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The impact of diet treatment on reproduction and thermophysiological processes in the land snails *Cornu aspersum* and *Helix pomatia*

Thèse soutenue à la station biologique de Paimpont, le 29 Juillet 2010

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To those needing a little bit deceleration in their life

Language

Because this work integrates a project about conservation and organic farming of *Helix pomatia* in Germany, the part concerning research in this species was written in German except for articles. The general introduction and conclusion as well as the abstract of the study that could not be published were also translated in English. The joint supervision thesis contract allows this duality in language choice.

Da diese Arbeit Teil eines Projektes zum Schutz und zur Schneckenzucht von *Helix pomatia* in Deutschland ist, wurde der Forschungsteil zu dieser Art auf Deutsch geschrieben, auβer die Artikel. Die allgemeine Einleitung und Schlussfolgerung sowie die Zusammenfassung der Studie, die nicht veröffentlicht werden kann, wurden auch ins Englische übersetzt. Der binationale Promotionsvertrag erlaubt die Auswahl dieser zwei Sprachen.

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PUBLICATIONS

Articles in international journals

Published article

Cryobiology 50 : 48-57 (2005)

A. Nicolai, P. Vernon, M. Lee, A. Ansart and M. Charrier
Supercooling ability in two populations of the land snail *Helix pomatia* (Gastropoda: Helicidae) and ice-nucleating activity of gut bacteria.

Submitted articles

Cryoletters
Ref.: Ansart et al. *Ca*-V

A. Ansart, **A. Nicolai**, P. Vernon and L. Madec
Do ice nucleating agents limit the supercooling ability of the land snail *Cornu aspersum*?

Canadian Journal of Zoology
Ref.: Nicolai et al. *Ca*-I

A. Nicolai, J. Filser, V. Briand, M. Charrier
Seasonally contrasting life history strategies in the land snail *Cornu aspersum*: physiological and ecological implications.

Journal of Experimental Zoology
Ref.: Nicolai et al. *Ca*-II

A. Nicolai, J. Filser, R. Lenz, V. Briand, M. Charrier
The contrasted effects of body stores on partial capital breeding in the land snail *Cornu aspersum.*

Naturwissenschaften
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Prepared articles

Ref.: Nicolai et al. *Hp*-I

A. Nicolai, A. Ansart, J. Filser, R. Lenz, M. Charrier
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A. Nicolai, J. Filser, R. Lenz, C. Bertrand, M. Charrier
Physiological adjustment to micro-environmental variations in the land snail *Helix pomatia*.

Ref.: Nicolai et al. *Ca*-III

A. Nicolai, R. Lenz, V. Briand, M. Charrier
Reproductive allocation of cholesterol in the terrestrial gastropod *Cornu aspersum.*

Planned article	
	A. Nicolai, J. Filser, R. Lenz, C. Bertrand, M. Charrier Seasonal changes in metabolite composition of haemolymph during growth of the land snail *Helix pomatia*.
	Article in a national journal
Horizonte 30 : 3-4 (2007)	**A. Nicolai** and R. Lenz. Weinbergschneckenzucht auf der Schwäbischen Alb – ein Beitrag zur Regionalentwicklung.
	Book
Ed. Region Aktiv (2006)	**A. Nicolai** and R. Lenz Die Albschneck®-Zucht auf der Schwäbischen Alb. ©Interessengemeinschaft Albschneck
	International Congresses
Poster, World Congress of Malacology, Belgium (2007)	**A. Nicolai**, S. Fournier, V. Briand and M. Charrier The allocation of energy to reproductive success in *Cornu aspersum* before and after hibernation.
Prevision	
Oral communication, World Congress of Malacology, Thailand (2010)	Seasonally contrasting life history strategies in the land snail *Cornu aspersum*: physiological and ecological implications.
	National Congress
Oral communication, Colloque national sur l'héliciculture, Ile de Ré (2009)	La résistance au froid et au chaud chez *Cornu aspersum*.

PART 1

The impact of diet treatment on reproduction and thermophysiological processes in *Cornu aspersum*

Resource acquisition and allocation is a basic task of all animals. Among physiological processes, those that influence resource use are the most likely to influence an animal's behaviour, its population dynamics, its role in biotic communities, and its contribution to ecosystem fluxes. Organisms often encounter environmental heterogeneity in space and time, and may adapt their life histories that have been described as heritable set of rules determining age-specific allocation to growth or storage for survival *versus* reproduction (Karasov & Martinez del Rio 2007). Energy allocation to each function depends on resource availability and on the interaction with other selective pressures and might be reflected in trade-offs between life history traits (Reznick *et al.* 1996; Kim & Thorp 2001; Roff 2002; Sandland & Minchella 2003; Lardies *et al.* 2004; Ter Maat *et al.* 2007).

When energy is allocated to reproduction, the unavailability of this energy for other functions represents the costs of reproduction (Begon *et al.* 1996). However the allocation of energy to offspring influences fitness via offspring survival, growth and future fecundity. Animals may answer to temporally uncertain environments with life history delays, variable age and size at maturity, and iteroparity, this is spreading their reproductive effort over several breeding periods (Beckerman *et al.* 2002; Wilbur & Rudolf 2006). Even if acquisition of energy stores is costly (Reznick & Braun 1987), it makes reproduction possible at times or places with limited access to food (Varpe *et al.* 2009).

Survival in uncertain environment might be costly when physiological adjustments are necessary beyond the optima. For instance thermal reaction norms can involve different trade-offs dependent on the variability and predictability of certain environmental factors to overcome costs of rate adjustment or stress tolerance (Parsons 1997).

Recently, many studies in ecology try to analyze impacts of the global changes on biodiversity and species abundance, like fluctuations in climatic factors, intensive land use and habitat fragmentation. However the underlying physiological relations are still not completely known and there is always a surprise when species do not do what we might have understood from earlier studies they should do (see some of your referee comments). Terrestrial mollusks have to cope more than other taxa with adverse environmental conditions due their low dispersal ability. Therefore, some questions raised about resource allocation focused on *Cornu aspersum*, because it is an ectothermic hermaphrodite species with determinate growth pattern subjected to a wide range of different climatic and trophic conditions because of its large distribution. Nowadays Cornu aspersum is considered as an invasive species colonizing new habitats of North America, Australia and Europe (Guiller *et al.* 2001). Studies on *C. aspersum* showed plasticity of quantitative reproductive traits related to environmental factors in one reproductive season (Baur 1994; Madec *et al.* 1998; Madec *et al.* 2000), partly genetic control of adult weight (Dupont-Nivet *et al.* 1997) and a susceptibility to freeze at subzero temperatures (Ansart *et al.* 2001a).

Aims of this study:

- To test the energy availability in food on allocation to seasonal time constrained reproduction by comparing reproductive strategies and their success.
- To analyze the influence of quality of body stores, which were built up during growth on reproductive investment.
- To know if a biosynthesized essential compound unavailable in food was allocated to offspring.
- To investigate the role of mineral source in egg shell composition in relation to heat stress of different population origins.
- To point out the origin and nature of freezing susceptible gut compounds.

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The garden snail *Cornu aspersum* Linnaeus 1758

Phylum:	Mollusca
Class:	Gasteropoda
Order:	Sigmurethra
Family:	Helicidae
Genus:	*Cornu* (former: *Helix)*
Species:	*aspersum*(former: *aspersa)*
Author:	Müller (1774)

This species has a variable habitat, often connected with human activity, e.g. parks and gardens but also dunes, forests, rocks and hedges (Kerney & Cameron 1979). Originating from the Mediterranean area, *Cornu aspersum* is also prevalent in Western Europe, along the Atlantic coast and at the Balkan (Figure *ca*1) (Chevallier 1977; Kerney & Cameron 1979). This species can adapt to different climatic circumstances and could therefore be found from the temperate climate to the tropics. Because of its gastronomic use this species also occurs in Africa, Oceania, America and Australia.

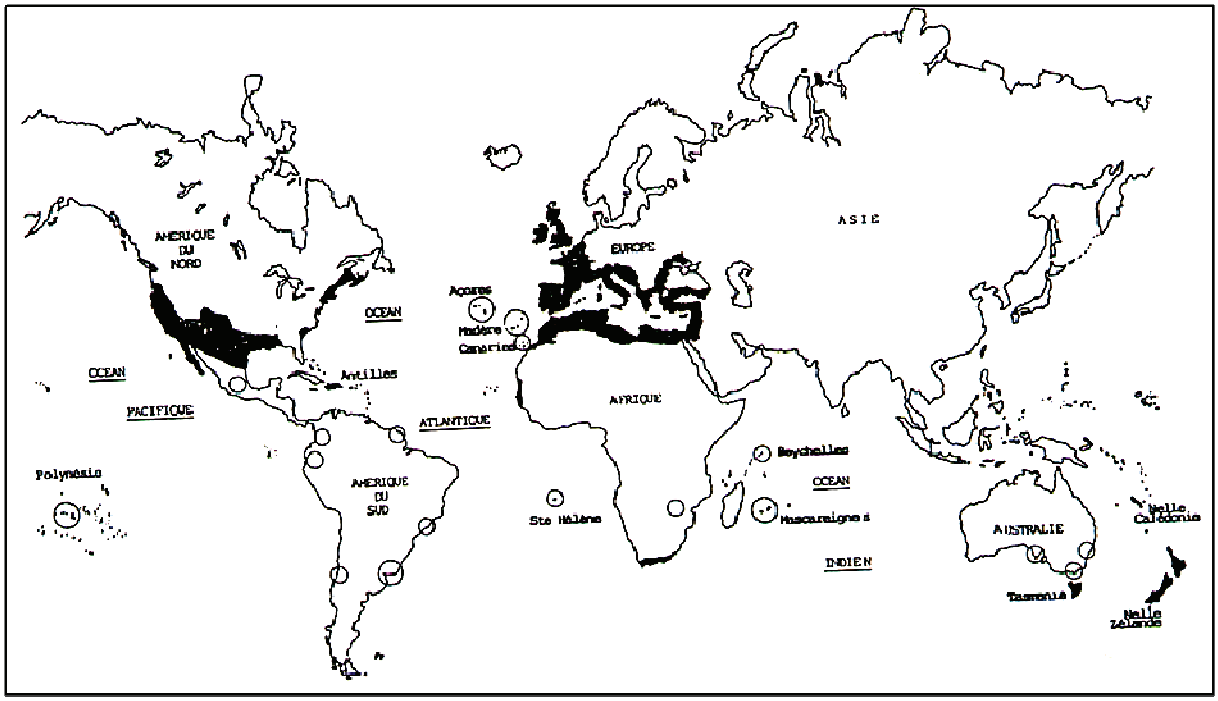


Figure *ca*1. Worldwide distribution of *Cornu aspersum*. Black marking: current area of distribution, circle: island like distribution (Madec 1989).

The land snail *Cornu aspersum* has a globular shell (height: 25 - 35 mm, diameter: 25 – 40 mm) with 4.5 - 5 spires (Figure *ca*2 A) (Kerney & Cameron 1979). The ombilicus is covered by the peristom. The shell with irregular growth lines is bright to dark brown with up to five spotted belts. The colouration of the tegument is dark brown to dark grey.

The physiological state of *Cornu aspersum* depends on climate conditions of habitat. When the temperatures drops under 7°C or exceeds 27°C and the relative humidity lays beyond the optimum of 75-95%, *Cornu aspersum* enters in a state of dormancy, hibernation or aestivation, respectively (Charrier & Daguzan 1980; Ansart *et al.* 2001a, b). The duration of dormancy and the moment of initiation vary in respect to the geographic region (Iglesias *et al.* 1996). At the initiation of dormancy the peristom is obturated by an epiphragm (Figure *ca*2 B), which is formed by the secretion of mucus rich in proteins and calcium (Ansart *et al.* 2001a, b).

Different stages of developpement have been described in *Cornu aspersum* by (Charrier & Daguzan 1978) according to the shell diameter:

- „Juvenile I“ with $3 \leq d < 22$mm and immature genital tract (Figure *ca*2 C)
- „Juvenile II“ with $22 \leq d < 27$mm and some maturated parts of the genital tract (albumin gland, mucus gland and penis)
- „Adult“ with $d > 27$mm and completely mature genital tract

The reinforcement of the shell and the reflection of the shell lip at the peristom take place at the end of growth, which corresponds with the sexual maturity of snails in captivity (Madec & Daguzan 1991). Snails in the nature can sexually mature before the end of growth.

Cornu aspersum is a simultaneous hermaphrodite with reciprocal fecundation (Figure *ca*2 D) (Adamo & Chase 1988). 80-140 eggs with a diameter of 3 mm surrounded by a mucous layer are placed in a burrow. The incubation time takes between 10 to 30 days. Egg cannibalism in the nest is characteristic for this species and enhances the chances of survival of the juveniles by accelerated growth (Desbuquois 1998).

Figure *ca*2. Adult *Cornu aspersum* (A), hibernating *Cornu aspersum* with epiphragm (B), juveniles of *Cornu aspersum* in the nest (C), and copulation of *Cornu aspersum* (D).

The period of breeding depends on the climate of the geographic region and is influenced by different environmental factors, like temperature, humidity, but particularly photoperiod. Thus, the species can reproduce one to several times per year before and after dormancy (hibernation or aestivation) (Attia 2004). In Western Europe the breeding period lasts from April to June and can repeat in September/October. In the Mediterranean region there are two breeding

periods: October to December and February to April. Multiple copulations and ovipositions within one reproduction period are common (Adamo & Chase 1988). Under natural conditions, juveniles of the first breeding period reach sexual maturity generally before growth termination, the next year before hibernation. Juveniles of the late reproduction period mature in the second year (Charrier 1980). Usually, there is one period of dormancy between sexual maturity and first reproduction.

Cornu aspersum is polyphageous, but the nettle *Urtica dioica* and plants of the family Poacae are mostly eaten (Chevalier *et al.* 2001). Some of the plant minerals appear repulsive, others, especially calcium, appear attractive to *Cornu aspersum* and can influence the choice of food depending on their concentration (Chevalier *et al.* 2003).

Rearing conditions

Origin of snails and food formulation

Breeders were taken from an outdoor snail farm (Corps-Nuds near Rennes, France) and reproduced in February 2004. Four clutches of different mothers laid on the 13th of February gave rise to 397 newborns on the 28th of February. Newly hatched snails were chosen at random and assigned to different diet treatments (sibling-split design).

Snails were fed *ad libitum* on a specific food for cultured snails, Helinove®, formulated by Idena (Sautron, France) and made by Berton Alimentation Animale (Le Boupère, France) (Table *ca*1). E0 represents the basis food used in the snail farm. The analysis of each mineral source in the Cas and Cam diet was done by Energy Dispersive Spectrometry (EDS, Oxford, INCA at CMEBA of University Rennes 1). The mineral content was then calculated for each diet (Table *ca*2).

Table *ca*1. Composition of the Helinove® diets in % of mass and energy content in kcal/kg. Prime materials of all diets were corn, soja, wheat, lucern, pea, minerals, and additives, like trace elements and vitamins. Added minerals were calcium phosphate, limestone type „Albacal 0/315 µm" from Ile-de-France in France, dried algae *Phymatolithon calcareum* from Brittany in France, and fossil oyster shells OYTA® from the Fjord Roskilde in Norway.

	TYPES OF DIET				
	Energy poor (E-)	Energy basis (E0)	Energy rich (E+)	Mixed Ca source (Cam)	Simple Ca source (Cas)
Lipids[1]	2.5	4.0	5.5	2.0	2.0
Proteins	15.0	15.0	15.0	15.5	15.5
Starch and sugars	24.9	25.12	24.7	26.5	26.9
Parietal polymers	12.8	11.23	10.8	9.84	9.9
Moisture	7.9	8.4	7.7	9.62	9.61
Plant minerals	3.1	3.0	3.0	2.5	2.5
Calcium phosphate	3.8	3.8	3.3	4.0	4.0
Limestone	30.0	30	30.0	10.0	30.0
Algae *P. calcareum*				10.0	
Fossil oyster shell				10.0	
Energy	2220	2320	2420	2240	2240

[1] Lipids are mainly soy oil (15% saturated FA, 24% mono-unsaturated FA, 60% poly-unsaturated FA with 52% linoleic acid of total FA) and flaxseed (with linoleic acid as the major FA). FA = fatty acids. Diets are cholesterol free.

Table *ca*2. Composition of alternative mineral sources in Helinove® diets in mean ± SE % of mass. The analysis was done on *N* samples, and the mineral content of each diet was calculated.

	TYPES OF MINERAL SOURCES IN DIET			TYPES OF DIET	
	Limestone *N* = 13	Algae *P. calcareum* *N* = 19	Fossil oyster shell *N* = 9	Mixed Ca source (Cam)	Simple Ca source (Cas)
O	57.75 ± 1.92	40.55 ± 3.26	54.20 ± 0.97	15.28	17.30
Ca	37.07 ± 3.46	24.53 ± 5.03	45.22 ± 1.02	10.67	11.10
Mg	0.18 ± 0.04	1.02 ±0.32	0.57 ± 0.10	0.18	0.05
Si	4.55 ± 3.85	19.13 ± 4.82		2.37	1.37
Al	0.40 ± 0.09	1.24 ± 0.63		0.16	0.12
Fe	0.04 ± 0.04	7.47 ± 5.51		0.75	0.01
S		2.41 ± 2.40		0.24	
Na		1.24 ± 0.49		0.12	
Ti		1.10 ± 1.10		0.11	
Cl		1.00 ± 0.24		0.10	
K		0.30 ± 0.15		0.03	

Growth conditions

The snails were individually tagged and isolated in plastic rearing cages with a constantly wet latex HR (high resilient) foam under controlled conditions (temperature: 20 ± 1°C, relative humidity: 60-80%, L/D = 12/12 h) until they reached maturity (reflected shell peristom), 14-15 weeks later (Figure *ca*3). Replicate cages (daily change of cage location in the rearing room) were used per sample to take possible cage effect into account. Additional cages with snails held in the same condition and density served as stock to replace dead snails over the whole experimental rearing conditions time.

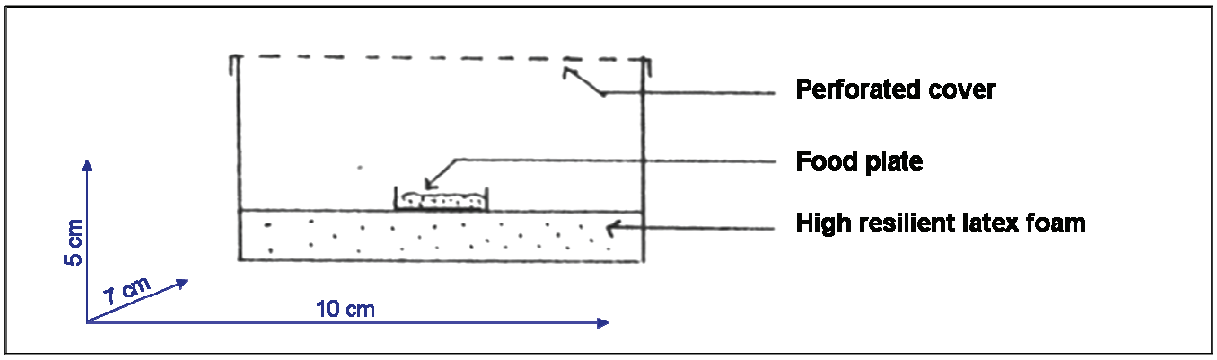


Figure *ca*3. Experimental cage disposition for growth of *Cornu aspersum.*

The dried food dishes were weighed with and without the food at the beginning and at the end of the week. Calculation of growth efficiency GE = G / I (in $g.g^{-1}.d^{-1}$) served to evaluate the capacity of the animal to use the energy of the food. G is the gain of snail weight per day and I the weight of ingested food.

Reproduction condition

For reproduction, five individually tagged snails were placed into a cage with a wet latex HR (high resilient) foam under controlled conditions (temperature: 20 ± 1°C, relative humidity: 60-80%, photoperiod: 16h light/8h dark). The individual density in cages (30 $snails.m^{-2}$) was chosen to be close to natural conditions. Four pottery pots filled with compost (Eco-Terre ®, pH 7.0, Avignon, France) were put in each cage for oviposition and replaced as soon as used (Figure *ca*4). Replicate cages (daily change of cage location in the rearing room) were used per sample to take possible cage effect into account. Additional cages with snails held in the same condition and density served as stock to replace dead snails over the whole experimental time.

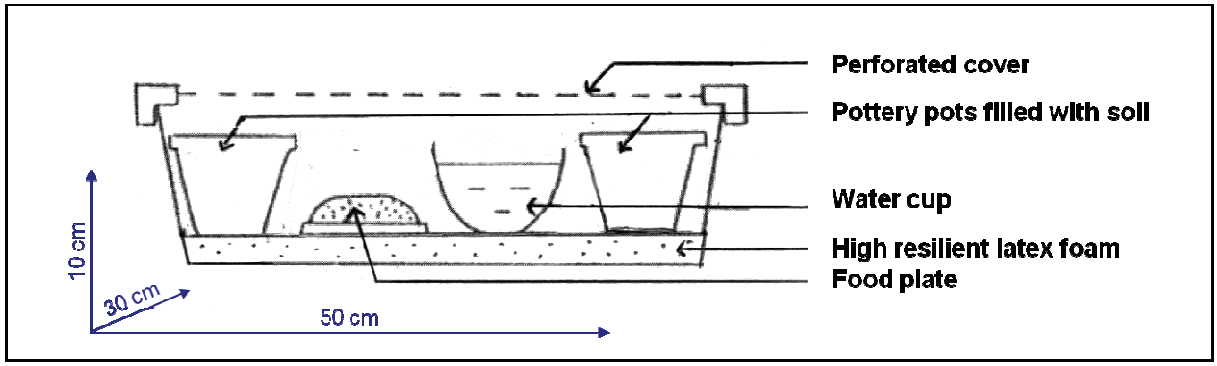


Figure *ca*4. Experimental cage disposition for reproduction of *Cornu aspersum.*

The individuals were observed daily and weighed at least twice per week, when water and food (*ad libitum*) were supplied. During mating and oviposition, snails were not disturbed. All copulations and ovipositions could be recorded. Each clutch was identified by its parentage, by laying and hatching dates. The eggs were counted, and 30 to 40 eggs were randomly chosen and weighed individually.

Egg incubation

The identified clutches could be incubated following two different methods according to the objectives:

1. Clutches were incubated at 20°C in a mixture of constantly humid soil (1/3 compost, 2/3 peat) after careful replacement in the same nest and pottery pot where they were laid (Figure *ca*5 A). The young snails hatched from eggs two to three weeks after egg-laying. The offspring were counted once all eggs had hatched or had been cannibalized, and 30 to 40 of them were randomly chosen and weighed. Hatching rate could directly be used for evaluating the degree of egg cannibalism since no dead eggs were found in the nests. Young cannibalistic snails were also directly observed at the time of hatching, devouring first the egg fluid, afterwards the egg shell.
2. Clutches were distributed in aggregates of five eggs in Petri dishes and incubated at 20°C on an imbued gaze with Marc's Modified Ringer buffer (MMR: NaCl 0.5M, KCl 0.2M, $CaCl_2$ 0.1M, $MgCl_2$ 0.1M and Hepes 23.8 $mg.ml^{-1}$, (Peng 1991) adjusted to pH 7.0 and to incubation temperature (Figure *ca*5 B). Preliminary tests showed the role of separate aggregates in preventing egg cannibalism as much as possible. This method is particularly useful for testing different temperature treatments on incubation time and hatchling rate. In this case, one subsample of the clutch served as control and was exposed to 20°C/24h. The other subsample was exposed to a daily fluctuating temperature cycle, like 20°C/20h - 35°C/4h or 20°C/16h - 35°C/8h, with a progressive temperature adjustment of 0.5°C/min (SANYO climatic chamber, MIR 153, Fisher Labosi, France).

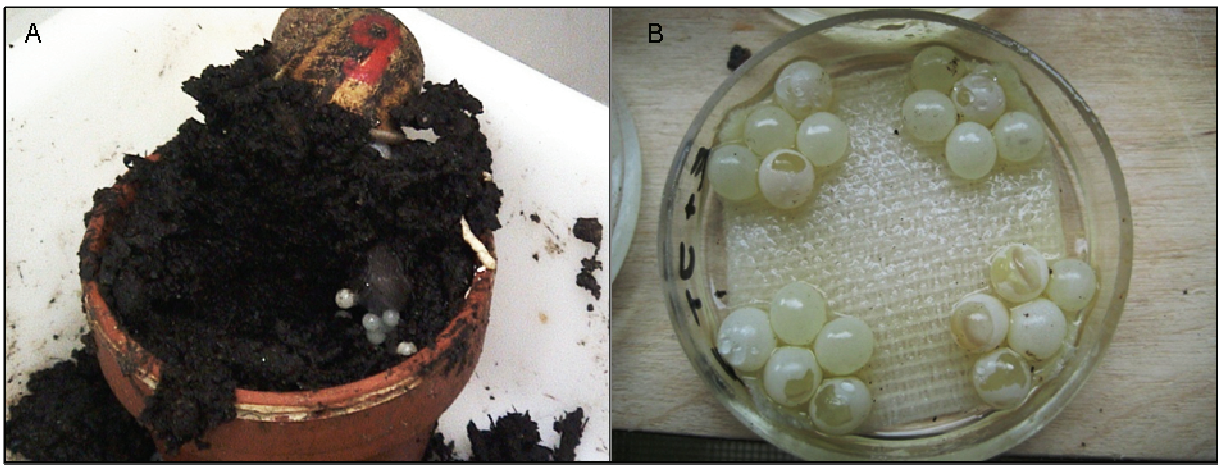


Figure *ca*5. Oviposition of *Cornu aspersum* in a potery pot that will be used for egg incubation (A) and egg incubation on a gaze imbued with buffer solution (B).

Aestivation condition

Snails in activity were kept under controlled conditions (temperature: 20 ± 1°C, relative humidity: 80 ± 5%, photoperiod: 16h light/8h dark) in a cage without the HR (high resilient) foam and without water supply. Aestivating individuals were starved for at least 4 months before beginning of assays.

Hibernation condition

Snails were progressively transferred to hibernation conditions over two weeks without feeding by stepwise decreasing temperature, humidity and photoperiod (Table *ca*3). After six months of hibernation under artificial conditions, they were transferred in the same way progressively to activity conditions corresponding also to reproduction condition.

Table *ca*3. Transfer of *Cornu aspersum* to hibernation and avctivity conditions. T - temperature (°C), RH - relative humidity (%), and PP - photoperiod ($h.day^{-1}$).

		DAY 1-3	DAY 4-6	DAY 7-9	DAY 10-12	DAY 13-15	FINAL CONDITION
Hibernation	T	17	14	11	8	5	5
transfer	RH	85	85	80	70	60	60
	PP	16	14	12	10	8	0
Activity	T	8	11	14	17	20	20
transfer	RH	60	70	80	85	85	85
	PP	8	10	12	14	16	16

Biometric measurements

During growth, weekly measurements were done. Shell breadth was used as body size (BS) measurement (Figure *ca*6 A) and fresh body mass (BM) was recorded (A200S, Sartorius AG, Göttingen, Germany). The residuals from linear regression of body mass on body size were used as body condition index, according to (Glazier 1999) The index values gave adequate measures of variations in body conditions pre-partum and post-partum.

To determine dry mass (DM), snails were killed by freezing and then dissected in separate fractions: shell, foot, genital tract, and digestive tract (Figure *ca*6 B, C). Accidentally cut organs that lost liquid were discarded from further manipulation. The organs were weighed (fresh mass, FM), then frozen and lyophilized in vacuum (Lyovac™ GT3, Leybold-Heraeus, Orsay, France) for 48 h. The dry mass of each dissected organ, ofr the whole body and of the shell were determined. The dry mass density of the body, expressed as DM mg.FM mg^{-1}.100%, represented the storage capacity (Ter Maat *et al.* 2007).

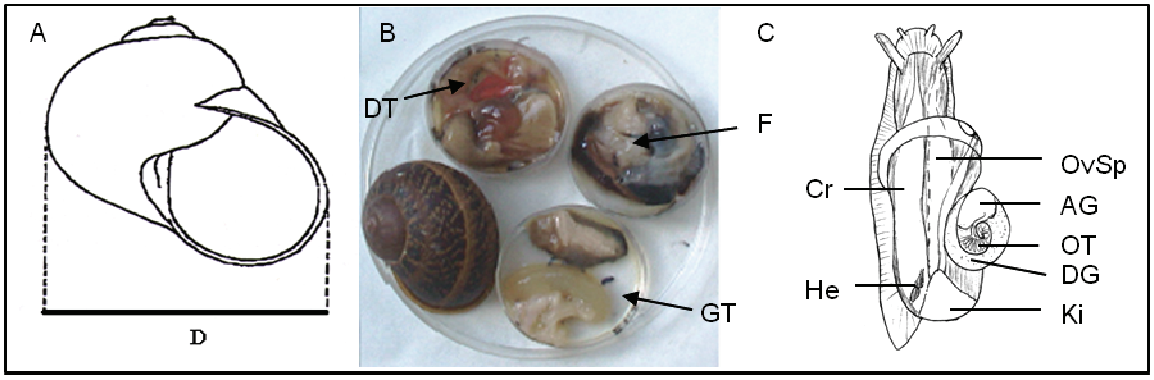


Figure *ca*6. Measurment of body size in *Cornu aspersum* (A), dissection and separation of organs in *Cornu aspersum*: DT – digestive tract, GT – genital tract, F- foot (B), anatomy of *Cornu aspersum*: Cr – crop, He – heart, OvSp – ovospermiducte, AG – albumen gland, OT – ovotestis, DG –digestive gland, Ki – kidney (Tixier & Gaillard 1969) (C).

Egg structure

Microstructure of the egg was examined using a Scanning Electron Microscope (JEOL JSM-6301 F, Tokyo, Japon), after dehydration in graded ethanol and acetone series (ethanol: 50° for 12h; 70°, 80°,90° for 2h each; 100° for 12h; and acetone for 1h) followed by critical point drying (Balzers CPD 010, Balzers, Lichtenstein) and a gold-palladium coating (JEOL JFS-1100, Tokyo, Japon). This preliminary analysis allowed get new insights in the egg structure and querying the earlier work of Bayne (1968) (Figure *ca*7, *ca*8). Egg shell thickness was measured on obtained pictures using the Mac Intosh Graphic Converter Version 5.6.2.

Figure *ca*7. Egg scans of *Cornu aspersum* obtained by a Scanning Electron Microscope at the CMEBA, University Rennes 1 (Photos: Joseph Le Lannic).

A – Broken egg showing two parts: an external compact shell matrix and an internal gelee (scale = 100 µm).

B – Smooth outer surface of the egg (scale = 100 µm).

C – Shell matrix observed in a transverse fracture (scale = 10 µm).

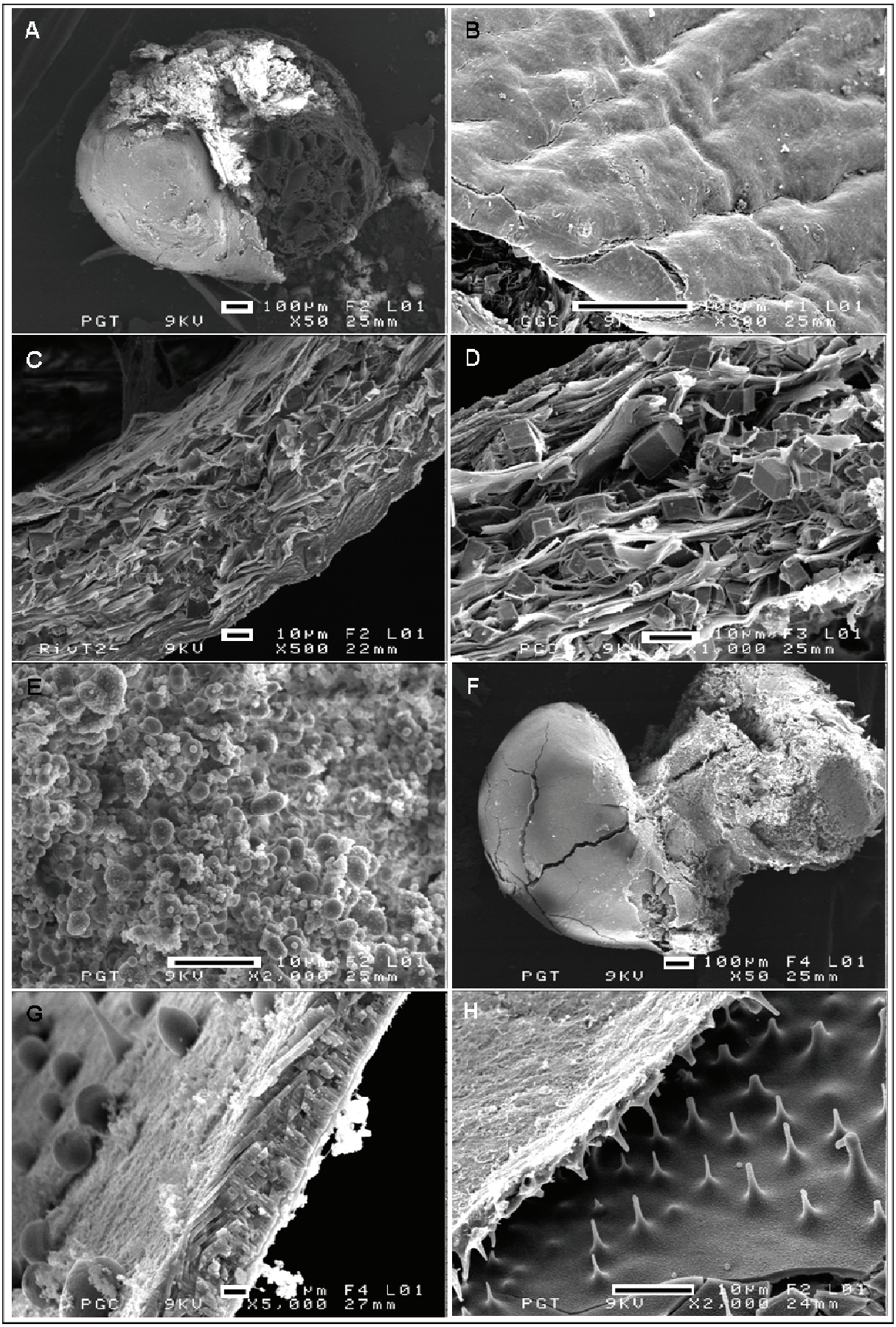
D – Lamellar structure of the shell: calcite rhomboedric crystals are irregularly aggregated between the foliated organic matrix (scale = 10 µm).

E – Granular aspect of the perivitelline fluid (scale = 10 µm).

F – General view of a larval stage developing inside the egg with the velum and the larval shell (protoconch) (scale = 100 µm).

G - Synthesis of the protoconch by the mantle cells (scale = 10 µm).

H – Juvenile shell just before hatching with aragonite needles and organic deposits of the mantle (scale = 5 µm).


A
100µm F2 L01
PGT 9KV X50 25mm
B
C
10µm F2 L01
9KV X500 22mm
D
E
10µm F2 L01
PGT 9KV X2,000 25mm
F
100µm F4 L01
PGT 9KV X50 25mm
G
PGC 9KV X5,000 27mm
H
10µm F2 L01
PGT 9KV X2,000 24mm

The mineral content in eggs was estimated by ash determination using a Muffle furnace (HORNO, Naber, Germany) at 550°C for 1h. Additionally, the analyses of the content of storage compounds in eggs were performed following the same protocol like for breeder tissues (Table *ca*4).

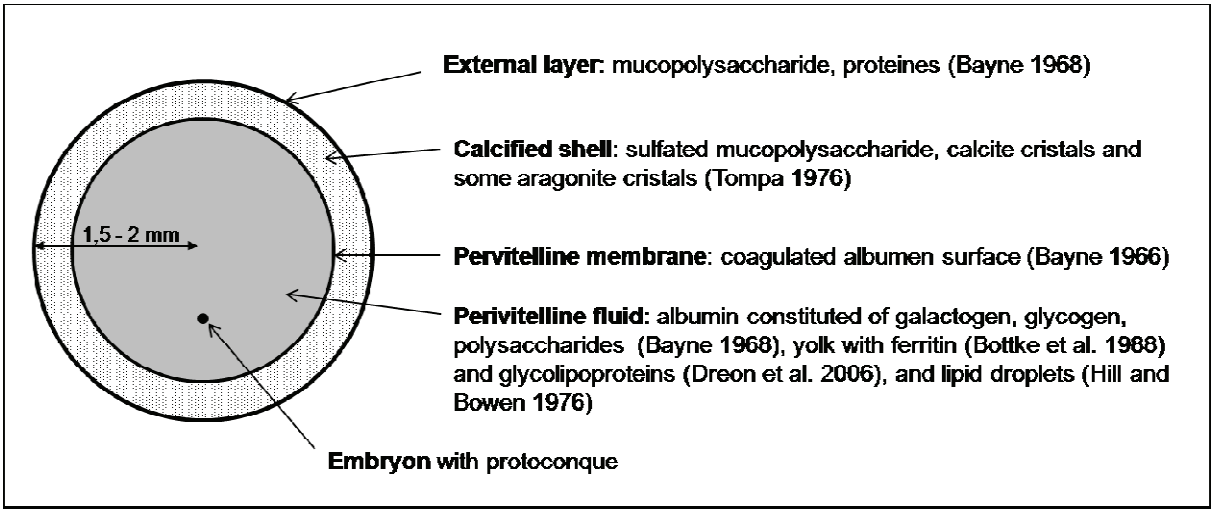


Figure *ca*8. Schema of the structure and composition of an egg in *Cornu aspersum*.

Biochemical analysis

Biochemical analysis was conducted on storage organs (digestive gland, albumen gland, and foot) and in eggs, after their dessication in vacuum (Lyovac™ GT3, Leybold-Heraus, Orsay, France). The main storage compounds (triglycerides, galactogen, and glycogen) and the main sterol (cholesterol) were analyzed on dry tissues that were homogenized by bead-beating using Tungsten beads of 3 mm diameter (4 x 30 s at 30 agitations. s^{-1}, Retsch™ MM301, Retsch GmbH, Haan, Germany). Lipid extraction was performed according to the protocol of (Hervant *et al.* 1999) and polysaccharide extraction according to the protocol of (Van Handel 1965). Then the extract was dried under nitrogen flow at 30°C for 1 min and dissolved for chemical revelation using micro plate spectrophotometer (VERSAmax™ microplate reader, Sunnyvale, CA, USA). For a summary of analyses protocols see table *ca*4. Storage compounds were quantified on the basis of calibration curves and the values were expressed in mass per body dry mass.

Table *ca*4. Summary of biochemical analysis on storage organs and eggs in *Cornu aspersum*.

	EXTRACTION MEDIUM	PHASE SEPARATION	DISSOLVING MEDIUM	CHEMICAL REVELATION
Triglycerides	methanol: chloroform (1:2 v/v)	KCl (2 $g.l^{-1}$), 5 min at 40°C	BSA (3% w/v, fatty acid free) - Triton (0.2% v/v)	Triglyceride Assay Kit (Cayman Chemicals, Ann Arbor, USA)
Cholesterol			Ethanol (95°)	Cholesterol RTU™ kit (Biomerieux, France)
Glycogen	Trichloroacetic acid (4%)	Centrifugation (5000 g, 5 min, 4°C), precipitation by ethanol (96°)	Water (12 h at 21°C)	Lugol
Galactogen			Hydrolysis with HCl 6M (6h at 110°C), neutralisation with KOH 6M	Enzyplus® EZS 784+ Lactose/D-Galactose Kit (BIOCONTROL, Bellevue, USA).

Supercooling point measurement

Supercooling is the process of lowering the temperature of a liquid below its freezing point, without change to solid. Below its standard freezing point, the crystallization will take place in the presence of a seed crystal or nucleus around which a crystal structure can form. At the temperature when the liquid turns into a solid, also called supercooling point or crystallization temperature, heat is released that can be recorded as the exotherm (Figure *ca*9 A) (Lee 1989).

In adult snail and egg samples the supercooling point was measured by attaching the sensor of an electronic thermometer (HI141JH, HANNA Instrument GmbH, Kehl am Rhein, Germany) to the sterilised sample surfaces (Figure *ca*9 B). Inserted into plastic tubes, samples were immerged in a cryostat (Polystat CC3, Huber Kältemachinen GmbH, Offenburg, Germany) filled with an antifreeze fluid maintained at 5°C. After acclimatization, the temperature of the bath was cooled at the rate of 0.5°C.min^{-1}, as recommended by Salt (1966).

From intestinal contents, mucous ribbons were extracted and placed with water in a microtube and the sensor of the thermometer attached to the tube surface, the whole inserted into a bigger tube. The same kind of treatment applied to an identical volume of sterile water provided control value.

In bulk samples (soil, food, faeces, eggshell) the relative ice nucleating activity was determined as the temperature necessary to provoke ice formation of a constant volume of sterile distilled water mixed to a constant volume of dehydrated substratum, compared to equivalent quantities of sterile water (Costanzo *et al.* 1998, 2000).

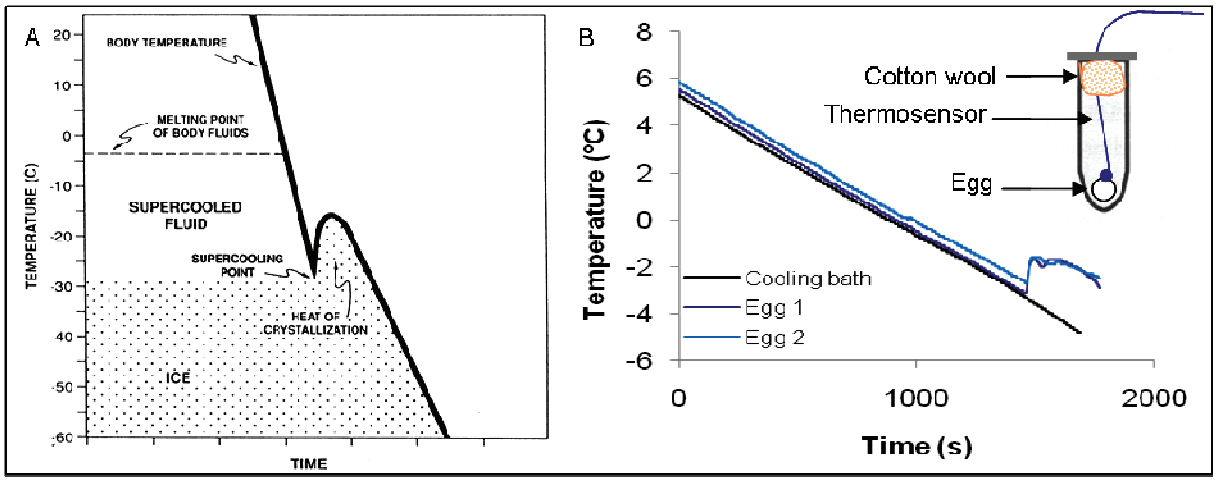


Figure *ca*9. Effect of the decrease of temperature in body fluids with corresponding supercooling point and exotherm in insects (Lee 1989) (A) and in eggs of *Cornu aspersum* (B).

Assessing ice nucleating activities in bacteria

Ice nucleating agents are particles, proteins, salts or bacteria that promote the formation of ice crystals in body fluids at temperatures between -10 to 0°C, limiting thereby the supercooling ability of some organisms (Vali 1995).

Under sterile microbiological conditions agar plates were cultured aerobically by spreading mucous ribbons, food, faeces, soil and eggs on the agar surface. Plates were sealed with Parafilm and incubated at 20°C for 5 days. Representative bacteria from all the colonies on a plate were removed with a sterile loop and mixed with sterile distilled water. If necessary, the microbial solution was diluted to obtain a comparable concentration of 10^8 bacteria/ml of sterile water, corresponding to a DO of 0.5 at 660 nm (VERSAmax™ microplate reader, Sunnyvale, USA).

The droplet method of Vali (1971) was used for assessing ice nucleating activity. 5 µl-droplets (N = 10) were deposited on an aluminium pan floating on the antifreeze solution surface in a cryostat (Polystat CC3, Huber Kältemachinen GmbH, Offenburg, Germany). A captor of an electronic thermometer (HI141JH, HANNA Instrument GmbH, Kehl am Rhein, Germany) was taped on a supplementary pan allowing to follow precisely the evolution of temperature at the pan surface (Figure *ca*10 A). The freezing of droplets cooled at 0.5°C/min was noted visually: when freezing, they turned from transparent to white opaque (Figure *ca*10 B). Data from a non-ice nucleating bacteria (*Escherishia coli*) and from an ice nucleating bacteria (*Pseudomonas syringae*) as well as from sterile distilled water were included for comparison. Cumulative ice nuclei spectra were then determined as recommended by Vali (1971), providing for each temperature the abundance of nucleating material dispersed in the fluid (Figure *ca*10 C).

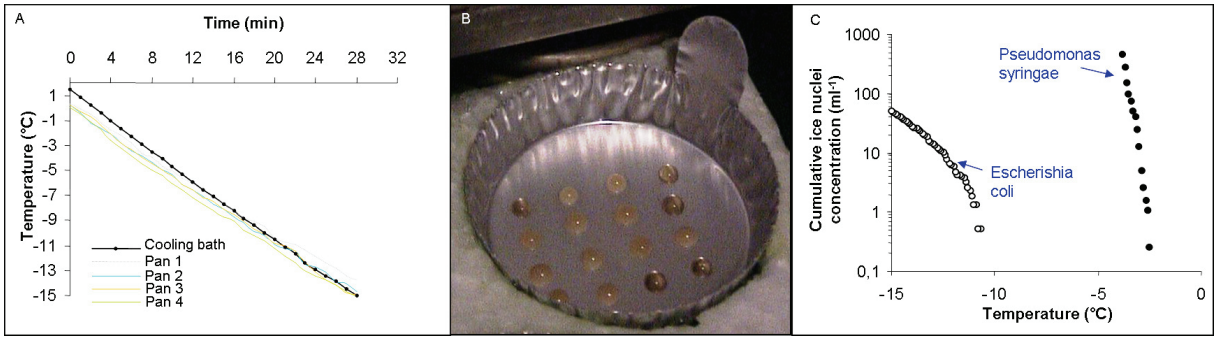


Figure *ca*10. Temperature decrease on each aluminum pan in the cooling bath (A), frozen (opaque) and unfrozen (transparent) droplets of a bacterial solution on the surface of an aluminum pan (B), and the cumulative ice nuclei spectra of non-ice nucleating bacteria (*Escherishia coli*) and from an ice nucleating bacteria (*Pseudomonas syringae*) (C).

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To breed or not to breed ?

