppe „Physiologische Ökologie der Tiere“ angefertigt.

Zuerst bedanke ich mich herzlichst bei meinen beiden Betreuern, Prof. Dr. Heinz-R. Köhler und Prof. Dr. Rita Triebskorn, dass sie mir die Möglichkeit zum eigenständigen wissenschaftlichen Arbeiten eröffneten und mir stets mit hilfreichen Ratschlägen und Hilfestellungen bei Problemen zur Seite standen.

Der Eberhard Karls Universität Tübingen und dem Land Baden-Württemberg danke ich für die Finanzierung über das '*Twinning project'.*

Für eine einzigartige Arbeitsatmosphäre möchte ich mich herzlichst bei allen aktuellen und ehemaligen Mitgliedern der Arbeitsgruppe „Physiologische Ökologie der Tiere“ bedanken (in ungeordneter Reihenfolge): Paul Thellmann, Carla Lorenz, Hannah Schmieg, Sabrina Willhelm, Katharina Peschke, Dr. Volker Scheil, Dr. Alexandra Scheil, Krisztina Vincze, Diana Maier und Andrea Demuth. Besonderen Dank geht an meine Projektpartnerinnen Dr. Sandra Maute (geb. Troschinski) und Dr. Maddalena Angela Di Lellis sowie an Stefanie Krais, Anja Henneberg und Simon Schwarz für tatkräftige Unterstützung während der Feldprobennahmen, für die Einweisungen im Labor und die Hilfestellung bei Fragen zur Statistik.

Bei meinen Kooperationspartnern an der Hochschule Esslingen, Ulf Fischbach, Markus Ludwig, Ulrich Gärtner und Stefan Rösler möchte ich mich für die produktive und stets freundliche Zusammenarbeit bedanken.

Für hilfreiche Diskussionen, neue Ideen, Zerstreuung der Alltagssorgen und ihre Freundschaft möchte ich mich herzlich bei Paul Thellmann, Carla Lorenz, Simon Schwarz, Hannah Schmieg, Mike Föll, Markus Lifka, Axel Zander, Jochen Gaiß, Reinhard Berron, Sebastian Kuhn, Jonas Müller und Tobias Korn, sowie deren Partner und Partnerinnen bedanken.

Bei meiner Familie und der Familie meiner Frau möchte ich mich herzlich für die liebevolle Unterstützung bedanken.

Zu guter Letzt danke ich meiner Frau Monika für ihre Liebe, Zuneigung und Hilfe in allen Lebenslagen.

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