Cornu aspersum uses environmental cues to predict the seasonal climate changes, and prepares the appropriate physiological and behavioural response. Hibernation is triggered by shortening of photoperiod (Ansart *et al.* 2001b), and reproduction is mainly triggered by temperature, but also by a long photoperiod as compensation for suboptimal temperature (Gomot & Gomot 1989; Flari & Edwards 2003). Reproduction before hibernation represents an additional cost enhancing the mortality rate during hibernation (Lazaridou-Dimitriadou & Kattoulas 1991), because winter survival depends on glycogen and lipid storage (Charrier 1995; Borges *et al.* 2004), synthesis of epiphragm (Fournie & Chetail 1984) and cryoprotectants (Karanova & Gakhova 2007). Moreover, juveniles from snails breeding right after maturity are prone to high winter mortality (Wolda & Kreulen 1973; Charrier & Daguzan 1980) if they don't reach a critical shell breath before hibernation (Biannic & Daguzan 1993). Delaying reproduction after hibernation in *Cornu aspersum* might represent a conservative bet-hedging strategy (Box *ca*1) to avoid the risk of an enhanced probability of semelparity, and to respond to an unpredictable outcome of the reproductive bout according to the uncertainty of winter arrival.

In *Cornu aspersum* the length of breeding period and the timing of mating and oviposition are notably labile (between April and October), according to unpredictable seasonal (and annual) fluctuations in climatic factors (Madec & Daguzan 1987). One or more clutches per year are produced, the second or third clutches being laid in late summer or autumn (Madec *et al.* 2000). Under natural conditions, young adult snails developed from early clutches in the year reach sexual maturity in late summer of the next year and reproduced before hibernation right after maturity (Charrier 1980) (Figure *ca*11). Young adults developed from late clutches in the year reach sexual maturity in autumn of the next year and did reproduce only after hibernation. There is an inter-generation variation in reproduction moment and reproductive strategies might represent phenotypic adjustments to time constraints.

Hypotheses of this study:

- Reproductive strategy depends on seasonal time constraints as response to probabilities of offspring survival
- Energy available in food could influence allocation to current reproduction and future hibernation

BOX *ca*1. Diversifying *versus* conservative risk spreading

Organisms often encounter environmental heterogeneity in space and time, and may adapt their life histories to temporally uncertain environments with life history delays, variable age at maturity, and iteroparity (Wilbur and Rudolf 2006). Many studies explain such strategies in the context of bet-hedging in a wide range of plants (e.g. Satake et al. 2001, Simons and Johnston 2003) and animals (e.g. Hopper 1999, Menu and Desouhant 2002 Laaksonen 2004). Bet-hedging in evolutionary ecology, is a strategy in unpredictably variable environments to reduce the variance in fitness at the cost of lower arithmetic mean fitness, thereby achieving higher geometric mean fitness (Seger and Brockman 1987). Two types of risk-spreading meet this criterion: (I) Diversifying risk-spreading: a single genotype produces multiple phenotypes to hedge against unpredictable environmental changes, (II) conservative risk-spreading: a genotype produces a single phenotype that avoids risk altogether either in a predictably fluctuating environment or in an uncertain environment (for a review see Hopper 1999). The conservative bet-hedging strategy might better named risk aversion strategy (e.g. obligate diapause or migration), because it will always bet that the environment will be adverse.
While diversifying risk-spreading was largely studied in the context of evolution in different taxa, including molluscs (e.g. Baur and Bengtsson 1987, Boletzky 1997, Krug 2001), conservative bet-hedging was sometimes considered suboptimal in fluctuating environment (Visser et al. 2003, Weatherhead 2005). This applies, for instance, in the timing of reproduction of semelparous species, when reproduction (trade-off between fecundity and growth) was restricted to a short early period in the year or delayed to the next year (Simons and Johnston 2003). If higher fecundity in the next season outweighs the risk of mortality during the cold season in temperate regions, the reproductive delay would be favoured over spreading reproduction over a longer time in the first reproductive season (de Jong et al. 1987). In many species a positive correlation has been found between age and fecundity (Freese and Zwölfer 1996, Roff 2002, Sandercock et al. 2005). Wilbur and Rudolf (2006) raised the question about reproductive delay and adult survival being either adaptations or constraints in the case of iteroparous species skipping opportunities for reproduction. Are adults foregoing a breeding season as a plastic response to uncertainty of the outcome of a reproductive bout (adaptation to hedge their evolutionary bets) or because they have not yet obtained a state (e.g. sequestration of sufficient resources or maturation of sexual organs) that tips the balance in favour of reproduction versus survival?

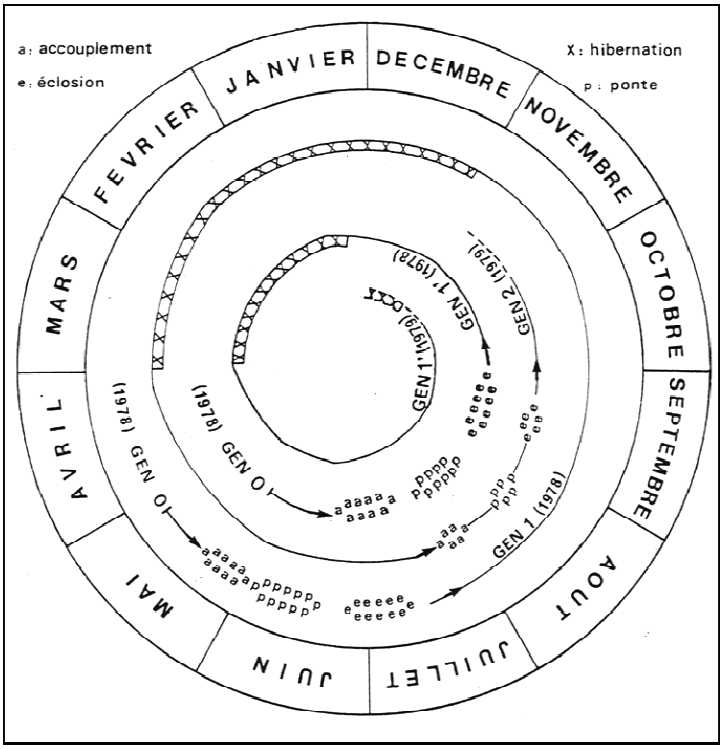

Figure *ca*11. Schema of the development cycle de Cornu aspersum in outdoor cages during the study of Charrier (1980). a – copulation, e – hatching, X – hibernation, p – oviposition.

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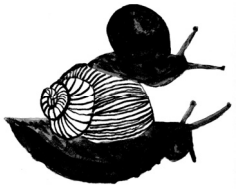

Seasonally contrasting life history strategies in the land snail *Cornu aspersum*: physiological and ecological implications

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Reference: Nicolai et al. *Ca*-I

Abstract: When a life history is characterized by both seasonality in reproduction and seasonality in offspring fitness, trade-offs in reproductive traits might be adjustments to seasonal time constraints in order to optimize reproductive success. Therefore, we compared in the laboratory the trade-offs in reproductive traits between early (after maturity) and delayed (after dormancy) reproduction in young land snails *Cornu aspersum* (Syn. *Helix aspersa*) depending on food quality. We also investigated the maternal investment in reproductive output in both breeding periods. After maturity, snails produced single clutches with many small eggs, which resulted, in contrast to previous studies, in large offspring with a low hatching rate due to high within-clutch cannibalism. The young cannibals may have a higher survival probability in the following hibernation. After hibernation snails produced smaller clutches of larger eggs, resulting in high quantity of lighter offspring. The clutch weight was positively correlated with maternal weight after maturity and negatively correlated after hibernation. Multiple oviposition occurred only after hibernation thereby enhancing reproductive success. An energy-rich diet did not affect reproductive strategies. Further studies should focus on seasonal plasticity of reproductive strategies in natural populations of *C. aspersum*, and investigate survival probabilities of breeders and juveniles in an evolutionary context.

Keywords: Delayed reproduction, Reproductive strategy, Maternal investment, Egg cannibalism, Energy allocation

Introduction

Life histories have been described as heritable set of rules that determine age-specific allocation to growth *versus* storage and reproduction (Karasov and Martinez del Rio 2007). Organisms often encounter environmental heterogeneity in space and time, and may adapt their life histories to temporally uncertain environments with life history delays, variable age and size at maturity, and iteroparity (Beckerman et al. 2002, Wilbur and Rudolf 2006). Iteroparous species in temperate regions may achieve reproductive success by hedging their bets and spreading their reproductive effort over several breeding periods (Wilbur and Rudolf 2006).

Reproductive delays of iteroparous species in temperate regions raise questions about their significance in evolutionary processes (De Jong et al. 1987, Simons and Johnston 2003, Wilbur and Rudolf 2006). If higher fecundity in the next season outweighs the risk of mortality during the cold season, the reproductive delay would be favored. However adults might forego a breeding season as a plastic response to uncertainty of the outcome of a reproductive bout (adaptation to hedge their evolutionary bets) or because they have not yet obtained a state (e.g. sequestration of sufficient resources or maturation of sexual organs) that tips the balance in favors of immediate reproduction *versus* survival. When a life history is characterized by both seasonality in reproduction and seasonality in offspring fitness, reproductive strategies might be adaptations to seasonal time constraints, generated by resource availability, risk of predation, and climatic factors (Kim and Thorp 2001, Potts 1975, Varpe et al. 2007, Weatherhead 2005). These reproductive strategies are compromises in resource allocation to brood size, egg size and parental care (Begon et al. 1996, Karasov and Martinez del Rio 2007). Parental care in ectotherms may range from none to extensive brooding, including a clutch size increase that allows some sibling to consume others, as reviewed in different species by Stearns (1987) and in mollusks by Baur (1994b), Little (1998) and Heller (2001). However the physiological link of food quality or availability to the pattern of reproductive allocation is still poorly understood (Karasov and Martinez del Rio 2007, Zera and Harshman 2001).

Little attention has been given until now to the trade-off between early (after maturation) and delayed (after dormancy) reproduction and the role of food quality in this trade-off in iteroparous ectotherms. Therefore we focused on the terrestrial gastropod *Cornu aspersum* (Syn. *Helix aspersa*), an obligate hermaphrodite and iteroparous snail with determinate growth pattern and a short life span (3-4 years, Madec et al. 2000). Previous studies showed plasticity in the phenotype of quantitative reproductive traits related to maternal energy investment in one reproductive season after hibernation, but not associated to age classes (Baur 1994a, Madec et al. 2000, Madec et al. 1998).

C. aspersum lives in temperate regions and exhibits cyclic behavioural rhythms with hibernation during low winter temperatures (Attia 2004). The length of breeding period and the timing of mating and oviposition are notably labile (between April and October), according to unpredictable seasonal (and annual) fluctuations in climatic factors (Heller 2001). The variability in oviposition frequency within a population consists of producing one or more clutches per year, the second or third clutches being laid in late summer or autumn (Madec et al. 2000). Under optimal natural conditions, young snails developed from early clutches in the year reach sexual maturity in late summer of the next year, and young adults developed from late clutches in the year reach sexual maturity in autumn of the next year (Charrier 1980).

C. aspersum uses environmental cues to anticipate the seasonal climate changes, and prepares the appropriate physiological and behavioral response. Cold hardiness is triggered by shortening of photoperiod (Ansart et al. 2001), and reproduction is mainly triggered by temperature, but also by a long photoperiod as compensation for suboptimal temperature (Gomot de Vaufleury 2001). *C. aspersum* may skip a reproductive period when low reproductive success becomes predictable. Winter survival in adults is considered a key factor in population dynamics of Helicidae in Europe (Cain 1983, Peake 1978), and many snails in populations of *C. aspersum* hibernate before first reproduction with a survival probability of 45% (Charrier 1980). Reproduction before hibernation represents an additional cost enhancing the mortality rate during hibernation (Lazaridou-Dimitriadou and Kattoulas 1991, Reznick 1992). In particular juveniles from snails breeding right after maturity are prone to high winter mortality if they don't reach a critical shell size before hibernation (Biannic and Daguzan 1993).

Roff (2002) pointed out that manipulation experiments are powerful means of demonstrating the presence of life history trade-offs and dissecting its causes in an evolutionary context. We hypothesize that reproductive strategies of *C. aspersum* are influenced by seasonal time constraints and by energy available in food. Therefore, we compared under constant conditions in the laboratory reproductive traits and maternal investment in the reproductive output between breeding periods and with respect to food quality

Materials and Methods

REARING CONDITIONS AND REPRODUCTION EXPERIMENTS

Breeders were taken from an outdoor snail farm (Corps-Nuds near Rennes, France) and reproduced in February 2004. Four clutches of different mothers laid on the 13th of February gave rise to 397 newborns on the 28th of February. 120 newly hatched snails were chosen at random and assigned to three diet treatments in samples of 10 individuals (sibling-split design). Four replicate cages (daily change of cage location in the rearing room) were used per sample to take possible cage effect into account. Additional cages with snails held in the same condition and density served as stock to replace dead snails over the whole experimental time. The snails were individually tagged and reared under controlled conditions (temperature: 20 ± 1°C, relative humidity: 60-80%, L/D = 12/12h) until they reached maturity (reflected shell peristom) 14-15 weeks later.

Snails were fed *ad libitum* on a specific food for cultured snails, Helinove®, formulated by Idena (Sautron, France) and made by Berton Alimentation Animale (Le Boupère, France). The Helinove® diets differed in their energy content (2220, 2320 and 2420 kcal/kg) owing to their differing fat content (2.5, 4.0 and 5.5% fat, respectively) despite sugar showing the opposite trend. Protein, cellulose and ash content were similar (15%, 3% and 37%, respectively). Snails were hydrated twice a week. Food and faeces were removed each week.

At maturity, two samples were made on the basis of body weight homogeneity (Fig. 1, five snails x four replicates per diet in both samples). The first sample (AM: after maturity) was kept under optimal conditions for reproduction (temperature: 20 ± 1°C, relative humidity: 60-80%, photoperiod: 16h light/8h dark) once sexual maturity was achieved (15 weeks after birth), which means without hibernating (Fig. 1). The second sample (AH: after hibernation) was progressively transferred to hibernation conditions over two weeks without feeding by stepwise decreasing temperature, humidity and photoperiod. No mating occurred in the AH samples

before hibernation since they were kept inside individual boxes. After six months of hibernation under artificial conditions (temperature 5 ± 1°C, relative humidity: 50%, 24h darkness), snails were progressively transferred to reproduction conditions over two weeks and fed once they awoke from hibernation (by the end of the second week). Copulations started right after attaining maturity in the AM sample and after arousal in AH sample. The first clutch was observed about two weeks after first copulation in each sample and determined day 1 of the oviposition period. The oviposition period was limited to one month like in the autumnal reproduction before hibernation and spring reproduction before aestivation in natural populations in warmer climatic conditions.

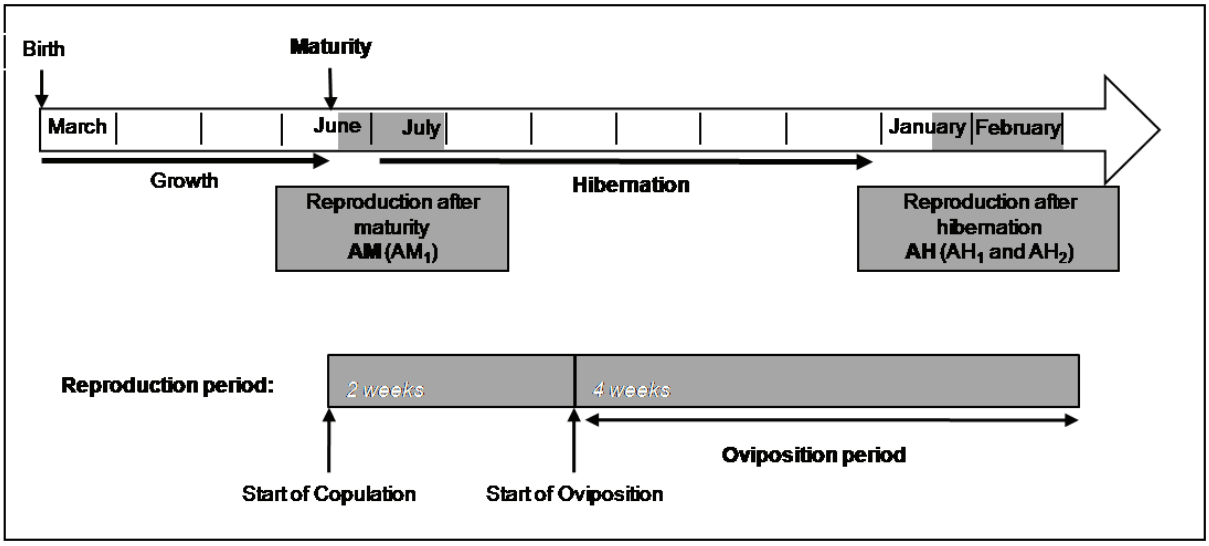

Figure 1. Experimental design of the reproduction experiment. AM: after maturity, AH: after a six-month period of hibernation. Snails of the AM and AH sample were kept in reproduction conditions for six weeks with an oviposition period of four weeks starting two weeks after the first copulation. AH snails aroused from hibernation during two weeks before reproduction period. AM snails laid only one clutch (AM_1), and AH snails laid one (AH_1) or two clutches (AH_2) during the oviposition period. Clutches of AH_2 were distinguished in first (AH_{21}) and second clutch (AH_{22}).

For reproduction, five tagged individuals of each replicate were placed into a cage with a wet latex HR (high resilient) foam. The individuals were observed daily and weighed at least twice per week, when water and food (*ad libitum*) were supplied. During mating and oviposition, snails were not disturbed. Four pottery pots filled with compost (Eco-Terre ®, pH 7.0, Avignon, France) were put in each cage for oviposition and replaced as soon as used. All copulations and ovipositions could be recorded. Each clutch was identified by its parentage, by laying and hatching dates. The eggs were counted, and 30 to 40 eggs were randomly chosen and weighed individually. The identified clutches were incubated at 20°C in a mixture of constantly humid soil (1/3 compost, 2/3 peat). The young snails hatched from eggs two to three weeks after egg-laying. The offspring were counted once all eggs had hatched or had been cannibalized, and 30 to 40 of them were randomly chosen and weighed.

Hatching rate could directly be used for evaluating the degree of egg cannibalism since no dead egg was found in the nests. Young cannibalistic snails were also directly observed at the time of hatching, devouring first the egg fluid, afterwards the egg shell.

DATA ANALYSES

Statistical analyses were carried out using "R" (2008). Prior to the experiment, we tested the samples for homogeneity on individual weight by using one-way ANOVA. Before analyzing the data, we performed separate hierarchical analyses (GLMM, LMM) with cage nested within each factor to detect possible cage effects. No cage effect was found (AM: clutch size: $F_{3,6} = 1.45$, $P = 0.38$; egg weight: $F_{3,6} = 0.35$, $P = 0.79$, AH: clutch size: $F_{3,6} = 1.55$, $P = 0.30$; egg weight: $F_{3,6} = 2.34$, $P = 0.17$). The weekly distribution of clutches between breeding periods (AM and AH) and diet treatments were compared by a Pearson-χ^2 test and a Fisher's F test, respectively. We tested the effects of breeding period and diet treatments in single clutches and their interactions on the reproductive traits using GLM procedures (Generalized Linear Models; Nelder and Weddenburn 1972) with fixed effects, followed by multiple comparisons (Tukey HSD test). After a first analysis, models were simplified by (i) backward elimination of non-significant effects from the full-model and (ii) aggregating factor levels that did not differ significantly from each other (Crawley 2007). As recommended by Crawley (2007), we corrected for overdispersion by using a quasibinomial (proportion data) or a quasipoisson (count data) distribution, and we performed F tests on the deviance reductions. The egg weights followed a normal distribution with identity link function. A quasipoisson distribution with a log-link function was used for clutch size whereas a gamma distribution with power (-1) link function was defined to analyse hatching weights. The hatching rate was analyzed with a quasibinomial procedure and a logit link. To egg weight we added clutch size and to offspring weight we added offspring number per clutch as a covariate. We performed Pearson correlations between the weight of reproductive output and breeder initial weight at the beginning of reproduction period as well as between reproductive traits and covariates.

Multiple oviposition in the AH sample was analyzed by Generalized Linear Mixed Models (GLMM), as described by Crawley (2007), using as fixed effects diet and clutch order and also with clutch order as random factor (repeated measurements: first and second clutches of one individual). For clutch size a poisson distribution with a log link function was used. Hatching rate followed a binomial distribution with a logit link function. Egg weight (with covariate clutch size) and offspring weight (with covariate offspring number) followed a normal distribution which permitted the use of a Linear Mixed Model (LMM) with the previously described random and fixed effects.

Results

The groups of five snails x four replicates did not differ in mean fresh weight (22.1 ± 3.1 g) at the beginning of each breeding experiment (ANOVA, $N = 120$, factor breeding period: $F_{1,92} = 2.919$, $P = 0.091$; factor diet: $F_{2,90} = 0.706$, $P = 0.496$, interaction breeding period x diet: $F_{2,88} = 1.437$, $P = 0.242$). Mortality during hibernation was high in the AH sample (43%), independent of diet treatment. The oviposition period started two weeks after maturity in the AM sample and four weeks after hibernation in the AH sample. Mortality during the reproductive period was weak (AM: 10% and AH: 5%). In the AM sample, 42 out of 60 snails laid clutches with no more than one clutch per snail (70% ovipositing snails, 0.7 clutches per snail). In the AH sample, 52 out of 60 snails laid 85 clutches (87% ovipositing snails, 1.4 clutches per snail). In the AH sample, 24 snails laid one clutch, 23 snails laid two clutches, and five snails laid three clutches. Because of their small sample size we did not analyze the third clutch. In the following, a

distinction is made between snails laying one or more clutches (for corresponding abbreviations see Fig. 1).

During the oviposition period, the breeding period significantly affected the weekly frequency of single clutches (AM_1 and AH_1, $\chi^2 = 10.346$, $DF = 3$, $P = 0.016$), the maximum reproductive activity being observed in the second week in AM_1 snails but a week later in AH_1 snails (Fig.2). The AH snails that started egg-laying within the first two weeks (clutches AH_{21}) laid a second clutch in the third and fourth weeks (clutches AH_{22}). Although the AM_1 snails reproduced as early as the AH_2 snails, they did not produce a second clutch.

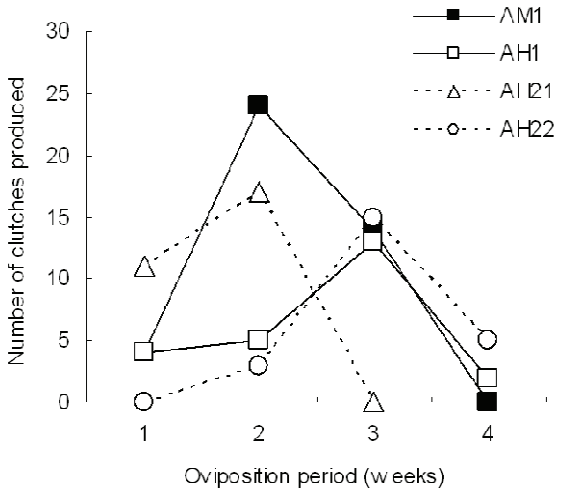

Figure 2. Development of clutch number produced in a sample during the oviposition period in N=120 snails of *C. aspersum*, according to breeding period (AM_1: single clutches after maturity, AH_1: single clutches after hibernation, AH_{21}: first clutches after hibernation, AH_{22}: second clutches after hibernation).

In single clutches of *C. aspersum*, diet quality had no effect on any of the parameters included in the models, except for egg weight (Table 1). AM_1 snails produced heavier eggs (42.35 ± 2.12 mg) with 5.5% fat diet than with the 2.5% and 4.0% fat diet (36.49 ± 1.14 mg and 36.98 ± 0.96 mg, respectively, Tukey HSD, $P < 0.05$). In the AH_1 sample eggs from both 4.0% fat diet and 5.5% fat diet fed snails were heavier (45.46 ± 1.24 mg and 42.44 ± 1.43 mg, respectively) than 2.5% fat diet fed snails (39.06 ± 1.26 mg, Tukey HSD, $P < 0.05$).

Table 1. Summary of the analysis of deviances on the reproductive traits observed in single clutches of *Cornu aspersum* after maturity (AM_1) and after hibernation (AH_1). We indicated the explained deviances from the final linear model, followed by *DF* (error variance degrees of freedom), *F*- and *P*- values. Initial linear model included as fixed effects: breeding period, diet treatment and interactions. Added covariates to the initial model: [1)] Clutch size, [2)] Offspring number.

Model factors (covariates as footnotes)	**Total deviance reduction (%)**	**Model term**	**Explained deviance (%)**	***DF***	***F***	***P***
Clutch size	11.44	Breeding period	100	1,64	8.84	0.0042
Egg weight [1)]	33.04	Breeding period	41.72	1,64	12.35	0.0008
		Diet	31.50	2,62	4.66	0.013
		Breeding period x diet	26.79	2,60	3.96	0.024
Offspring weight [2)]	46.59	Breeding period	50.67	1,64	27.93	<0.0001
		Offspring number	49.33	1,63	27.19	<0.0001
Hatching rate	16.14	Breeding period	100	1,64	12.79	<0.0007

The breeding period had a large effect on clutch size, hatching rate, egg weight and offspring weight (Table 1, Fig. 3). A trade-off existed between clutch size and egg weight (Pearson, $r = –0.48$, $DF = 120$, $P < 0.0001$). While the breeders after maturity (AM_1) invested more in larger clutches with light eggs, the converse happened after hibernation (AH_1) with heavier eggs in smaller clutches (Fig. 3). The hatching rate was significantly lower in AM_1 snails than in AH_1 snails (Fig. 3). The degree of egg cannibalism corresponds to the difference of the hatching rate to 100%, since no dead eggs were found. Therefore, the egg cannibalism was higher in AM_1 clutches (33%) than in AH_1 clutches (17%). For this reason, the offspring from the light eggs in the AM_1 snails had a higher weight than the offspring from the heavy eggs in the AH_1 snails (Fig.3). A negative correlation was observed between offspring number per clutch and offspring weight (Pearson, $r = -0.38$, $DF = 120$, $P <0.0001$). A positive relationship between breeder weight at the beginning of reproduction period and weight of reproductive output was found in AM_1 breeders and a negative one in AH_1 breeders whereas no correlation occurred in AH_2 breeders (Fig. 4).

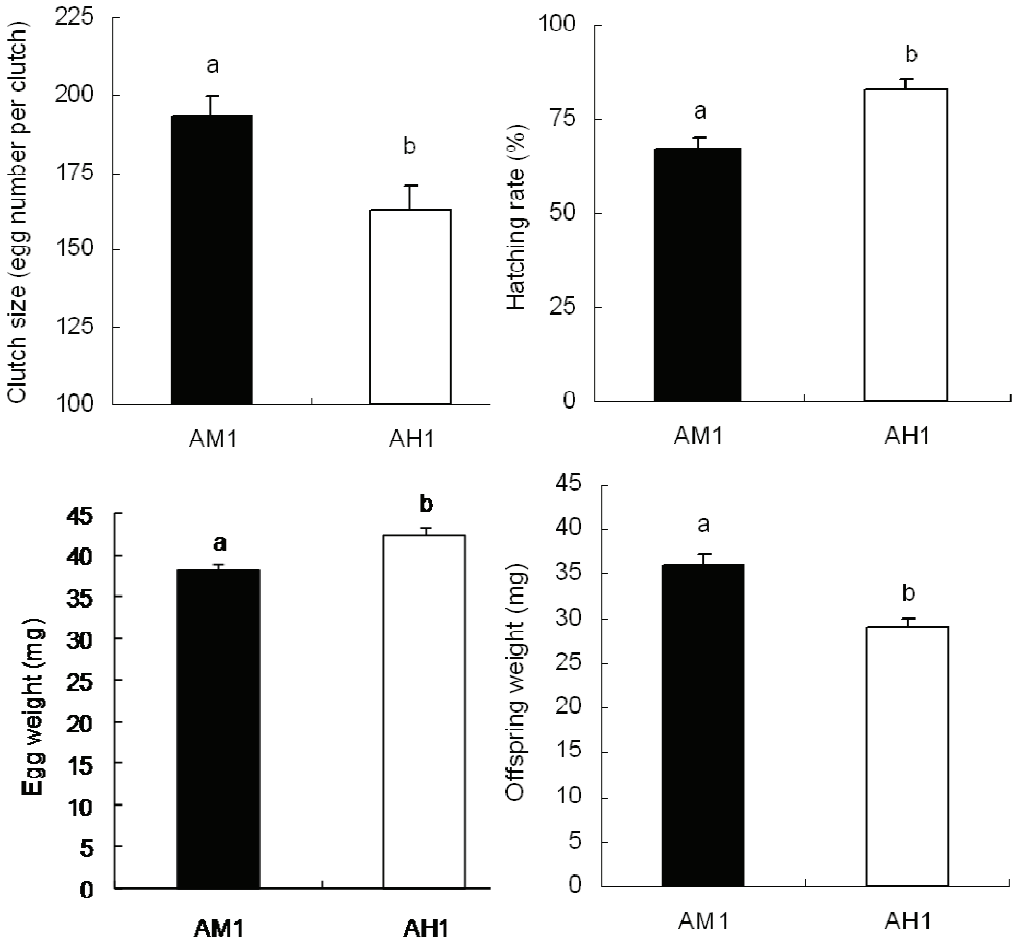

Figure 3. Reproductive traits in *C. aspersum* laying single clutches after maturity (AM_1) and after hibernation (AH_1). Error bars denote standard errors. Dissimilar letters indicate significant differences for the factor breeding period as indicated in table 1 (GLM, $P < 0.05$, N=42[24] clutches for AM_1[AH_1] respectively).

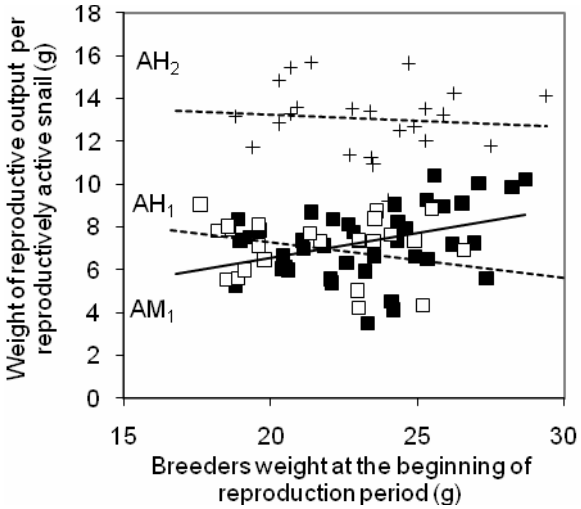

Figure 4. Weight of reproductive output per reproductively active snail in relation to breeder weight in *C. aspersum*, depending on breeding period (after maturity: AM and after hibernation: AH) and the number of clutches per snail (one clutch AM_1: black squares, one clutch AH_1: white squares, and two clutches AH_2: crosses). Pearson Correlation: AM_1: $r = 0.37$, $DF = 40$, $P = 0.007$, AH_1: $r = -0.38$, $DF = 22$, $P = 0.03$, AH_2: $r = -0.10$, $DF = 21$, $P = 0.33$. Reproductively active snails: N=89 with AM_1=42 and AH=24[23], for AH_1[AH_2] respectively. Proportion of reproductively active snails AM = 70% and AH = 87%.

The reproductive success was measured by the offspring number per reproductively active snail and was mainly influenced by the clutch number produced per snail and the breeding period (GLM, breeding period: $F_{1,87} = 35.74$, $P < 0.0001$, diet: ns, clutch number: $F_{1,86} = 53.23$, $P < 0.0001$, reproductively active snails: N=89 with AM_1=42 and AH=24[23], for AH_1[AH_2] respectively, proportion of reproductively active snails AM = 70% and AH = 87%.). After maturity, the mean offspring number per reproductively active snail (129 ± 7) did not differ from that produced after hibernation (135 ± 8) by snails with one clutch (Tukey HSD between AM_1 and AH_1, $P = 0.95$). With two clutches after hibernation (AH_2), the offspring number per reproductively active snail reached the mean value of 244 ± 20 (Tukey HSD between AM_1 and AH_2, $P < 0.0001$, Tukey HSD between AH_1 and AH_2, $P < 0.0001$).

Multiple oviposition occurred only after hibernation (Table 2). Diet quality had no effect on the measured reproductive traits. Clutch order (AH_{21} *vs.* AH_{22}) did neither have an effect on egg weight nor on hatching rate, but clutch size and offspring weight were significantly lower in the second clutch (AH_{22}) than in the first clutch (AH_{21}).

Table 2. Reproductive traits for multiple oviposition after hibernation (AH_2: N=28) in *Cornu aspersum*. Mean ± SEM are represented for first clutches (AH_{21}) and second clutches (AH_{22}). Dissimilar letters indicate significant differences for the final model (clutch size: GLMM, $F_{2,26} = 3.50$, $P = 0.045$; hatching rate: GLMM, $F_{2,26} = 2.23$, $P = 0.13$; egg weight: LMM, $F_{2,26} = 2.83$, $P = 0.08$; offspring weight: LMM, $F_{2,26} = 21.75$, $P < 0.0001$). Initial model included also diet treatment as fixed effect, but it appeared not to be significant.

Clutch	Clutch size	Hatching rate	Egg weight	Offspring weight
AH_{21}	153.0 ± 5.5 [a]	81.1 ± 2.3 [a]	45.37 ± 0.90 [a]	31.18 ± 0.59 [a]
AH_{22}	138.6 ± 6.1 [b]	86.1 ± 2.6 [a]	43.55 ± 1.05 [a]	27.12 ± 0.55 [b]

Discussion

In life-history theory, trade-offs in quantitative reproductive traits reflect the balance of gains and costs of reproduction. *C. aspersum* adopted two different reproductive strategies in respect to seasonal time constraints: a "many-small-eggs strategy" after maturity and a "few-large-eggs strategy" after hibernation. Producing large eggs represents a greater investment of energy for the mother (Baur 1994b, Krug 2001) and is usually considered more beneficial for hatchlings, because egg weight is positively related to hatchling weight and consequently to survival, growth and fecundity (Madec et al. 1998, Moran and Emlet 2001). Two phenomena may explain the existence of AM and AH breeders: (i) phenotypic plasticity of reproductive traits; (ii) a genetically fixed polymorphism. If juveniles from early clutches reach maturity constantly early, they would keep the early reproduction phenotype, which could lead to phenotypic isolation by life history constraints and result in stable polymorphism (i). Our results support the hypothesis of within-generation variability (ii), because snails reaching maturity at the same time reproduced either after maturity or after hibernation (70% and 87% ovipositing snails, respectively) and adjusted their reproductive strategy to the seasonal time constraint.

In contrast to previous studies, we found that the "many-small-eggs strategy" (after maturity) was associated with a lower hatching/higher cannibalism rate and bigger offspring compared to the "few-large-eggs strategy" (after hibernation). The offspring weight provides a better picture than the hatchling weight of what happens in natural conditions at the moment of nest leaving. This consideration highlights the role of within-clutch egg cannibalism in reproductive success of *C. aspersum*. Desbuquois (1997) showed that cannibalistic hatchlings were 1.3 times heavier than non-cannibalistic hatchlings, and their survival rate was enhanced (100% *versus* 75.8%). The selection-arena hypothesis (Stearns 1987) states that in larger clutches sib-competition is higher and will result in selection of high quality offspring. A large clutch with a certain degree of asynchrony of hatching provides a fertile ground for sib-competition through non-random egg cannibalism (Baur 1993). As a reason for hatching asynchrony, Stearns (1987) suggests differential maternal investment in egg quality (Baur 1994a, Dziminski and Alford 2005, Krug 2001). If early juvenile body size or weight affects growth, the reproductive strategies may have consequences on population dynamics through cohort effects (Beckerman et al. 2002). Larger juveniles should more quickly reach the critical shell size of 20 mm, below which they are unable to hibernate (Biannic and Daguzan 1993), and have a higher survival probability during the following hibernation. In contrast, hatching in spring after hibernation coincides with flush of vegetation and provides a sufficient energy supply for growth (Fournier et al. 1999, Karasov and Martinez del Rio 2007), which has a positive influence on offspring survival, even if the offspring is small.

Hibernation is often considered as a physiological syndrome, an obligate dormancy. The first hibernation period before reproduction might be a part of the life cycle, not only triggered by environmental factors, but maybe also by endocrine signals acting on maturation of the genital tract (Flari and Edwards 2003). Several authors described the necessity of hibernation as a refractory period to enhance the reproductive success in land snails (Bonnefoy-Claudet and Deray 1984, Gomot de Vaufleury 2001). This might correspond to what we have observed in snails after hibernation, some snails laid two clutches thereby enhancing their reproductive success. In snails that reproduce after maturity, the tendency to postpone the oviposition period (Fig. 2) might therefore correspond to a physiological constraint, like further maturation or

resource acquisition (Wilbur and Rudolf 2006). No study actually describes how physiological constraints act on the reproductive patterns.

Maternal effects may influence life history of the brood (Mousseau and Fox 1998) through changes in energy allocation to reproduction (e.g. Wallace et al. 2007). Madec et al. (2000) found a positive correlation between breeder size and egg size in *C. aspersum*. Within the "many-small-eggs-strategy" of AM breeders clutch weight increased with breeder weight, whereas the opposite happened in the AH_1 breeders, but not in the AH_2 breeders. This means with increasing weight AM and AH snails invest oppositely in the reproductive output Therefore the reproductive strategies of AM and AH breeders might also differ by the investment strategy of body stores into reproduction.

Food availability influences the trade-offs in reproductive traits (Kim and Thorp 2001, Krug 2001, Lardies et al. 2004). Breeders invest generally either in the number or in the weight of eggs, reducing or keeping constant one of both parameters, depending on energy available in their environment (Qian 1994). Energy-rich diet oriented maternal investment in heavier eggs in both breeding periods, after maturity and after hibernation, but without affecting reproductive strategies and offspring weight. It could be possible that the snail food was of too high quality to bring out any differences in the dietary treatment.

Individuals of *C. aspersum* showed great differences in reproductive success between early and delayed reproduction due to a difference in oviposition frequency. Multiple oviposition is a common strategy in *C. aspersum*, but within populations single oviposition may occur (Madec et al. 2000). However, reproductive success is not sufficient for fitness calculations which should include different aspects of life- history, costs of reproduction and environmental conditions: (I) Generation time in snails reproducing after maturity may be shorter than after hibernation. (II) Offspring of second AH clutches, being smaller (Table 2), might have a lower survival probability (Desbuquois 1997). (III) A higher reproductive success in delayed reproduction (AH snails) seems to outweigh the costs of winter mortality (De Jong et al. 1987, Simons and Johnston 2003). A single oviposition after maturity (AM snails) could lower the costs of reproduction and act on the trade-off between reproduction and adult winter survival (Reznick 1992). (IV) Juvenile survival strongly depends on unpredictable environmental factors such as predation, climatic variation, and resource availability (Madec et al. 2000, Potts 1975), creating within-generation variability (Menu and Desouhant 2002). Therefore, further studies should focus on plasticity of reproductive strategies in natural populations of *C. aspersum*, and especially investigate survival probabilities of autumnal breeders and their juveniles during hibernation *versus* spring breeders and their juveniles, which would be relevant to evaluate fitness and evolutionary consequences of both strategies.

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Too rich for a baby boom

In *Cornu aspersum*, reproductive strategies depend on seasonal time constraints, but egg weight was influenced by energy availability in food (Nicolai et al. *Ca*-I). There might be differential egg investment affecting growth and survival of offspring. Generally, it is admitted that reproductive investment depends on body condition, this is the quantity of stored energy which in turn depends on available energy in food. Two opposite possibilities have been studied in the past: (1) the state-dependent theory: better body condition leads to higher reproductive investment (McNamara & Houston 1996), (2) the terminal-investment theory: worse body condition leads to higher reproductive investment with even a maximal investment and subsequent mortality (Fischer *et al.* 2009) (Box *ca*2, Figure *ca*12). However (2) is based on a dynamic model including (1).

Lipids are particularly interesting in investment studies, because triglycerides store nine times more energy as glycogen because of their higher per mass energy content and the anhydrous storage. In the past, biologists and biochemists have ignored lipids and their role in animal welfare for a long time because of the bad rap from pop nutrients (Karasov & Martinez del Rio 2007). In gastropods, glycogen is the main storage polymer, but it can be transformed into triglycerides for longer storage (Arakelova *et al.* 2004). Assuming a human-like process, triglycerides could be stored within lipid droplets which serve exclusively as a pool of metabolic energy (Martin & Parton 2006). Bayne (1968) did not found lipids in the perivitellin fluid of eggs, but described galactogen as the main energy store in eggs, that would be transferred from the albumen gland to eggs (Tompa 1984). Nevertheless, recently some authors suggested lipids to have a significant role during reproduction in gastropods (Borges *et al.* 2004; Giokas *et al.* 2007), and Barre et al. (1991) and Luchtel et al. (1997) proposed the transfer of lipid yolk droplets from the digestive gland to the closely situated ovotestis for incorporation in oocytes before fecundation.

Hypotheses of this study:

- High lipid content in food leads to a high lipid storage in snails
- The amount of stored lipids influences lipid allocation to eggs and investment in reproduction (Figure *ca*12) either following the state-dependant theory (right side from threshold) or following the terminal investment-theory (left side of the threshold)

Box *ca*2. Terminal-investment theory
Fischer et al. (2009) predicted a maximal investment in reproduction, even with a high probability of subsequent mortality, when energy availability is low. With increasing energy availability the investment in reproduction drops either down to a minimum in low stochastic and high predictable environment or nearly vanishes in a highly variable environment independent of the degree of predictability. This minimum of reproductive investment is observed at a threshold value of energy availability, above which the investment in reproduction increases. Empirical evidences for the left side of the threshold in this model were given by a study on rotifers (Stelzer 2001). They maximized their reproductive investment at low food concentration, which was followed by death after reproduction, and decreased reproductive investment when food concentrations were improved. On the right side of the threshold, empirical evidences were given by a study on the insect parasitoïd *Asobara tabida* that increased reproductive allocation at the detriment of allocation to maintenance and survival when nutrient availability increased (Ellers and van Alphen 1997). The model of Fischer et al. (2009) also showed that small changes in energy availability can lead to major variations in optimal energy allocation to reproduction. However, the authors restricted the application of the model to "income breeders", which can acquire energy for reproduction and maintenance only during the current reproductive season (Jönsson 1997).

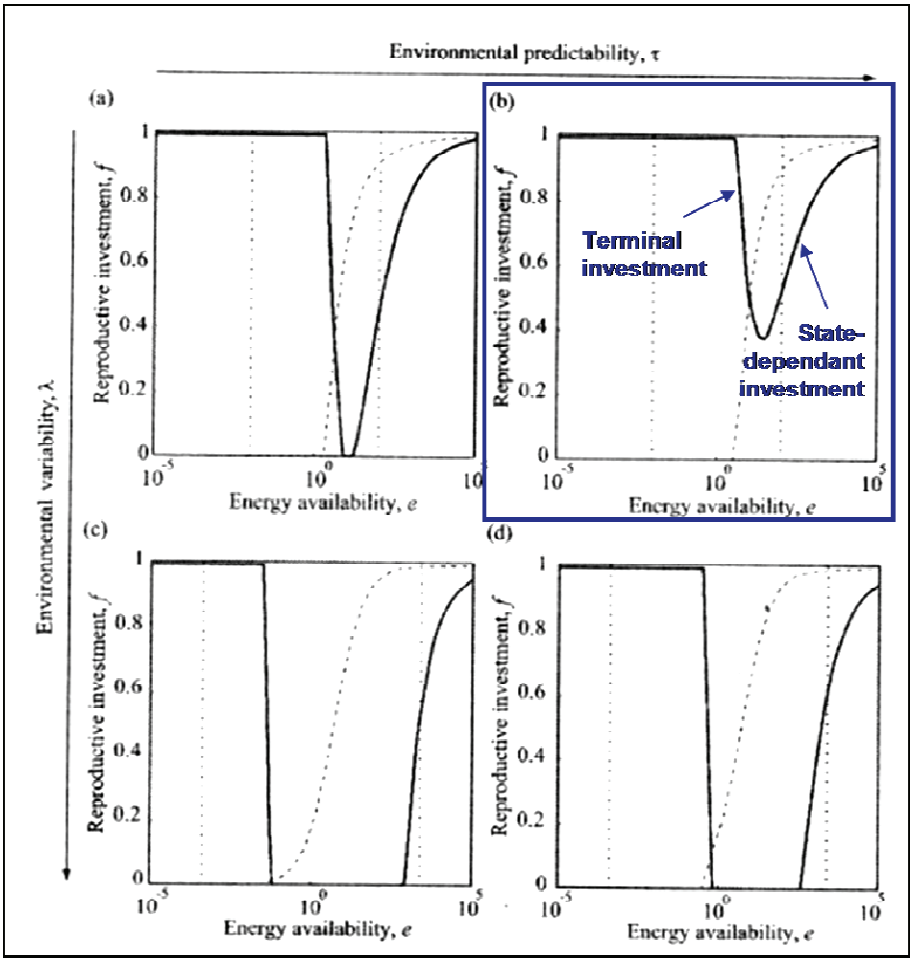

Figure *ca*12. Model of the terminal envestment theory under different environmental conditions (Fischer et al. 2009).

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The contrasted effects of body stores on partial capital breeding in the land snail *Cornu aspersum*

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Reference: Nicolai et al. *Ca*-II

Abstract: The success of a life history strategy is calculated by the energy allocation to growth, maintenance and reproduction, so that individual fitness is maximized. Resource quality can influence the pre-reproductive build-up of body stores during growth, thereby affecting the reproductive investment partial capital breeders. In this study on the gastropod *Cornu aspersum* we investigated the effect of energy availability in food (E+: lipid rich diet, E-: lipid poor diet) on growth and the building up of body stores usable in capital breeding. We estimated the income during breeding period and investment in brood, and we analyzed trade-offs in reproductive traits. E+ snails incorporated more nutrients from the food and reached higher triglyceride content than E- snails. Their allocation to reproduction was oriented in clutch size instead in egg weight (related to triglyceride content), and was negatively correlated to somatic allocation. Residual stores of E+ snails were not invested in a second clutch within the same breeding period. E- snails invested more nutrient stores in their brood than did E+ snails, and depleted income and partially capital stores. They maximized their reproductive investment by high clutch size whatever somatic allocation without affecting egg weight. This result was consistent with the terminal investment theory and suggests a threshold value to induce reproduction, below which energy is allocated to survival. Future studies should consider hatchling survival to evaluate the fitness in *C. aspersum* as a function of energy availability in natural populations.

Keywords: Income breeding, Galactogen, Gastropod, Glycogen, Growth, Maternal effects, Triglyceride, Energy availability, Body storage

Introduction

A life-history strategy with production of many small eggs is adopted by small invertebrates (Andersen et al., 2008). The success of this strategy is calculated by the energy allocation to growth, maintenance and reproduction, so that individual fitness is maximized (Roff, 2002). When energy is allocated to reproduction, the unavailability of this energy for other functions represents the costs of reproduction (Begon et al., '96). Energy allocation to each function depends on resource availability and on the interaction with other selective pressures (Reznick et al., '96, Kim and Thorp, 2001, Roff, 2002, Sandland and Minchella, 2003, Lardies et al., 2004, Ter Maat et al., 2007).

The acquisition of energy stores is costly (Reznick and Braun, '87), but makes reproduction possible at times or places with limited access to food (Varpe et al., 2009). In ectothermic animals the accumulation, maintenance and utilization of body energy stores does not seem to be as costly (energetic and demographic costs) as in endothermic animals (Jonsson, '97, Bonnet et al., '98). This makes capital breeding very common in ectothermic animals comprising the most extreme examples that tend to semelparity, where the capital reserves are invested in one single reproductive event (Bonnet et al., '98). Because offspring of most ectotherms is nutritionally independent at hatching, a female's total energetic investment in her brood is contained within the clutch. Therefore the resource allocation to offspring is accomplished during ovulation without any interruption for feeding, and makes income breeding in the strict sense of Jönsson ('97) impossible. Bonnet et al. ('98) also pointed out that income breeders should store at least for a short time their acquired nutrients, because biochemical pathways used in the allocation of nutrients for reproduction pass through storage organs. Hence, the frontier between strict income and strict capital breeding strategies becomes blurred.

Resource quality can influence the pre-reproductive build-up of body stores during growth thereby affecting reproductive investment in those ectotherms relying on capital or partial capital breeding. Several studies on ectotherms have shown that growth and quantitative reproductive traits were influenced by the protein *versus* carbohydrate content in food (Lardies et al., 2004), by the quality of fatty acids or lipids (Wacker and von Elert, 2003, Wacker, 2005) or by the quantity of proteins (Sandland and Minchella, 2003, Wacker and Baur, 2004). Therefore, we examined in this laboratory study how the availability of energy in food, through differences in lipid content, influenced the quality of body stores and the allocation of energy to reproduction in the terrestrial invasive gastropod *Cornu aspersum* Müller (Syn. *Helix aspersa*). This species is especially suitable for this kind of study, because it is an ectothermic hermaphrodite species with determinate growth pattern and can be easily reared and handled in the laboratory. (Borges et al., 2004) suggested the use of lipids as an energetic source during the reproductive phase in *C. aspersum.* In the gastropod *Codringtonia helenae*, the lipid content of the body is the highest during the reproduction period (Giokas et al., 2007). Studies on Helicidae, especially *C. aspersum*, showed plasticity of quantitative reproductive traits related to environmental factors in one reproductive season (Baur, '94b, Madec et al., '98, Madec et al., 2000). Therefore we tested the following consecutive hypotheses:

1. The amount and quality of body stores depends on energy availability in food. The addition of lipids in the snail diet would lead to a higher storage capacity during growth independently of body size (Wacker, 2005).

2. *C. aspersum* may rely on recently acquired energy stores for oviposition (income breeding), due to a feeding period between copulation and oviposition. Then the stores acquired and shortly stored during this period could cover at least the investment in brood. Because snails do not take care of their offspring further, the nutrient investment in clutch represents the highest part of physiological reproductive costs and the most beneficial for offspring (Reznick, '92, Baur, '94a, Baur, '94b, Bonnet, Bradshaw and Shine, '98). Residual body stores after reproduction could be used for further reproduction or be allocated to maintenance/survival during hibernation.
3. There is a trade-off between resource allocation to reproduction and to soma (Stearns, '92, Begon et al., '96, Roff, 2002). According to the classic theory of Van Noordwijk and De Jong ('86), this correlation is negative if inter-individual variation in resource allocation exceeds that in resource acquisition, usually the case in laboratory experiments (Glazier, '99). Since the reproductive investment depends on energy availability in food (Fischer et al., 2009), two opposing predictions are investigated:
   (a) Brooding individuals with fewer triglyceride stores, due to lower lipid (= energy) availability in food, invest more energy in reproduction than individuals with more triglyceride stores (terminal investment theory, Ellers and van Alphen, '97, Fischer etal., 2009). Maternal effects on egg quality could be observed in snails fed with lipid poor diet and would either lead to a trade-off between egg quality and number (Glazier, '92, Reznick, Callahan and Llauredo, '96) or to an increase of both, egg quality and number (Venable, '92, Mousseau and Fox, '98).
   (b) Brooding individuals with more triglyceride stores, due to higher lipid (= energy) availability in food, invest more energy in reproduction than individuals with less triglycerides stores (state-dependant theory, McNamara and Houston, '96, Kim and Thorp, 2001). Maternal effects on egg quality could be observed in snails fed with lipid rich diet and would either lead to a trade-off between egg quality and number (McNamara and Houston, '96, Kim and Thorp, 2001) or to an increase of both, egg quality and number (Mousseau and Fox, '98).
   Whichever strategy the snails followed, (a) or (b), without maternal effects on egg quality only an increase in egg number would optimize reproductive investment (optimal offspring investment theory, Smith and Fretwell, '74, Glazier, 2000).

Materials and Methods

Care and use of animals in the following experiments comply with all relevant local animal welfare laws, guidelines and policies.

REARING CONDITIONS AND GROWTH EXPERIMENT

Breeders were taken from a snail farm (Corps-Nuds near Rennes, France) and reproduced in February 2004. Four clutches laid on the 13th of February gave rise to 397 newborns on the 28th of February. Hundred newly hatched snails were assigned to two different diet treatments in samples of 50 individuals by a sibling-split design. Forty further snails were used to constitute a stock for each treatment. Twice a week, they were fed *ad libitum* with two types of Helinove® snail food, formulated by Idena (Sautron, France) and made by Berton Alimentation Animale

(Le Boupère, France). The Helinove® diets differed in energy value due to their respective fat content (Table 1).

Table 1. Composition (in %) of the Helinove® diets of increasing energetic values (in kcal.kg^{-1}) due to different percentages in lipids.

	TYPES OF DIET	
	Energy poor (E-)	Energy rich (E+)
Lipids[1]	2.5	5.5
Proteins	15.0	15.0
Starch and sugars	24.9	24.7
Parietal polymers	12.8	10.8
Minerals	36.9	36.3
Moisture	7.9	7.7
Energetic value	2220	2420

[1] Lipids are mainly soy oil (15% saturated FA, 24% mono-unsaturated FA, 60% poly-unsaturated FA with 52% linoleic acid of total FA) and flaxseed (with linoleic acid as the major FA). FA = fatty acids.

The snails were reared individually in plastic cages with a wet latex HR (high resilient) foam under controlled conditions (temperature: 20 ± 1°C, relative humidity: 60-80%, L/D = 12/12 h) until they reached maturity. During growth, 20 snails of each diet treatment were weighed and sized individually at the end of every week. We used shell breadth as body size measurement. Dead individuals were noted and replaced from the stock. The dried food dishes were weighed with and without the food at the beginning and at the end of the week. Calculation of growth efficiency GE = G / I (in g.g^{-1}.d^{-1}) served to evaluate the capacity of the animal to use the energy of the foodt, where G is the gain of snail weight per day and I the weight of ingested food.

At maturity, when the maximal body size was reached (reflected shell lip), body weight was determined, and ten breeder snails from the stock of each diet treatment were slowly frozen and then dissected to separate the foot, the albumen gland and the digestive gland. Accidentally cut organs that lost liquid were discarded from further manipulation. The frozen organs were weighed (fresh mass, FM) and lyophilized in vacuum (Lyovac™ GT3, Leybold-Heraeus, Orsay, France) for 48 h. The dry mass (DM) was determined before storage at -80°C for further biochemical analysis. The dry weight density (DM mg.FM mg^{-1}.100%) of the body represents the storage capacity (Ter Maat et al., 2007).

REPRODUCTION EXPERIMENT

Twenty snails per diet sample were chosen on the basis of body size homogeneity and maturity synchrony in the totality of manipulated snails and were kept under optimal conditions for reproduction (temperature: 20 ± 1°C, relative humidity: 60-80%, photoperiod: 16 h light/8 h dark). Four replicate cages with five individually tagged snails (daily change of cage location in the rearing room) were used per sample to take possible cage effects into account. The individual density in cages (30 snails.m^{-2}) was chosen to be close to natural conditions. Ten further snails were kept in the same conditions and served as stock to replace dead individuals.

During the reproduction period the individuals were monitored and weighed daily to evaluate the body weight before and after oviposition. During mating and oviposition, snails

were not disturbed. Four pottery pots filled with compost (Eco-Terre ®, pH 7.0, Avignon, France) were put in each cage for oviposition and replaced as soon as used. All copulations and ovipositions could be recorded. The first clutch observed in each sample determined day one of the oviposition period, which was limited to one month like in the autumnal reproduction after maturity and before hibernation in natural populations. For the schedule of the reproduction period see figure 1(upper part).

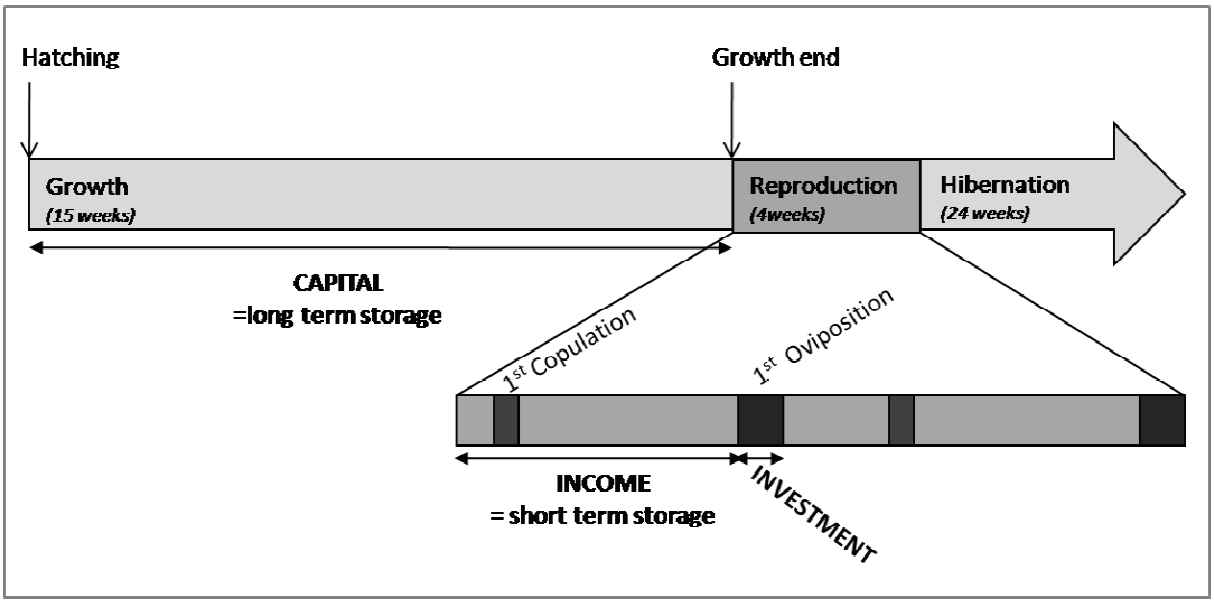

Figure 1. Schedule of growth and reproduction in *C. aspersum*. The acquired energy stored during growth represents the capital. The acquired energy during breeding period that could be stored at least for a short time from growth end to oviposition is defined as income ($E_{acquired}$ - $E_{expended}$ = E_{stored} = Income). Energy invested in clutches (investment) was depleted from energy stores pre-partum (income + capital).

Each clutch was weighed and identified by its parentage and by laying dates. The eggs were counted, and 40 eggs were randomly chosen and weighed individually. These eggs from each diet sample were frozen and lyophilized in vacuum (Lyovac™ GT3, Leybold-Heraeus, Orsay, France) for 48h, weighed again, and then stored at -80°C for further biochemical analysis. The dry weight density was deduced (mg DM.mg^{-1} FM.100%).

BIOCHEMICAL ANALYSIS

For one measurement, we took 20-30 mg of organ tissues per individual, and we pooled eggs from clutches (one egg from each clutch, six repetitions). Triglycerides (TG), glycogen (GLY) and galactogen (GAL) in the perivitellin fluid of eggs and in tissues were analyzed according to the protocol of Hervant et al., ('99) for lipid extraction and Van Handel, ('65) for polysaccharide extraction. Homogenisation of lyophilized material was done by bead beating with Tungsten beads of 3 mm diameter (4 x 30 s at 30 agitations.s^{-1}, Retsch™ MM301, Retsch GmbH, Haan, Germany) followed by the addition of 1.5 ml Folch solution (methanol:chloroform 1:2 v/v) for lipid extraction and 2.5 ml Trichloroacetic acid (4%) for polysaccharide extraction to the weighed tissues or eggs. For TG analysis 12 h incubation at –20°C was performed before separation of the inferior lipid phase from the superior aqueous phase by adding 300 µl KCl (2 g.l^{-1}) and heating for 5 min at 40°C. Polysaccharides, contained in 2 ml supernatant after

centrifugation (5000 g, 5 min, 4°C, Sigma® 2-16K, Fisher Bioblock Scientific, USA) were precipitated with 4 ml ethanol (96°). After centrifugation, the pellet was dissolved in 1 ml water and precipitated again with 2 ml ethanol (96°) followed by centrifugation. A part of the extracted lipid phase (500 µl) was dried under nitrogen flow at 30°C for 1 h before suspension in 300 µl solution BSA (3% w/v, fatty acid free) - Triton (0.2% v/v) for TG analysis. For GLY analysis the pellet of polysaccharides was suspended in 1 ml water (incubation 12 h at 21°C). For GAL analysis the pellet was hydrolyzed in galactose residues with 500 µl HCl (6M) during 6h at 110°C followed by neutralization with 500 µl KOH (6M). The content of TG, GLY and galactose was determined by measuring the absorbance with a micro plate spectrophotometer (VERSAmax™ microplate reader, Sunnyvale, CA, USA), using the Triglyceride Assay Kit (Cayman Chemicals, Ann Arbor, Michigan, USA) and the Enzyplus® EZS 784+ Lactose/D-Galactose Kit (BIOCONTROL, Bellevue, WA, USA). The aqueous GLY solution was centrifuged before dying with 10 µl Lugol for absorbance measurement at 425 nm.

DATA ANALYSIS

Statistical analyses were carried out using "R" (Team, 2007). The NLME procedure (non linear mixed effect model) with a four parametric logistic equation was applied to the somatic growth curves and to the cumulated GE curves, both obtained on individual data. Coefficients of each adjusted curve were used to compare between diet treatments by Student *t*-test. Growth time to reach maturity was compared between diet treatments using a Mann-Whitney test (count data).

Energy of body stores was calculated using average caloric values of 17.2 $kJ.g^{-1}$ for carbohydrates (CH = GLY + GAL) and 39.0 $kJ.g^{-1}$ for TG (Karl et al., 2007). Analyzed storage compounds and the energy of body stores were compared between diet treatments by Student *t*-test.

Prior to the reproduction experiment, we tested the samples for homogeneity on individual size by using Student *t*-test, and we performed separate hierarchical analyses LME (linear mixed effect models), as described by Crawley (2007), with cage nested within each factor to detect possible cage effects. In statistical analysis of reproductive traits, we tested the effects of diet treatment and the covariates breeder size, egg weight and clutch size using GLM procedures (generalized linear models, Nelder and Weddenburn, '72). After a first analysis, models were simplified by (i) backward elimination of non significant effects from the full-model and (ii) aggregating factor levels that did not differ significantly from each other (Crawley, '07). As recommended by Crawley (2007), we corrected for overdispersion by using quasipoisson distribution with a log-link function on count data (clutch size) and we performed F tests on the deviance reductions. A gamma distribution with power (-1) link function was defined to analyze clutch weight. Egg weight was analyzed with Gaussian distribution and identity link function. We performed Pearson correlations between reproductive traits and significant covariates on the whole dataset.

The negative resource allocation trade-off between soma and reproduction can be visualized by a correlation between offspring investment and body condition post-partum. We could not sacrifice snails to measure the stored nutrients post-partum because we expected further ovipositions of the same individuals during the reproductive period. However, body condition would be well described by body weight on body size, if body energy stores were positively correlated to body weight (Glazier, '99). Therefore we calculated logarithmic values

(ln) of body size, body weight, and body energy at the end of growth. Then we performed linear regressions of ln(body energy) on ln(body size) as well as ln(body weight) on ln(body size), in order to verify the supposed positive correlation between body energy and body weight by using the obtained regression residuals. Afterwards we used body weight per body size to compare body conditions at the end of growth, before oviposition, and the investment in offspring between diet treatments using Student *t*-test. The paired t-test was performed to compare income and investment in offspring. In order to describe the pattern of maternal effects on egg quality and the variation of clutch size, we used linear regression residuals of ln(egg weight) on ln(body size) and ln(clutch size) on ln(body size) to correlate with linear regression residuals of ln(body weight post-partum) on ln(body size).

Results

GROWTH AND GROWTH EFFICIENCY

Somatic growth of *C. aspersum* and the cumulated GE during growth followed a logistic pattern (Fig. 2).The asymptotic size did not differ significantly between diet treatments, but in the E- sample half the asymptotic size was reached about two days later than in the E+ sample (x_{mid} E- > x_{mid} E+) and the growth rate (1/*s*) in the E- sample was lower than in the E+ sample (Table 2). In contrast, in the E-sample the asymptotic GE was lower and half the cumulated GE was reached two days earlier than in the E+ sample (x_{mid} E- < x_{mid} E+, Table 2). The parameter *s* of the GE curve did not differ between diet treatments.

Snails reached maturity at 13 weeks in both diet treatments, with the first quartile at 12 weeks and the third quartile at 13.25 weeks (Mann-Whitney, Z = 0.32, N = 20, P = 0.75). Mortality during growth was low with two dead individuals in each sample.

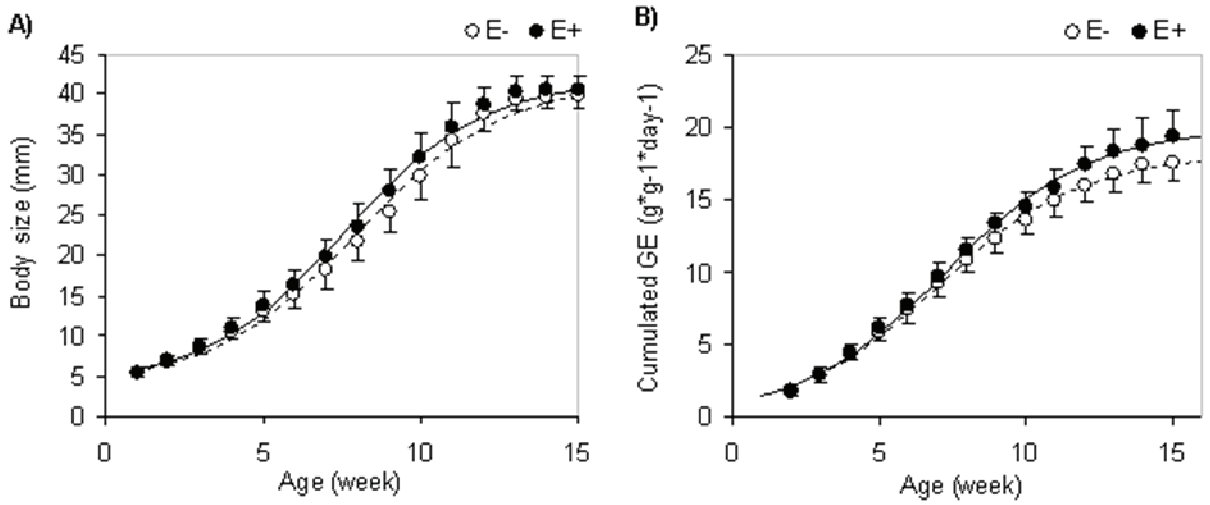

Figure 2. Theoretical adjustment of somatic growth (body size), A): $Y_{SG} = 4.00 + (a - 4.00) / [1 + \exp(-(x - x_{mid})/s)]$ and of cumulated growth efficiency, B): $Y_{GE} = a / [1 + \exp(-(x - x_{mid})/s)]$ in *Cornu aspersum*, where Y_{SG} is the body size and Y_{GE} the growth efficiency at a given age *x*, *a* is the asymptote, *s* is the numerical scale parameter on the x axis, and x_{mid} is the inflection point at *a*/2. The somatic growth curve was adjusted by a left asymptote of 4.00 mm, the lowest body size observed at hatching. The coefficients of determination between the theoretical adjusted curve and the observed data are >0.95 for each curve. Points represent observed data (mean ± s.e.m., N = 20) for different diet treatments: (E-: lipid poor diet, E+: lipid rich diet).

Table 2. Results of *t*-test for comparison of model variables (mean ± s.e.m., $N = 20$) used for theoretical adjustment of somatic growth curves and growth efficiency curves between diet treatments (E-: lipid poor diet, E+: lipid rich diet) in *Cornu aspersum*. See figure 1 for abbreviations of model variables. Degrees of freedom appear as subscript of *t*.

	Asymptote	x_{mid}	*s*
Somatic growth (mm)			
E-	39.76 ± 1.44	7.97 ± 0.07	2.26 ± 0.05
E+	40.46 ± 1.80	7.59 ± 0.08	2.20 ± 0.08
t-test	$t_{38} = 1.32$	$t_{38} = 15.20$	$t_{38} = 3.01$
	$P = 0.19$	$P < 0.0001$	$P = 0.005$
Growth efficiency ($g.g^{-1}$)			
E-	18.11 ± 0.28	7.01 ± 0.06	2.45 ± 0.04
E+	19.92 ± 0.35	7.25 ± 0.07	2.46 ± 0.05
t-test	$t_{38} = 4.04$	$t_{38} = 2.65$	$t_{38} = 0.15$
	$P = 0.0002$	$P = 0.0117$	$P = 0.88$

REPRODUCTIVE TRAITS

The two newly composed samples (E- and E+), from growth experiment samples and stock, did not differ in mean body size (40.06 ± 0.36 mm, *t*-test, $t_{38} = 0.70$, $P = 0.49$, $N = 40$). Mortality during the reproductive period was low (E- = 2, E+ = 3 dead individuals). In the E– sample 18 of 20 and in the E+ sample 14 of 20 snails laid one clutch.

Variations in all reproductive traits (Table 3, Fig. 3) were well described by the model (total deviance reduction due to the model ranged from 33.48% to 72.10%). No cage effect was detected (LME, clutch weight: $F_{3,3} = 2.38$, $P = 0.25$, clutch size: $F_{3,3} = 1.45$, $P = 0.38$, egg weight: $F_{3,3} = 0.35$, $P = 0.79$). Significant differences in reproductive traits between diet treatments were recorded, but no correlation existed with breeder size. Clutch size and egg weight were mainly influenced by diet (explained deviances of 74.56% and 65.30%, respectively). The significant correlation between both traits revealed a trade-off represented by a negative correlation on the whole dataset (Pearson, $r = –0.55$, $DF = 30$, $P = 0.0012$, $N = 18[14]$ clutches for E-[E+], respectively). E+ snails laid smaller clutches of bigger eggs to the detriment of clutch weight, while E- snails laid larger clutches of smaller eggs. The clutch weight was mainly influenced by clutch size (explained deviance of 66.81%), and consequently E- snails laid heavier clutches because of bigger clutch size.

Table 3. Summary of the analysis of deviances on reproductive traits observed in *Cornu aspersum*. We indicated the explained deviance from the final linear model after backward simplification (Crawley, 2007), followed by *F* and *P*- values, ns – not significant ($p > 0.05$). $N = 14[18]$ for E+[E-], respectively.

	Total deviance reduction (%)	Model term	Explained deviance (%)	*F*	*P*
Clutch weight	72.10	Diet	13.15	13.57	0.001
		Clutch size	66.81	68.95	<0.0001
		Egg weight	20.04	20.69	<0.0001
		Breeder size			ns
Clutch size	33.48	Diet	74.56	12.80	0.0012
		Egg weight	25.44	4.37	0.0455
		Breeder size			ns
Egg weight	33.86	Diet	65.30	9.69	0.0041
		Clutch size	34.70	5.15	0.0308
		Breeder size			ns

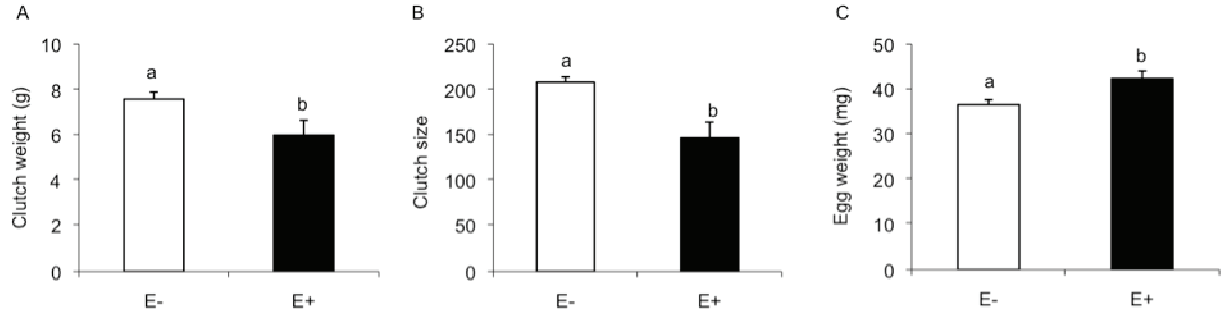

Figure 3. Reproductive traits of *C. aspersum* as a function of diet treatment (E-: lipid poor diet, E+: lipid rich diet). Error bars denote standard errors. Dissimilar letters indicate significant differences: GLM (table 4), $P < 0.05$, $N = 18[14]$ clutches for E-[E+] respectively.

STORAGE COMPOUNDS IN BREEDER TISSUES AND EGGS

TG was mainly stored in the digestive gland, while the TG content in the albumen gland was low and not influenced by the diet treatment (Table 4). E+ snails differed from E- snails by a higher TG content in digestive gland and foot, and by a higher release of TG in their eggs.

Table 4. Storage compounds and dry weight density (mean ± s.e.m.) in tissues ($N = 10$) and eggs ($N = 6$ for TG, GLY and GAL, N = 18[14] for dry weight density of E-[E+], respectively) according to diet treatment (E-: poor energy diet, E+: rich energy diet). Results of the Student *t*-test are indicated between diet treatments. Degrees of freedom appear as subscript of *t*. DM: dry mass, np: not present.

	FOOT	GLANDS		EGGS
		Digestive	Albumen	
Triglycerides (µg*mg^{-1} DM)				
E-	7.87 ± 0.77	13.10 ± 1.51	5.29 ± 1.00	11.68 ± 0.85
E+	11.86 ± 1.20	19.90 ± 2.24	4.53 ± 0.41	17.22 ± 2.02
t-test	$t_{18} = 3.19$ $P = 0.005$	$t_{18} = 3.09$ $P = 0.006$	$t_{18} = 0.86$ $P = 0.40$	$t_{10} = 2.53$ $P = 0.030$
Glycogen (µg*mg^{-1} DM)				
E-	55.04 ± 6.04	17.62 ± 1.76	87.19 ± 13.41	26.32 ± 6.57
E+	72.96 ± 11.18	21.08 ± 2.64	68.47 ± 12.48	23.69 ± 9.50
t-test	$t_{18} = 1.73$ $P = 0.10$	$t_{18} = 1.34$ $P = 0.20$	$t_{18} = 1.25$ $P = 0.23$	$t_{10} = 0.23$ $P = 0.82$
Galactogen (µg*mg^{-1} DM)				
E-	np	np	140.07 ± 25.11	133.62 ± 17.43
E+			113.15 ± 21.62	135.18 ± 21.44
t-test			$t_{18} = 1.00$ $P = 0.33$	$t_{10} = 0.06$ $P = 0.96$
Dry weight density (%)				
E-	12.7 ± 0.3	18.0 ± 1.2	21.6 ± 1.1	23.85 ± 1.24
E+	14.3 ± 0.4	19.9 ± 1.6	23.2 ± 1.0	18.81 ± 1.02
t-test	$t_{18} = 3.20$ $P = 0.005$	$t_{18} = 0.94$ $P = 0.36$	$t_{18} = 1.07$ $P = 0.30$	$t_{30} = 2.18$ $P = 0.037$

Diet treatment did not influence GLY storage in body tissues and eggs (Table 4). GLY predominated in albumen gland and foot tissue. GAL, only present in albumen gland and eggs, was stored equally in both diet treatments. Besides water GAL was the major compound in eggs, stored at a mean level which was six times as high as GLY content (Table 4).

The dry weight density in the foot of E+ snails was significantly higher, whereas E+ eggs had a lower dry weight density due to higher water content (Table 4). The dry mass of eggs was not affected by diet treatments (7.75 ± 0.25 mg, *t*-test, $t_{28} = 0.54$, $P = 0.59$), but E+ eggs had a higher water mass (35.17 ± 1.92 mg) than E- snails (26.94 ± 1.24 mg, *t*-test, $t_{58} = 3.61$, $P = 0.0006$). The dry weight density of glands was not influenced by diet treatment.

ALLOCATION TO SOMA AND REPRODUCTION

Although the quality of body stores differed between diet treatments (Table 4), the energy content of analyzed body stores (TG + CH) per individual was not significantly different between diet treatments (3.92 ± 0.30 kJ/snail, *t*-test, $t_{18} = 1.45$, $P = 0.16$, $N = 20$). Moreover, in both diet treatments we observed a significant positive correlation between body energy per body size and body weight per body size (Fig. 4), which allowed us to use body weight per body size as indicator for body condition.

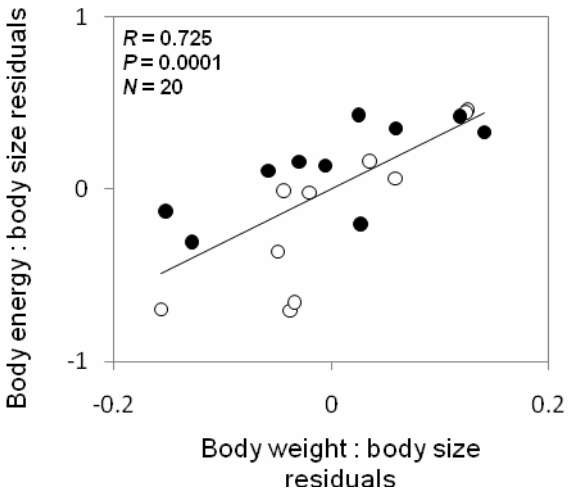

Figure 4. At growth end measured body energy stores in relation to body weight (both are residuals of ln[values] regressed against ln[body size]) in *C. aspersum* fed with two different diet treatments (E-: lipid poor diet, E+: lipid rich diet). Note that there was no treatment effect, so Pearson's correlation coefficient is indicated for the whole dataset.

At the end of growth the breeding period started (Fig. 1, lower part), and snails were observed copulating for several hours (8-10 h) and several times. Snails continued feeding afterwards until oviposition (1-1.5 weeks later). During copulation and oviposition (egg formation and egg laying) snails do not feed. The energy acquired during growth could be considered as the capital available for breeding. From the end of growth until oviposition snails could acquire further energy stores referred to as income (Fig. 1, lower part). If any acquired energy was directly used to cover energy expenditure during this time, especially for copulation and spermatophore production, or to fill up used energy stores, the income would represent the difference between "brut income" and energy expenditure, and would be available for oviposition. The income in breeding snails is energy that should be stored at least a short time for

further utilization. Therefore, the condition of the snails before oviposition (pre-partum) is obtained by addition of capital and shortly stored income. The capital and income were equal in both diet treatments (Fig. 5), so that body condition before oviposition (capital + income) did not differ between diet treatments (0.66 ± 0.08 g.mm^{-1}, *t*-test, $t_{30} = 0.30$, $P = 0.76$). The investment represents the parental energy investment in offspring, because snails do not take further care of offspring. Nevertheless, the high investment in offspring of E- snails could not be covered by the income (paired t-test, $t_{17} = 8.06$, $P < 0.0001$, $N = 18$) by contrast to E+ snails (paired t-test, $t_{13} = 1.94$, $P = 0.07$, $N = 14$).

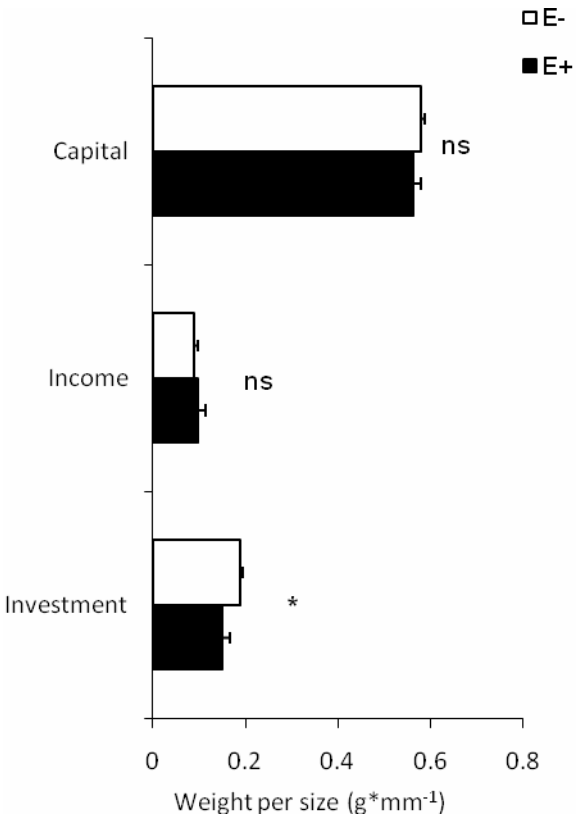

Figure 5. Body storage condition at the end of growth (capital), acquisition of stores during breeding period (income), and allocation of stores to brood (investment) in *C. aspersum* as a function of diet treatment (E-: lipid poor diet, E+: lipid rich diet). Error bars denote standard errors. Significant differences between diet treatments are indicated by * ($P < 0.05$), and ns: not significant. Student *t*-test, capital: $t_{30} = 0.79$, $P = 0.43$, income: $t_{30} = 0.42$, $P = 0.68$, investment: $t_{30} = 2.19$, $P = 0.037$. $N = 18[14]$ brooding snails for E-[E+] respectively.

MATERNAL EFFECTS ON REPRODUCTIVE TRAITS

Reproduction allocation increases when somatic allocation decreases in the case if inter-individual variation in resource allocation exceeds that in resource acquisition (Van Noordwijk and De Jong, '86, Glazier, '99). Consequently, the negative correlation between clutch size and body conditions post-partum in E+ snails (Fig. 6) indicates increasing allocation to reproduction oriented in clutch size with decreasing somatic allocation. In contrast, E- snails invested at the same level in clutch size whatever somatic allocation. The same is true for the relation with pre-partum body conditions (E-: Pearson, $r = 0.203$, $DF = 16$, $P = 0.21$, $N = 18$; E+: Pearson, $r = -0.534$, $DF = 12$, $P = 0.02$, $N = 14$). TG rich eggs in E+ snails were heavier than eggs in E- snails

(Fig. 3, Table 4), but no maternal effects on egg weight could be revealed in both diet samples (Fig. 6).

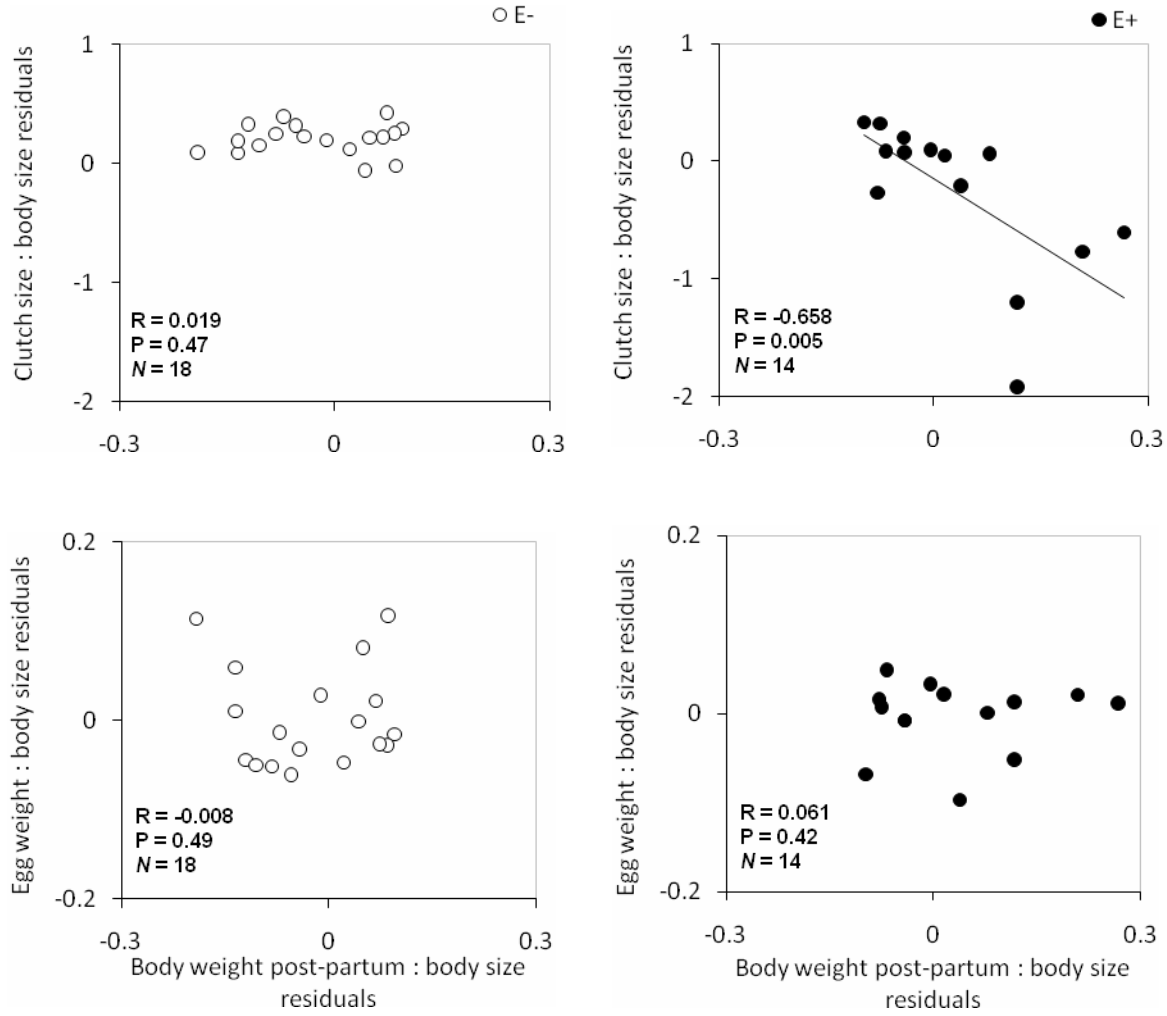

Figure 6. Reproduction allocation in relation to somatic allocation (both are residuals of ln[values] regressed against ln[body size]) of individual brooding *C. aspersum* with two different diet treatments (E-: lipid poor diet, E+: lipid rich diet). Pearson's correlation coefficient is indicated on the graph.

Discussion

For most organisms, where one or more resources are limited, they are allocated to enhance either growth and adult survival, or offspring production and their survival (Barnes et al., 2001). The model of Fischer (2009) predicted a maximal investment in reproduction when energy availability is low, and a decrease of energy investment in reproduction until a threshold when energy availability in the environment increases. Accordingly, E- snails fed with low energy diet depleted all their income stores and partially capital stores for reproduction, which could lead to a higher risk of mortality after reproduction or during hibernation and consequently a higher probability of semelparity (Begon et al., '96, Bonnet et al., '98, Stelzer, 2001). E+ snails, supplied with high energetic food invested less in reproduction than did E- snails.

Body weight per body size could estimate accurately body storage condition (Glazier, '99), because acquired energy stores per body size were positively correlated to body weight per

body size. The lipid rich diet E+ did rather enhance TG storage than GLY or GAL storage, as observed in the gastropod *Helisoma trivolvis* (Schneck et al., 2003). TG can liberate fatty acids as source of energy (Singh et al., 2009). GLY is transformed into TG for longer storage in the gastropod *Littorina saxatilis* (Arakelova et al., 2004), and GAL appears only during the reproductive phase in the albumen gland by transformation of GLY in *C. aspersum* (Tompa, '84). In agreement with growth observations made in the gastropod *Arianta arbustorum* (Wacker, 2005), E+ diet accelerated growth rate but did neither affect the time to reach maturity nor body size at growth end. Nevertheless, as expressed in the cumulating curve of GE, E+ snails gained more weight per unit of ingested food than did E- snails, resulting in higher foot dry density (Bonnet et al., '98, Varpe et al., 2009). However, the higher incorporation of TG in E+ snails did not lead to significant higher energy content of body stores, so that the quality of body stores seemed to greatly influence reproductive allocation rather than the energy content of body stores.

The investment in clutch did not take into account other reproductive costs, e.g. for egg-laying itself, nest-building or secretion of mucus, which could affect egg development and hatchling survival (Baur, '94b). The investment in clutch represents that part of physiological cost of reproduction (Calow, '79, Reznick, '92) attributed to offspring development and feeding in the nest. In snails, hatchlings feed usually on egg shell remains and often rely on egg cannibalism during the first week of life (Baur, '93). In the present study, investment in clutch was not covered by the income incorporated during breeding period. This results led us to refer *C. aspersum* to a partial capital breeder (Bonnet et al., '98).

To estimate the whole amount of physiological costs, we should have evaluated storage compounds in pre- and post-partum tissues of brooding snails. Instead we expected further copulations and ovipositions during the breeding period, but they did not occur, leading to the conclusion that E- snails exhausted available energy through oviposition. The decision, whether to put all the resources into reproduction or only a part, seems to depend on the amount of energy stores. Stearns, ('92) postulated the existence of a threshold level of body reserves needed for induction of reproduction, which has been demonstrated in the capital breeding snake *Vipera aspis* with strong semelparous tendency (Naulleau and Bonnet, '96). In contrast, situated above the threshold for reproduction induction, E+ snails adjusted their reproductive investment as a function of body condition and resource availability in order to optimize the trade-off between current and future reproduction and between reproductive investment and survival or longevity. Furthermore, they may forego another copulation and oviposition because they had not yet obtained the threshold (sequestration of sufficient resources) by the end of reproduction period (McNamara and Houston, '96, Wilbur and Rudolf, 2006). Poor individual condition is thought of as the main causes for skipped reproduction (Rideout et al., 2005), which is expected to occur when future reproductive success outweighs the benefits of immediate reproduction (Wilbur and Rudolf, 2006).

Reproductive strategies evolve to maximize maternal fitness (Smith and Fretwell, '74, Karasov and Martinez del Rio, 2007) and are characterized by the relation between egg size and number. Maternal effects on egg investment might enhance survival probability of offspring and could influence reproductive success of the next generation (Baur, '94a, McNamara and Houston, '96, Mousseau and Fox, '98). Larger juveniles of *C. aspersum* should reach more quickly the critical shell size of 20 mm, below which they have a low probability to survive hibernation (Biannic and Daguzan, '93). If early juvenile body size or weight affects growth and juvenile survival, reproductive strategies may also have consequences on population dynamics through

cohort effects (Beckerman et al., 2002). In our study, eggs in both diet treatments differed in egg weight and TG content, suggesting that egg weight reflects TG content. Therefore, the absence of any correlation between egg weight and individual body condition post-partum shows that there are no maternal effects on egg TG content with increasing reproductive allocation, since the later is negatively correlated to somatic allocation (Van Noordwijk and De Jong, '86; Glazier, '99). In this case, egg number per clutch should increase with reproductive investment, according to the optimal offspring investment theory (Smith and Fretwell, '74). Consistently, clutch size of E+ snails was negatively correlated to body conditions, as observed in the gastropod *Lymnaea stagnalis*, where fecundity was high when body dry weight density (storage) was low (Ter Maat et al., '07). In contrast, E- snails with few body TG stores maximized their reproductive investment by high clutch size whatever their individual body condition.

Despite its low content compared to CH, TG in eggs could significantly influence offspring survival and growth. Larger TG rich E+ eggs contained more water, a relation already observed in the butterfly *Bicyclus anynana* (Karl et al., 2007). TG stores in eggs are depleted just before hatching and in newly hatched offspring of the gastropod *Pomacea canaliculata* (Dreon et al., 2006). While TG are stored anhydrously, the lipase is hydrosoluble and enhances its activity with increasing water-TG interface (Horton et al., '93). Considering these facts, TG content with related egg weight might well indicate egg quality and might ensure a high hatching rate and high hatchling survival to compensate for low fecundity in E+ snails. This is in accord with works of Madec et al. ('98) and Baur ('94a) who observed larger offspring hatching from bigger (heavier) eggs of small clutches in the gastropods *C. aspersum* and *Arianta arbustorum*, respectively. By contrast, the selection-arena hypothesis (Stearns, '87) states that large clutches of many small eggs have a significant degree of asynchrony of hatching and might therefore provide a fertile ground for sib competition through non-random egg cannibalism resulting in selection of high quality offspring (Baur, '93). In an earlier study (Nicolai et al. *Ca*-I) we have observed that the degree of egg cannibalism and hatchling size increased with clutch size. Desbuquois ('97) also showed that cannibalistic hatchlings were 1.3 times heavier than non-cannibalistic hatchlings, and they had higher survival rates (100% *versus* 75.8%).

In conclusion, the quality of energy stores which means the type of nutrient available in food and stored in the body, greatly influenced reproductive investment. Further studies should consider hatchling survival and growth as well as breeder survival to evaluate the fitness in *C. aspersum* depending on energy and nutrient availability in food. Moreover, the adjustment of life history traits to resource accessibility in natural environment and their consequences on population dynamics is particularly interesting in studying the invasive potential of the species as a function of changing habitat characteristics.

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Homemade delicacy

When *Cornu aspersum* is fed with lipid rich diet, more triglycerides are invested in each individual egg albeit reducing the overall investment in one reproductive event by decreasing clutch size (Nicolai et al. *Ca*-II). Now, it would be interesting to know if essential nutrients that are not found in food, but synthesized *de novo*, are also allocated at a higher level to eggs when energy availability in environment is high.

Cholesterol represents 86-92% of total sterols in slugs and snails (Voogt 1983; Zhu *et al.* 1994) and 10-15% or 20-30% of total lipids in the digestive gland or foot of land snails, respectively (Arakelova 2008). It can be synthesized *de novo* from dietary lipid precursors (Zhu *et al.* 1994) or from acetate provided by intestinal bacteria (Charrier *et al.* 2006). It is one of the most important compounds in an organism since it regulates fluidity of cell membranes (Figure *ca*13) and thermal adaptation through membrane-stabilizing effects (Robertson & Hazel 1997; Crockett 1998; Hochachka & Somero 2002). That is why this sterol has attracted more attention especially in species that depend on dietary availability and are prone to thermal destabilizing effects through increasing temperature in their habitat (Wacker & Martin-Creuzburg 2007; Sperfeld & Wacker 2009). In the aquatic food web keystone *Daphnia* sp. dietary cholesterol is limited. Higher amounts are needed for growth and reproduction under higher thermal regimes, thereby indicating possible influences on ecosystem processes and community structures under global warming.

Hypotheses of this study:

- Cholesterol is allocated at a higher level to eggs when snails are fed with lipid rich diet
- Allocation of cholesterol to eggs is generally low because of the biosynthesis capacity in snails

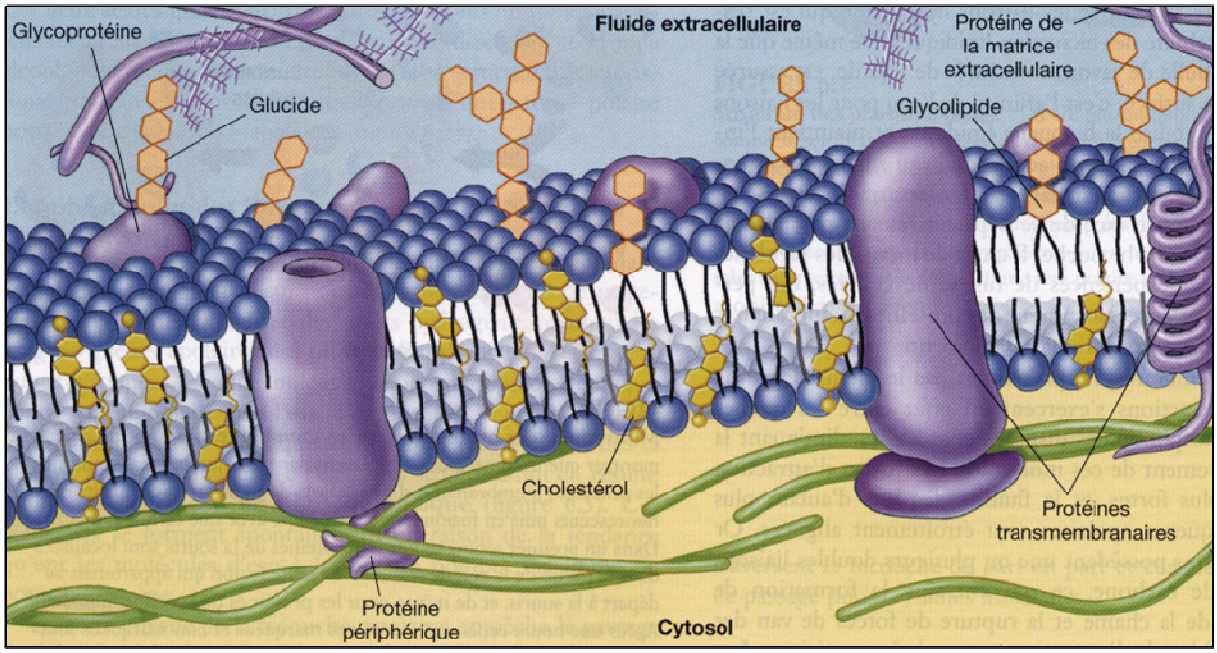

Figure *ca*13. The fluid mosaic of the cell membrane. Cholesterol is intergrated in the double phospholipid layer (Raven *et al.* 2005).

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Reproductive allocation of cholesterol in the terrestrial gastropod *Cornu aspersum*

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Reference: Nicolai et al. *Ca*-III

Abstract: Cholesterol, the predominant sterol in land snails, can be synthesized *de novo* from dietary precursors like acetate. When individuals of *Cornu aspersum* were fed with lipid rich diet, they enhanced their cholesterol synthesis in the foot, but not in the digestive gland and deposited less cholesterol in eggs. The overall allocation of cholesterol to the brood was lower than 1% of the whole body cholesterol synthesis. This result emphasizes the direct transfer of cholesterol within lipid yolk droplets from the digestive gland to the ovotestis and the ability of the embryo and of the hatchling to synthesize cholesterol by their own. Since cholesterol is involved in the thermal regulation of membrane functioning, further studies should focus on this biosynthesis ability of some ectotherms under thermal stress.

Keywords: Cholesterol, Nutrition, Gastropod, Reproductive allocation, Biosynthesis

Introduction

Cholesterol, the predominant sterol in animals (Goad 1981), is an indispensable structural component of cell membranes, regulating the function of membrane-bound proteins and plays a role in thermal adaptation through membrane-stabilizing effects (Crockett 1998; Hochachka and Somero 2002; Robertson and Hazel 1997). Furthermore, it serves as precursor for numerous steroid hormones in invertebrates (Goad 1981) among which those involved in reproductive activity in gastropods (Flari and Edwards 2003).

Cholesterol represents 86-92% of total sterols in slugs and snails (Voogt 1983; Zhu et al. 1994) and 10-15% or 20-30% of total lipids in the digestive gland or foot of land snails, respectively (Arakelova 2008). *C. aspersum*, a terrestrial hermaphrodite gastropod with determinate growth pattern, contains 10% lipids of the dry mass (Gomot 1998). In contrast to other invertebrates, gastropods can synthesize cholesterol *de novo* using acetate (Addink and Ververgaert 1963, Flari and Edwards 2003) provided by intestinal bacteria (Charrier et al. 2006). The main precursor is desmosterol which was found to be abundant in land snails, but not in plants that constitute the diet of these snails (Zhu et al. 1994). Cholesterol is transported with the aid of a high density lipoprotein in the haemolymph (Pollero et al. 1992) and could be stored within lipid droplets (Martin and Parton 2006).

Several studies in gastropods have shown that quantitative reproductive traits were influenced by the quality of fatty acids (Wacker 2005) or by the quantity of lipids in food (unpublished), and some authors suggested lipids to be important for reproduction (Borges et al. 2004; Giokas et al. 2007). Besides some studies about cholesterol allocation of *Daphnia* sp. limited in dietary cholesterol (Sperfeld and Wacker 2009; Wacker and Martin-Creuzburg 2007), no study has provided empirical data about cholesterol allocation in species that could synthesise cholesterol *de novo*. We hypothesize that the addition of lipids in the snail diet will lead to higher cholesterol production without affecting cholesterol allocation to brood.

Materials and Methods

Care and use of animals in the experiments comply with all relevant local animal welfare laws, guidelines and policies.

100 newly hatched snails from four clutches of different mothers of an outdoor snail farm (Corps-Nuds near Rennes, France) were randomly assigned to two different diet treatments in samples of 50 individuals. The Helinove® diets, formulated by Idena (Sautron, France) and made by Berton Alimentation Animale (Le Boupère, France), differed in their energy content (2220 and 2420 kcal/kg) owing to their differing fat content (2.5 and 5.5% fat, respectively) despite partial polymers showing the opposite trend (13 and 10%, respectively). Protein, sugar/starch and ash content were similar (15%, 25% and 37%, respectively). Both diets were cholesterol free. The snails were reared under controlled conditions (temperature: 20 ± 1°C, relative humidity: 60-80%, L/D = 12/12 h) until they reached maturity (reflected peristom of shell).

At maturity ten snails from each diet treatment were dissected and the frozen organs were lyophilized in vacuum (Lyovac™ GT3, Leybold-Heraeus, France) for 48 h. 20 further snails per diet sample were chosen on the basis of body size homogeneity and maturity synchrony and were kept under optimal conditions for reproduction (temperature: 20 ± 1°C, relative humidity:

60-80%, L/D = 16/8 h). Pottery pots filled with constantly humid compost (Eco-Terre ®, pH 7.0, Avignon, France) were put in each cage for oviposition. All copulations and ovipositions could be recorded. The first clutch observed in each sample determined day 1 of the oviposition period, which was limited to one month like in the in natural populations. The eggs of each clutch were counted and weighed. Frozen eggs from each diet sample were lyophilized in vacuum for 48h (Trivac, Oerlikon Leybold Vacuum GmbH, Köln, Germany) For cholesterol analysis we took one egg from each clutch, pooled and homogenized them (repetition six times).

To analyze cholesterol content in the perivitellin fluid of eggs and in tissues, we used the protocol of Hervant et al. (1999) for lipid extraction. Cell lyses was done by bead beating (4 x 30 s at 30 agitations*s^{-1}, Retsch™ MM301, Haan, Germany) after adding 1.5 ml Folch solution (methanol:chloroform 1:2 v/v) to the weighed tissues or eggs. After 12 h incubation at –20°C the lipid phase separation was performed by adding 300 µl KCl (2 g*l^{-1}) and heating for five minutes at 40°C. 500 µl of the extracted lipid phase were dried under nitrogen flow at 30°C for 1 h before suspension in 10 µl Ethanol (95°) and absorbance measurement with a micro plate spectrophotometer (VERSAmax™, CA, USA), using the Cholesterol RTU™ kit (Biomerieux, France) and a calibration curve. Data were analyzed with *t*-test using the software "R" (R Core Team 2008).

Results

The two samples, E- and E+, did neither differ in mean body size (40.06 ± 0.36 mm, *t*-test, $t_{38} = 0.70$, $P = 0.49$, $N = 40$) nor in mean body mass (22.94 ± 0.47 g, t-test, $t_{38} = 0.96$, $P = 0.34$, N = 40). Mortality during the reproductive period was low (E- = 2, E+ = 3 dead individuals). In the E– sample 18 of 20 and in the E+ sample 14 of 20 snails laid one clutch.

E+ snails laid smaller clutches of bigger eggs to the detriment of clutch weight, while E- snails laid larger clutches of smaller eggs, however enhancing significantly clutch weight (Table 1). The significant correlation between both traits revealed a trade-off represented by a negative correlation on the whole dataset (Pearson, $r = –0.55$, $DF = 30$, $P = 0.0012$, $N = 18[14]$ clutches for E-[E+], respectively).

Table 1. Summary of the analysis of deviances on reproductive traits observed in *Cornu aspersum*. We indicated explained deviance from the final linear model after backward simplification (Crawley, 2007), followed by *P* and *F*- values, ns – not significant (p > 0.05). *N* = 14[18] for E+[E-], respectively.

	E-	**E+**	**Total Deviance Reduction (%)**	**Model term**	**Explained Deviance (%)**	***F***	***P***
Clutch size (egg number)	208 ± 6	147 ± 18	33.48	Diet Egg weight Breeder size	74.56 25.44	12.80 4.37	0.0012 0.0455 ns
Egg weight (mg)	36.5 ± 1.1	42.3 ± 1.7	33.86	Diet Clutch size Breeder size	65.30 34.70	9.69 5.15	0.0041 0.0308 ns
Clutch weight (g)	7.57 ± 0.31	5.98 ± 0.66	33.86	Diet Clutch size Egg weight Breeder size	13.15 66.81 20.04	13.57 68.95 20.69	0.001 <0.0001 <0.0001 ns

Cholesterol was not equally distributed in the tissues and in eggs (Table 2). It was very abundant in the foot, but low in glands and eggs. While cholesterol content of the foot in E+ snails was significantly higher than in E- snails, the digestive gland and eggs of E+ snails contained less cholesterol than E- snails. The content in the albumen gland was not influenced by the diet treatment. The estimated cholesterol allocation to the clutch reached only 0.054 ± 0.002 mg in E- snails and 0.021 ±0.002 mg in E+ snails (*t*-test, t_{30} = 10.10, $P < 0.0001$, N = 18[14] clutches for E-[E+], respectively), which corresponds to 0.36% and 0.13% of the whole individual cholesterol content, respectively.

Table 2. Cholesterol content (mean ± s.e.m. expressed as µg.mg^{-1} DM) in tissues and eggs according to diet treatment. Results of the Student *t*-test between diet treatments are indicated. E- = poor energy diet, E+ = rich energy diet, DM = dry mass. N = 10 for tissues and N = 6 for eggs.

	Foot	Glands		Eggs
		Digestive	Albumen	
E-	17.16 ± 0.60	0.44 ± 0.06	1.30 ± 0.05	0.030 ± 0.003
E+	21.74 ± 1.36	0.26 ± 0.03	1.21 ± 0.07	0.019 ± 0.003
t-test	T_{18} = 3.77	T_{18} = 3.24	t_{18} = 1.29	t_{10} = 2.59
	P = 0.0014	P = 0.0046	P = 0.21	P = 0.027

Discussion

Our results showed that *C. aspersum* allocated only a small part of its somatic cholesterol to brood, even though lipid rich diet enhanced cholesterol synthesis. In *Daphnia* sp. that are not able to produce cholesterol *de novo*, the content in eggs is kept constant even under poor food quality conditions and on the detriment of somatic allocation, suggesting high importance for early juvenile growth (Wacker and Martin-Creuzburg 2007). In contrast, *C. aspersum* allocated < 1% of its somatic cholesterol to the whole clutch, slightly increasing the cholesterol content in eggs when lipid availability in food was low. Nevertheless, the difference in cholesterol content could be significant for embryo development and hatching success, and thus contribute to a higher maternal investment in reproduction observed under poor energy availability in this species (Nicolai et al. *Ca*-II). In fact, investment in clutches in terms of weight was higher in snails fed with lipid poor diet.

E+ breeders stored more cholesterol in the digestive gland than E- breeder, whereas the content in the albumen gland in both diet treatments did not differ. The albumen is mainly constituted of polysaccharides (galactogen and glycogen), without lipids (Bayne 1968). Lipids are rather furnished within the yolk protein complex (Barre et al. 1991; Luchtel et al. 1997). Hence, our results emphasize the transfer of lipid yolk droplets from the digestive gland to the closely situated ovotestis for incorporation in oocytes before fecundation.

Enhanced cholesterol requirements with increasing temperature have been observed in some ectothermic animals, thereby indicating possible influences on population dynamics under thermal stress (Robertson and Hazel 1995; Robertson and Hazel 1997; Sperfeld and Wacker 2009). Further studies should also take into account the ability of cholesterol producing species to adapt their synthesis to the physiological demand under increasing thermal regimes.

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How to dress when it's getting warmer?

Cornu aspersum does not allocate an essential compound for growth, like cholesterol, at a high level to offspring when biosynthesis is possible (Nicolai et al *Ca*-III). Small differences in egg cholesterol content might nevertheless influence embryo development. Besides energy storage compounds, growth factors and enzymes in the perivitellin fluid of eggs, the survival of an egg and the embryo development might also depend on protective function of some egg components against environmental disturbances, especially egg shell that depends in turn on mineral allocation of the breeder (Wolda 1963; Beeby & Richmond 1998).

The egg shell of *C. aspersum* is composed of a mucopolysaccharide-glycoprotein jelly with individual crystals of calcite or aragonite (Tompa 1976), containing around 0.6 mg of calcium per egg (Tompa & Wilbur 1977). The partly calcified eggs of *C. aspersum* showed the fastest development at constant temperature between 20 and 25°C and the upper lethal temperature at constantly 27.5°C (Le Calvé 1995). Drought was described to be the most important cause of death in eggs (Wolda 1965; Pollard 1975). Since *Cornu aspersum* is widely distributed in France, it encounters different thermal regimes in respect to population origin and may adjust egg constitutions for better physical protection as well as thermal reaction norms for embryo development (Box *ca*3, Figure *ca*14). This makes empirical studies on heat tolerance so important in order to elaborate prediction models for species range distribution under climate change or for species invasion potential in newly colonized habitats.

Hypotheses of this study:

- Breeder size and fecundity depend on thermal regime of population origin
- Egg shell formation is influenced by the type of mineral source in food
- Population origin and egg shell composition influence embryo development

Box *ca*3. Thermal reaction norms

Biological rates in life history of ectotherms, like development rate, survival rate, and growth rate, follow a function that could be well described as followed (Briere et al. 1999; Kingsolver 2009): the rate curve accelerates at temperatures near the lower threshold, increases constantly at intermediate temperatures and decelerates as temperatures are approaching the optimum, above which rate decreases rapidly to zero until the upper lethal limit. According to an evolutionary perspective, when fitness is reduced above the optimal temperature, exposing to heat represents a stress (Bijlsma and Loeschcke 1997). According to the temperature-size-rule, higher temperature at development results in smaller adult body size (Atkinson 1994; Kingsolver 2009). In evolutionary terms, this rule indicates a negative slope for the thermal reaction norm that relates temperature to adult size, but reversal does exist (Mousseau 1997, Walters and Hassall 2006). Larger individuals tend to have higher performances, like greater fecundity, greater survival, and greater mating success, which underlie directional selection for larger body size (Kingsolver and Huey 2008). Thermal reaction norms can involve different trade-offs dependent on the variability and predictability of certain environmental factors to overcome costs of rate adjustment (Angilletta et al. 2003) or stress tolerance (Parsons 1997). Bennett and Lenski (1997) advanced that regardless of the width of the thermal niche, there is asymmetry in performance and functional capacity with upper and lower extremes, higher temperatures being more stressful than lower ones, and individuals living close to the temperature optimum having narrower thermal tolerances (Kingsolver 2009).

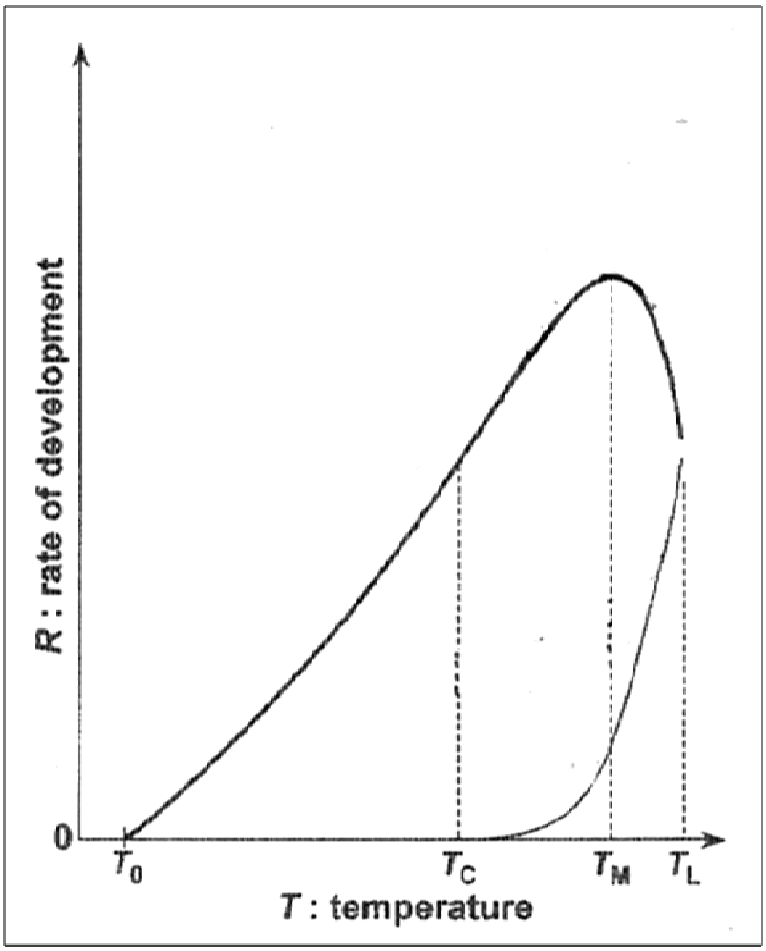

Figure *ca*14. Thermal reaction norm for development rate. T_0 – development zero, T_C– critical (optimum) temperature, T_M – temperature of maximum rate, T_L – upper lethal limit for development (Briere et al. 1999).

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Well wrapped eggs: effects of egg shell composition on heat resistance and hatchling size in the invasive land snail *Cornu aspersum*

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Reference: Nicolai et al. *Ca*-IV

Abstract: In ectotherms, the temperature changes that accompany climate shifts, microhabitat changes, and species range extensions can have profound effects on the performance of organisms. The temperature-size-rule describes in evolutionary terms the negative relationship between thermal regime of population habitat and body size, and larger individuals have greater fecundity and survival. Biological rates in life history of ectotherms are most often positively influenced by temperature increase. However above optimal temperature fitness is reduced signalling heat stress. The aim of this laboratory study on the terrestrial invasive gastropod *Cornu aspersum* was to investigate reproductive traits of two populations in different climatic regions of France and the effect of dietary calcium source on egg shell formation and heat resistance of eggs. Up to date no literature is known about heat stress in calcified ectothermic eggs while exposed to optimal humidity and natural occurring summer heat temperature during alternating thermal regimes. Despite the climatic adaptation of breeder body size and reproductive strategy in the southern population (bigger body size and higher fecundity), we could not observe any relation between heat resistance of eggs and climate of population origin. However, a long exposition to summer heat temperature resulted in the death of all clutches, and at shorter heat exposition incubation time of eggs was less variable in both populations at the detriment of hatchling mass. The latter depended on population origin and the type of mineral source in diet, like egg shell thickness where we observed the same pattern. In the southern population, fed on limestone $CaCO_3$ source, we found the smallest hatchlings. Limestone represents the most accessible Ca source for snails, however responsible for thinner and more mineralised egg shells in our study, whereas egg shell thickness could play a nutritional role for hatchlings. Further studies should focus on fitness consequences of the interaction between climatic factors and mineral availability in soil and food in natural populations of this species.

Keywords: Income breeding, Galactogen, Gastropod, Glycogen, Growth, Maternal effects, Triglyceride, Energy availability, Body storage

Introduction

In ectotherms, the temperature changes that accompany seasonal cycles, climate shifts, and species range extensions can have profound effects on the performance of organisms with given morphology (Koehl 1996). The effects of temperature on biological and ecological processes, such as development rate and life history pattern, are well documented, and imply a multitude of interactions influencing fitness (Arnold 1983; Grant and Porter 1992; Huey and Kingsolver 1993; Angilletta, Sears et al. 2003; Kingsolver 2009).

Biological rates in life history of ectotherms, like development rate, survival rate, and growth rate, follow a function that could be well described as followed (Briere, Pracros et al. 1999; Kingsolver 2009): the rate curve accelerates at temperatures near the lower threshold, increases constantly at intermediate temperatures and decelerates as temperatures approaching the optimum, above which rate decreases rapidly to zero until the upper lethal limit. According to an evolutionary perspective, when fitness is reduced above the optimal temperature, exposing to heat represents a stress (Bijlsma and Loeschcke 1997). In order to model phenologies and to approach natural conditions when testing effects of temperature on fitness, alternating thermal regimes were used, revealing increasing development rates of eggs and increasing growth rates of larvae in the insect *Chrysophtharta agricola* (Nahrung, Allen et al. 2004) or heat and cold stress reactions in the insect *Drosophila* sp. (Petavy, David et al. 2001).

According to the temperature-size-rule, higher temperature at development results in smaller adult body size (Atkinson 1994; Kingsolver 2009). In evolutionary terms, this rule indicates a negative slope for the thermal reaction norm that relates temperature to adult size, but reversal does exist (Walters and Hassall 2006). Larger individuals tend to have higher performances, like greater fecundity, greater survival, and greater mating success, which underlie directional selection for larger body size (Kingsolver and Huey 2008).

Thermal reaction norms can involve different trade-offs dependent on the variability and predictability of certain environmental factors to overcome costs of rate adjustment (Angilletta, Sears et al. 2003) or stress tolerance (Parsons 1997). Bennett and Lenski (1997) advanced that regardless of the width of the thermal niche, there is asymmetry in performance and functional capacity with upper and lower extremes, higher temperatures being more stressful than lower ones, and individuals living close to the temperature optimum having narrower thermal tolerances (Kingsolver 2009). This makes empirical studies on heat tolerance so important in order to elaborate prediction models for species range distribution under climate change or for species invasion potential in newly colonized habitats.

In fact, heat and aridity besides hunger are the most important stress encountered by land snails (Schmidt-Nielsen, Taylor et al. 1971; Riddle 1975). Nevertheless, the land snail *Cornu aspersum* Müller (Syn. *Helix aspersa*) is nowadays colonizing new habitats of North America, Australia and Europe (Guiller, Coutellec-Vreto et al. 2001), thereby developing morpho-anatomic, physiological and behavioural adaptations (Arad, Goldenberg et al. 1989). Studies on *C. aspersum* showed plasticity of quantitative reproductive traits related to environmental factors in one reproductive season (Madec, Guiller et al. 1998; Madec, Desbuquois et al. 2000) and partly genetic control of adult weight (Dupont-Nivet, Mallard et al. 1997).

The viability of eggs in terrestrial gastropods depends on the provision in nutrients, including minerals (Beeby and Richmond 1998), and on the given microclimate (Wolda 1963). These findings suggest that besides buffering climatic fluctuations through nest building and

mucus deposit (Baur 1994), the formation of egg shell could play an important role in egg survival under variable environmental conditions, comparable to insect cocoons (Danks 2004). Although Tompa (1974) could not attribute an ecological signification to the degree of egg calcification in *Anguispira alternata*, he distinguished three types of egg shell on the basis of the degree of calcification in Stylomatophora snails (Tompa 1976): (i) uncalcified eggs, (ii) partly calcified eggs with individual crystals of $CaCO_3$, and (iii) heavily calcified eggs with fused $CaCO_3$ crystals similar to bird eggs. The optimal egg incubation temperature depends on thermal regime of the population (Riddle 1983). Using constant thermal regimes in laboratory, the partly calcified eggs of *C. aspersum* showed the fastest development at 20 - 25°C and the upper lethal temperature at 27.5°C (Le Calvé 1995). Drought was described to be the most important cause of death in eggs (Wolda 1965; Pollard 1975) and an increasing egg size to attenuate this effect (Bayne 1968). No literature is known about the egg shell protecting against heat stress while exposed to optimal humidity.

The aim of this laboratory study was to investigate the reproductive strategy adopted by two geographically distant populations of *C. aspersum* and to compare egg performances in relation to thermal conditions. Therefore we tested the following two hypotheses:

1. Populations from two different climatic regions, Mediterranean and western Atlantic, adapted their reproductive strategy to temperature constraints. According to the temperature-size-rule (Atkinson 1994), we should observe differences in body size and subsequently in fecundity (Kingsolver and Huey 2008) that could be related to thermal conditions prevailing in the regions.
2. Eggs from southern regions should be more resistant to thermal stress than those from western oceanic sites. Using a natural occurring temperature of summer heat peak, we tested the heat resistance of eggs from individuals of different population origins under alternating thermal regimes.We also addressed the question of a physical role of egg shell in thermal protection of the embryo by varying the mineral composition in diet of both populations.

Materials and Methods

Between September and October 2006, 40 adult snails of *C. aspersum* were collected in two regions of France: 20 snails in the north-western region around Rennes (REN, 48°04'N, 1°43'W) and 20 snails in the southern region around Rivesaltes (RIV, 42°44'N, 2°52'E). The REN population habitat is characterized by western Atlantic climate, while the RIV population habitat is characterized by Mediterranean climate (Figure 1).

Snails were individually tagged and transferred progressively to hibernation conditions without feeding. After six months of hibernation (temperature 5 ± 1°C, relative humidity: 50%, 24h darkness), snails of each population sample were reared in two plastic cages (10 individuals per cage) with a wet latex HR (high resilient) foam and progressively transferred to reproduction conditions (temperature: 20 ± 1°C, relative humidity: 60-80%, photoperiod: 16h light/8h dark).

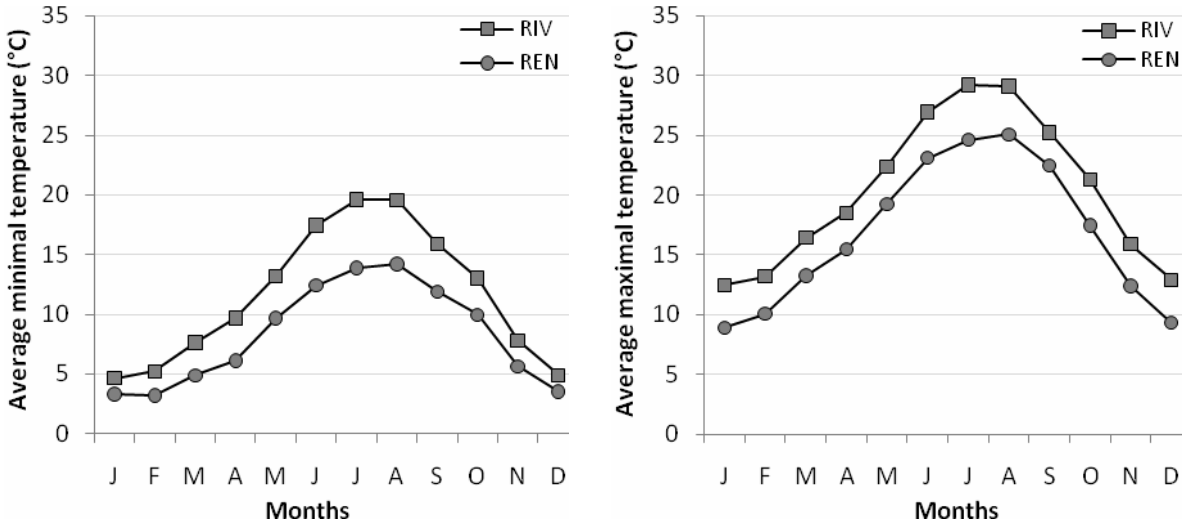

Figure 1. Ambient temperature in the sampling region of Rivesaltes, southern France (RIV), and of Rennes, northern France (REN). Data were collected from 1996 to 2006 and furnished by Meteo France. The average temperature of each month over 10 years was calculated on the average daily minimal and maximal recorded temperature per month of each year.

Twice a week, water was supplied *ad libitum* in a small pot, and snails were fed *ad libitum* on Helinove®, formulated by Idena (Sautron, France) and made by Berton Alimentation Animale (Le Boupère, France). Each population sample received two Helinove® diets which differed by their source of Calcium (Cas: simple source of calcium, Cam: multiple source of calcium), but not by their energy content (Table 1). The mineral composition of each mineral source added to the Helinove® diets was analyzed by Energy Dispersive Spectrometry (EDS, Oxford, INCA) on crushed samples of 1 mg (Table 2).

Table 1. Composition of the Helinove® diets in % of mass and energy content in kcal/kg. Prime materials of both diets are corn (36%), soja (27.5%), minerals (34%), and additives, like trace elements and vitamins (2.5%). Added minerals were calcium phosphate, limestone type „Albacal 0/315 µm" from Ile-de-France in France, dried algae *Phymatolithon calcareum* from Brittany in France, and fossil oyster shells OYTA® from the Fjord Roskilde in Norway.

	TYPES OF DIET	
	Mixed Ca source (Cam)	**Simple Ca source (Cas)**
Lipids	2.0	2.0
Proteins	15.5	15.5
Starch and sugars	26.5	26.9
Parietal polymers	9.84	9.9
Moisture	9.62	9.61
Plant minerals	2.5	2.5
Calcium phosphate	4.0	4.0
Limestone	10.0	30.0
Algae *P. calcareum*	10.0	
Fossil oyster shell	10.0	
Energy	2240	2240

Table 2. Composition of alternative mineral sources in Helinove® diets in mean ± SE % of mass. The analysis of each mineral source was done by Energy Dispersive Spectrometry (EDS, Oxford, INCA) on *N* samples, and the mineral content of each diet was calculated.

	TYPES OF MINERAL SOURCES IN DIET			TYPES OF DIET	
	Limestone *N* = 13	**Algae *P. calcareum*** *N* = 19	**Fossil oyster shell** *N* = 9	**Mixed Ca source (Cam)**	**Simple Ca source (Cas)**
O	57.75 ± 1.92	40.55 ± 3.26	54.20 ± 0.97	15.28	17.30
Ca	37.07 ± 3.46	24.53 ± 5.03	45.22 ± 1.02	10.67	11.10
Mg	0.18 ± 0.04	1.02 ±0.32	0.57 ± 0.10	0.18	0.05
Si	4.55 ± 3.85	19.13 ± 4.82		2.37	1.37
Al	0.40 ± 0.09	1.24 ± 0.63		0.16	0.12
Fe	0.04 ± 0.04	7.47 ± 5.51		0.75	0.01
S		2.41 ± 2.40		0.24	
Na		1.24 ± 0.49		0.12	
Ti		1.10 ± 1.10		0.11	
Cl		1.00 ± 0.24		0.10	
K		0.30 ± 0.15		0.03	

During reproduction, four pottery pots filled with compost (Eco-Terre ®, pH 7.0, Avignon, France) were put in each cage for oviposition and replaced as soon as used. The eggs of each clutch were counted, and 30 to 40 randomly chosen eggs were weighed individually. Two subsamples per clutch, at least 25 eggs each, were distributed in aggregates of five eggs in Petri dishes. Preliminary tests showed the role of separate aggregates in preventing as much as possible delayed eggs from cannibalism. Egg incubation took place on an imbued gaze with Marc's Modified Ringer buffer (MMR: NaCl 0.5M, KCl 0.2M, $CaCl_2$ 0.1M, $MgCl_2$ 0.1M and Hepes 23.8 $mg.ml^{-1}$, Peng 1991) adjusted to pH 7.0 and to incubation temperature. For incubation treatment, one subsample of the clutch served as control and was exposed to 20°C/24h, the other subsample was exposed to a daily fluctuating temperature cycle, either 20°C/20h - 35°C/4h (treatment 35_{4h}) or 20°C/16h - 35°C/8h (treatment 35_{8h}) with a progressive temperature adjustment of 0.5°C/min (SANYO climatic chamber, MIR 153, Fisher Labosi, France). Hatching rate, incubation time were recorded, and hatchling mass was measured in both subsamples. Eggs that were not used in incubation experimentation were pooled per diet and population. Four replicates of 10 eggs per diet and per population served for ash determination using a Muffle furnace (HORNO, Naber, Germany) at 550°C for 1h. Microstructure of egg shell was examined in 15 eggs per diet and per population using a Scanning Electron Microscope (JEOL JSM-6301 F, Tokyo, Japon), after dehydration in graded ethanol and acetone series (ethanol: 50° for 12h; 70°, 80°,90° for 2h each; 100° for 12h; and acetone for 1h) followed by critical point drying (Balzers CPD 010, Balzers, Lichtenstein) and a gold-palladium coating (JEOL JFS-1100, Tokyo, Japon).

For statistical analysis of breeder size and reproductive traits we used "R" (Team 2008), and we tested the effects of population origin and diet treatment using GLM procedures (generalized linear models; Nelder and Weddenburn 1972). After a first analysis, models were simplified by (i) backward elimination of non significant effects from the full-model and (ii) aggregating factor levels that did not differ significantly from each other (Crawley 2007). We performed an analysis of deviance reduction on the final model of each reproductive trait and did multiple comparisons *a posteriori* using Tukey HSD (Gaussian distribution) or a contrast matrix procedure with esticon analysis (Gamma and Poisson distribution). As recommended by

Crawley (2007), we used Poisson distribution with a log-link function on count data (clutch size) with egg mass as covariate. We corrected for overdispersion by using quasiPoisson distribution with a log-link function on count data (clutch size) and we performed *F*-tests on the deviance reductions. Gaussian distribution with identity link function was used for breeder size, egg mass with clutch size as covariate, and ash content in eggs, while a Gamma distribution with power (-1) link function was used for hatching mass with egg mass as covariate and for egg shell thickness. We performed Pearson correlations between reproductive traits to detect trade-offs.

In order to compare incubation time, hatchling mass and hatching rate between the control and the treatment subsample within the same clutches, we performed LME procedure (linear mixed effect models, Crawley 2007) on means (M) and coefficients of variation (CV) of standard deviation (SD) calculated as followed on five aggregates of five eggs in each clutch subsample: CV % = SD/M*100. We proceeded for model simplification as in GLM (Crawley 2007). Population origin and diet were fixed effects and incubation treatment was the random factor. To compare between the control subsamples used for the 35_{4h} and 35_{8h} treatment a Student *t*-test was applied.

Results

We observed significant differences between populations in breeder size and in clutch size, but not between diet supplement (Cas, Cam) of each population (Figure 2). The importance of population origin was high for variation in breeder size, but less pronounced in clutch size (TDR 50.12% *versus* 13.16%, respectively, table 3). No significant correlation between clutch size and egg mass was found (Pearson, $r = 0.022$, $DF = 28$, $N = 30$, $P = 0.91$).The egg laying frequency was REN-Cas = REN-Cam = RIV-Cam: 7 clutches/ 10 snails, RIV-Cas: 9 clutches/ 10 snails, with a fecundity of one clutch per snail.

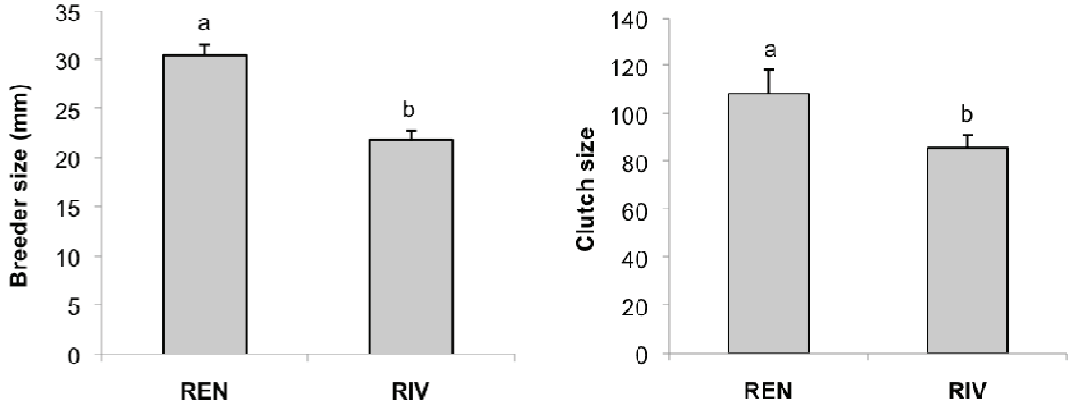

Figure 2. Breeder size and clutch size (mean ± SE) in two populations of *Cornu aspersum* (REN = Rennes, RIV = Rivesaltes). Dissimilar letters indicate significant differences for the final model of GLM procedure (table 3). Breeder size: $N = 20[20]$ for REN[RIV], respectively, clutch size: $N = 14[16]$ for REN[RIV], respectively.

Egg mass did not differ between population origins, but eggs from Cas snails where smaller than eggs from Cam snails (Figure 3, Table 3). Hatchling and egg mass were positively correlated (Pearson, $N = 30$, $r = 0.71$, $DF = 28$, $P < 0.0001$). The covariate egg mass exerted the same influence on hatchling mass as diet supplement (ED 39.28% and 38.72 %), while population origin was less important with REN hatchlings being bigger than RIV hatchlings

(24.94 ± 1.05 mg *versus* 21.77 ± 1.03 mg, Table 3). Significant smaller hatchlings emerged from Cas eggs from the RIV population (Figure 3).

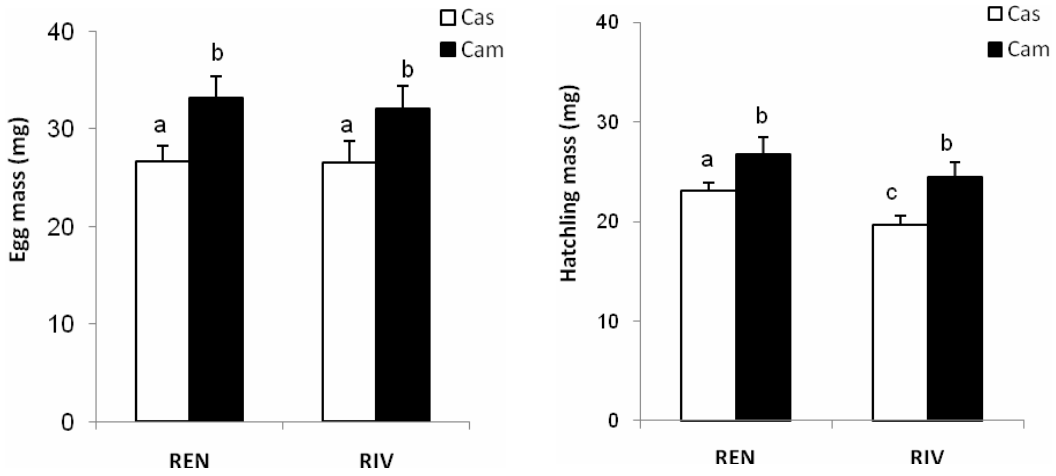

Figure 3. Egg and hatchling mass at 20°C (mean ± SE) in two populations of *Cornu aspersum* (REN = Rennes, RIV = Rivesaltes) as a function of diet treatment (Cas: simple source of Calcium, Cam: multiple source of Calcium). Dissimilar letters indicate significant differences (Tukey HSD and contrast matrix-esticon, respectively, with $P < 0.05$) for the final model of GLM procedures (table 3). N_{REN} = 7[7] and N_{RIV} = 9[7] for Cas[Cam], respectively.

The variation in egg shell thickness showed a comparable pattern as egg and hatchling mass (Figure 4, Table 3). Also egg shell thickness was influenced by population origin, and even more importantly by diet supplement (ED 15.83% *versus* 84.17%, respectively, Table 3). Eggs from Cas snails had thinner egg shells than eggs from Cam snails, but Cas eggs from RIV population had the thinnest egg shell (Figure 4). Nevertheless, ash content representing the mineral content of an egg was higher in Cas snails than in Cam snails whatever the population origin (Figure 4).

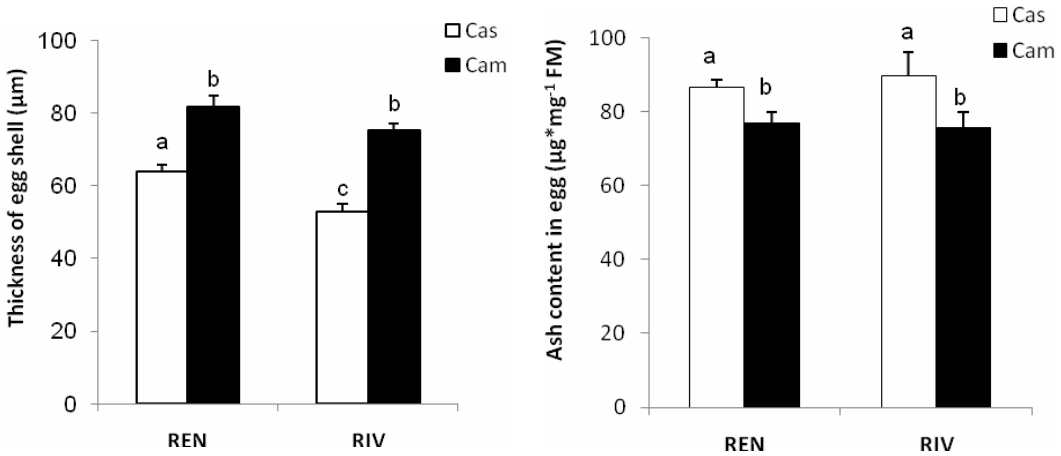

Figure 4. Thickness of egg shell and egg ash content (mean ± SE) in two populations of *Cornu aspersum* (REN = Rennes, RIV = Rivesaltes) as a function of diet treatment (Cas: simple source of Calcium, Cam: multiple source of Calcium). Dissimilar letters indicate significant differences (contrast matrix-esticon and Tukey HSD, respectively, with $P < 0.05$) for the final model of GLM procedures (table 3). Egg shell thickness: $N_{REN} = N_{RIV}$ = 15[15] for Cas[Cam], respectively, ash mass: $N_{REN} = N_{RIV}$ = 4[4] for Cas[Cam], respectively.

Table 3. Summary of the analysis of deviances on reproductive traits observed in *Cornu aspersum.* We indicated total deviance reduction due to the model (TDR) and explained deviances (ED) from the final linear model after backward simplification (Crawley 2007), followed by degree of freedom (*DF*), *F* and *P* values. Two way interactions origin x diet were not significant (ns).

	TDR (%)	**Model term**	**ED (%)**	***DF***	***F***	***P***
Breeder size	50.12	Origin	100	38	38.19	< 0.0001
		Diet				ns
Clutch size	13.16	Origin	100	28	4.31	0.047
		Diet				ns
		Cov. egg mass				ns
Egg mass	22.79	Origin				ns
		Diet	100	28	8.27	0.0076
		Cov. clutch size				ns
Hatchling mass	68.12	Origin	22.01	28	11.62	0.0021
		Diet	38.72	27	20.45	0.0001
		Cov. egg mass	39.28	26	20.75	0.0001
Egg shell thickness	61.83	Origin	15.83	58	14.48	0.0003
		Diet	84.17	57	76.99	< 0.0001
Egg ash content	30.06	Origin				ns
		Diet	100	14	9.02	0.0095

The structure of the outer egg shell with calcite crystals being integrated in mucopolysaccharide layers shows differences in layer and crystal amount (Figure 5).

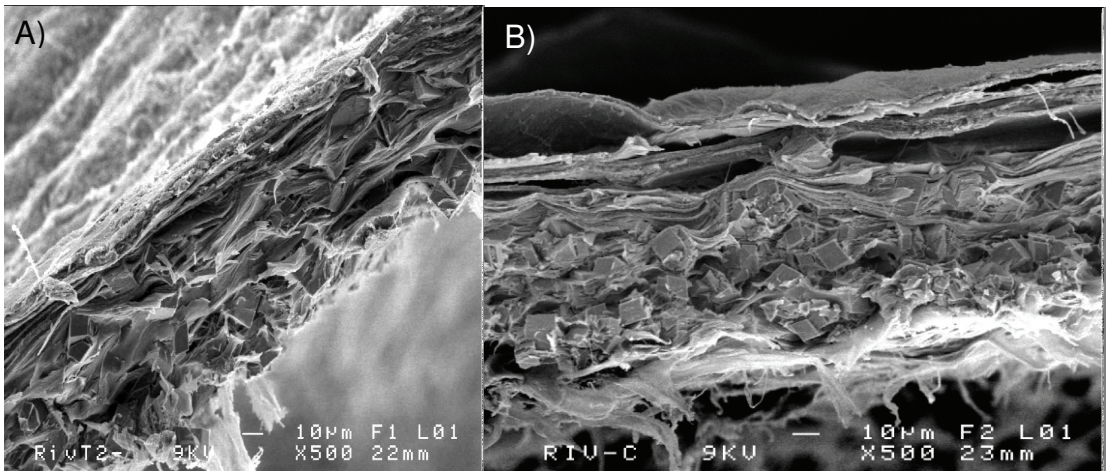

Figure 5. Scanning electron micrographs of egg shell of *C. aspersum*: layers of mucopolysaccharides with integrated calcium crystals in RIV snails. A) Cas: diet with simple Ca source, B) Cam: diet with mixed Ca source.

When eggs were exposed to a fluctuating temperature treatment 35_{4h}, they did not accelerate their development, the mean of incubation being neither affected by treatment nor by diet nor by population origin (Figure 6, Table 4). However, the coefficient of variation diminished when peaks of heat occurred for 4h per day (control, $N = 21$: CV ± SE = 6.86 ± 2.51 *% versus* 35_{4h}, $N = 21$: CV ± SE = 1.82 ± 0.80 %, Table 4). The 35_{4h} treatment of eggs decreased

significantly the mean of hatching rate (Figure 6, Table 4), whereas the coefficient of variation of hatching rate was positively influenced (control, N = 21: CV ± SE = 18.19 ± 2.96 % *versus* 35_{4h}, N = 21: CV ± SE = 41.26 ± 8.27 %, Table 4). Hatchling mass was not affected by the 35_{4h} treatment of eggs, only population origin and diet made smaller offspring hatching from Cas eggs of the RIV population (Figure 6, Table 4). The coefficient of variation of hatchling mass was low and not affected by any factor (whole dataset, N = 42: CV ± SE = 9.10 ± 0.70 %, Table 4). We detected a positive correlation between hatchling mass and incubation time under 35_{4h} treatment (Pearson, N = 21, r = 0.50, DF = 19, P < 0.011), but not in control subsamples (Pearson, N = 21, r = 0.30, DF = 19, P < 0. 1).

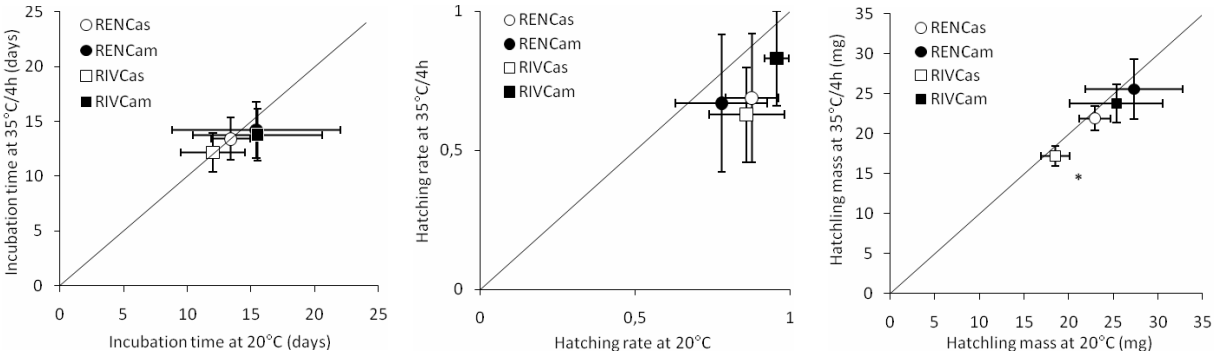

Figure 6. Incubation time, hatchling mass and hatching rate (mean ± SD) at 20°C and 35°C/4h/day in two populations of *Cornu aspersum* (REN = Rennes, RIV = Rivesaltes) as a function of diet treatment (Cas: simple source of calcium, Cam: multiple sources of calcium). The star indicates a significantly different mean value (contrast matrix-esticon P < 0.05) from the final model of LME procedures (Table 4) with incubation treatment (20°C and 35°C/4h/day on eggs of the same clutch) as random effect. N_{REN} = 5[5] and N_{RIV} = 7[4] for Cas[Cam], respectively. The line represents the absence of treatment effect (P = 1).

Table 4. Results of Linear Mixed Effect Model (LME) performed on means and variation coefficients of standard deviation for hatching traits observed in *Cornu aspersum.* The random effect was incubation treatment: control (20°C/24h/) and 35_{4h} (20°C/20h - 35°C/4h) on eggs of the same clutch. We indicated the degree of freedom (*DF*), *F* and *P* values for the final model after backward simplification (Crawley 2007). Interactions between model terms were not significant (ns).

	Model term	**Mean**			**Variation coefficient**		
		DF	***F***	***P***	***DF***	***F***	***P***
Incubation time	Origin	19	3.28	0.09			ns
	Diet			ns			ns
	Random: treatment			ns	20	4.46	0.02
Hatching rate	Origin			ns			ns
	Diet			ns			ns
	Random: treatment	20	12.47	0.002	20	7.89	0.011
Hatchling mass	Origin	18	11.45	0.004			ns
	Diet	18	19.41	0.0003	19	0.94	0.34
	Random: treatment			ns			ns

The 35_{8h} treatment of eggs led to the death of all eggs in every tested clutch (2 clutches/diet treatment/population), while the control parts of theses clutches showed a mean hatching rate of 0.88 ± 0.05, N = 8, which is equal to the mean hatching rate observed in the control part of all 35_{4h} clutches (0.86 ± 0.02, N = 25, Student t-test, t_{31} = -0.41, P =0.68).

Discussion

Reproductive strategies of *C. aspersum* followed the temperature-size-rule, which describes in evolutionary terms the negative relationship between thermal regime of population habitat and body size (Atkinson 1994; Kingsolver 2009), and larger individuals are favoured by selection because of greater fecundity and survival (Kingsolver and Huey 2008). In our study, smaller RIV snails laid smaller clutches than the bigger REN snails without maternal effects on egg mass. Therefore the investment in reproduction was maximized through larger clutch size in the western Atlantic population (optimal offspring investment theory, Smith and Fretwell 1974; Glazier 2000), which was due to climatic adaptation of body size (Irie and Fischer 2009; Omori, Sogabe et al. 2009).

In both populations, egg mass was only influenced by the diet treatment. The bigger eggs with the Cam diet had also a thicker egg shell, but surprisingly a lower mineral content. The egg shell of *C. aspersum* is composed of a mucopolysaccharide-glycoprotein jelly with individual crystals of calcite or aragonite (Tompa 1976), containing around 0.6 mg of calcium per egg (Tompa and Wilbur 1977). During egg shell formation, the mucus which is first fixed on perivitellin membrane facilitates Ca crystal nucleation (Marin, Smith et al. 1996). Afterwards, the mucus stays solubilized and acts as inhibitor of calcification to avoid overcalcification. Sulfated proteins and mucopolysaccharides are key participants in the control of shape and structure of mineralized tissues (Dauphin, Cuif et al. 2005). Carbohydrates and proteins are essentially present in the albumen that surrounds the embryo (Bayne, 1968), and lipids are furnished with egg yolk (Barre, Bride et al. 1991, Luchtel, Martin et al. 1997), the whole being protected by a perivitellin membrane (Bayne 1966).

The high egg mass with Cam diet could be explained by a higher nutrient content associated to higher water content, since the water content in eggs of *C. aspersum* was observed to vary between 75% and 85% depending on the lipid content in diet as well as in eggs (unpublished). Since the carbohydrate, protein and lipid contents did not differ between diets, egg mass would rather be linked to egg shell thickness. Egg shell calcification depends on the same organic precursors as body shell calcification (Marin, Smith et al. 1996). When calcium supply is low, body shell is thicker, but less rich in calcium crystals (Fournie and Chetail 1984). Our results are consistent with these observations, suggesting a high amount of organic layers and a deficiency of minerals in egg shell from Cam snails.

The calcification of egg shell occurs progressively during the transit of the egg in the oviduct (Tompa 1976). Mobilized calcium is transported to calcium cells in the oviduct epithelium via the haemolymph (Tompa and Wilbur 1977) for incorporation in egg shell layers (Wilbur and Tompa 1979). Calcium embedded in $CaCO_3$ spherules can be easily mobilized, whereas calcium of $CaMgP_2O_7$ granules is less available (Davis, Dove et al. 2000). In the slug *Deroceras reticulatum*, both Ca sources are used for oviposition (Fournie and Chetail 1982). The seasonal restricted Ca take up in terrestrial gastropods involves first uptake of environmental $CaCO_3$ by the sole (soil) or by intestine (nutrition), second transportation of calcium ions bound

to organic compounds, and third precipitation in vesicles of calcium cells (Fournie and Chetail 1984, Almendros and Porcel 1992). Even though total calcium content was equal in both types of diet, the threefold limestone content in Cas diet seemed to enhance the mineral content of eggs. Crowell (1973) observed egg production doubling when $CaCO_3$ was supplied to breeders of *C. aspersum* exposed to calcium-poor soil. Snails retained 20% of the ingested calcium for egg production. Consequently, it could be that the $CaMgP_2O_7$ crystals bound in the organic matrix of algae *P. calcareum* and oyster shell of the Cam diet were less available for calcium ion extraction by the snail intestine in order to fill up calcium reserves in the body.

The egg shell represents also a nutrient source for the embryo and hatchlings. During embryo development the calcareous egg shell is used to form the protoconch, and after hatching the egg shell and albumen remains are ingested by the hatchling (Tompa and Wilbur 1977). From Cas eggs of RIV population with the thinnest egg shell hatched the smallest offspring, which may prove that the egg shell mucopolysaccharides have a nutritional value besides the albumen. A $CaCO_3$ rich nutrition allows a high precipitation of calcium crystals in a still thin organic matrix of egg shell through the affinity of mucopolysaccharides for calcium. Their inhibitor function for the calcification process to avoid overcalcification (Marin, Smith et al. 1996) may depend on precipitation rate and crystal amount. Cam snails seemed not to be able to precipitate rapidly a high amount of calcium crystals in the egg shell, but continued secreting mucopolysaccharides. The well wrapped Cam eggs had therefore bigger hatchlings. This has consequences on offspring fitness since there are positive correlations between hatchling mass and offspring survival, growth and fecundity (Madec, Guiller et al. 1998; Moran and Emlet 2001), which may also have consequences on population dynamics through cohort effects (Beckerman, Benton et al. 2002).

The survival of eggs at 35°C depended on the duration of exposition. Despite the choice of a natural occurring heat peak, eggs could not withstand a prolonged exposition (35_{8h}) even not at optimal humidity. This is consistent with observations on *Deroceras reticulatum*, where the proportion of viable eggs decreased with temperature increase at optimal humidity (Willis, Bohan et al. 2008). The shorter heat treatment (35_{4h}) induced a heat stress expressed by a lower mean hatching success with greater variability within populations. The decrease in hatching rate with increasing constant incubation temperature has been observed in *C. aspersum* (Le Calvé 1995) and is consistent with the rate-temperature curve approaching the upper lethal temperature, a zone referred to as stressful (Bennett and Lenski 1997). In selected lines of *Drosophila* sp. heat stress resistance was negatively correlated to origin climate (Loeschcke, Krebs et al. 1997). In our study, eggs from warmer climate (RIV) seemed not to better resist heat periods than eggs from colder climate (REN), and neither thicker organic matrix (Cam eggs) nor higher calcification degree (Cas eggs) in egg shell seemed to improve temperature buffering of the embryo. However, hatchling mass of surviving eggs was not affected by heat periods and showed the same distribution pattern as hatchling mass at 20°C incubation temperature, with RIV hatchlings being the smallest ones.

The positive effect of increasing temperature on embryo development rate was proved in some insects, especially *Drosophila* sp., which served to establish the model describing the relation between several reproductive rates and temperature (McKenzie 1978; Briere, Pracros et al. 1999; Petavy, David et al. 2001). Shortening of embryo development with increasing incubation temperature was also observed in *Deroceras sturanyi* eggs (Kosinska 1980). Higher heterogeneity of hatching moment within the clutch at low temperatures has been described in *A.*

arbustorum for constant thermal regimes (Baur and Baur 1986), and a similar impact occurred in our study. Independently of diet or population origin, incubation time was less variable when eggs were exposed to heat periods during the day. This means that some heat resistant eggs even accelerated embryo development to the detriment of hatchling mass, as shown by the positive correlation between hatchling mass and incubation time. This suggests an allocation trade-off between development rate and embryo growth under a stressful thermal regime (Angilletta, Sears et al. 2003).

In conclusion, the Mediterranean RIV population is experiencing heat peaks around midday during the reproduction period. Even if eggs are laid in temperature buffering soil holes, they are susceptible to be exposed to such heat peaks or even more stressful events, e.g. through anthropic deterioration of habitat. Despite the climatic adaptation of breeder body size and reproductive strategy in the RIV population, we could not observe any relation between heat resistance of eggs and climate of population origin. After the shorter heat exposition, incubation time of eggs was less variable in both populations at the detriment of hatchling mass which depended on population origin and mineral availability in environment affecting egg shell thickness. In the RIV population we found the smallest hatchlings with limestone $CaCO_3$ source, considered to be the most accessible Ca source for snails (Fournie and Chetail 1984). Therefore the expansion of this species depends on the interaction of climatic factors and food availability or mineral content of soil. Studies on the colonization of this invasive species as well as about responses of populations to climate changes in the actual species' range should consider the complex interaction of abiotic and biotic environmental factors acting on reproduction and consequently on population dynamics (Calow 1989, Kingsovler, Massie et al. 2007).

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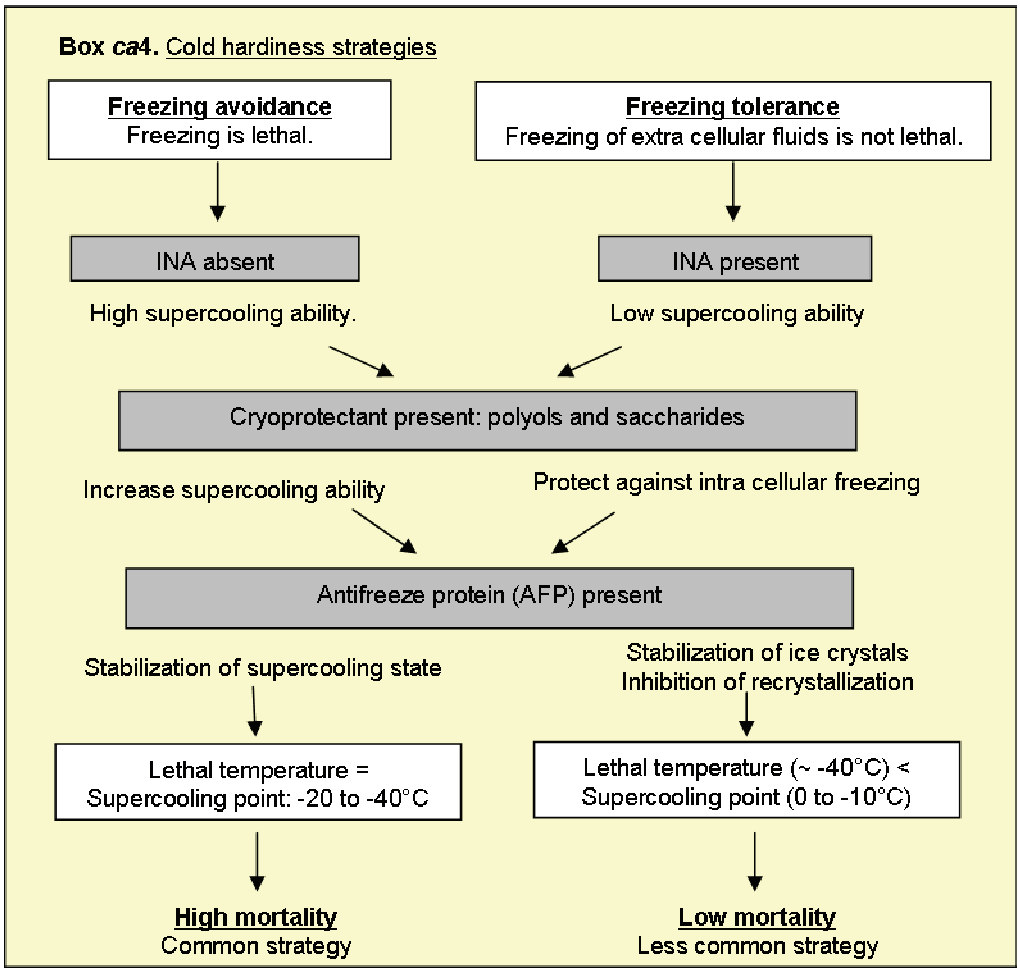
Freezing cohabitants

The accessibility of mineral nutrients in food can have a positive effect on the constitution of egg shell with consequences on hatchling mass, but not on heat resistance (Nicolai et al. *Ca*-IV). Egg survival under shortly occurring heat peaks during the day (like in summer) is reduced whatever egg shell constitution. Another issue in a snail's annual cycle is the influence of food on cold hardiness during winter. If food particles or bacteria, ingested with food or by soil scraping, remain in the snail gut during hibernation, they could lower the freezing resistance under sub-zero temperatures.

Survival at sub-zero temperatures is commonly divided into two alternative strategies, freezing avoidance and freezing tolerance, largely described by different authors (e.g. (Lee 1989; Block 1991; Vernon & Vannier 2002) and determined by differential supercooling ability (Box *ca*4 and Figure *ca*15). Supercooling ability refers to the process of lowering the temperature of body fluids below its freezing point, without becoming solid. However intermediary strategies frequently gain in importance (Sinclair 1999), as it is the case for *Cornu aspersum* termed by (Ansart *et al.* 2001a) a partially freezing tolerant species that could only withstand a very short freezing period (2 – 4 h at -10°C). Water loss during hibernation is associated to an increase in haemolymph osmolality which might enhance the cryoprotectant content and subsequently supercooling ability (Nicolai *et al.* 2005). In fact the body supercooling point (temperature of crystallization of a supercooled fluid) in *Cornu aspersum* was –4.8°C in winter and –2.7°C in spring and related to body water mass (Ansart *et al.* 2002). Body size also influences supercooling capacity. The larger the animal, the greater its water volume and the higher the probability that ice nucleators in body fluids will catalyse freezing (Lee & Costanzo 1998). Such ice-nucleating agents (INA) limit the ability to supercool by producing freezing nuclei at temperatures > –10°C (Vali 1971). INAs include food and dust particles in the gut, ice-nucleating proteins (INPs) or lipoproteins and ice-nucleating microorganisms (Lee *et al.* 1991; Lee *et al.* 1993).

Hypotheses of this study:

- INAs are ingested by snails with food or by soil scraping
- INAs are mainly of organic origin, particularly of bacterial origin



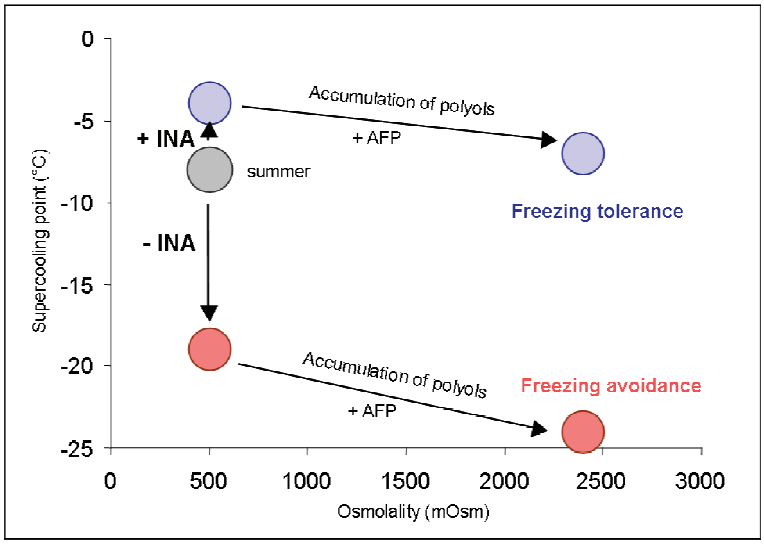


Figure *ca*15. Alternative strategies of cold hardiness expressed as supercooling ability as a function of INA (ice nucleating agents), AFP (Antifreeze proteins) and cryoprotectants (like polyols) expressed as osmolality of body fluids.

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Do ice nucleating agents limit supercooling ability of the land snail *Cornu aspersum*

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Reference: Ansart et al. *Ca*-V

Abstract: The supercooling ability of adults and eggs of the partially freezing tolerant land snail *Cornu aspersum* remains limited to high subzero temperatures (ca. -5°C) whatever the conditions, suggesting the presence of ice nucleating agents (INAs). In this study, we investigated the nucleation activity of the digestive tract of adult snails, eggs and their direct environment: food, faeces and soil. The mucous ribbon always present in the distal intestine of adults exhibited a heat-sensitive (i.e. organic) nucleation activity, close to that of the entire snails, whatever their metabolic status (active, aestivating or hibernating). However, a microbial nature of these INAs could not be established in inactive snails. The food provided to active snails contained ice nucleating bacteria, which followed the digestive tract to be found in the intestine and in the faeces, but with a decreasing concentration along the transit. Eggshells also presented a heat-sensitive nucleation activity, which could be related to its structure. Moreover, eggs are laid directly in the soil which contained both organic and mineral INAs. This study is the first to demonstrate the implication of organic INAs in the cold hardiness of a terrestrial gastropod.

Keywords: cold hardiness, ice nucleating agents, ice nucleating bacteria, gastropod

Introduction

Ectothermic animals are generally classified as “freezing avoidant” or “freezing tolerant”, the first avoiding lethal ice formation in their bodily fluids by supercooling, the second bearing body ice by controlling its formation thanks to ice nucleating agents (for more information see reviews, e.g. 19, 31, 40).

Ice nucleating agents (INAs) are any substance of inorganic nature as mineral crystals or organic nature as proteins, lipoproteins, microorganisms, leading to heterogeneous nucleation of water molecules. However, the term is usually used only for substances inducing nucleation at temperatures higher than ca. –10°C, i.e. corresponding to nuclei of biological interest (20, 24). Contrary to inorganic ice nuclei, organic ones are heat labile, inactivated by heavy metal and sensitive to high pressure (35).

INAs have been shown to occur naturally in both the hemolymph (23, 27) and the gut (18, 21) of freezing tolerant and freezing avoidant ectotherms. Hemolymph ice nucleators are generally proteins or lipoproteins which are seasonally produced, whereas gut nucleators can be food particles and ice nucleating bacteria. Known since the 1970’s, ice nucleating bacteria catalyse ice nucleation at temperatures as high as 1 to 2 degrees below zero (13). Over the past decades, many have been identified, notably from atmosphere, soil and decaying vegetation (22, 25), suggesting that they may be common in the environment.

Prior to winter, INAs are generally evacuated or inactivated from freezing avoidant species, whereas they are maintained or produced in freezing tolerant species. INAs can also be found in organisms where freezing accrues no advantage or can be lethal. In these cases, they are considered as incidental (19, 24).

In molluscs, both avoidance and tolerance to freezing are encountered, depending on the habitat (intertidal, freshwater or terrestrial), and the organism size, reflecting water mass, a critical parameter to molluscs. Relatively few studies concern terrestrial gastropods, and the mechanisms implicated are generally unknown (see articles cited in 3).

Widely distributed in Europe, the land snail *Cornu aspersum* Müller 1774 (syn. *Helix aspersa*) has been described as partially freezing tolerant, having a limited ability to supercool (ca. –5°C) together with a limited ability to survive freezing (only few hours) during winter (5). In their natural habitat, overwintering snails seek in rock crevices or dig in the soil, avoiding extreme temperatures this way. In *C. aspersum*, the slight decrease of temperature of crystallisation (Tc) observed for whole individuals during hibernation (approximately from –2 to –5°C) is probably sufficient to escape body ice formation. It has been shown to be associated with gut emptying and water content decrease, both related to metabolic depression (1). The fact that Tc remained limited even in individuals inactive for several months could suggest the presence of INAs. Previous results showed that no INA was present in the hemolymph of inactive individuals. However, providing starved *C. aspersum* with an antibiotic solution contributed to a slight but significant enhancement of their supercooling ability (4); moreover, a mucous ribbon, persisting in the distal intestine even after long fasting period, exhibited a heat-sensitive nucleating activity, close to that of whole individuals (1). This mucous ribbon has been described as harbouring a permanent bacterial population, which would mainly be of exogenous origin (8).

Previous results also showed that eggs of *C. aspersum*, laid in spring and autumn, were freezing avoidant and had a limited ability to supercool, ca. –5.5°C (2). This supercooling ability

is poor, compared to that of arthropods' eggs, generally lower than –20°C (6, 30, 36). Eggs of *C. aspersum* are relatively heavy (40-60 mg) and their surface is moist, which can explain a great part of that difference, however, the Tc revealed to be constant whatever the mass, age or incubation temperature of the eggs, which suggests the possible existence of INAs (2). These INAs could be inorganic crystals at the egg surface as shown in turtle's eggshell fragments by Costanzo et al. (10). They could also be of an organic nature, as eggs are laid in the soil, where there is a high probability that they will be contaminated by ice nucleating bacteria. Moreover, most parents lay a faecal ribbon in contact with the clutch nest (17), which could also be a source of microbial contamination.

The aim of the present study was to look for the presence of INAs in eggs and in the gut of adult *C. aspersum*, depending on their metabolic status (active, aestivating, hibernating), as well as in their direct environment: soil, food and faeces. We aimed to determine the nature of INAs (organic/inorganic) by autoclaving and bacterial culture.

Materials and Methods

All dishes used for sample preparation were sterilised by autoclaving (120°C, 20min) or alcohol washing. Water used in the various assays was distilled water, previously sterilised by autoclaving.

ANIMALS AND REARING CONDITIONS

All adult individuals used for this study were born and raised in a farm ("L'Escargotier", Corps-Nuds, France), where their growth was achieved in outside conditions.

They were separated into three rearing conditions: activity (n=150), aestivation (n=100), hibernation (n=100).

Active and aestivating individuals were placed by groups of 15 animals in plastic cages maintained in constant conditions of temperature (20 ± 1°C), humidity (RH 80 ± 5%) and photoperiod (LD: 16-8). For active animals only, the cage bottom was covered with a layer of moist synthetic foam, food (alimentary powder, Etablissements Berton, Le Boupère, France) and water being provided once a week. Aestivating individuals were starving for at least 4 months before beginning of assays. Hibernating individuals were kept in a room maintained at 5°C, RH 70% and with total darkness for at least 4 months before beginning of assays.

EGGS AND FAECES

Two soil jars were placed in cages containing active individuals, after observing mating activity. Only one clutch from each cage was randomly collected, assuring a different parental origin for each clutch.

To collect faeces, 15 active individuals whose shell was thoroughly washed with alcohol were placed in individual sterilised cages for 24 hours. Faeces were then placed in sterile microtubes and immediately frozen (-20°C) before use.

MEASUREMENT OF TC

Whole individuals and eggs. For each rearing condition, Tc of 20 whole individuals was measured. Active snails were starved two days before assays to standardize gut content.

Before measurements, the shell diameter was sized with a calliper (±0.01mm) and shell was washed with alcohol to eliminate potential external ice nucleating agents. A thermocouple (HANNA instrument) was attached to snail shell in the cardiac region and animals were individually inserted into plastic tubes immerged in a cryostat (Huber Polystat CC3) filled with an antifreeze fluid maintained at 5°C. After half an hour, the temperature of the bath was cooled at the rate of 0.5°C/min. The exotherm recorded by the thermocouple indicated spontaneous freezing of the animal, the Tc being the lowest temperature at the start of the exotherm.

Tc of 30 eggs from 3 different clutches (10 eggs per clutch) was measured. The thermic captor was placed in contact with the egg in a microtube plunged in the antifreeze solution and Tc was measured as described for whole snails. Prior to assays, eggs were washed three times in sterile distilled water.

Mucous ribbon. For 20 animals from each rearing condition, the mucous ribbon, always present in the distal intestine, was collected after rapid dissection. Each mucous ribbon was directly placed in a 500µl-microtube containing 100 µl of sterile distilled water. Samples were frozen (-20°C) before use. Tc was measured for the whole sample (water and mucous ribbon): a thermic captor was taped to the exterior of the 500 µl-microtube, itself inserted into a 1500 µl-microtube placed in the cryostat. Tc was determined for 10 untreated and 10 autoclaved samples. For comparison, Tc of 100 µl of sterile distilled water (n=10) was similarly evaluated.

Bulk samples. Following the method described by Costanzo et al. (11, 12), the relative ice nucleating activity of different solid materials (soil, food, faeces, eggshell) was determined as the temperature necessary to provoke ice formation of a constant volume of sterile distilled water mixed to a constant volume of dehydrated substratum, compared to the Tc of equivalent quantities of water.

To obtain eggshells, 800 eggs from 10 different clutches (80 eggs per clutch) were emptied of their content, shells being washed several times with sterile distilled water.

All materials were dehydrated for 48 hours at 40°C and crushed into powder. Then, 100 mm^3 of substrate were placed in a 500µl-microtube, 25 µl of sterile distilled water were added and samples were briefly vortexed to homogenize water and material.

Tc of ten untreated and ten autoclaved samples of each material as well as Tc of ten 25 µl-samples of sterile H_2O_d were measured.

ICE NUCLEATING ACTIVE BACTERIA

All assays were performed under secure microbiological conditions. For each sample type (eggs, mucous ribbons, food, faeces, soil), 10 sterile nutrient agar plates were cultured aerobically. On each plate, five plots containing the powdered material (soil, food or faeces) or five eggs were equally spaced. For mucous ribbons, a whole ribbon was spread on the agar surface (10 plates per rearing condition). Plates were sealed with Parafilm and incubated at 20°C for 5 days.

On each incubated plate, microbial growth was observed. Representative bacteria from all the colonies on a plate were removed with a sterile loop and mixed with sterile distilled water. If necessary, the microbial solution was diluted to obtain a comparable concentration of 10^8 bacteria/ml of sterile water, corresponding to a DO of 0.5 at 660 nm (spectrophotometer Versamax, Molecular Devices). For each microbial solution, ten 5 µl-droplets were deposited on an aluminium pan floating on the antifreeze solution surface; a thermocouple was taped on a supplementary pan allowing to follow precisely the evolution of temperature at the pan surface.

The freezing of droplets cooled at 0.5°C/min was noted visually: when freezing, they turned from transparent to white opaque. Cumulative ice nuclei spectra were then determined as recommended by Vali (34), providing for each temperature the abundance of nucleating material dispersed in the fluid.

Data from a non-ice nucleating bacteria (*Escherishia coli*) and from an ice nucleating bacteria (*Pseudomonas syringae*) were included for comparison, as well as Tc of 5 µl-droplets of sterile distilled water (i.e. lacking INA, n=100).

STATISTICAL ANALYSES

Prior to the analyses of data, normality and homoscedasticity of the error terms of the model were checked. Box-Cox transformation was applied to the data for which observed deviations from group means lacked normality (Tc of mucous ribbon). One-way ANOVAs followed by multiple comparisons (Tukey test) were applied to test for the effect of clutch on Tc of eggs, the effect of rearing conditions on Tc of whole individuals, size being introduced into the model as a covariate (ANCOVA). Two-way ANOVA was used to test the effect of condition, treatment and their interaction on Tc of the mucous ribbon. t-test were performed for comparison of data with H_2O_d controls and for comparison of untreated and autoclaved bulk samples. Nested ANOVA (droplet<individual<condition) was performed to test variation of the freezing of bacterial solutions obtained from mucous ribbons. Results are presented as mean and standard deviation. Analyses were performed with Minitab 15.

Results

WHOLE INDIVIDUAL AND EGG TC

Individual Tc was significantly different between the three rearing conditions (ANCOVA; $F_{(2, 59)}$=16.8; p<0.0001). Active animals presented a very weak ability to supercool (-2.6 ± 2.3°C, n=20), slightly enhanced in aestivation (-4.1 ± 1.4°C, n=20) and maximal during hibernation (-6.0 ± 3.0°C, n=20). Inter-individual variability was high, particularly for active (-0.3° to –7.8°C) and hibernating (-2.1° to –10.2°C) snails.

Mean Tc of eggs (-4.8 ± 0.6°C, n=30) was relatively high, not different between clutches (ANOVA; $F_{(2, 29)}$=1.48; p=0.25), and inter-individual variability was weak.

MUCOUS RIBBON

The mean Tc of untreated mucous ribbon differed significantly between active animals (–3.9 ± 1.1°C) and hibernating ones (–5.7 ± 1.7°C), and whatever the modality, autoclaving decreased significantly the Tc of mucous ribbons (ca. –10°C; Two-way ANOVA, interaction 'conditionxtreatment', $F_{(2,59)}$=4.26, p=0.02; Tukey test, p<0.05; fig. 1).

The mean Tc of 100 µl of H_2O_d was –15.9 ± 2.2°C, differing from that of samples containing mucous ribbon, whatever the condition and treatment (t-test, p<0.0001).

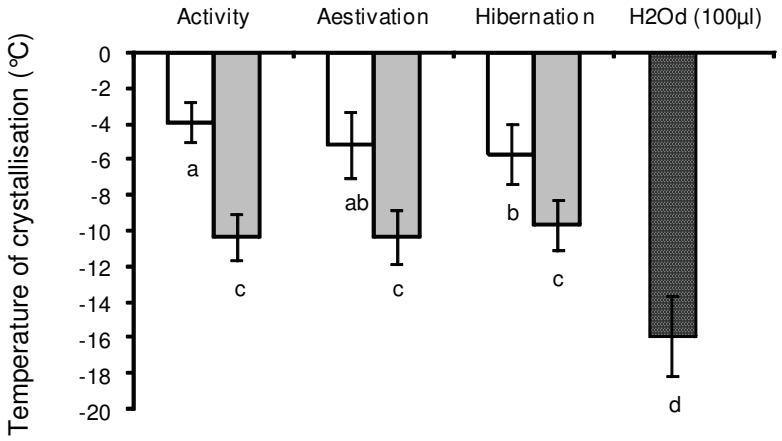

Figure 1. Mean (± SD) temperature of crystallisation (°C) of mucous ribbons collected from adult *Cornu aspersum* reared under different conditions (n=10). White bars: untreated mucous ribbons; grey bars: autoclaved mucous ribbons, black bar: control, 100µl of sterile distilled water. Dissimilar letters under bars indicate a significant difference between data (p<0.05).

BULK SAMPLES

The mean Tc ranged from –2.7 ± 0.7°C (soil) to –6. 7 ± 1.6°C (eggshell) for untreated bulk samples, and from –6.0 ± 1.5°C (soil) to –12.6 ± 1.4°C (food) for autoclaved samples (fig. 2). Differences were significant between treatments whatever the substrate (t-test, p<0.0001). The Tc of 25µl of H_2O_d was –16.7 ± 1.6°C, significantly lower than that of water mixed with any substrate (t-test, p<0.0001).

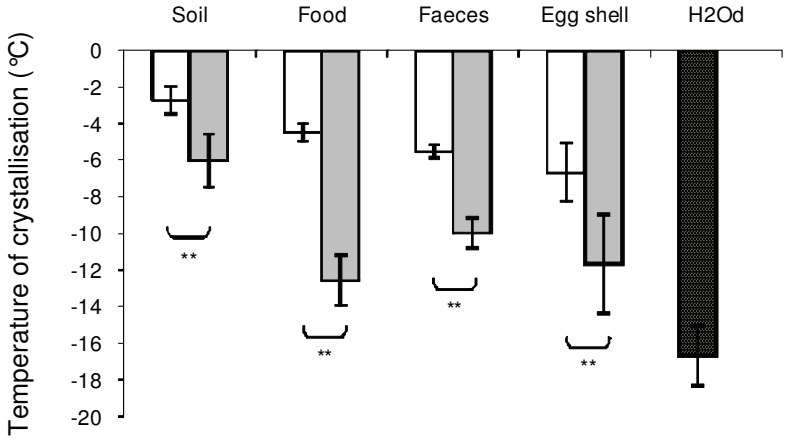

Figure 2. Mean (± SD) temperature of crystallisation (°C) of bulk samples of food, soil, faeces and eggshell of *Cornu aspersum* (n=10). White bars: untreated bulk samples; grey bars: autoclaved bulk samples, black bar: control, 25µl of sterile distilled water. ***: significant differences between data, t-test, p<0.0001.

BACTERIAL SOLUTIONS

For each sample type and control, the temperatures at which the first droplet froze and at which 50% and 90% of the droplets have frozen are given in Table 1.

Table 1. Ice nucleating activity of 5 µl-droplets (n=100) of bacterial suspensions. T_{max}, T_{50} and T_{90} indicate respectively the temperatures at which the first droplet froze, 50% or 90% of droplets have frozen.

	Temperature (°C)			
	Mean ± SD	**T_{max}**	**T_{50}**	**T_{90}**
Control				
H_2O_d	-17.8 ± 0.7	-14.7	-17.4	-20.6
E. coli	-19.7 ± 1.0	-17.3	-19.5	-21.5
P. syringae	-3.5 ± 0.2	-3.0	-3.5	-3.7
Mucous ribbon				
Activity	-14.1 ± 3.8	-5.5	-15.3	-19.3
Aestivation	-15.1 ± 1.6	-10.8	-15.0	-17.0
Hibernation	-16.7 ± 2.0	-11.7	-16.6	-19.5
Substrate				
Soil	-16.5 ± 1.7	-11.2	-17.0	-18.7
Food	-11.3 ± 4.2	-5.3	-11.5	-17.6
Faeces	-15.5 ± 1.6	-6.9	-16.1	-18.3
Eggshell	-16.5 ± 0.9	-11.5	-16.6	-18.6

For bacterial solutions obtained from mucous ribbons, significant differences were found between rearing conditions, with a high inter-individual variation within each modality (Nested ANOVA; condition: $F_{(2,299)}$=75.68, p<0.0001; individual(condition): $F_{(29,299)}$=23.42, p<0.0001; Fig. 3). Whereas bacterial solution droplets coming from hibernating and aestivating snails froze below –10°, 18% of the droplets from active snails froze above –10°C. However, this result was obtained from only two of the ten mucous ribbons cultivated.

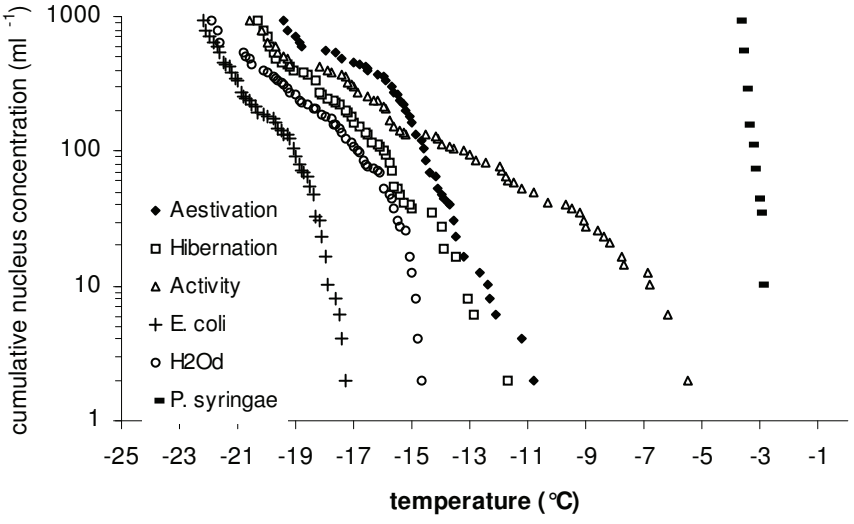

Figure 3. Cumulative ice nucleation spectra of suspensions of the mucous ribbon bacteria from *Cornu aspersum* reared under different conditions. Positive (*P. syringae*) and negative (*E. coli*, H_2O_d) controls are also represented.

Droplets of bacterial solutions obtained from eggshell and soil froze below –11°C (fig. 4, table 1). All droplets from faeces cultivation, except four from two different plates, froze below –10°C. In contrast, 45% of droplets scattered between seven of the ten plates with food were frozen above –10°C.

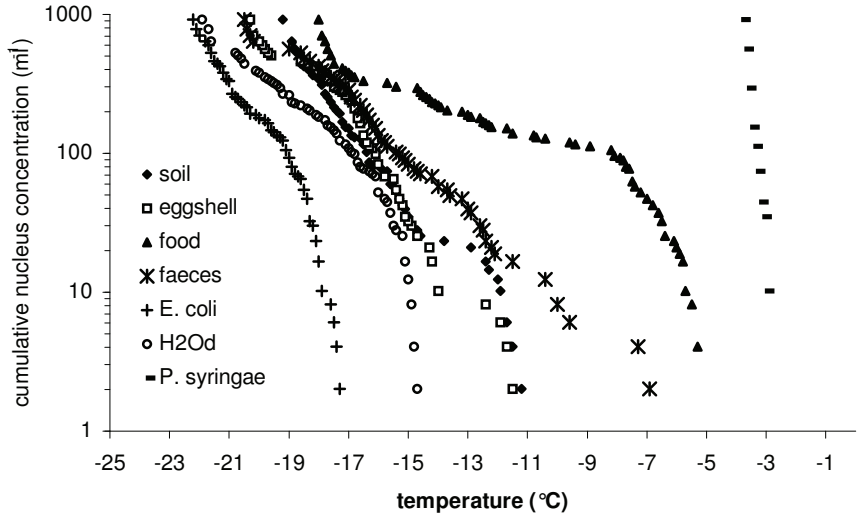

Figure 4. Cumulative ice nucleation spectra of bacterial suspensions obtained from different substrates. Positive (*P. syringae*) and negative (*E. coli*, H_2O_d) controls are also represented.

Discussion

INAS IN THE DIGESTIVE TRACT, FOOD AND FAECES

Lee et al. (21) and Kaneko et al. (15) were the first to prove the existence of ice nucleating bacteria as normal flora in insect gut (respectively, in the beetles *Ceratoma trifurcata* and *Hippodamia convergens*, and in the moth *Plutella xylostell*a) and to demonstrate their capacity to depress the Tc of overwintering animals. To enhance supercooling, either these bacteria must be evacuated from the gut prior to overwintering, or the expression of this phenotype in the gut flora must be diminished or eliminated (19). Presence of ice nucleating microbes was also demonstrated in the gut of freezing tolerant species (18, 33). They could then perform the same function as hemolymph ice nucleating proteins, by limiting ice formation to extracellular compartments, suggesting a mutualistic association between the microbe and its host (20). However, to determine if an ice nucleator is adaptive or incidental can be difficult, as the ice nucleating trait may be incidental to the primary role of these microorganisms (19, 24). For example, in the land snail *Helix pomatia*, Nicolai et al. (28), described the strain *Kluyvera* sp as an ice nucleator of weak efficiency, but triggering nucleation at temperatures well below the Tc of active or hibernating snails.

The existence of organic INAs was previously demonstrated in the mucous ribbon of starved *C. aspersum* (1). In the present work, whatever the metabolic status (activity, aestivation, hibernation), mucous ribbons exhibited a heat-sensitive ice nucleation activity (i.e. of an organic nature). In dormant snails, this Tc is close to that of the entire animal, which confirms that the digestive tract is the first site of nucleation in *C. aspersum*, as shown in the Heteroptera

Pyrrhocoris apterus (14) and in the Hymenoptera *Trichiocampus populi* (29). In active snails, the Tc of entire animal is higher than that of the mucous ribbon, which can be explained by a full digestive tract and a higher water mass (1).

C. aspersum is known to house a permanent bacterial population, essentially constituted of aerobes and facultative anaerobes, dominated by Aeromonacae and Enterobacteriacae. Although starvation and hibernation triggered a fall in bacterial counts, a great diversity of bacteria survived in the snail gut during these periods (9, 16, 38). In particular, growth of the flora is observed in the mucous ribbon of hibernating *C. aspersum*, as it could be a nutritive medium for bacteria (8). However, mixed bacterial solutions obtained from mucous ribbons culture did not allow to detect the presence of ice nucleating bacteria in hibernating and aestivating snails. In active animals, only two of the ten plates gave evidence for ice-nucleating bacteria in the digestive tract.

In their description of the aerobic microflora of *C. aspersum*, Kiebre-Toe et al. (16) showed that, even if permanent, the composition of this microflora evolved over time and especially, they pointed to *Pseudomonas* species, which presence was sporadic, particularly during hibernation. This irregular presence of INAs in the digestive tract of active individuals could partially explain the high variation observed between samples. However, if our results lack evidence of ice nucleating bacteria in the gut of inactive animals, the conclusion is not definitive. Some authors pointed to the difficulty to attest nucleating activity from cultures, given the propensity for ice nucleating microbes to lose their activity under culture (12, 39). Thus, a shift in temperature, the culture age, culture media and diets are known to affect the ice nucleation activity of the bacteria (26, 39).

Moreover, our study concerned only bacteria growing in aerobic conditions. Only one strictly anaerobe *Clostridium* sp. has been identified in *C. aspersum* by Charrier et al. (9). However, as emphasized by these authors, a fraction of the microbial community escaped cultivation. We then cannot exclude the possibility that an ice nucleating bacteria present in the mucous ribbon did not grow or did not exhibit nucleation activity under the culture conditions, as well as we cannot exclude the possibility that these organic INAs might be of a non-microbial nature, e.g proteins, lipoproteins associated with mucus or remaining food particles.

Active snails were fed with an alimentary powder constituted of grain flour complemented with vitamins and calcium. This material revealed a high, heat-sensitive, nucleating activity (Tc: -4.5°C) and the presence of ice nucleating bacteria. Faeces collected in active individuals also possessed heat-sensitive, organic INAs (Tc: -5.5°C), and a weak concentration of ice nucleating bacteria.

Bacteria from *C. aspersum* gut have been shown to be mainly of exogenous origin, picked up from the environment during feeding (38), then following the progression of food mass in the digestive tract, found in the intestine (mucous ribbon), where they are progressively digested, or voided with the faeces (8). Several works have proved that the ingestion of either living or killed ice nucleating bacteria causes an immediate elevation of the Tc in adults and larvae of insects (e.g. 7, 32). In the clover leaf weevil *Hypera punctata*, Watanabe (37) found a positive correlation between the number of live *P. syringae* present in the gut after ingestion and the Tc of larvae. Most of these ingested ice nucleating bacteria were excreted with the faeces within a few days, resulting in the decrease of the larval Tc. In *C. aspersum*, a full-filled gut has been shown to be related to a reduced supercooling ability (1), which can be explained by the ingestion of food containing ice nucleating bacteria. The loss of nucleation activity during the

transit in digestive tract is expressed by the sporadic presence of ice nucleating bacteria in mucous ribbon and faeces.

INAS IN THE EGGS AND SOIL

Generally, eggs of invertebrates exhibit a strong ability to supercool, between –25° and –50°C, as a consequence of their small size, spherical form and elevated concentrations of sugars. This phenomenon has been demonstrated for insects overwintering as eggs (30), as well as for insects which eggs emerge before winter (6, 36). *C. aspersum* reproduces from spring to autumn, depending on regions, and eggs hatch before winter, being very sensitive to low temperatures, with a low supercooling ability close to that of adults. Our results on eggshells revealed the presence of moderately active organic INAS (Tc<-5°C), i.e. slightly lower that the Tc of entire eggs. In the turtle *Chrysemys picta*, eggshells also proved to be weakly active, with a nucleation temperature of –8.4°C, but this activity was not reduced by autoclaving (10). No ice nucleating bacteria has been detected in the bacterial solutions tested from egg culture in *C. aspersum*. Eggshells are constituted of insoluble calcium carbonate crystals associated with a mucopolysaccharid matrix, surrounded by a mucus layer. The structure of the shell in itself could be the source of nucleation.

Costanzo et al. (11, 12) identified two classes of nucleating agents present in the nest soil of hatchling turtles: one class was represented by ice nuclei that were active in bulk samples of autoclaved soil (mineral particles); the other was represented by extremely small nuclei that were water extractable and whose activity was largely destroyed by autoclaving (organic compounds, possibly microorganisms). In our study, bulk samples of soil (commercial compost) also revealed the presence of heat sensitive INAs provoking nucleation at high subzero temperatures (-2.7°C) as well as the presence of autoclave-resistant nuclei, triggering freezing at –6.0°C. Indeed, organic and inorganic ice nucleating agents were present in the soil used for experiments. However, droplets assay on bacterial solution did not prove the microbial nature of these organic INAs. Assay for nucleating activity of nest soil culture by Costanzo et al. (12) also gave largely negative results, even if numerous organic INAs were present.

CONCLUSION

This study proved that organic INAs are present eggshells, but their nature needs to be defined. The structure of the eggshell could be directly connected to the egg supercooling ability. Moreover, eggs are laid in the soil and a mucous faecal ribbon is generally deposited over the clutch nest, both being source of nucleating agents. We can then hypothesize that eggs are largely subjected to surrounding sources of anticipated nucleation in their environment. However, it is worth considering that our study focused on isolated eggs, whereas clutches are formed of a compact mass of eggs surrounded by a thick layer of mucous, which may considerably change their interaction with direct environment. Moreover, study of species overwintering as eggs would be of great interest in a comparative point of view.

In active adult snails, ingestion of food rich in ice nucleating bacteria leads to the presence of these bacteria in the intestine (mucous ribbon) and, in lesser quantity, in the faeces. In nature, active *Cornu aspersum* feeds with surrounding vegetation, digs into the soil and eats soil and faeces, all sources of various INAs. The present results emphasize the importance of gut emptying in evacuation of potent INAs prior to hibernation. We also demonstrated that the digestive tract was the first site of nucleation whatever the metabolic status of snails, due to the

presence of organic INAs, probably limiting the whole animal Tc. Even if we failed to demonstrate a microbial nature of these INAs, we can not exclude the possibility that such bacteria did not grow or did not express their nucleating phenotype under the experimental conditions.

This study is the first demonstrating the implication of organic INAs in the cold hardiness of a terrestrial gastropod. However, as emphasized by Lee et al. (19) and Lundheim (24), the presence of INAs, whatever their nature, in this partially freezing tolerant species during hibernation, is not clear. We can hypothesize (i) that in *Cornu aspersum*, whose distribution is limited to temperate region and whose overwintering strategy is dependent on behavioural avoidance of extreme temperature, such INAs have not been counter-selected and/or (ii) that their ice nucleation trait is incidental to their primary role and that their presence might be a limitation to the geographical expansion of this species.

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The use of acquired energy might greatly influence an animal's population dynamic and its contribution to community structure and ecosystem functioning. The understanding of resource related life history strategies and underlying mechanism for responses to environmental variation is essential to analyze and predict a species distribution and colonizing capacity, especially in invasive species like *Cornu aspersum*.

Net energy that results from resource acquisition after the transit through the digestive tract could be allocated to work, maintenance, and to production, which comprises growth, storage and reproduction (Karasov & Martinez del Rio 2007). In this study on *Cornu aspersum* the influence of energy or mineral content in food on these aspects of production has been studied (Box *ca*5).

Energy rich diet accelerated growth and enhanced body storage. The acquired body stores were partially allocated to reproduction, especially when income body stores after growth termination were not sufficient for reproductive allocation. High energy availability, in terms of lipids in food, led to high allocation of lipid storage compounds to larger eggs, except for cholesterol which can be synthesized *de novo*. The contrary was observed for mineral allocation to eggs, where a thicker egg shell made of fewer minerals enhanced egg weight. However there is a trade-off in quantitative reproductive traits, this is between egg weight and egg number per clutch, generally reflecting the balance of gains and costs of reproduction in life-history theory. Producing large eggs represents a greater investment of energy for the mother (Baur 1994; Krug 2001) and is usually considered more beneficial for hatchlings, because egg weight is positively related to hatchling weight and consequently to survival, growth and fecundity (Madec *et al.* 1998; Moran & Emlet 2001).

Under low energy availability, snails invested more in reproduction by maximizing clutch size, emphasizing the optimal offspring investment theory (Smith & Fretwell 1974; Glazier 2000). Higher fecundity might compensate for lower offspring survival or be beneficial for newly hatched snails that feed on unborn eggs thereby rapidly gaining weight and improving survival (Desbuquois 1997). This has been observed in individuals reproducing after maturity adopting a "many-small-eggs strategy" *versus* individuals reproducing after hibernation adopting a "few-large-eggs strategy". Offspring weight at nest leaving was higher in bigger clutches than in smaller clutches according to a high degree in egg cannibalism in the selection arena theory (Stearns 1987). Consistently, the absence of any consequences of high egg weight (rich energy diet treatment) on hatchling size in both breeding seasons might be due to egg cannibalism within each breeding period.

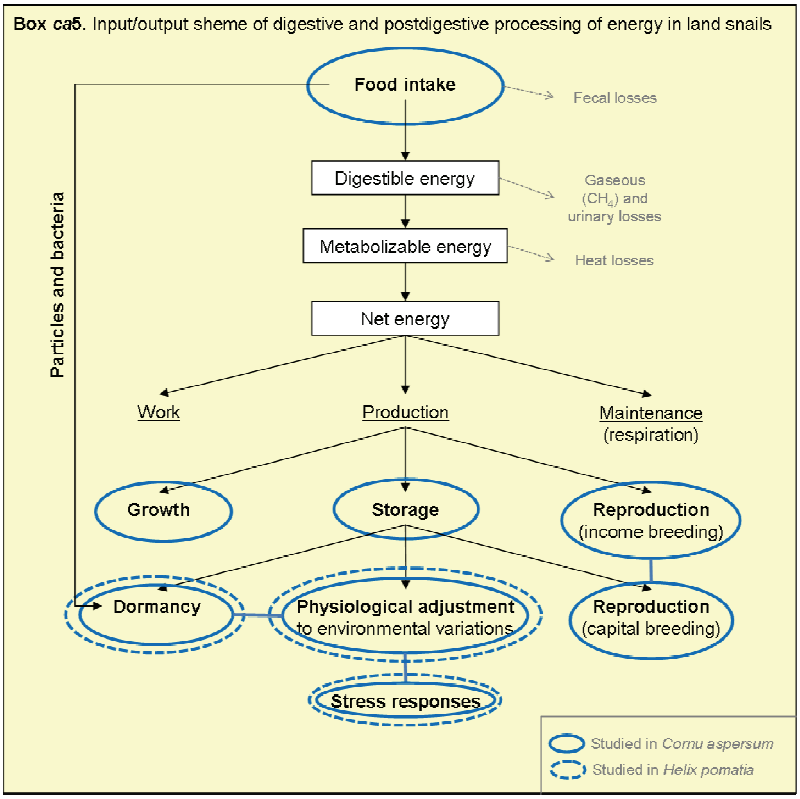
Storage compounds could also be allocated to physiological adjustments in variable environments or even used in stress protection or stress responses (Angilletta *et al.* 2003). The higher mineral allocation to eggs following the ingestion of food with a highly accessible mineral source did not influence thermal adjustment of development rate and hatching rate of

eggs. The lower incubation time variation induced by daily occurring heat peaks was only compensated by lower hatching rate suggesting an allocation trade-off between development rate and embryo growth under a stressful thermal regime (Angilletta *et al.* 2003). This means that neither highly mineralized thin egg shells nor thick slightly mineralized egg shells were able to attenuate the effect of heat under constant humidity.

Physiological adjustments also occur in dormancy and energy stores are depleted for maintenance during this period (Rees & Hand 1993; Storey 1997). Besides this aspect of energy resource, food can also have a particular effect, especially on cold hardiness in hibernation, through the presence of particles and bacteria with ice nucleating acticity in gut, like observed in insects (Shimada 1989; Lee *et al.* 1993; Hodkova & Hodek 1997). Mucous ribbons exhibited a heat-sensitive ice nucleation activity (i.e. of an organic nature) close to that of the entire animal, which confirms that the digestive tract is the first site of nucleation. However, the bacterial origin could not be confirmed, because (i) the study was restricted to aerobe culture method, and (ii) bacteria have the propensity to change their activity in respect to culture condition (Worland & Block 1999; Costanzo *et al.* 2000). On the other hand, food and faeces harboured ice nucleating active bacteria which shows that these bacteria might be ingested with food.

These studies show that life history traits are influenced by resource acquisition. Resource availability can fluctuate in space and time and animals have to adapt their life history strategies in order maximise fitness. Actually, responses of land snails to seasonal climatic fluctuations are still poorly understood, although these species have a wide range in temperate regions. Therefore, some more aspects concerning dormancy and the adjustment of physiological processes have been studied in *Helix pomatia* in the following part of this work (Box *ca*5).

Box *ca*5. Input/output sheme of digestive and postdigestive processing of energy in land snails



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TEIL 2

Thermophysiologische Prozesse und der Einfluss der Ernährung auf Wachstum und Fortpflanzung von *Helix pomatia*

Ein Beitrag zur Begleitforschung der Schneckenzucht in Deutschland

Weinbergschneckenzucht auf der Schwäbischen Alb – ein Beitrag zur Regionalentwicklung

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Short translation: The land snail *Helix pomatia* is of high economic value as it is a delicacy and used in the professional gastronomy. A specific area for high quality land snails is in the Swabian Alps (South West Germany) (Lenz 2005). Traditionally the land snails were collected from natural sites after the egg deposition around the 25th of June. Then they were fed up in artificial "snail gardens" and sold after their formation of the calcified epiphragms in late autumn. Specific herbs growing on the heathland and specific climatic conditions lead to inimitable specific nut aromatic taste and to the reputation of being the tastiest snails. This is displayed by the image "Ulm or Swabian oyster".

50 – 500 barrels per year, consisting of up to 15 million individuals, were traded between the Swabian Alps, Vienna, Budapest and Paris in the 18th, 19th and early 20th century. In 1970 4000 t land snails were exported from Germany. The export ceased at the end of the 1970ies. The annual consumption of snails in Germany could only be assured by imports from Poland, Hungary, Yugoslavia and Romania in the years 1980 to 1995 and later from France, Thailand and Belgium. In 2001 the consumption of snails in Germany was 2500 t and consisted of around 98% of wild collected *Helix ssp.*

Human activities like intensive agriculture led to a decrease of habitats of the land snails, and thus also to a strong decrease of the land snail populations in Europe. It was potentiated by the uncontrolled collection of the land snails. As a consequence the species is protected in Europe since 2002 by the appendix III of the Bern convention and standing to benefit from a general collection interdiction in Germany since February, the 18th 2005 (§42 Abs. 1 Nr.1 BNatSchG).

Breeding facilities for land snails were established to respond to the high demand of snails for the gastronomy sector in Germany. As no experiences in the breeding of snails exist in Germany, the facilities are following the intensive rearing model from Italy using breeder snails imported from Italy. A new breeding concept for land snails was developed in the Swabian Alps focusing on peasant structures and native snails. Within the low input production the land snails are living at their natural habitats and substrates and are feed up at least one year with the local specific herbs to ensure the traditional high quality product from the Swabian Alps. It is brought to the market as a regional delicacy with the label Albschneck® (www.Albschneck.de), which guarantees the control of the referred guidelines. It is furthermore included in the Ark of Taste, an international catalogue of heritage foods in danger of extinction, in the international network Slow Food®.

The guidelines of the low input farming on land snails in the Swabian Alps are matching the principles of organic farming. Thus the new breeding concept has high potential for nature conservation and sustainable regional development with specific high quality products thereby creating economic carriers in the rural area.

Furthermore it contributes highly to the consumer protection. Pesticides, fertilizers and heavy metals are ingested by the snails and accumulate in their tissues (Dallinger *et al.* 2001). The degrees of contaminations of the snails, collected from natural sites, are unknown or need to be checked by cost intensive tests due to different high degrees of environmental pollutions. The Albschneck® farming can guarantee a product free of harmful substances for the consumer as the farming site is checked and a contamination is avoided afterwards.

The operating efficiency of the land snail farming is the requirement for the capability of competing at the open market. The high quality product Albschneck® based on the low input farming requires therefore a good knowledge in land snail breeding and the annual biological cycle. Long-term experiences in snail breeding are absent in Germany and the Albschneck® farming is a new breeding concept. Thus, investigations on the snail breeding are essential to get the expertises for the optimisation of the low input snail farming.

Aims of this study: (1) Testing the impact of energy rich and energy poor food on the fitness of *Helix pomatia* in snail farm conditions, (2) Analysing the dynamic of the intestinal bacteria community structure and their influence on hibernation process, (3) Investigating cryoprotectants and heatprotectants in relation to environmental factors and mortality during hibernation and aestivation

Im 18., 19. und frühen 20. Jh. wurde in Bereichen der Mittleren Schwäbischen Alb intensiver Weinbergschneckenhandel mit Wien, Budapest und Paris betrieben. Ca. 50 - 500 Fässer/Jahr, was ungefähr bis zu 15 Millionen Schnecken ergibt, wurden verkauft. Die Schnecken wurden erst nach der Eiablage ab dem 25. Juli in großen Mengen gesammelt und bis zum Herbst in Schneckengärten gemästet, um anschließend die überwinternden Deckelschnecken zu verkaufen. Die Deckelschnecken weisen einen niedrigen Kalkgehalt im Fleisch auf, haben einen nussig-aromatischen Geschmack durch die speziellen Wildpflanzen und das Klima der Alb sowie den „Winterspeck“. Eine Art Hochburg der Schneckenproduktion ist dabei eindeutig die Mittlere Schwäbische Alb (Lenz 2005). Diese Schnecken, meist über Ulm entlang der Donau gehandelt - galten unter dem Namen „Ulmer oder Schwäbische Austern“ als die schmackhaftesten aller Schnecken, was sich bis zum heutigen Zeitpunkt bewährt hat.

1970 exportierte Deutschland noch 4000 t Weinbergschnecken im Jahr. Gegen Ende der 70er Jahre des 20. Jh. wurden die letzten Mastanlagen geschlossen und der Export eingestellt. In den Jahren 1980 bis 1995 konnte der Bedarf an Schnecken in Deutschland nur noch durch Importe gedeckt werden. Die wichtigsten Lieferanten waren Polen, Ungarn, Jugoslawien und Rumänien. Laut den letzten offiziellen Daten aus dem Jahre 1992 wurden 4617t Schnecken eingeführt. Nach 1995 wurde der Bedarf an Schnecken in Deutschland durch gefrorene Ware aus Frankreich, Thailand und Belgien gedeckt. Der Konsum in Deutschland im Jahre 2001 wird auf ca. 2500 t geschätzt. 98% der konsumierten Schnecken, ausschließlich der Gattung *Helix* spp., wurden gesammelt.

Naturstörungen durch den Menschen (intensive Landwirtschaft, Rückgang von Feldgehölzen, Hecken, natürlichen Waldrändern etc.) wirkten sich bereits negativ auf die Biomasse der Art *Helix pomatia* aus. Aufgrund des unkontrollierten Sammelns sind die Populationen noch stärker dezimiert worden. Seit dem 18.02.2005 gilt in Deutschland ein generelles Sammelverbot.

Bereits Anfang des 21. Jh. wurden einige Schneckenzuchtanlagen nach dem intensiv bewirtschafteten italienischen Modell in Deutschland gegründet. Diese Anlagen werden mit Zuchttieren aus Italien besetzt. Auf der Schwäbischen Alb wurde dagegen ein völlig neues Schneckenhaltungssystem für Kleinbauern entwickelt. Es ist ein ökologisches, extensives ganzjähriges Verfahren, in dem die Schnecken von der Schwäbischen Alb auf ihrem natürlichen Substrat mit begrenzter Zufütterung gehalten werden, um die sich im Überwinterungsprozess befindende Deckelschnecke als regionale Spezialität (Albschneck® - Mitglied in der Arche des Geschmacks des internationalen Netzwerks Slow Food®) zu vermarkten. Das eingetragene Markenzeichen Albschneck® steht für die Wiederbelebung der traditionellen Schneckenproduktion auf der Schwäbischen Alb und für die Garantie der Anwendung von Qualitätskriterien. Die Weinbergschnecke mit Herkunft aus dem Naturraum Schwäbische Alb muss dazu mindestens ein Jahr lang in extensiver Haltung aufgezogen und überwiegend mit Wildpflanzen gefüttert werden. Nur Deckelschnecken werden lebend, konserviert oder tiefgefroren zu festen Preisen angeboten.

Die Interessengemeinschaft Albschneck® garantiert und überwacht diese Richtlinien. Sie besteht aus einer Reihe von Erzeugern, einem Verarbeiter, aus Gastronomen und Touristikern. Sie fördern und unterstützen die vorhandene Produktion und Kommunikation, aber auch das Erweitern der Erzeugeranzahl, die Versorgung mit professionellen Daten, die Einbindung in einen ökologischen sanften Tourismus sowie die Förderung der Wahrnehmung und Bevorzugung von Spitzenqualität durch den Verbraucher.

Die Albschneck®-Gärten verschiedener Betreiber garantieren somit einen nachhaltigen Schutz der Art *Helix pomatia*, da das artenschutzwiderrechtliche Sammeln sinnvoll und ökonomisch rentabel ersetzt werden kann. Zusammengefasst in der Interessengemeinschaft Albschneck®, stellt die Produktion von Deckelschnecken ein Potential im Ökolandbau dar. Sie trägt ebenso zur naturschutzorientierten nachhaltigen Regionalentwicklung mit regionalspezifischen Markenprodukten bei und stärkt den ländlichen Raum durch die Schaffung zusätzlicher Einkommensquellen (s. auch www.albschneck.de).

Der ausschließliche Verkauf von Schnecken aus Zuchtanlagen garantiert ebenso ein höheres Maß an Verbraucherschutz. Wenn Schnecken gesammelt und direkt verkauft werden, kann die Qualität nicht immer durch einen Herkunftsnachweis zugesichert werden. Ein Problem stellt an dieser Stelle die Verschmutzung der Umwelt mit xenobiotischen Stoffen (Pestizide, Dünger, Schwermetalle etc.) dar, die von den Schnecken in ihren Geweben angereichert werden (Dallinger *et al.*, 2001). Da ein Sammler nicht weiß, wo sich die Schnecke in ihrem Leben aufgehalten hat, d.h. keine Kenntnisse über spezifische Bodenstoffe, die Wasserqualität und die Nahrung der Schnecke hat, ist ohne detaillierte Untersuchungen schwer nachzuweisen, in welcher Menge für Menschen schädliche Stoffe im Gewebe der Schnecke enthalten sind. Bei einer Zuchtschnecke kann allein durch ihre Herkunft die Schadstofffreiheit des Endprodukts durch die Schadstofffreiheit in der Erstmaterie nachgewiesen werden.

Die Wirtschaftlichkeit einer Zuchtanlage ist Vorraussetzung für die Marktanteilnahme und kann nur durch ein gut funktionierendes ökologisches Haltungssystem gewährleistet werden, bei dem das Produkt eine ausgezeichnete Qualität aufweist, aber auch die Fortführung der Schneckenzucht über eine längere Periode garantiert ist. Aus diesem Grund soll die Weinbergschneckenzucht wissenschaftlich begleitet werden. Die Begleitforschung wird im Rahmen einer binationalen Promotion an der Universität Bremen und an der Université Rennes 1 durchgeführt, wobei die Freilanduntersuchungen in den Schneckengärten der Schwäbischen Alb unter Mitarbeit der Hochschule für Wirtschaft und Umwelt Nürtingen-Geislingen vertreten durch Prof. Dr. Roman Lenz vom Projekt Albschneck® durchgeführt werden. Der Titel der Promotion lautet: „Einfluss der Ernährung auf thermophysiologische Prozesse und die Fitness der Weinbergschnecke *Helix pomatia* L. (Gastropoda: Pulmonata) in den Zuchtanlagen auf der Schwäbischen Alb mit spezieller Betrachtung der geographischen Herkunft und der Haltungsdichte.“ Prof. Dr. Juliane Filser vom UFT an der Universität Bremen betreut die Ausarbeitung der Zusammenhänge und die Extrapolierung der studierten Aspekte im Promotionsvorhaben. Laboruntersuchungen im Rahmen der Thermobiologie werden an der Université Rennes 1 stattfinden. Das dortige UMR EcoBio, vertreten durch Dr. Dr. Maryvonne Charrier, verfügt über die entsprechende Labortechnologie sowie über langjährige Erfahrungen in der Forschung zur Ökologie und Zucht von Landschnecken.

Für die erfolgreiche Zucht der *Helix pomatia* wird besondere Bedeutung der spezifischen Boden- und Klimabeschaffenheit des auserwählten Zuchtareals beigemessen. Ein kalkhaltiger Boden ist Vorraussetzung für die Detoxifikation (Almendros & Porcel, 1992) sowie die Schaffung des Gehäuses und der Eier während der Fortpflanzung (Chétail & Krampitz, 1982). Mit der Lage und Ausrichtung des Zuchtareals kann Einfluss auf das Mikroklima in der Anlage genommen werden, denn Wachstum und Vermehrung hängen von der Überdauerung der Trocken- und Kälteperiode ab, was durch eine begrenzte Austrocknungsresistenz (Rees & Hand, 1993) und Kälteresistenz (Nicolai, 2002; Ansart &Vernon, 2004) bedingt ist. Jedoch wird erst mit der Auswahl der geeigneten Zuchtpopulation der Art *Helix pomatia*, die vom

Mittelmeerraum bis nach Schweden vertreten ist, die Vorraussetzung für eine erfolgreiche Schneckenzucht geschaffen, da die Populationen an das in ihrem Lebensraum bestehende Klima angepasst sind (Nicolai *et al.*, 2005). Allerdings ist noch nichts über den Einfluss der Ernährung auf die Fitness (Wachstum der Jungtiere bis hin zum Fortpflanzungserfolg), auf die Kälte- und Trockenheitsresistenz und die organoleptische Qualität der Deckelschnecken in der Schneckenhaltung bekannt. Im Bezug auf die Kälte- und Austrocknungsresistenz sind die Auswirkungen klimatischer Veränderungen, mit denen das naturnahe Zuchtsystem in Zukunft noch stärker konfrontiert wird, bei der Weinbergschnecke *Helix pomatia* noch nicht erforscht.

Mit Ende der Promotion im Herbst 2009 stehen die Ergebnisse den Schneckenzüchtern in Deutschland zur Verfügung. Ziel des Forschungsvorhabens ist es, den Betreibern der Albschneck®-Gärten Leitlinien für die ökologisch-wirtschaftliche Haltung von *Helix pomatia* geben zu können.

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Die Weinbergschnecke *Helix pomatia* Linnaeus 1758

Stamm:	Mollusca
Klasse:	Gasteropoda
Ordnung:	Sigmurethra
Familie:	Helicidae
Gattung:	*Helix*
Art:	*pomatia*
Autor:	Linné (1758)

Die Landschnecke *Helix pomatia* bewohnt lichte Haine und Büsche, hauptsächlich in warmen Niederungen, bei günstigen Bedingungen im Bergland auch bis zu einer Höhe von 2000m. Sie bevorzugt kalkhaltigen Boden mit guter Wasser- und Wärmeleitfähigkeit. Oft ist sie auf Kulturflächen Mittel- bis Südosteuropas zu finden und hat sich auch in Südengland und Südskandinavien nach der Einführung durch die Römer im 4. Jh. angesiedelt. (Abb. *hp*1, (Kerney & Cameron 1979; Pfleger 1984).

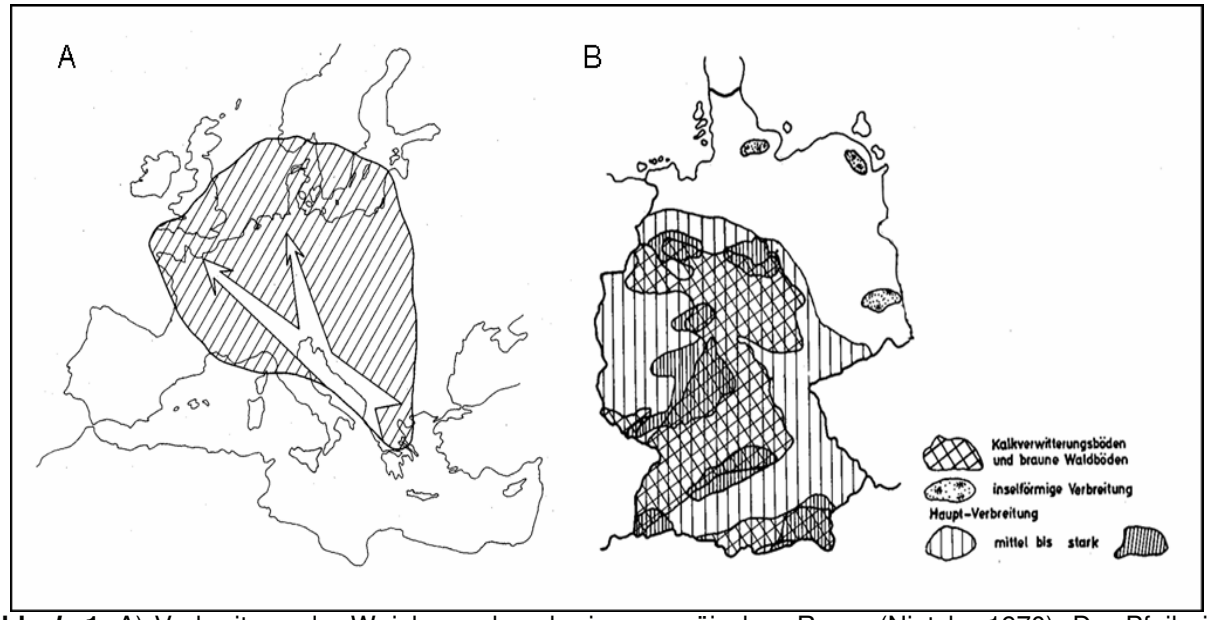

Abb. *hp*1. A) Verbreitung der Weinbergschnecke im europäischen Raum (Nietzke 1970). Der Pfeil gibt die Ausbreitungsrichtung an. B) Verbreitung der Weinbergschnecke in Deutschland (Nietzke 1970).

Helix pomatia besitzt ein kugeliges Gehäuse mit ausgeprägtem, kegelförmigem Gewinde und feinen, unregelmäßigen Längslinien (Abb. *hp*2). Es hat 4,5-5 stark gewölbte, schnell und regelmäßig wachsende Umgänge. Das Peristom ist wenig verbreitert, mit flacher weißlicher bis bräunlicher Lippe. Das Gehäuse selbst ist weißgrau bis hell gelbbraun, mit schwach

angedeuteten bis dunkelvioletten Bändern. Die Gehäusebreite variiert von 32-50mm, ebenso die Höhe von 30-50mm.

Bei einem Absinken der Temperatur und einer Verkürzung der Tageslichtdauer vergraben sich die Weinbergschnecken bis zu 50cm tief in die Erde und bilden ein stark mit Kalk angereichertes Epiphragma am Rand des Gehäusemundes (Abb. *hp*2, Cook 2001). Bei lang anhaltenden Trockenperioden mit hohen Temperaturen im Laufe eines Jahres tritt die Weinbergschnecke erneut in eine Ruhephase, die Ästivation, wobei sie an Steinen oder Baumstämmen durch die Bildung eines Sommerhäutchens haften bleibt (Kilias 2004).

Helix pomatia ist zwittrig mit gegenseitiger Befruchtung. Nach 3-4 Wochen werden 40-70 Eier mit einem Durchmesser von 4-5mm in ein Erdloch gelegt. Die Inkubationszeit beträgt 20-30 Tage. Die Fortpflanzungsphase erstreckt sich von Mai bis August, mehrere Eiablagen sind möglich bis in den September hinein (Lind 1988). Bei der zweiten Fortpflanzungsperiode überwintern die Jungtiere meist im Nest. In den ersten 4 Lebensmonaten verachtfacht sich das Geburtsgewicht der Jungtiere. Anschließend bilden sich die Geschlechtsorgane aus. Erst nach 3-4 Jahren sind die Tiere ausgewachsen und geschlechtsreif, am Peristom bildet sich die Lippe aus (Kilias 2004).

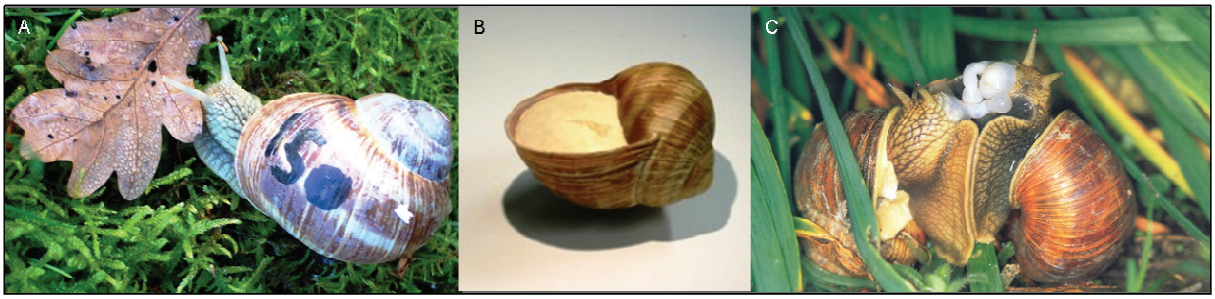

Abb. *hp*2. Ausgewachsene *Helix pomatia* (A), winterschlafende *Helix pomatia* (B), *Helix pomatia* bei der Fortpflanzung (C).

Helix pomatia ist herbivor, jedoch ohne spezifische Nahrungsbeziehung zu Pflanzen. Sie ernährt sich von Grünpflanzen, wobei sie mineralhaltige Pflanzen bevorzugt. Die in Pflanzen enthaltenen Carbonate, Nitrate, Sulfate, Phosphate und Chlorate braucht sie für den Bau des Gehäuses und des Deckels, für die Fortpflanzung und andere physiologische Prozesse. Mit der Nahrung nimmt sie auch Bakterien auf, die eine gute Eiweißquelle darstellen (Kilias 2004).

Herkunft der Schnecken und natürliche Bedingungen

Alle wilden Schnecken wurden mit der Erlaubnis §2 Abs. 2 BNatSchG für das Jahr 2006 und 2007 auf der Schwäbischen Alb, im Rheintal und in der Nähe von Cherasco (Italien) gesammelt.

Schwäbische Alb (ALB)

Lage: Weiler (Indelhausen), 9° 29' 47'' E, 48° 18' 7'' N, 600m ü. N.

Boden: Im September 2008 wurden an drei verschiedenen Stellen des Sammelgebietes Bodenproben entnommen. Nach Schlichting *et al.* (1994) wurde die Bodenart bestimmt und die Proben auf den Carbonatgehalt, den Glühverlust, die Lagerungsdichte sowie den pH-Wert und die Leitfähigkeit untersucht (Tab. *hp*1).

Tab. *hp*1. Methoden und Ergebnisse der Bodenuntersuchung von September 2008 nach Schlichting *et al.* (1994) im Sammelgebiet der Wildpopulationen von Helix pomatia auf der Schwäbischen Alb (ALB) und im Rheintal (RHE).

	METHODE	ALB	RHE
pH-Wert	Lösung aus Feinerde und 0,01M $CaCl_2$	5,76 ± 0,79	7,20 ± 0,04
Leitfähigkeit (µS)	Lösung aus Feinerde und Wasser	195 ± 77 µS	273 ± 11
Glühverlust (%)	Verglühen (430°C) nach Trocknung (60°C) von Feinerde	14,63 ± 5,25	4,85 ± 1,83
Lagerungsdichte ($g.cm^{-3}$)	Substanzgewicht durch Trocknung (60°C) eines Erdvolumens	1,06 ± 0,01	1,34 ± 0,11
Bodenart	Fingerprobe, DIN 4220	Sandiger Ton (Schluff: 0-15%, Ton: 35-45%, Sand: 40-65%)	Sandiger Lehm (Schluff: 0-15%, Ton: 35-45%, Sand: 35-55%)
Carbonatanteil	Zugabe von 10%iger HCl	0,5-2%	10-25%

Vegetation: **Rotbuche** (*Fagus sylvatica*), Gemeine Esche (*Fraxinus excelsior*), Sommerlinde (*Tilia platyphyllos*), **Schwarzerle** (*Alnus glutinosa*), Feldahorn *(Acer campestre*), Bergahorn (*Acer pseudoplatanus*), Stieleiche (*Quercus robur*), Brennnessel (*Urtica dioica*), Gewöhnlicher Erdrauch (*Fumaria officinalis*), Gewöhnliches Hexenkraut (*Circaea lutetiana*), **Großes Springkraut** (*Impatiens non-tangere*), Ruprechtskraut (*Geranium robertianum*), Waldveilchen (*Viola reichenbachiana*), Märzveilchen (*Viola odorata*), Buschwindröschen (*Anemone nemorosa*), **Zwiebel-Zahnwurz** (*Dentaria bulbifera*), Stechender Hohlzahn (*Galeopsis tetrahit*)

Klima: Das Klima im Raum Stuttgart ist durch hohe Niederschlagsmengen von Mai bis August charakterisiert. Die tiefsten Temperaturen werden im Januar erreicht mit -10°C im Durchschnitt, die Höchsttemperaturen im Juli mit 25°C (Abb. *hp*3). Um erzielte Ergebnisse der Freilandversuche in Bezug zum Klima zu bringen, wurden zusätzlich Wetterdaten erhoben, die in den entsprechenden Abschnitten zu finden sind.

Rheintal (RHE)

Lage: Grünstadt-Asselheim, 8° 9' 46''E, 49° 33' 14'' N, 150m ü. N.

Boden: Im September 2008 wurden an drei verschiedenen Stellen des Sammelgebietes Bodenproben entnommen. Nach Schlichting *et al.* (1994) wurde die Bodenart bestimmt und die

Proben auf den Carbonatgehalt, den Glühverlust, die Lagerungsdichte sowie den pH-Wert und die Leitfähigkeit untersucht (Tab.).

Vegetation: **Hainbuche** (*Carpinus betulus),* **Robinie** (*Robinia pseudoacacia),* Sommerlinde (*Tilia platyphyllos*), Schwarzerle (*Alnus glutinosa*), Feldahorn *(Acer campestre*), Stieleiche (*Quercus robur*), Fichte (*Picea abies*), **Brombeere** (*Rubus fruticosus*), Kratzbeere (*Rubus caesius*), Schneebeere (*Symphoricarpos rivularis*), Brennnessel (*Urtica dioica*), **Großes Springkraut** (*Impatiens non-tangere*), Ruprechtskraut (*Geranium robertianum*), Stechender Hohlzahn (*Galeopsis tetrahit*), Scharbockskraut (*Ranumculus ficaria*), **Schöllkraut** (*Chelidonium majus*), Wald-Erdbeere (*Fragaria vesca*)

Klima: Das Klima im Rheintal (Abb. *hp*3) ist durch eine hohe Niederschlagsmenge in kälteren Monaten gekennzeichnet und durch Trockenperioden im Frühjahr und Herbst. Die Höchsttemperaturen liegen bei 27°C und die Tiefsttemperaturen bei –7°C.

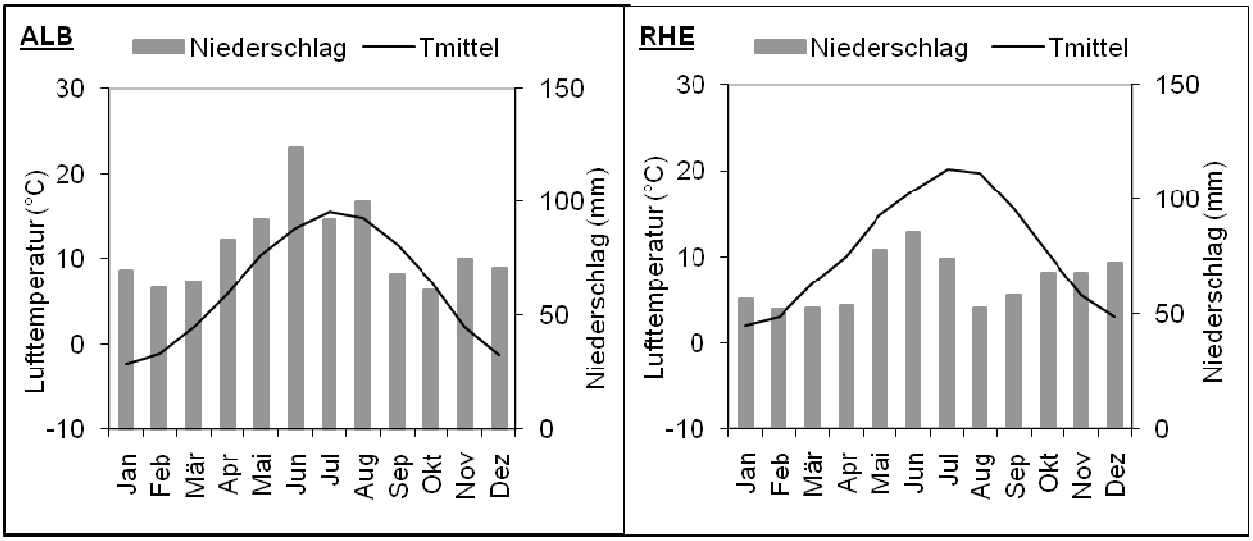

Abb. *hp*3. Durchschnittliche Klimadaten für den Zeitraum 1971-2000 im Raum Münsingen (ALB) und Raum Mannheim (RHE) (http://www.klimadiagramme.de).

Italien, Piemont (IT)

Lage: Natürliches Habitat und Schneckenzuchtinstitut in Cherasco (44° 38' 60''N, 7° 50' 60''E, 200m ü.N.)

Boden: keine Information erhältlich

Vegetation: **Hainbuche** (*Carpinus betulus*) and **Stieleiche** (*Quercus robur*), Feldahorn (*Acer campestre*), Traubeneiche (*Quercus petraea*), Rotbuche (*Fagus sylvatica*) und Gemeine Esche (*Fraxinus excelsior*), **Große Sternmiere** (*Stellaria holostea*), Gemeiner Efeu (*Hedera helix*), **Brombeere** (*Rubus fruticosus*) und Waldgeißblatt (*Lonicera periclymenum*)

Klima: Nur Temperaturdurchschnittswerte vom Sammeljahr 2006 wurden gefunden: Jahresdurchnittstemperatur = 12.9°C, Jahrestiefstemperatur = -3.9°C, Jahreshöchsttemperatur = 32°C (http://www.ilmeteo.it/portale/archivio-meteo/Cherasco).

Haltungsbedingungen

Natürliche Bedingungen am Projektstandort Tachenhausen

Natürliche Bedingungen am Projektstandort Tachenhausen

Lage: Hofgut Tachenhausen (Oberboihingen), Schwäbische Alb, 9° 23' 37'' E, 48° 38' 54'' N, 350m ü. N.

Boden: Da die Weinbergschnecken in direktem Kontakt zum Oberboden leben, wurden im Juni 2007 an drei verschiedenen Stellen des Versuchsgeheges vor dem Einsetzen von Schnecken Bodenproben bis in 20cm Tiefe entnommen. Im September 2008 wurden nach der Nutzung durch Schnecken erneut Bodenproben an den gleichen Stellen entnommen, um eine eventuelle Auswirkung der Schneckenhaltung auf die Bodenverhältnisse abschätzen zu können, die Gegenstand weiterführender Forschung werden könnten. Nach Schlichting *et al.* (1994) wurde die Bodenart bestimmt und die Proben auf den Carbonatgehalt, den Glühverlust, die Lagerungsdichte sowie den pH-Wert untersucht (Tab. *hp*2).

Tab. *hp*2. Methoden und Ergebnisse der Bodenuntersuchung nach Schlichting *et al.* (1994) im Versuchsschneckengarten in Tachenhausen.

	METHODE	SCHNECKENGARTEN	
		vor den Versuchen	nach den Versuchen
pH-Wert	Lösung aus Feinerde und 0,01M $CaCl_2$	6,87 ± 0,13	7,20 ± 0,06
Leitfähigkeit (µS)	Lösung aus Feinerde und Wasser		487,4 ± 10,66
Glühverlust (%)	Verglühen (430°C) nach Trocknung (60°C) von Feinerde	6,24 ± 0,52	4,91 ± 0,67
Lagerungsdichte ($g.cm^{-3}$)	Substanzgewicht durch Trocknung (60°C) eines Erdvolumens	2,20 ± 0,57	0,99 ± 0,06
Bodenart	Fingerprobe, DIN 4220	Tonig-sandiger Lehm (Schluff: 20-35%, Ton: 25-45%, Sand: 25-60%) mit Kalksplitt	
Carbonatanteil	Zugabe von 10%iger HCl	25-50%	

Vegetation und Nahrung: Im Versuchsgehege und in der Albschneck®-Anlage wachsen verschiedene Arten von Gräsern aus der Familie der Poaceae. Außerdem sind Löwenzahn *Taraxacum* sp., Butterblume *Ranunculu*s sp., Klee *Trifolium* sp., Pfefferminze *Mentha arvensis* und Ruprechtskraut *Geranium robertianum* vertreten. Teilweise im Gehege ausgesät wurden Kulturpflanzen, die den Schnecken als Nahrung dienen. Dazu gehören Raps *Brassica napus*, Spinat *Spinacia oleracea*, Mangold *Beta vulgaris*, Salat *Lactuca sativa*, Rübsen *Brassica rapa* und Ölrettich *Raphanus sativus.*

Ausrichtung und Gestaltung: Das Gehege der Versuchsanlage war parallel zu einer NW-SO ausgerichteten Baumreihe (*Quercus* sp.) angelegt, so dass die Längsseiten nach NO zeigten. Im Inneren wurden Zweige angehäuft, Kalksteine, Moos und Holzstücke ausgebreitet um Unterschlupfmöglichkeiten zu bieten (Abb. *hp*4 A)

Klima: Neben der Versuchsanlage ist eine Wetterstation installiert, die Daten zur Lufttemperatur und relativen Luftfeuchtigkeit an der Oberfläche (+20cm), zur Bodentemperatur (-20cm) sowie Daten zur Niederschlagsmenge liefert. Es wird ebenfalls ein individueller Temperaturdatenlogger (Tinytalk TK-4023-PK mit externem Sensor, Spectra Computersysteme GmbH, Reutlingen, Deutschland) an der Erdoberfläche im Versuchsgehege installiert. Die Daten, die über den Versuchszeitraum von Oktober 2006 bis Januar 2008 gesammelt wurden, zeigen Höchsttemperaturen im Juli und August und Tiefsttemperaturen im Januar 2007 und Dezember 2007. Im Boden ist die Temperatur nur im Januar 2007 deutlich gepuffert, da in diesem Monat 1 Woche lang Schnee lag (Abb *hp*4 B).

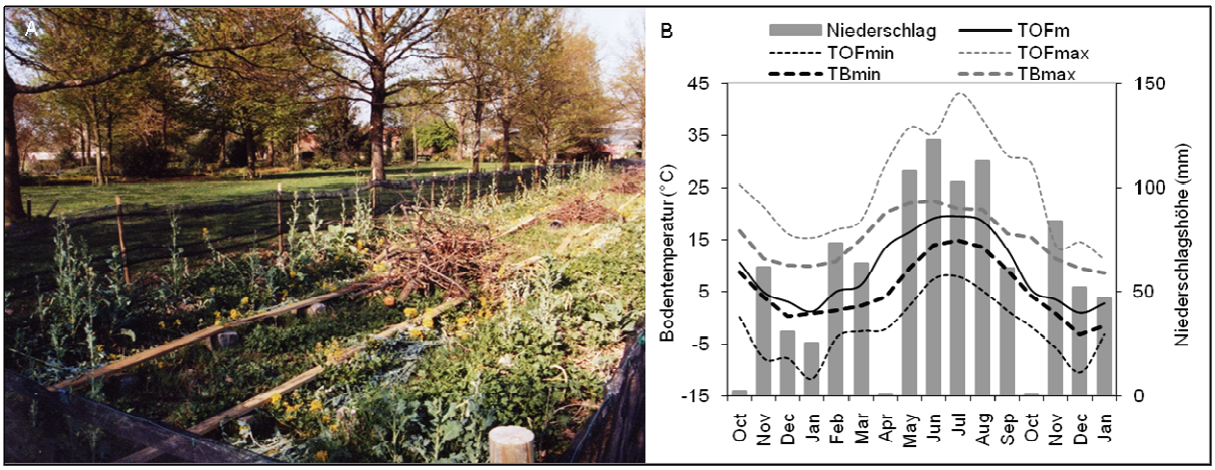

Abb. *hp*4. Gestaltung der Gehege der Versuchsanlage Tachenhausen (A) und durchschnittliche Klimadaten für den Beobachtungszeitraum von Oktober 2006 bis Januar 2008 in Tachenhausen (B). TOF – Temperatur an der Oberfläche (+20cm), TB – Bodentemperatur (-20cm).

Futterzusammenstellung

Während der Versuche wurde neben den natürlich vorhandenem Pflanzenbewuchs auch zugefüttert, wofür jährlich nach Richtlinien des biologischen Anbaus Raps *Brassica napus*, Sonnenblumen *Helianthus annuus*, Topinambour *Helianthus tuberosus*, Erbsen *Pisum sativum*, Luzerne *Medicago sativa*, Rübsen *Brassica rapa*, Ölrettich *Raphanus sativus*, Futterbohnen *Phaseolus* sp. und Rotklee *Trifolium pratense* angebaut wurden.

Teilweise wurden Versuche ohne Pflanzenbewuchs sondern mit Schneckenfutter in Puderform der Marke Helinove® durchgeführt, das von Idena (Sautron, France) zusammengestellt und von Berton Alimentation Animale (Le Boupère, Frankreich) hergestellt wurde (Tab. *hp*3).

Tab. *hp*3. Zusammensetzung des Futters (in Prozent %) und Energiegehalt (kcal/kg). Das Futter ist pflanzlich, aus Mais, Soja, Erbse, Weizen, Luzerne und Rüben mit Vitaminen zusammengestellt. Der Kalziumgehalt ergibt sich aus der Zugabe von gemahlenem Kalkgestein des Typs „Albacal 0/315 µm“ aus dem Departement Ile-de-France (Frankreich) und Kalziumphosphat.

	FUTTERTYPEN		
	Energiearm (E-)	Energie-Basis (E)	Energiereich (E+)
Fett[1]	1.5	2.0	7.0
Eiweiße	15.5	15.5	15.5
Stärke und Zucker	19.3	26.9	22.9
Zellwandbestandteile	19.2	9.9	10.9
Feuchtigkeit	9.0	9.6	9.0
Pflanzliche Mineralien	3.1	2.9	3.1
Kalziumphosphat	4.0	4.5	4.5
Kalziumkarbonat	28.5	29	28.0
Energy	2000	2250	2500

[1] Fette bestehen hauptsächlich aus Fettsäuren (FS) von Sojaöl (15% gesättigte FS, 24% einfach ungesättigte FS, 60% mehrfach ungesättigte FS mit 52% Linolfettsäure von allen FS).

Fortpflanzung unter kontrollierten Bedingungen (Generation F0, F1)

Die individuell markierten wildgesammelten Schnecken im Frühjahr 2006 wurden zu jeweils fünf Individuen in drei Plastikkästen gleicher Größe mit feuchtem Schaumstoff HR (high resilient) und zwei Behältern mit Erde (vom Projektstandort) bei experimentellen Klimabedingungen (20°C, 85% relative Luftfeuchtigkeit, 16h Licht/8h Dunkelheit) gehalten (Abb. *hp*5). Die Kästen wurden regelmäßig getauscht, um einen Käfigeffekt zu vermeiden, regelmäßig befeuchtet und einmal wöchentlich gesäubert. Die Muttertiere wurden *ad libitum* mit Löwenzahn und dem Futtertyp E0 ernährt. Nach jeder Eiablage wurden die Erdbehälter erneuert, so dass in jedem Behälter nur ein Gelege war. Die Eier wurden gezählt und gewogen.

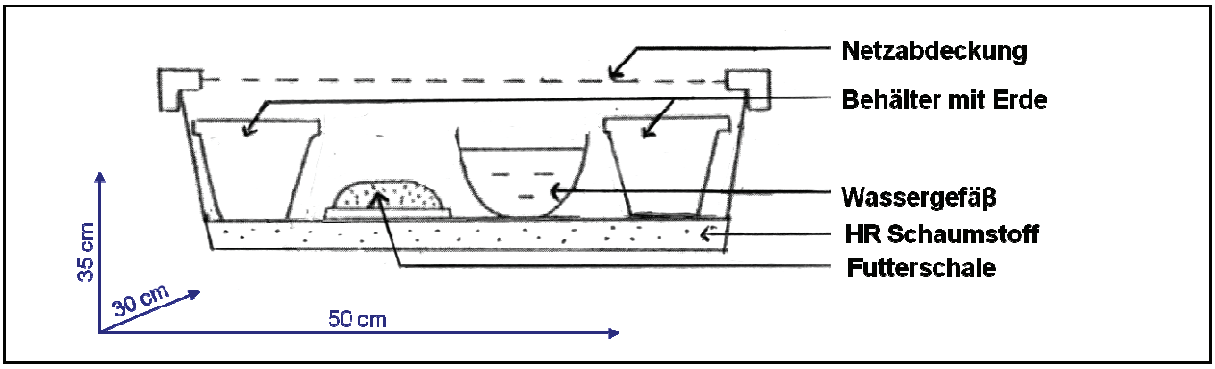

Abb. *hp*5. Anordnung des Versuchsgeheges für die Fortpflanzung von *Helix pomatia* unter kontrollierten Bedingungen.

Jungtieraufzucht und Wachstumsbeobachtungen (Generation F1)

Die Erdbehälter mit den Gelegen wurden bei 20°C und konstanter Feuchtigkeit gehalten. Regelmäßig wurden die Erdbehälter auf geschlüpfte Jungtiere untersucht ohne diese jedoch zu

stören. Nach Erscheinen der Jungtiere an der Erdoberfläche wurden sie gewogen und gemessen, anschließend eine Woche lang mit Löwenzahn *Taraxacum officinale* angefüttert.

Die geschlüpften Jungtiere wurden in der 2. Lebenswoche auf Plastikkästen zu je 17 Individuen verteilt, wobei jedes Gelege gleichmäßig auf alle Kästen verteilt wurde (sibling-split design). Die Kästen enthielten kleine Erdbehälter und in Wasser getränkten Schaumstoff HR. Die Jungtiere wurden *ad libitum* mit Schneckenfutter und *Taraxacum officinale* gefüttert (Abb. *hp*6).

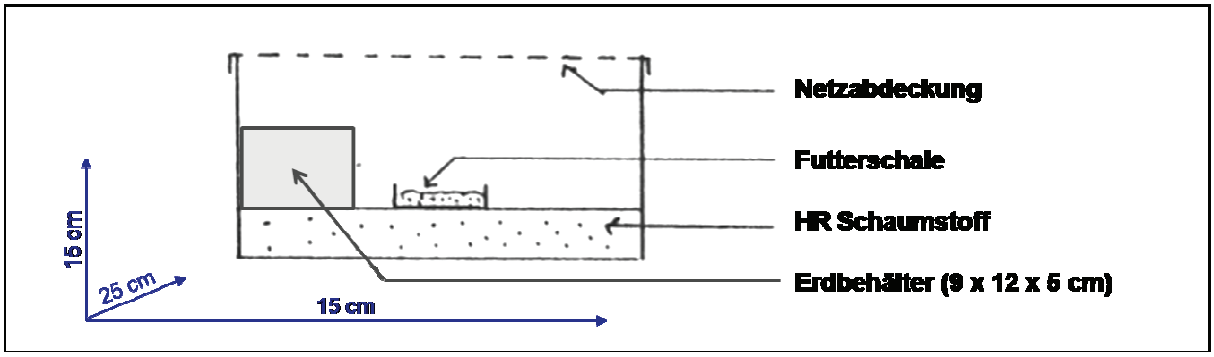

Abb. *hp*6. Anordnung des Versuchsgeheges für die Aufzucht von Jungtieren von *Helix pomatia* unter kontrollierten Bedingungen.

Nach 8 Wochen Haltung im Labor mit wöchentlicher Wachstumsuntersuchung wurden die individuell markierten Jungtiere ins Freilandgehege versetzt und markiert. Dabei wurden Parzellen mit jeweils 4 Individuen und Parzellen mit jeweils 12 Individuen besetzt. Die Haltungsdichten in den Parzellen betrugen 57 bzw. 171 Individuen/m². Diese Haltungsdichten sollten Annäherungen an die Haltungsdichten für Jungtiere (50 Individuen/m²) in der Albschneck®-Anlage bzw. an die Haltungsdichte für Jungtiere in intensiv bewirtschafteten Zuchtanlagen (150 Individuen/m²) darstellen. Aus technischen Gründen mussten die Dichten in den Versuchen leicht erhöht werden. In den Parzellen aus Plastiknetzen wurden die Schnecken auf dem natürlichen Substrat ohne Pflanzenbewuchs gehalten. Moos und Holzstücke standen ihnen als Schutz vor Kälte und Trockenheit zur Verfügung (Abb. *hp*7).

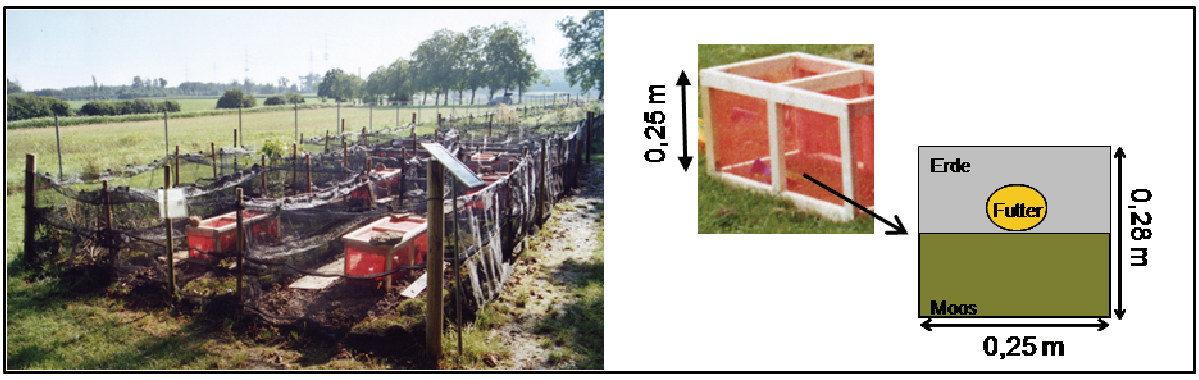

Abb. *hp*7. Anordnung und Gestaltung der Versuchsgehege für die Aufzucht der Jungtiere von *Helix pomatia* unter natürlichen Bedingungen.

Die Jungtiere wurden weiterhin *ad libitum* mit Schneckenfutter ohne pflanzliche Zufütterung ernährt. Um eine erhöhte Sterberate durch ungünstige Witterungsbedingungen zu vermeiden, wurden die Gehege gegen Frost mit Laub und Gartenvlies geschützt und im Sommer bei zu lang anhaltender Trockenheit bewässert. Die Schnecken verblieben in diesen Gehegen bis zur geschlechtlichen Reife und wurden dann zur Fortpflanzung gebracht.

Inkubation (Generation F2)

Die Gelege wurden der entsprechenden Mutterschnecke zugeordnet, die Eier gezählt, jeweils 20 Eier gewogen, und mindestens 10 Eier wurden in Erde zur Inkubation (20°C und konstanter Feuchtigkeit) gelegt. Die restlichen Eier wurden für spätere biochemische Analysen bei -80°C gelagert. Sobald die Jungtiere an der Erdoberfläche erschienen wurden auch sie bei -80°C für biochemische Analysen gelagert.

Haltung und Beobachtung adulter Schnecken

Weinbergschnecken der Wildpopulation wurden im Herbst 2006 auf der Schwäbischen Alb gesammelt und zu 25 ausgewachsenen Individuen in eine mit Pflanzen bewachsene Parzelle auf natürliches Substrat gesetzt (Abb. *hp*8). Die Fütterung erfolgte ausschließlich mit Grünfutter *ad libitum*. Die Schnecken wurden nicht vor Frost und Trockenheit geschützt. Allerdings standen Unterschlupfmöglichkeiten wie Moos, Holz und Pflanzenmaterial zur Verfügung. Alle zwei Wochen wurden die Schnecken auf ihre Gewichtsveränderung und auf ihren Aktivitätszustand untersucht. Eventuell auftretende tote Schnecken wurden notiert und ersetzt.

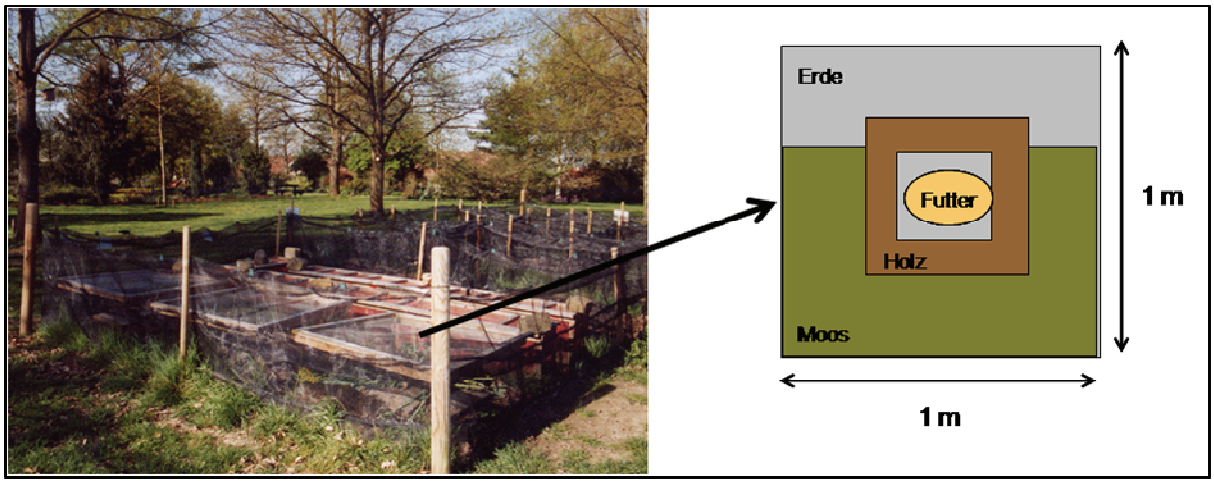

Abb. *hp*8. Gestaltung der Parzelle für 25 ausgewachsene *Helix pomatia* in der Versuchsanlage Tachenhausen.

Um Schnecken monatlich einsammeln zu können, wurden in weitere Gehege jeweils 120 ausgewachsenen Individuen gesetzt (Abb. *hp*9). Die Haltungsdichte von 25 Individuen/m² ist die Idealdichte für ausgewachsene Zuchttiere in den Albschneck®-Anlagen und wurde auch hier zu Beginn des Versuches beibehalten.

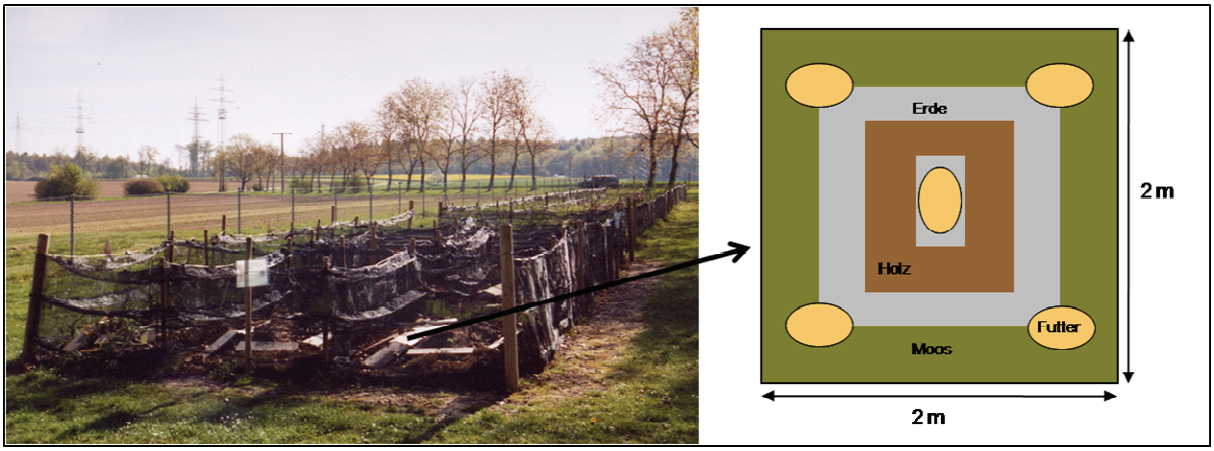

Abb. *hp*9. Abtrennung verschiedener Gehege für Versuche mit monatlichem Einsammeln von *Helix pomatia* und Gestaltung des Innenraumes.

Kontrollierte Hibernationsbedingungen

Wild gesammelte Schnecken wurde progressiv über 2 Wochen ohne Fütterung in den Hibernationszustand versetzt, wobei Temperatur, Luftfeuchtigkeit und Photoperiode schrittweise abgesenkt wurden (Tab. *hp*4). Nach 3 Monaten wurden diese Schnecken für Untersuchungen im Hibernationszustand genutzt.

Tab. *hp*4. Progressiver Übergang zu kontrollierten Hibernationsbedingungen bei *Helix pomatia.*

	Tage 1-3	Tage 4-6	Tage 7-9	Tage 10-12	Tage 13-15	Hibernationsbedingung
Temperatur (°C)	17	14	11	8	5	5
Relative Luftfeuchtigkeit (%)	85	85	80	70	60	60
Photoperiode (h.Tag^{-1})	16	14	12	10	8	0

Biometrische Messungen

Um das Wachstum zu verfolgen wurde der Gehäusedurchmesser mit einem digitalen Messschieber (TCM Digital Caliper, Bremen, Deutschland) ermittelt und die gesäuberte Schnecke auf einer digitalen transportablen Waage (CT200-S, OHAUS, Nänikon, Schweiz) gewogen.

Zum Bestimmen des Wassergehaltes der Schnecken wurden diese im Labor gewogen (S1202, Sartorius AG, Göttingen, Deutschland). Körper und Gehäuse wurden getrennt in einem Präzisionsbrutschrank (BKE 30-80, Memmert GmbH & Co. KG, Schwabach, Deutschland) bei

60°C drei Tage lang getrocknet und anschließend gewogen (angepasste Methode nach Baust *et al.* 1979).

Wassermasse (WM) in g: WM = FM – GM - TM

M: Masse des Körpers mit Gehäuse

Tm: Trockenmasse des Körpers

GM: Gehäusemasse

Messungen der Kristallisationstemperatur

Kristallisationstemperatur bei adulten Schnecken

Die Kristallisationstemperatur (im Englischen: supercooling point) entspricht der negativen Temperatur, bei der die unterkühlte Körperflüssigkeit gefriert. In diesem Moment wird latente Wärme freigesetzt, die zur plötzlichen Erhöhung der Körpertemperatur (Exotherm) führt (Abb. *hp*10) (Salt 1961, Block 1991).

Basierend auf der an Schnecken angepassten Technik von Ansart (2001) wurde der Gefrierpunkt durch Einführen eines Thermosensors (K-type: Chromel-Alumel, Fischer Scientific, USA) durch ein 1mm großes Loch im Gehäuse gemessen, welches mit einem Präzisionsbohrer (S2000, Georg Schick Dental GmbH, Schemmenhofen, Deutschland) angefertigt wurde. An jeder Schnecke wurden zwei Löcher gebohrt, eins über der Mantelhöhle und eins über dem Herz-Nieren-Bereich, wobei verwundete Tiere von der Untersuchung ausgeschlossen wurden. Der Thermosensor wurde durch ein Klebeband in der richtigen Position gehalten und war mit einem mehrkanaligen Temperaturaufzeichnungsgerät (Tempscan C8600, Comarck Electronics Ltd., Hertfordshire, UK) verbunden, das bis zu 12 Messungen gleichzeitig (1 Messung pro Sekunde) durchführen kann. Die Daten wurden auf einen Drucker (HP deskjet 500, Hewlett-Packard Company, Palo Alto, CA, USA) übertragen und aufgezeichnet.

Vor der Messung wurden die Schnecken 15min bei 5°C aufbewahrt und anschließend in einem Probebeutel in das Kältebad eines Kompaktthermostates (MT 3, Lauda GmbH & Co. KG, Lauda-Königshofen, Germany), gefüllt mit Temperierflüssigkeit aus Silikonöl (Kryo 51, Lauda GmbH & Co. KG, Lauda-Königshofen, Germany), getaucht, das mit einem Durchlaufkühler (DLK 10, Lauda GmbH & Co. KG, Lauda-Königshofen, Germany) verbunden war. Zu Beginn betrug die Temperatur des Kältebades 5°C. Sobald die Schnecken ihre Körpertemperatur auf 5±1°C stabilisiert hatten, wurde die Abkühlung des Kältebades auf -15°C eingeleitet. Bei einer Zimmertemperatur von 20°C erreichte das Kältebad eine Minimaltemperatur von -12°C, die weit unter dem zu erwartenden Gefrierpunkt von *Helix pomatia* lag (Abb. *hp*10) (-6,4°C, Nicolai *et al.* 2005). Die Abkühlungsgeschwindigkeit des Kältebades und von *Helix pomatia* waren nahezu gleich, rund 0,6°C/min, was ungefähr dem von Salt (1966) vorgeschlagenen Wert von 0,5°C/min entspricht.

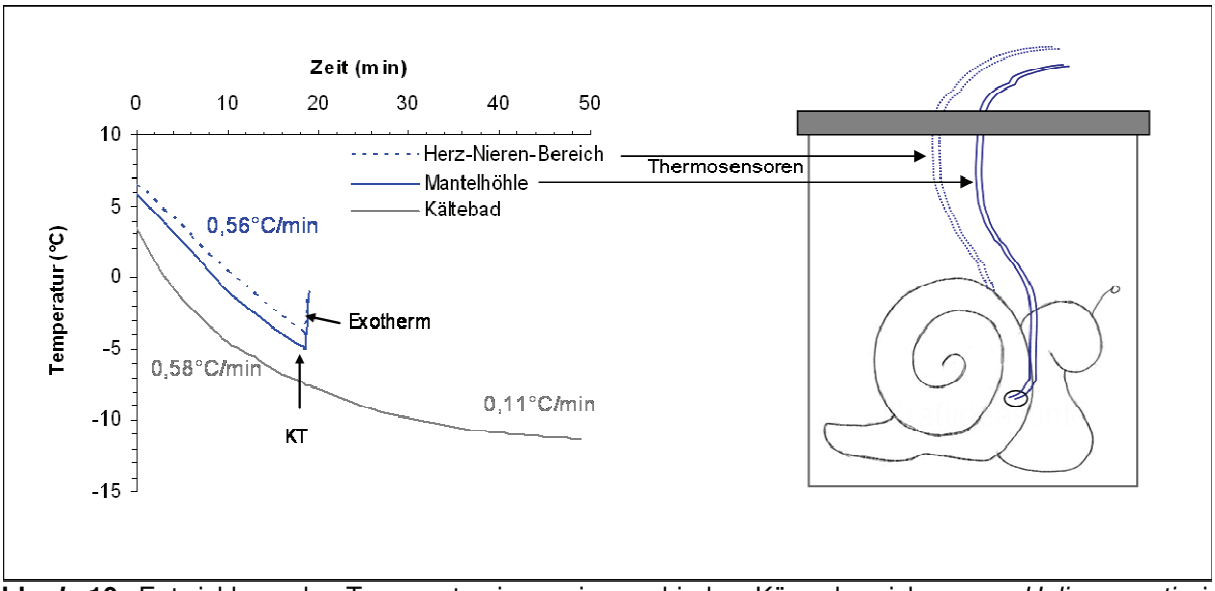

Abb. *hp*10. Entwicklung der Temperatur in zwei verschieden Körperbereichen von *Helix pomatia* im Verlauf der Unterkühlung im Kältebad. Dieser Test wurde bei 20°C Zimmertemperatur durchgeführt. Die Abkühlungsgeschwindigkeit des Kältebades und von *Helix pomatia* bis zur Messung der Kristallisationstemperatur KT (für das Kältebad auch danach) ist angegeben.

Kristallisationstemperatur der Hämolymphe

Das Gehäuse wurde im Herz-Nieren-Bereich entfernt und die Hämolymphe durch das Einstechen einer Pasteurpipette in die Lungenader nahe der Herzkammer entnommen (Abb. *hp*11 A). Somit konnten 250 – 300µl Hämolymphe entnommen werden, die bis zur Weiterverwendung bei -25°C in Kryoröhrchen aufbewahrt wurden. Um den Gefrierpunkt zu bestimmen, wurden jeweils 5 Tropfen (5µl) unbehandelter Haemolymphe auf eine Aluminiumschale gegeben, die dann in einem Kältebad gekühlt wurden (angelehnt an die Tröpfchenmethode, Vali 1971). Das Absinken der Temperatur des Kältebades (Polystat CC3, Huber Kältemachinen GmbH, Offenburg, Germany) erfolgte von 5°C auf bis zu –25°C mit einer Geschwindigkeit von 0,5°C/min. Ein Thermosensor (K-type: Chromel-Alumel, Fischer Scientific, USA) wurde auf einer Aluminiumschale fixiert, um die Temperatur an dessen Oberfläche ablesen zu können, die nicht mit der angezeigten Temperatur des Kältebades übereinstimmte. Der Thermosensor war mit einem mehrkanaligen Temperaturaufzeichnungsgerät (Tempscan C8600, Comarck Electronics Ltd., Hertfordshire, UK) verbunden. Das Gefrieren eines Tropfens konnte mit den Augen beobachtet werden, da die Tröpfchen „milchig“ wurden (Abb. *hp*11 B).

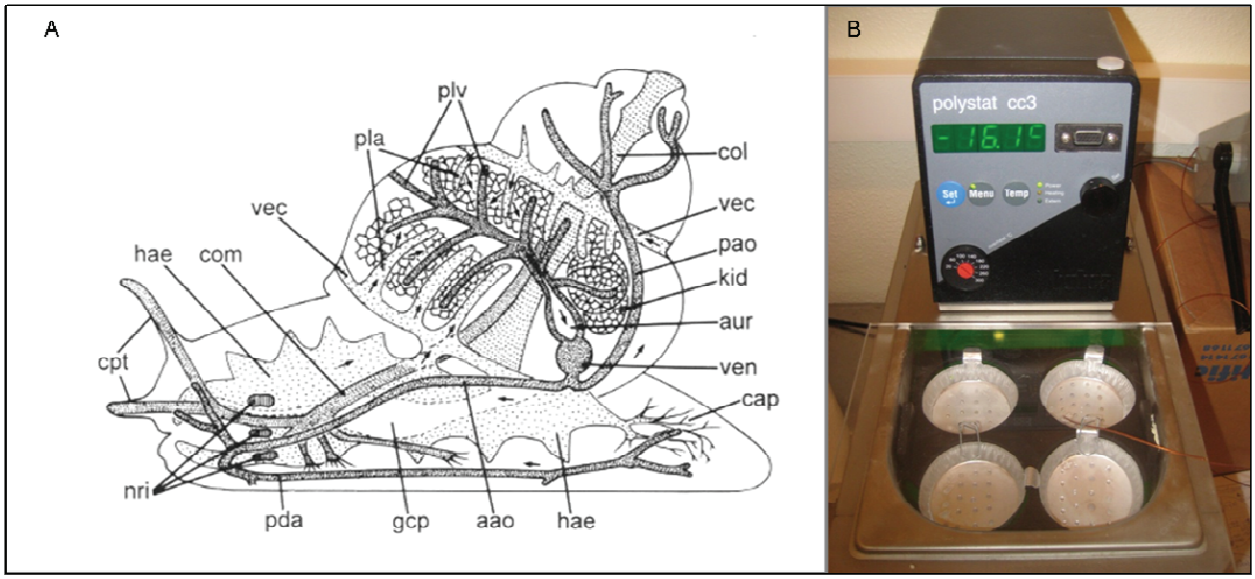

Abb *hp*11. A) Zirkulationssystem und haemolymphatische Höhlen von *Helix pomatia*. Aao – vordere Aorte, aur – vordere Herzkammer, ven – hintere Herzkammer, cap – Kapillare, col – Gehäusesäule, com – Muskel der Gehäusesäule, cpt – Tentakel, gcp – Magen, hae - haemolymphatische Höhle, kid – Niere, nri – Nervenring, pao – hintere Aorte, pda – Fußader, pla – Lungenader, plv – Lungenvene, vec – Venenring (after Borrodaile et al. 1958). B) Versuchsanordnung zur Bestimmung der Kristallisationstemperatur der Hämolymphe von *Helix pomatia*.

Biochemische Analysen

Unter Vakuum getrocknete Eier und Jungtiere (Lyovac™ GT3, Leybold-Heraeus, Orsay, France) wurden mit Bead Beating (4 x 30 s, 30 Bewegungen.s^{-1}, Retsch™ MM301, Retsch GmbH, Haan, Germany) unter Nutzung von 3-mm-großen Tungsten-Perlen zerkleinert und homogenisiert. Hemolymphe wurde direkt für die Analysen genutzt. Triglyceride und Cholesterin wurden entsprechend dem Protokoll von Hervant *et al.* (1999), Glykogen und Galaktogen entsprechend dem Protokoll von Van Handel (1965), Aminosäuren und Karbohydrate entsprechend dem Protokol von Renault et al. (2010) extrahiert. Das extrahierte Material wurde unter Vakuum getrocknet (miVac DNA concentrator, Genevac, Suffolk, UK) und anschließend mit dem Reaktionsmedium versetzt, das der quantitativen Bestimmung diente. Erläuterungen zu den einzelnen Extraktionen sind in der Tabelle *hp*5 zu finden.

Zur quantitativen Bestimmung von Triglyceriden, Cholesterin, Glykogen, Galaktogen, Glukose und Galaktose wurde ein Mikroplattenspektrometer (VERSAmax™ microplate reader, Sunnyvale, CA, USA) und Kalibrationskurven genutzt. Die Analyse der Karbohydrate wurde mit einem GC-MS (Gas Chromatography and Mass Spectrometry) (Thermo Fischer Scientific Inc, Waltham, MA) durchgeführt (Box *hp*1 und Abb. *hp*12). Das Gerät besteht aus einem TriPlus Autosampler, Trace GC Ultra Chromatograph und Trace DSQII Quadrupole Massenspektrometer. Chromatogramme wurden mit dem Programm AMDIS v2.65 (http://chemdata.nist.gov/mass-spc/amdis/) und der NIST Datenbank ausgewertet. Die Analyse von Aminosäuren erfolgte mit einem UPLC (Ultra Performance Liquid Chromatography) (Acquity UPLC® system, Waters Corporation, Milford, MA) (Box *hp*2 und Abb. *hp*13).

Tab. *hp*5. Zusammenfassung der biochemischen Analysen an Eiern und Jungtieren von *Helix pomatia*.

	EXTRAKTIONS-MEDIUM	SEPARATION DER PHASEN	LÖSUNGSMEDIUM	REAKTIONSMEDIUM
Triglyceride	Methanol: Chloroform (1:2 v/v)	KCl (2 $g.l^{-1}$), 5 min bei 40°C	BSA (3% w/v, fettsäurefrei) – Triton (0.2% v/v)	Triglyceride Assay Kit (Cayman Chemicals, Ann Arbor, USA)
Cholesterin			Ethanol (95°)	Cholesterol RTU™ kit (Biomerieux, France)
Glykogen	Trichloressig-säure (4%)	Centrifugation (5000 g, 5 min, 4°C), Ausfällung mit Ethanol (96°)	Wasser (12 h bei 21°C)	Lugol
Galaktogen			Hydrolyse mit HCl 6M (6h bei 110°C), Neutralisation mit KOH 6M	Enzyplus® EZS 784+ Lactose/D-Galactose Kit (Biocontrol, Bellevue, USA).
Glukose	Methanol: Chloroform (2:1 v/v)	Wasser (V_{Wasser} = $V_{Methanol}$)	Wasser	Enzyplus® EZS 784+ D-Glucose Kit (Biocontrol, Bellevue, USA).
Galaktose				Enzyplus® EZS 784+ Lactose/D-Galactose Kit (Biocontrol, Bellevue, USA).
Aminosäure				AccQ•Tag Ultra Derivitization Kit (Waters Corporation, Milford, MA)
Sonstige Karbohydrate			Methoxyamin-hydrochlorid - Pyridin (20 mg/ml)	MSTFA (Sigma, #394866)

Box *hp*1. GC-MS

Die Gaschromatographie (GC) ist eine Trennmethode, bei der eine Probe mit Hilfe eines Gasstromes über eine stationäre Phase geleitet wird. Dabei wird die Probe in ihre Einzelkomponenten aufgetrennt. Das inerte Trägergas ist in der Regel Helium, und die stationäre Phase besteht aus einer 10-50 m langen Quarzsäule mit einem Innendurchmesser von etwa 0,2 mm, die von Innen mit einem speziell entwickelten dünnen Film eines Trennmaterials belegt ist. Die Einzelkomponenten der Probe verlassen nach einer bestimmten Zeit (Retentionszeit) die Trennsäule und können dann mit einem Massenspektrometer (MS) auf ihre Molekülmassen analysiert werden. Ein Massenspektrometer besteht aus einer Ionenquelle, in der die gasförmigen Moleküle ionisiert werden, einem Massenanalysator, der die Ionen hinsichtlich ihres Masse/Ladungszahl-Verhältnisses (m/z) auftrennt und einem Detektor, der die Intensität der erzeugten Ionen misst. Als Ergebnis der Analyse wird ein für jede Substanz charakteristisches Massenspektrum erstellt, aus dem hervorgeht, welche Ionen in welchen relativen Mengen gebildet worden sind.

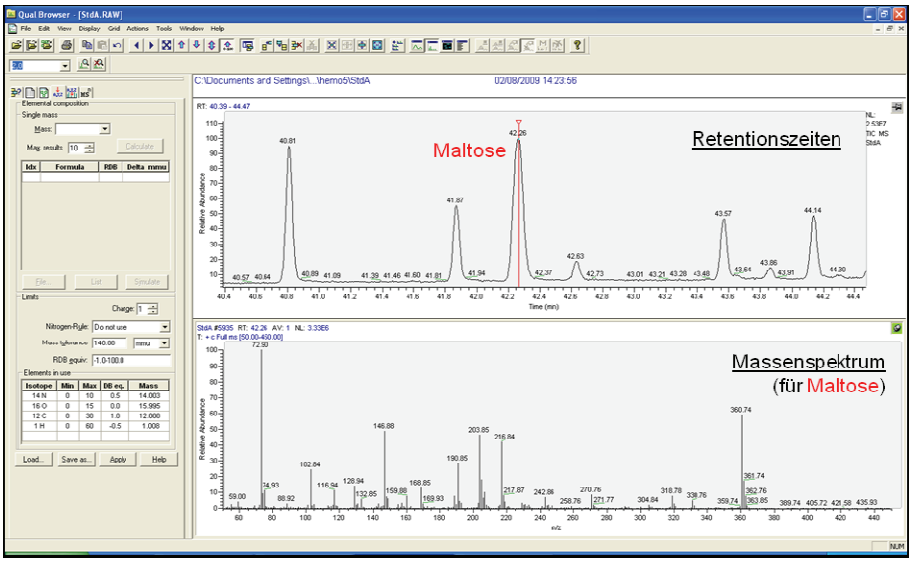

Abb. *hp*12. Beispiel für erhaltenes Karbohydratprofil während eines GC-MS-Durchlaufs. Jede Spitze entspricht einer Substanz (Bsp. Maltose), das ein spezifisches Massenspektrum aufweist.

Box *hp*2. UPLC/HPLC
UPLC (Ultra performace liquid chromatography) ist eine Herstellerbezeichnung für ihre Hochleistungsflüssigkeitschromatographie (engl. high performance liquid chromatography, HPLC). Das Flüssigchromatographie-Verfahren, mit dem man nicht nur Substanzen trennt, sondern diese auch über Standards identifizieren und quantifizieren kann, ist im Unterschied zur Gaschromatographie (GC) eine sehr gute Trennmethode für nicht flüchtige Substanzen. Mit der GC dagegen werden verdampfbare Stoffe analysiert werden. Die zu untersuchende Substanz wird zusammen mit einem Elutionsmittel, der mobilen Phase, durch eine sogenannte Trennsäule, welche die stationäre Phase enthält, gepumpt wird. Eine Trennsäule in einem HPLC-Gerät ist zwischen 18 und 300mm lang und hat zumeist einen Innendurchmesser von 2 bis 4,6mm. Je nach Stärke der Wechselwirkungen von den Bestandteilen der zu untersuchenden Substanz erscheinen die Bestandteile zu verschiedenen Zeiten (den Retentionszeiten) am Ende der Trennsäule, wo sie dann mit einem geeigneten Detektor nachgewiesen werden können.

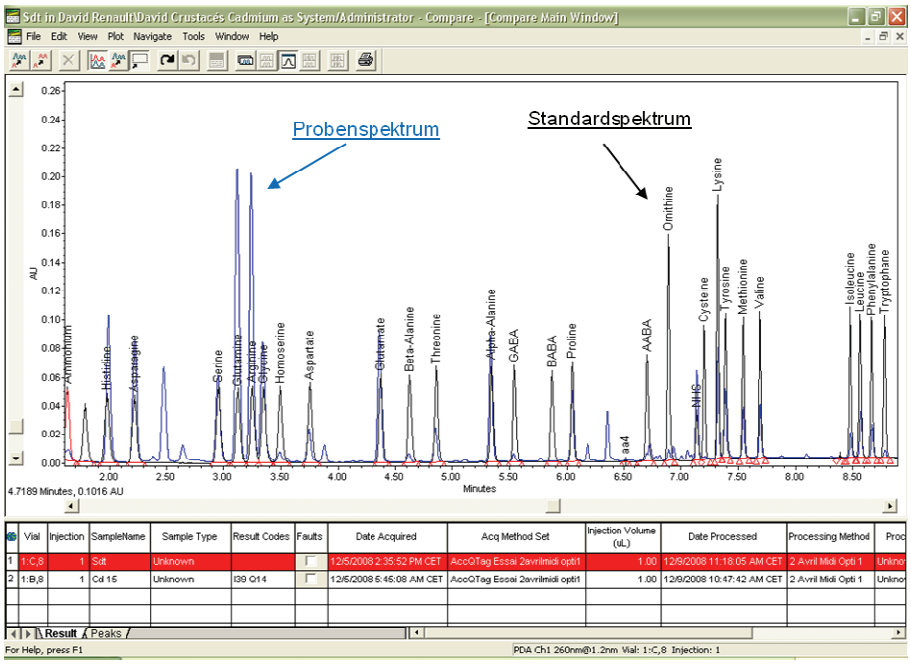

Abb. *hp*13. Beispiel für erhaltenes Aminoacidprofil einer Probe während eines UPLC-Durchlaufs. Jede Spitze entspricht einer Aminosäure und wird anhand der Retentionszeiten eines Standards identifiziert.

Untersuchung der Darmbakterienflora

Die bakterielle Darmflora wurde im Hepatopancreas, im proximalen und distalen Teil des Darmes untersucht. Nach dem Sezieren der Schnecken wurde die 16S rDNA entsprechend der Methode von Schmitt-Wagner et al. (2003) extrahiert. Vor der Isolation von bakteriellen DNA-Fragmenten mit Hilfe der Techniken PCR-DGGE (Polymorphism Chain Reaction - Denaturation Gradient Gel Electrophoresis) wurde die DNA zweimal gereinigt (Kit MicroSpin S-400 HR, Bioscience, UK). Anschließend wurde die Menge an extrahierter DNA mit einem Nanospektrophotometer (NanoDrop® ND-1000 ROCHE, Nanodrop Technologies, USA) bestimmt. Die PCR wurde mit einem universellen Primer 518-r (ATT-ACC-GCG-GCT-GCT-GG) und einem GC-clump-Primer gc-338-f (CGC-CCG-CCG-CGC-GCG-GGC-GGC-GGC-GCG-GGG-GCA-CGG-GGG-GAC-TCC-TAC-GGG-AGG-CAG-CAG) sowie der Taq-Polymerase und dNTP (puReTaq Ready-To-Go PCR Beads, Illustra™, GE Healthcare, UK) durchgeführt. Der PCR-Zyklus (Mastercycler personal Eppendorf®, USA) setzte sich aus 3 Phasen zusammen: (1) Initiale Denaturierung, (2) 35 x Zyklus Denaturierung, Primerhybridisierung, Polymerisation, (3) Endpolymersiation. Die somit gewonnen DNA-

Fragmente sind 230pb groß und wurden durch eine Elektrophorese auf 2% Agarosegel (55min, 100V, Wanne B1 OWLO, USA) mit einem Marker (100bp Molecular Ruler, BIORAD, Kanada) überprüft.

Für die DGGE (15h, 75V, Dcode System, BIORAD, Kanada) wurde ein 8% Acrylamidgel mit einem Gradienten von 40 - 55% Harnsäure hergestellt, der die DNA-Fragmente entsprechend der Anzahl und Lage von GC-Verbindungen denaturiert und somit Fragmente gleicher Länge aufgrund ihrer Beschaffenheit trennt („DNA-Fingerabdrücke“) (Abb. *hp*14 A). Ein DNA Fragment, das sich an entsprechender Höhe im Gel befindet, entspricht generell einer Bakterienart. Die intenivsten Fragmente wurden sequenziert und mit Sequenzen der Datenbank „GenBank“ (http://blast.ncbi.nlm.nih.gov/Blast.cgi) verglichen. Die taxonomische Zuordnung erfolgte mit dem „RDP classifier“ (http://rdp.cme.msu.edu/index.jsp).

Die DGGE ist dadurch limitiert, dass sie nur dominante Bakterienarten zeigt (Claesson *et al.* 2009), deren Zusammensetzung teilweise durch die Wahl der Primer orientiert wird (Malin & Illmer 2008). Außerdem können Bacterien Sequenzen haben, die sich höchstens um 1% unterscheiden (Acinas *et al.* 2004) und somit zu verschiedenen DNA-Fragmenten führen können. Solch leichte Sequenzvariationen können sich aber auch bei der PCR ergeben. (Speksnijder *et al.* 2001). Das Protokoll ist in Abb. *hp*14 B zusammengefasst.

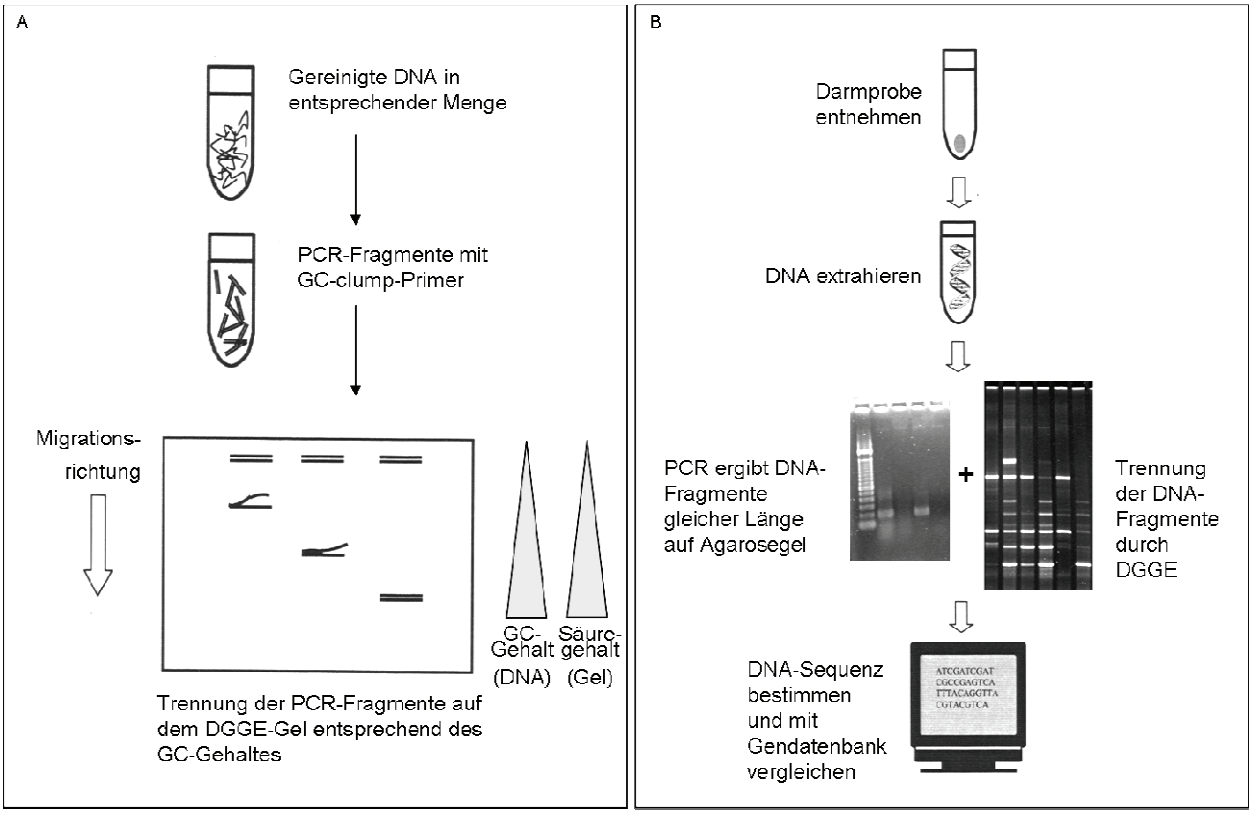

Abb. *hp*14. Prinzip der PCR-DGGE (A) und Zusammenfassung des Protokolls zur „DNA-Fingerabdruck“-Methode PCR-DGGE (B) angewendet auf die Bakteriengemeinschaften im Darm von *Helix pomatia*.

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Macht Fett fit ?

In der Schneckenzucht werden die Gehege mit wilden und kultivierten Pflanzen bepflanzt, wobei sich auf Erfahrungen aus der italienischen Schneckenzucht berufen wird. Es ist dabei wichtig zu wissen, dass in der Familie der Helicidae die quantitative Nahrungsaufnahme von der Pflanzenart abhängt (Frömming 1957). Im natürlichen Lebensraum wählt z. B. *Cornu aspersum* die Nahrung nach organischen (Iglesias & Castillejo 1999) und inorganischen Inhaltsstoffen (Chevalier *et al.* 2003) aus, aber nicht nach dem Energiegehalt (Chevalier *et al.* 2001). Unter den stimulierenden Substanzen befinden sich Calcium (Charrier & Daguzan 1980, Chevalier *et al.* 2003) und Proteine der Brennnessel *Urtica dioca* (Iglesias & Castillejo 1999). Die Bepflanzung der Anlagen sowie die Zufütterung mit Pflanzenmaterial oder kommerziellen Schneckenfutter sollte daher die Bedürfnisse der Schnecken berücksichtigen

Analytische Daten zur Zusammensetzung des Schneckenfleisches von *Helix pomatia* (Wildform) und *Cornu aspersum* (Zuchtform) (Tab. *hp*6, Lubell 2004) zeigen, dass *Helix pomatia* proteinreich ist. Der relativ geringe Fettgehalt ist jedoch noch höher als der Karbohydratgehalt. Da eine Vielzahl an Elementen mit der Nahrung aufgenommen wird, ist es wichtig, mit den Futterpflanzen eine angemessene Zusammenstellung an Ressourcen zu liefern. Gomot (1994) präzisiert den Bedarf an verschiedenen Elementen in der Nahrung entsprechend dem Entwicklungsstadium der Jungtiere von *Cornu aspersum* und betont ein relativ hohes Bedürfnis an Fetten. Diese haben meist keine Auswirkungen auf das Wachstum, verringern aber die Sterberate und verbessern die Fortpflanzung (Wacker 2005).

Ein weiterer kritischer Punkt ist die Haltungsdichte, die in den italienischen Zuchtanlagen sehr hoch ist (~150 – 200 Schnecken pro m^2). In Anlehnung an ein naturnahes Haltungskonzept wird diese Haltungsdichte immer wieder hinterfragt. Ebenso kritisch erscheint die Aufstellung einer Zuchtpopulation, die sich an die Haltungsbedingungen über Generationen angepasst haben könnten. Deswegen sollen an der wilden Albschneckpopulation und an einer italienischen Zuchtpopulation Auswirkungen von fettarmen und fettreichen Futter sowie Haltungsdichten auf Wachstum und Fortpflanzung getestet werden.

Hypothesen dieser Studie:

- Fettreiches Futter hat keinen Einfluss auf das Wachstum in beiden Populationen.
- Überlebensrate während des Wachstums ist mit fettreichem Futter höher in beiden Populationen.
- Während der Fortpflanzung können angereicherte Fette im Körper beider Populationen in Eier investiert werden und zu einer höheren Geburtsgröβe der Jungtiere führen.
- Eine geringe Haltungsdichte hat einen positiven Einfluss auf alle getesteten Prozesse

Tab *hp*6. Zusammensetzung des Schneckenfleisches bei *Cornu aspersum* (Zuchtform) und *Helix pomatia* (Wildform) nach Lubell, 2004. Bei *Cornu aspersum maxima* findet Gomot (1998) nur 13,8% an Kohlenhydraten. TM – Trockenmasse.

	CORNU ASPERSUM	*HELIX POMATIA*
Allgemeine Zusammensetzung (in % TM)	(Gomot 1998)	(Miletic et al, 1991)
• Wasser	83,7	81,9
• Proteine	65,2	70,6
• Fette	10,0	6,7
• Kohlenhydrate	15,4	4,4
• Asche	9,4	4,4
Essentielle Aminosäuren (in mg/100g TM)	(Gomot 1994)	(Miletic et al. 1991)
• Glutamin	6,3	9,8
• Asparagin	5,0	6,2
• Leucin	3,4	4,9
• Valin	2,2	6,1
Essentielle Fettsäuren (* in % TM, in mg/100g FM)**	(für *C. aspersum maxima*, Milinsk et al. 2003)*	(Scherz et al. 2000)**
• Ölsäure 18:1n-9	19	178
• Linolsäure 18:2ω-6	18	118
• Arachidsäure 20:4ω-6	10	65
• Linolsäure 18:3ω-3	1,7	18

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Einfluss der Ernährung und Haltungsdichte auf die Fitness von *Helix pomatia* verschiedener Herkunft in den Schneckengärten auf der Schwäbischen Alb

Short translation: The impact of diet treatment and of the density in cages on the fitness of Helix pomatia in the snail farms of the Swabian Alps in respect to population origin

Energy available in the food could either be invested in growth or in maintenance. This trade-off might be particularly important in *Helix pomatia* that has to overcome two to three hibernation periods during growth. Energy that is invested in growth and survival could not be invested in reproduction after growth termination. Since in organic snail farms fast growth, high survival and high reproductive output are important to sustain productivity, we investigated the impact of energy content in food on growth, survival and reproduction.

Wild snails from the Swabian Alps (ALB) and in farmed snails from Italy (IT) were reared in the test snail farm with two densities corresponding to organic and intensive snail farming. The energy content in food was obtained by adding lipids (E+) or plant cell wall polymers (E-) to a basic food type (E).

Growth rate was enhanced with lipid rich food in ALB snails but led to higher mortality during growth and hibernation as well as to smaller body size at the end of growth. ALB snails might not be able to extract energy from this lipid rich food and/or invested more in growth rate than body stores that could serve for maintenance and hibernation survival. The basic food gave the best results for final body size and survival in the ALB snails. In IT snails, food type had no influence on growth and survival, and final body size was the same as in ALB snails with basic food. All snails reached maturity one year earlier than in the wild. During reproduction ALB snails adopted the "many-small-eggs strategy" and maximized their reproductive success by increasing clutch size, according to the optimal offspring investment theory, when energy availability in food decreased. E- ALB snails showed the highest energy investment in clutch from all studied snails, which is consistent with a terminal investment followed probably by a high mortality. E ALB snails enhanced glycogen and cholesterol allocation to eggs, though in a small amount that did not affect energy content per egg. However, hatchlings from these eggs were bigger and have maybe a higher survival probability. E+ ALB snails did not reproduce at all.

IT snails adopted a "few-big-eggs strategy" and maximized their reproductive success by enhancing galactogen and subsequently energy content in eggs with increasing energy availability in food. E+ and E IT snails had therefore bigger and energy richer offspring than E- snails, which is consistent with the state-dependant theory, indicating higher investment in brood when body conditions are high. Density of individuals in cages had an opposite effect on growth and reproduction.

However, observations about growth and reproduction were poorly supported by statistical results, because high mortality reduced the number of analyzable individuals. Therefore, this chapter should only serve as information for snail farmers and show them the importance of a well balanced snail alimentation and a mid density of individuals in cages. Further studies should rather focus on hibernation or extreme weather survival and give some indications how to diminish mortality in organic snail farms.

Einleitung

Schnecken der Familie Helicidae sind herbivor, wobei sie eine große Anzahl an verschiedenen Pflanzen konsumieren (Chevalier *et al.* 2001). Das Vorhandensein von Ressourcen ist ein limitierender Faktor für die Auswahl der Nahrung, denn allein schon der Energieverbrauch (23% bei *Acmea* spp., Davies *et al.* 1990) für die Absonderung von Schleim (8,2% Glycoproteine bei *Helix pomatia,* Denny 1983) zur Fortbewegung auf der Suche nach anderer Nahrung ist enorm hoch.

Die Energie, die durch die Nahrung im Lebensraum zur Verfügung gestellt wird, kann einerseits in das Wachstum und die Anlagerung von Reserven oder in überlebenswichtige Prozesse investiert werden (Karasov und Martinez del Rio 2007). Dieser Kompromiss der Ressourcenzuteilung ist besonders bei *Helix pomatia* von Bedeutung, die eine Wachstumsphase von 3-4 Jahren hat mit 2 – 3 Überwinterungsphasen. Gewonnene Energie, die in Wachstum investiert wurde, kann nicht mehr für physiologische Hibernationsprozesse genutzt werden, was

Kosten in Form von erhöhter Sterberate darstellt. Ebenso kann Energie, die in Hibernationsprozesse investiert wurde, nicht mehr in spätere Fortpflanzung investiert werden.

Proteinreiche Nahrung bewirkt bei *Arianta arbustorum* ein schnelleres Wachstum und eine kürzere Dauer bis zum Erreichen der geschlechtlichen Reife bei einem größeren Durchmesser, wobei der Calciumgehalt der Nahrung keine Auswirkungen auf das Gehäusewachstum hat. Dagegen führt eine protein- und calciumarme Ernährung zu einer höheren Sterberate, obwohl die Schnecken mehr Nahrung konsumieren (Wacker & Baur 2004).

Fette und Cholesterin sind essentiell für verschiedene Lebensprozesse. *Arianta arbustorum* verringert ihre Konsumation und hat eine höhere Sterberate, wenn zu wenig Cholesterin oder zu wenig ungesättigte Fettsäuren in der Nahrung enthalten sind. Ebenso wirkt sich der Mangel an ungesättigten Fettsäuren negativ auf das Paarungsverhalten aus, wobei der Cholesterinmangel keine Auswirkungen hat. Dagegen haben Fette und Cholesterin keinen Einfluss auf das Wachstum, die Dauer bis zum Erreichen der geschlechtlichen Reife, die Samenbildung und die Anzahl der gelegten Eier bei *Arianta arbustorum* (Wacker 2005).

Bei *Cornu aspersum* konnte gezeigt werden, dass sich das Wachstum nicht vom Fettgehalt beeinflussen lässt, aber dass Fette in größere Eier investiert werden, was die Überlebenschancen der Jungtiere erhöhen kann (Nicolai et al. *Ca*-II). Allgemein wird die Fortpflanzungsinvestition verringert, was zu einer höheren Überlebenschance der Muttertiere im folgenenden Winter führen kann.

In der französischen Forschung zur Schneckenzucht wurden seit 1980 verschiedene industriell hergestellte Schneckenfutter auf Basis von Getreide, Soja und anderen Pflanzen bei *Cornu aspersum* getestet, was Aufschluss über den Energiebedarf und den Einfluss bestimmter Inhaltsstoffe auf die Entwicklung der Schnecke gibt (Charrier & Daguzan 1980, Charrier 1981). Gomot (1994, 1998) hat festgestellt, dass Milchproteine besonders wichtig für die Entwicklung der frisch geschlüpften Jungtiere sind, und Gomot (1994) präzisiert den Bedarf an verschiedenen Elementen in der Nahrung entsprechend dem Entwicklungsstadium der Jungtiere von *Cornu aspersum*: 12,8-13,4% Proteine, 3,4-4,3% Fette, 2,2-2,5% Zellulose, 34,6-35,2% Mineralien davon 12,1-12,8% Calcium. Milchproteine führten bei Jungtieren zu einem höheren Fettgehalt im Schneckenfleisch und bei adulten Schnecken zu einer Anreicherung von Galaktogen in der Eiweißdrüse des Fortpflanzungsapparates (Gomot 1994, 1998).

Bisher wurden noch keine Ernährungsstudien an *Helix pomatia* durchgeführt, obwohl eine artgerechte Schneckenhaltung dessen bedarf. Für diese Studie wurden ein fettreiches und ein fettarmes Futter entwickelt, das sich im Energiegehalt unterscheidet, wobei der Proteingehalt konstant bleibt. Milinsk *et al.* (2003) haben gezeigt, dass eine Anreicherung von Leinöl im Futter zu einer Erhöhung von Linolsäure im Muskel von *Cornu aspersum maxima* führt. Es ist davon auszugehen, dass der unterschiedliche Energiegehalt der Nahrung sich nicht gleichermaßen auf Wachstum und Fortpflanzung in einer wilden und einer Zuchtpopulation auswirkt, sondern eventuell bereits gewonnene Adaptationen zu besseren Wachstums- und Fortpflanzungsergebnissen führen. In diesem Sinne könnten auch Haltungsdichten von Bedeutung sein. Daher wurden in der folgenden Studie nachstehende Hypothesen getestet.

(1) Fettreiches Futter hat keine Auswirkung auf das Wachstum von *Helix pomatia* einer wilden Population oder einer Zuchtpopulation, erhöht aber Überlebenschancen in den Hibernationen während des Wachstums.

(2) Fettreiches Futter verringert die Investition in die Fortpflanzung, erhöht aber den Energiegehalt in den Eiern und damit die Geburtsgröβe der Jungtiere.

(3) Die italienische Zuchtpopulation zeigt allgemein bessere Überlebenschancen und höhere Fortpflanzungserfolge als die wilde Albschneckpopulation.
(4) Eine geringere Dichte führt bei der wilden Albschneckpopulation zu einem höheren Fortpflanzungserfolg und besseren Überlebenschancen als eine hohe Dichte.

Material und Methoden

Adulte Schnecken *Helix pomatia* wurden auf der Schwäbischen Alb gesammelt. In der Region Piemont wurden aus Schneckenzuchtinstitut in Cherasco italienische Zuchtschnecken für die Versuche genutzt. 15 Muttertiere wurden im Frühjahr 2006 im Labor zur Fortpflanzung gebracht. Die Schnecken wurden zu jeweils fünf Individuen in drei Plastikkästen gleicher Größe mit feuchtem Schaumstoff HR (high resilient) und einem Behälter mit Erde (vom Projektstandort) bei experimentellen Klimabedingungen (20°C, 85% relative Luftfeuchtigkeit, 16h Licht/8h Dunkelheit) gehalten. Die Kästen wurden regelmäßig getauscht, um einen Käfigeffekt zu vermeiden, regelmäßig befeuchtet und einmal wöchentlich gesäubert. Die Muttertiere wurden *ad libitum* mit Löwenzahn und dem Futtertyp E ernährt (Tab. 1). Nach jeder Eiablage wurden die Erdbehälter erneuert, so dass in jedem Behälter nur ein Gelege ist. Regelmäßig wurden die Erdbehälter auf geschlüpfte Jungtiere untersucht ohne diese jedoch zu stören. Nach Erscheinen der Jungtiere an der Erdoberfläche werden sie eine Woche lang mit Löwenzahn *Taraxacum officinale* angefüttert.

Tab. 1. Zusammensetzung des Futters (in Prozent %) und Energiegehalt (kcal/kg). Das Futter ist pflanzlich, aus Mais, Soja, Erbse, Weizen, Luzerne und Rüben mit Vitaminen zusammengestellt. Der Kalziumgehalt ergibt sich aus der Zugabe von gemahlenem Kalkgestein des Typs „Albacal 0/315 µm“ aus dem Departement Ile-de-France (Frankreich) und Kalziumphosphat.

	Futtertypen		
	Energiearm (E-)	**Energie-Basis (E)**	**Energiereich (E+)**
Fett[1]	1.5	2.0	7.0
Eiweiße	15.5	15.5	15.5
Stärke und Zucker	19.3	26.9	22.9
Zellwandbestandteile	19.2	9.9	10.9
Feuchtigkeit	9.0	9.6	9.0
Pflanzliche Mineralien	3.1	2.9	3.1
Kalziumphosphat	4.0	4.5	4.5
Kalziumkarbonat	28.5	29	28.0
Energy	2000	2250	2500

[1] Fette bestehen hauptsächlich aus Fettsäuren (FS) von Sojaöl (15% gesättigte FS, 24% einfach ungesättigte FS, 60% mehrfach ungesättigte FS mit 52% Linolfettsäure von allen FS).

Die geschlüpften Jungtiere wurden in der 2. Lebenswoche auf 12 Plastikkästen zu je 17 Individuen verteilt. Auf drei Plastikkästen wurden die verbleibenden Jungtiere verteilt, die als Reserve für verstorbene Jungtiere dienen sollten. Die Kästen enthielten kleine Erdbehälter und in Wasser getränkten Schaumstoff HR. Die Jungtiere wurden *ad libitum* mit drei verschiedenen Futtertypen ernährt (vier Replikate je Futtertyp). Die Futtertypen unterschieden sich in ihrem Energiegehalt (E+, E, E-), was durch die Anreicherung von Fett oder Zellwandbestandteilen erreicht wurde (Tab 1). Nach 8 Wochen Haltung im Labor mit wöchentlicher Wachstumsuntersuchung wurden die Jungtiere ins Freilandgehege versetzt und markiert. Dabei

wurden 12 Parzellen mit jeweils 4 Individuen und 12 Parzellen mit jeweils 12 Individuen besetzt. Die Haltungsdichten in den Parzellen betrugen 57 bzw. 171 Individuen/m². Diese Haltungsdichten sollen Annäherungen an die Haltungsdichten der Jungtiere (50 Individuen/m²) in der Albschneck®-Anlage bzw. an die Haltungsdichte von Jungtieren in intensiv bewirtschafteten Zuchtanlagen (150 Individuen/m²) darstellen. Aus technischen Gründen mussten die Haltungsdichten in den Versuchen leicht erhöht werden. In den Parzellen aus Plastiknetzen wurden die Schnecken auf dem natürlichen Substrat ohne Pflanzenbewuchs gehalten. Moos und Holzstücke standen ihnen als Schutz vor Kälte und Trockenheit zur Verfügung. Um eine erhöhte Sterberate durch ungünstige Witterungsbedingungen zu vermeiden, wurden die Gehege gegen Frost im Winter mit Laub und Gartenvlies geschützt und im Sommer bei zu lang anhaltender Trockenheit bewässert. Die Jungtiere wurden weiterhin *ad libitum* mit den drei verschiedenen Futtertypen ernährt (Tab. 1), so dass je Haltungsdichte und Futtertyp vier Replikate untersucht werden konnten.

Alle zwei Wochen wurden Messungen an den markierten Schnecken durchgeführt. Um Wachstum oder Auswirkungen von saisonalen Wetterveränderungen dokumentieren zu können, wurde der Gehäusedurchmesser mit einem digitalen Messschieber (TCM Digital Caliper, Bremen, Deutschland) ermittelt und die gesäuberte Schnecke auf einer digitalen Waage (OHAUS CT200-S, Nänikon, Schweiz) gewogen. Etwaige tote Tiere wurden notiert, um die Sterberate zu bestimmen.

Sobald die Schnecken die geschlechtliche Reife erreicht hatten, wurden sie zu jeweils 10 Individuen in Plastikkästen im Labor entsprechend des bereits beschriebenen Protokolls zur Fortpflanzung gebracht. Ab der ersten Eiablage Mitte Juni 2008 wurde die Fortpflanzungsperiode auf einen Monat beschränkt, um die Tiere dann wieder in natürliche Bedingungen zu versetzen, wo zu diesem Zeitpunkt die Ästivation einsetzte. Die Gelege wurden der Mutterschnecke zugeordnet, die Eier gezählt, jeweils 20 Eier gewogen, und mindestens 10 Eier wurden in Erde zur Inkubation gelegt. Die restlichen Eier wurden für spätere biochemische Analysen bei -80°C gelagert. Nach dem Schlüpfen der Jungtiere wurden diese gewogen und für spätere biochemische Analysen bei -80°C gelagert.

Für biochemische Analysen wurden Eier verschiedener Gelege gemischt und homogenisiert. Aus diesem Gemisch wurden 6 Analysen jeder Sustanz durch geführt. Triglycerides (TG), Cholesterin (Chol), Glykogen (GLY) und Galaktogen (GAL) wurden entsprechend dem Protokoll von (Hervant *et al.* 1999) für Fette und von (Van Handel 1965) für Polysaccharide extrahiert. Zellen wurden mit Bead Beating (4 x 30 s at 30 agitations.s^{-1}, Retsch™ MM301, Retsch GmbH, Haan, Germany) unter Nutzung von 3-mm-großen Tungsten-Perlen durchgeführt nachdem 1.5 ml Folch-Lösung (Methanol:Chloroform 1:2 v/v) für Fettextraktion und 2.5 ml Trichloroacetic acid (4%) für Polysaccharidextraktion zu den getrockneten und gewogenen Eiern gegeben wurde. Für die TG- und Chol-Analyse wurde das Gemisch 12 h lang bei –20°C gelagert, um die Fettextraktion zu maximieren. Anschließend wurde die untere Fettphase von der oberen wasserlöslichen Phase durch Zugabe von 300 µl KCl (2 g.l^{-1}) unter 5-minütiger Erwärmung bei 40°C getrennt. Polysaccharide, die in 2 ml flüssiger Phase nach der Zentrifugation (5000 g, 5 min, 4°C, Sigma® 2-16K, Fisher Bioblock Scientific, USA) enthalten waren, wurden mit 4 ml Ethanol (96°) ausgefällt. Nach einer weiteren Zentrifugation wurde die Ausfällung in 1 ml Wasser aufgelöst und nochmals mit 2 ml Ethanol (96°) gefolgt von einer Zentrifugation ausgefällt. 500 µl der extrahierten Fettphase wurden unter Stickstoffzufuhr bei 30°C für 15 min getrocknet und dann in 300 µl BSA (3% w/v, fatty acid

free) - Triton (0.2% v/v) für die TG-Analyse und in 10 µl Ethanol (95°) für die Chol-Analyse aufgelöst. Für die GLY-Analyse wurde die Polysaccharidablagerung in 1 ml Wasser aufgelöst und 12 h bei 21°C gelagert, um die Auflösung der Polysaccharide im Wasser zu maximieren. Für die GAL-Analyse wurde die Ablagerung mit 500 µl HCl (6M) für 6 h bei 110°C in Galaktose hydrolisiert und anschließend mit 500 µl KOH (6M) neutralisiert. Der Gehalt von TG, Chol, GLY und Galaktose wurde durch das Messen der Absorption mit einem Mikroplattenspectrophotometer (VERSAmax™ microplate reader, Sunnyvale, CA, USA) bestimmt. Hierzu wurde das Triglyceride Assay Kit (Cayman Chemicals, Ann Arbor, Michigan, USA), das Cholesterol RTU™ Kit (Biomerieux, Craponne, France) und das Enzyplus® EZS 784+ Lactose/D-Galactose Kit (BIOCONTROL, Bellevue, WA, USA) genutzt. Die GLY-Lösung wurde zentrifugiert und mit 10 µl Lugol versetzt, um die Absorption bei 425 nm zu messen. Mit Kalibrierungskurven wurde der Gehalt relativ zur Absorption bestimmt.

Alle statistischen Analysen erfolgten mit dem Programm „R“ Version 2.5.0 (2007) unter Nutzung der Hinweise von Crawley (2007). Für die Wachstumskurven von der 2. bis 8. Woche wurde ein „Mixed Effect Model“ verwendet. Für die gesamte Wachstumskurve (2. Woche bis Wachstumsende, Hibernationsperioden ausgeschlossen) wurde eine logarithmisches Modell verwendet und die Parameter mit *T*-Tests verglichen. Als Varianzanalyse von Endgrößen wurde das „Generalized Linear Model“ verwendet. Für die Sterberate wurde eine „Survival Analysis“ mit Weibull Distribution durchgeführt und für die Fortpflanzungsdaten wurden ANOVA und *T*-Tests genutzt.

Ergebnisse

Da das Gewicht stark mit der Größe (D) der Schnecken korreliert (Madec 1989), werden nur Ergebnisse zur Größe dargestellt. 204 Jungtiere der ALB-Population und 68 Jungtiere der IT-Population schlüpften drei Wochen nach der Eiablage. Erst eine Woche bzw. zwei Wochen später erschienen sie an der Erdoberfläche und begannen Löwenzahn zu fressen. Nach dieser einwöchigen Anfütterungsphase begannen sie auch das Versuchsfutter zu akzeptieren. Zum Zeitpunkt des Erscheinens an der Erdoberfläche hatten die Jungtiere eine Größe von: 7,67 ± 0,07 mm (ALB) und: 7,10 ± 0,12 mm (IT) mit einer geringen Standardabweichung (T-test, $t_{270} = 3{,}97$, $P < 0{,}0001$, $N = 272$).

In der 8. Lebenswoche erreichten die Jungtiere unterschiedliche Größen von 12,6 ± 3,5 mm für ALB und 10,3 ± 1,9 mm für IT unabhängig vom Futtertyp (Abb. 1, Generalized Linear Model, Gamma distribution, inverse link function, total deviance reduction 10,07%, Herkunft $F = 16{,}19$, $P < 0{,}0001$, Futter und Interaktion $P > 0{,}05$ ns.). Die Standardabweichung stieg mit dem Alter und der Größe der Jungtiere an. Der Wachstumsverlauf unterschied sich zwischen den Populationen (Mixed Effect Model, repeated measurments, Herkunft, $F = 99{,}71$, $P < 0{,}0001$, Futter und Interaktion $P > 0{,}05$ ns.).

Die Überlebensrate innerhalb der ersten 8 Lebenswochen unterschied sich signifikant zwischen den Populationen und den Futtertypen (Abb. 2, Survival Analysis, Weibull distribution, Herkunft $P < 0{,}05$, Futter $P < 0{,}05$, Interaktion $P < 0{,}05$). Bei den Albschnecken war sie bis zum Tag 32 stabil und fiel erst danach bis auf 61% bei den Futtertypen E+ und E ab und auf 32% bei E- (Survival Analysis, Weibull distribution, Futter E-≠E=E+, $\chi^2 = 19{,}03$, $P < 0{,}0001$). Bei den italienischen Jungtieren verhielt es sich entgegengesetzt. Ab dem 32. Tag fiel

die Überlebensrate auf 50% unabhängig vom Futtertyp (Survival Analysis, Weibull distribution, Futter E+ ≠ E = E-,χ^2 = 2,78, *P* = 0,25).

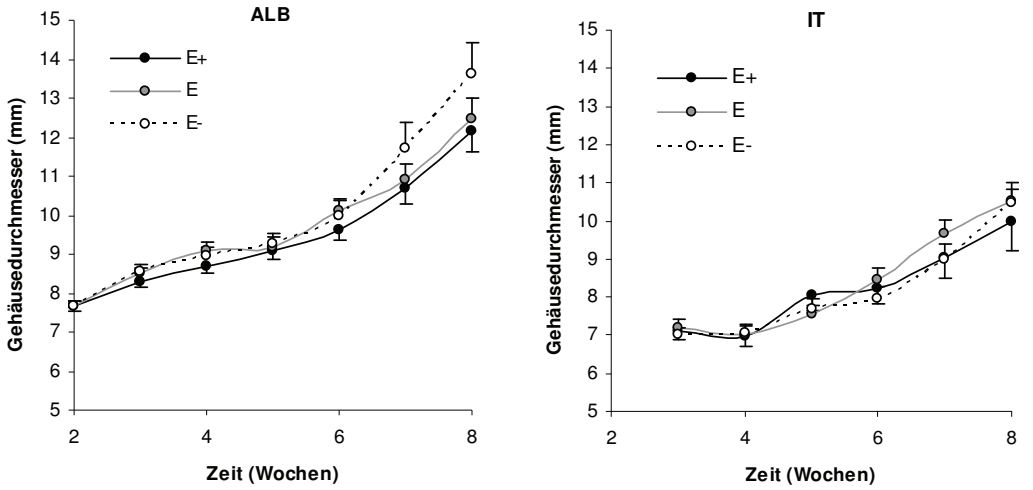

Abb. 1. Wachstum der ersten acht Lebenswochen der Jungtiere von *Helix pomatia* von der Schwäbischen Alb (ALB) und aus Italien (IT). ALB: N=45/44/25 für E+/E/E-, IT: N=11/13/14 für E+/E/E-.

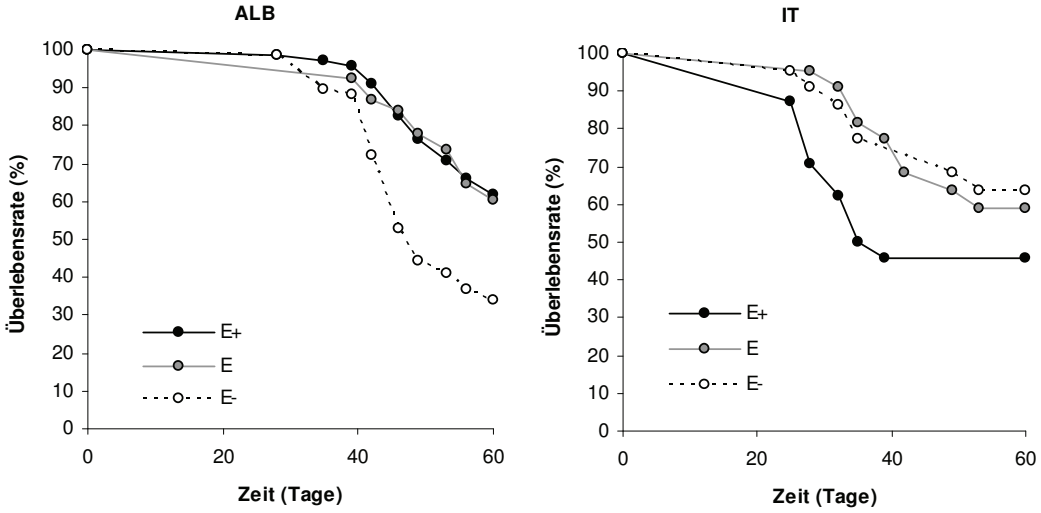

Abb. 2. Kumulierte Sterberate der Jungtiere von *Helix pomatia* von der Schwäbischen Alb (ALB) und aus Italien (IT) im Verlauf der ersten 8 Lebenswochen. ALB: N=45/44/25 für E+/E/E-, IT: N=11/13/14 für E+/E/E.

Nach dem Aussetzen der Jungtiere ins Freilandgehege stagnierte das mittlere Wachstum beider Populationen nach einigen Wochen bis zum Einsetzen der Überwinterung (Abb. 3). Vom Sterben betroffen waren hauptsächlich Jungtiere, die mit ihrer erreichten Größe unterhalb der mittleren Wachstumskurve lagen. Im Frühjahr 2007 (35. bis 50. Woche) setzte ein starkes Wachstum ein und Schnecken starben, wenn sie unterhalb der Wachstumskurve lagen. Zwischen Sommer und Winter stagnierte das Wachstum, um dann wieder im Frühjahr 2008 (90. bis 100. Woche) einzusetzen. Bereits im Frühjahr 2008 erreichten die Schnecken die geschlechtliche Reife, was im natürlichen Lebensraum erst ein bis zwei Jahre später einsetzen würde (Kilias 1985). Während den Überwinterungen (15. – 35. Woche und 75. -90. Woche) waren Tiere aller Größe vom Sterben betroffen.

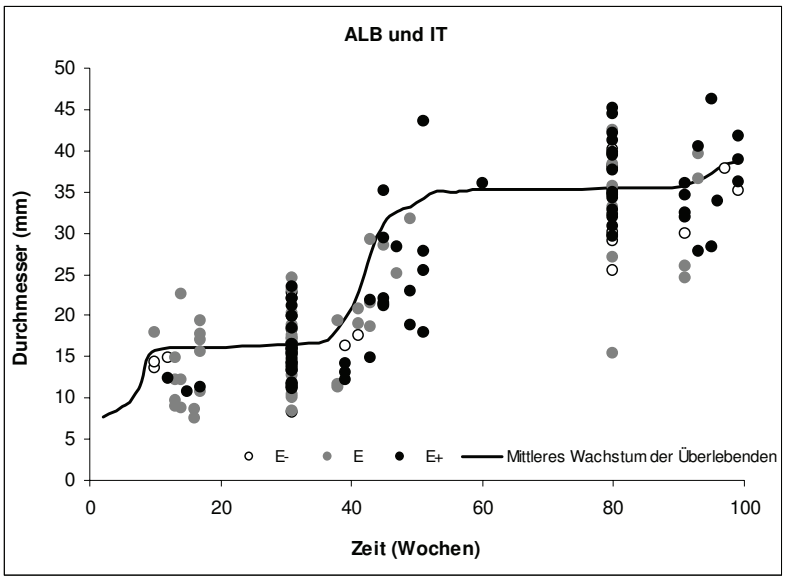

Abb. 3. Größe der gestorbenen Schnecken im Verlauf der Wachstumsperiode und allgemeine Wachstumskurve aller beobachteten Schnecken (ALB und IT in allen Dichten und mit allen Futtertypen) für *Helix pomatia*. *N* = 52 für die Wachstumskurve.

Die simulierten Wachstumskurven zeigen eine hohe Variabilität im Wachstum der einzelnen Schnecken (Abb. 4, Tab. 2 - 5). Energiereiches Futter (E+) bewirkt ein schnelleres Wachstum mit geringerer Maximalgröße bei den Albschnecken in hoher Haltungsdichte. Bei geringer Haltungsdichte ist die Maximalgröße mit diesem Futtertyp allerdings höher als bei hoher Haltungsdichte. Dennoch erreichen Schnecken mit dem Basisfutter (E) die größte Maximalgröße bei geringer Haltungsdichte. Gegenüber der hohen Haltungsdichte haben sie eine geringere Wachstumsrate und erreichen eine Woche später die halbe Maximalgröße. Die italienischen Schnecken haben mit allen Futtertypen die gleiche Größe, so dass mit E+ die Maximalgröße höher als bei den Albschnecken mit gleichem Futter ist. Auch sie haben eine geringere Wachstumsrate als die Albschnecken.

Tab. 2. Parameter der theoretischen Wachstumskurven $Y_D = a / [1 + \exp(-(x - x_{mid})/s)]$ von *Helix pomatia* zweier Populationen (ALB – Schwäbische Alb und IT – Italien) in verschiedenen Haltungsdichten und verschiedener Nahrungstypen. Y_D ist die Größe der Schnecken, *a* ist die Asymptote (Maximagröße), *s* ist Scal (die umgekehrte Wachstumsrate), *x* ist die Zeit, und x_{mid} ist der Zeitpunkt des Erreichen der Hälfte der Maximalgröße. Das Wachstum wurde von der zweiten Lebenswoche an bis zum Wachstumsende der Jungtiere simuliert. *N* ist in der Tabelle gegeben

Herkunft	Dichte	Futtertyp	N	Asym (a)	Scal (s)	x_{mid}
ALB	hoch	E-	5	37,56 ± 0,72	0,50 ± 0,03	13,18 ± 0,58
		E+	10	34,03 ± 0,49	0,51 ± 0,02	12,48 ± 0,39
		E	11	37,51 ± 0,49	0,52 ± 0,02	13,20 ± 0,36
	niedrig	E-	2	37,48 ± 0,59	0,63 ± 0,03	11,24 ± 0,37
		E+	4	36,05 ± 0,57	0,49 ± 0,02	12,49 ± 0,44
		E	6	40,78 ± 0,47	0,59 ± 0,02	12,95 ± 0,30
IT	niedrig	E-	4	39,40 ± 0,43	0,59 ± 0,02	13,89 ± 0,31
		E+	4	40,65 ± 0,51	0,68 ± 0,03	12,74 ± 0,35
		E	6	40,15 ± 0,56	0,60 ± 0,03	14,05 ± 0,39

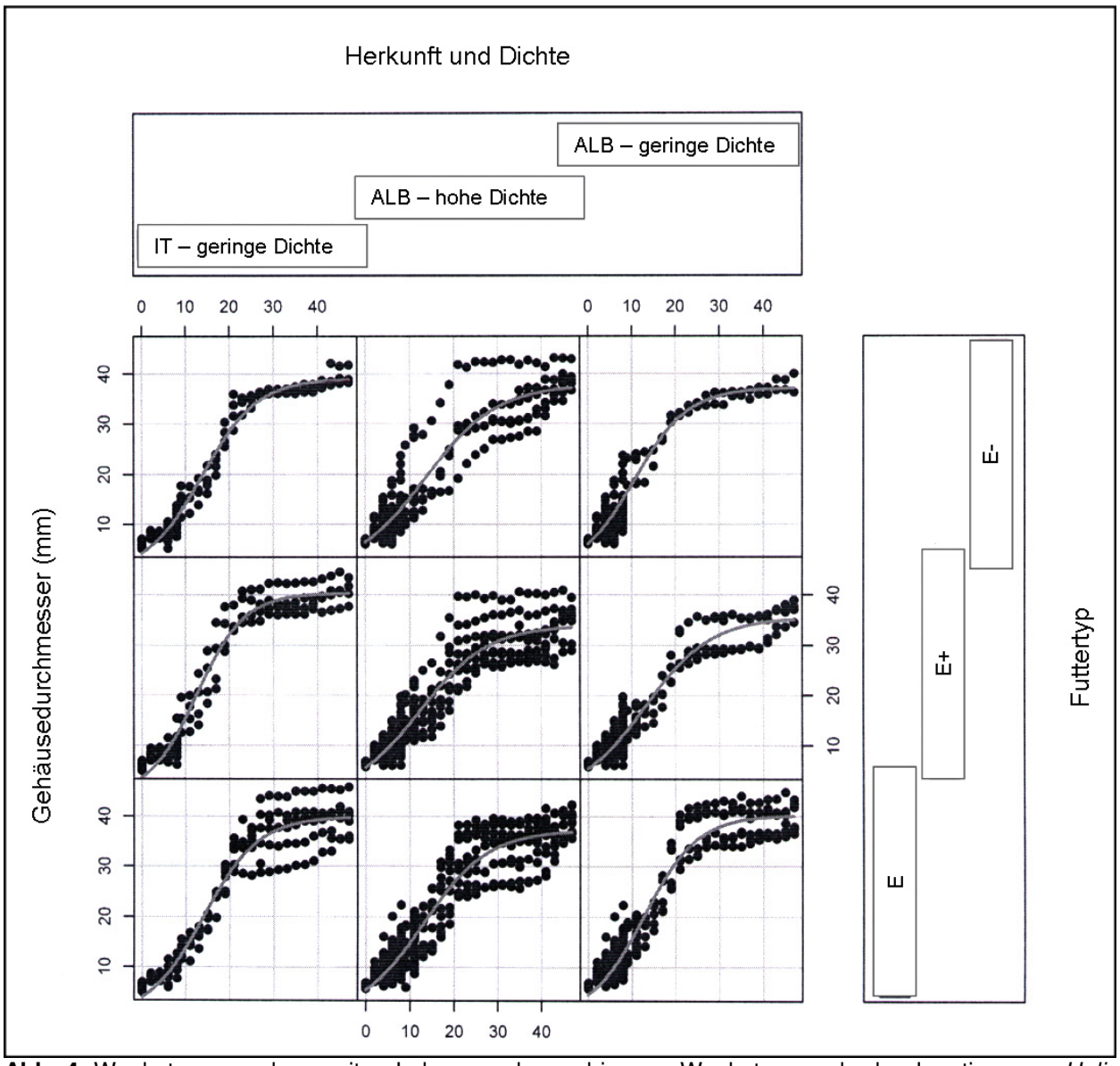

Abb. 4. Wachstum von der zweiten Lebenswoche an bis zum Wachstumsende der Jungtiere von *Helix pomatia* von der Schwäbischen Alb (ALB) und aus Italien (IT). ALB-geringe Dichte: N=6/4/2 für E+/E/E-, ALB-hohe Dichet: N=11/10/5 für E+/E/E- und IT: N=6/4/4 für E+/E/E-. Theoretische Wachstumskurven $Y_D = a / [1 + \exp(-(x - x_{mid})/s)]$ sind in grau dargestellt Y_D ist die Größe der Schnecken, a ist die Asymptote (Maximagröße), s ist Scal (die umgekehrte Wachstumsrate), x ist die Zeit, und x_{mid} ist Zeitpunkt des Erreichen der Hälfte der Maximalgröße.

Tab. 3. Ergebnisse der T-tests für scal (umgekehrte Wachstumsrate) der Wachstumskurven von *Helix pomatia* zweier Populationen (ALB – Schwäbische Alb und IT – Italien) in verschiedenen Haltungsdichten und verschiedener Nahrungstypen. ALB-geringe Dichte: N=6/4/2 für E+/E/E-, ALB-hohe Dichet: N=11/10/5 für E+/E/E- und IT: N=6/4/4 für E+/E/E-. Theoretische Wachstumskurve $Y_D = a / [1 + \exp(-(x - x_{mid})/s)]$: Y_D ist die Größe der Schnecken, a ist die Asymptote (Maximagröße), s ist Scal (die umgekehrte Wachstumsrate), x ist die Zeit, und x_{mid} ist der Zeitpunkt des Erreichen der Hälfte der Maximalgröße.

		Herkunft	**ALB**						**IT**		
		Dichte	**hoch**			**Niedrig**					
Herkunft	**Dichte**	**Futtertyp**	**E-**	**E+**	**E**	**E-**	**E+**	**E**	**E-**	**E+**	**E**
ALB	**hoch**	**E-**				*T* = 2,508 *P* > 0.05					
		E+	*T* = 0,329 *P* > 0,05				*T* = 0,576 *P* > 0,05				
		E	*T* = 0,583 *P* > 0,05	*T* = 0,288 *P* > 0,05				***T* = 2,195** ***P* = 0,044**			
	niedrig	**E-**									
		E+				***T* = 3,593** ***P* = 0,023**					
		E				*T* = 0,992 *P* > 0,05	***T* = 3,225** ***P* = 0,012**				
IT		**E-**				*T* = 0,956 *P* > 0,05					
		E+					***T* = 4,801** ***P* = 0,003**		*T* = 2,242 *P* > 0,05		
		E						*T* = 0,335 *P* > 0,05	*T* = 0,224 *P* > 0,05	*T* = 1,817 *P* > 0,05	

Tab. 4. Ergebnisse der T-tests für Asymptote (theoretisch maximal erreichte Größe) der Wachstumskurven von *Helix pomatia* zweier Populationen (ALB – Schwäbische Alb und IT – Italien) in verschiedenen Haltungsdichten und verschiedener Nahrungstypen. ALB-geringe Dichte: N=6/4/2 für E+/E/E-, ALB-hohe Dichet: N=11/10/5 für E+/E/E- und IT: N=6/4/4 für E+/E/E-. Theoretische Wachstumskurve $Y_D = a / [1 + \exp(-(x - x_{mid})/s)]$: Y_D ist die Größe der Schnecken, a ist die Asymptote (Maximagröße), s ist Scal (die umgekehrte Wachstumsrate), x ist die Zeit, und x_{mid} ist der Zeitpunkt des Erreichen der Hälfte der Maximalgröße.

		Herkunft	**ALB**						**IT**		
		Dichte	**hoch**			**Niedrig**					
Herkunft	**Dichte**	**Futtertyp**	**E-**	**E+**	**E**	**E-**	**E+**	**E**	**E-**	**E+**	**E**
ALB	**hoch**	**E-**				*T* = 0,056 *P* > 0,05					
		E+	***T* = 3.835** ***P* = 0,002**				***T* = 2.205** ***P* = 0,048**				
		E	*T* = 0,56 *P* > 0,05	***T* = 4,769** ***P* > 0,0001**				***T* = 4,087** ***P* = 0,001**			
	niedrig	**E-**									
		E+				*T* = 1,287 *P* > 0,05					
		E				***T* = 3,239** ***P* > 0,018**	***T* = 5.723** ***P* = 0,0004**				
IT		**E-**				*T* = 2,133 *P* > 0,05					
		E+					***T* = 5,187** ***P* = 0,002**		*T* = 1,611 *P* > 0,05		
		E						*T* = 0,789 *P* > 0,05	*T* = 0,871 *P* > 0,05	*T* = 0,557 *P* > 0,05	

Tab. 5. Ergebnisse der T-tests für x_{mid} (Zeitpunkt des Erreichen der Hälfte der Maximalgröße) der Wachstumskurven von *Helix pomatia* zweier Populationen (ALB – Schwäbische Alb und IT – Italien) in verschiedenen Haltungsdichten und verschiedener Nahrungstypen. ALB-geringe Dichte: N=6/4/2 für E+/E/E-, ALB-hohe Dichet: N=11/10/5 für E+/E/E- und IT: N=6/4/4 für E+/E/E-. Theoretische Wachstumskurve $Y_D = a / [1 + \exp(-(x - x_{mid})/s)]$: Y_D ist die Größe der Schnecken, a ist die Asymptote (Maximagröße), s ist Scal (die umgekehrte Wachstumsrate), x ist die Zeit, und x_{mid} ist der Zeitpunkt des Erreichen der Hälfte der Maximalgröße.

		Herkunft	**ALB**						**IT**		
		Dichte	**hoch**			**Niedrig**					
Herkunft	**Dichte**	**Futtertyp**	**E-**	**E+**	**E**	**E-**	**E+**	**E**	**E-**	**E+**	**E**
ALB	**hoch**	**E-**				$T = 1,746$ $P > 0,05$					
		E+	$T = 0,963$ $P > 0,05$				$T = 1,526$ $P > 0,05$				
		E	$T = 0,026$ $P > 0,05$	$T = 1,302$ $P > 0,05$				$T = 0,431$ $P > 0,05$			
	niedrig	**E-**									
		E+				$T = 2,703$ $P > 0,05$					
		E				***T* = 2,644** ***P* = 0,038**	$T = 0,935$ $P > 0,05$				
IT		**E-**				***T* = 4,288** ***P* = 0,013**					
		E+					$T = 1,161$ $P > 0,05$		$T = 2,160$ $P > 0,05$		
		E						$T = 2,029$ $P > 0,05$	$T = 0,262$ $P > 0,05$	$T = 2,110$ $P > 0,05$	

Die reellen Größenunterschiede der Schnecken zu Wachstumsende waren signifikant, hauptsächlich durch die Fütterung hervorgerufen (Generalized Linear Model, Gaussian distribution, identity link function, total deviance reduction 32,78%, Herkunft $P = 0,0039$ und deviance reduction 18,31%, Futter $P = 0,04$ und deviance reduction 49,40%, Dichte $p > 0,05$ ns., Interaction Herkunft x Futter $P = 0,03$ und deviance reduction 32,29%). Dabei ist die energiereiche Fütterung gegenüber der Basisfütterung bei den Albschnecken in beiden Haltungsdichten für eine geringere Größe verantwortlich (geringe Dichte: E = 39,96 ± 2,96 mm und E+ = 37,01 ± 1,84 mm, Tukey HSD, $P = 0,0039$; hohe Dichte: E = 39,28 ± 2,20 mm und E+ = 35,10 ± 3,38 mm, Tukey HSD, $P = 0,044$). Bei geringer Haltungsdichte erreichten die Albschnecken auch eine geringere Größe als die italienischen Schnecken bei gleicher energiereicher Fütterung (ALB: E+ = 37,01 ± 1,84 mm, IT: E+ = 40,78 ± 2,41 mm, Tukey HSD $P = 0,023$).

Die Entwicklung der Überlebensrate von der achten Lebenswoche bis zum Wachstumsende (Abb. 5) bei geringer Haltungsdichte unterscheidet sich nicht signifikant zwischen den Populationen für alle Futtertypen (Survival Analysis, Weibull distribution, Herkunft und Futter $P > 0,05$ ns.). Innerhalb der iatlienischen Population hat der Futtertyp keine Auswirkung auf die Überlebensrate (Survival Analysis, Weibull distribution, $P > 0,05$ ns.). Bei den Albschnecken hängt die Überlebensrate nicht von der Haltungsdichte ab (Survival Analysis, Weibull distribution,Dichte, $P > 0,05$ ns). Bei hoher Dichte hat der Futtertyp keine Auswirkung auf die Überlebensrate (Survival Analysis, Weibull distribution, Futter $P > 0.05$). Bei geringer Haltungsdichte dagegen erreicht die Überlebensrate 20%, auβer beim Basisfutter (E), wo sie 40% erreicht (Survival Analysis, Weibull distribution, Futter $P = 0,034$). Wogegen die Überlebensrate bei hoher Haltungsdichte gleichmäßig abfällt, sind bei geringer Dichte deutlich zwei Phasen zu unterscheiden. Die erste Phase stärkeren Absinkens für E- und E endet mit dem

Erwachen aus der ersten Hibernation (40. Woche). Die zweite Phase stärkeren Absinkens gilt nur für E+ während der zweiten Hibernation (Erwachen in der 90. Woche).

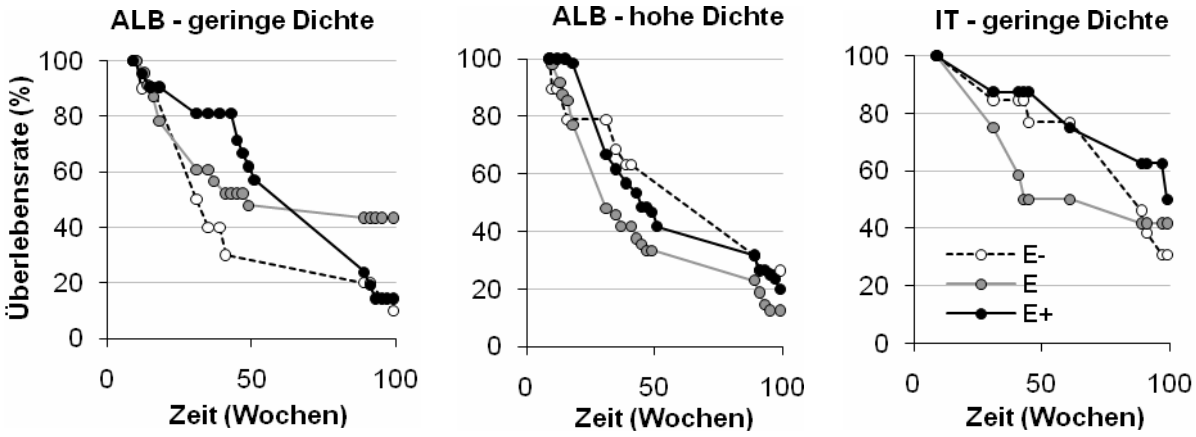

Abb. 5. Kumulierte Sterberate der Jungtiere von *Helix pomatia* von der Schwäbischen Alb (ALB) und aus Italien (IT) bei geringer und hoher Haltungsdichte mit verschiedenen Futtertypen gefüttert. ALB – geringe Dichte: N=6/4/2 für E+/E/E-, ALB – hohe Dichte: N=11/10/5 für E+/E/E- und IT: N=6/4/4 für E+/E/E-.

Die Fortpflanzungsaktivität war nur gering oder sogar nicht existierend (E+), bedingt durch die niedrige Anzahl an überlebenden Schnecken. Dennoch wurden Unterschiede in der Eiablage und der geschlüpften Jungtiere erkannt (Tab. 6). Die Albschnecken legten unabhängig vom Futter und der Dichte eine größere Anzahl an Eiern als die italienischen Schnecken. Während bei den italienischen Schnecken der Energiegehalt eines Geleges positiv zum Energiegehalt des Futters variiert, bei gleich bleibender Gelegegröße, hängt der Energiegehalt der Gelege der Albschnecken von der Gelegegröße ab und variiert negativ zum Energiegehalt des Futters. Diese Einschätzungen können nicht statistisch unterstützt werden, da die Anzahl der Gelege zu gering ist.

Tab. 6. Angaben zur Fortpflanzung bei *Helix pomatia* verschiedener geographischer Herkunft und verschiedener Haltungsmethoden (niedrige und hohe Haltungsdichte) ernährt mit energiearmen (E-) und energiereichen (E+) Nahrung, sowie mit Nahrung mittlerem Energiegehaltes (E).

	ALB						**IT**		
	Geringe Dichte			**Hohe Dichte**			**Geringe Dichte**		
	E-	E	E+	E-	E	E+	E-	E	E+
fortgepflanzende Schnecken	-	4	-	3	4	-	1	2	4
Anzahl der Gelege		4		4	4		2	4	4
Mittlere Anzahl der Eier im Gelege		59		84	61		38	42	36
Geschätzte Energie für Karbohydrate und Fette pro Gelege (kJ)		0.76		1.61	1.13		0.49	1.15	1.51
Mittlere Geburtenrate		0		0.8	0.5		0.6	0.7	0.3

Die Analyse von Reservestoffen in den Schneckeneiern ergab Unterschiede in der Eifeucht- und Eitrockenmasse sowie im Gehalt von Glykogen und Galaktogen, allerdings nicht im Gesamtfettgehalt (Tab. 7, 8). Die Ergebnissdarstellung erfolgte entsprechend der beeinflussenden Faktoren: Nahrungsenergiegehalt, Haltungsdichte und geographische Herkunft.

Nahrungsenergiegehalt: Die Albschnecken legten Eier der gleichen Größe mit geringerer Trockenmasse und höherem Wassergehalt wenn sie mit der Nahrung E gefüttert wurden,

verglichen zu der Nahrung E-. Galaktogen ist der Hauptreserveteil der Schneckeneier, der sich allerdings nicht zwischen den Nahrungstypen unterschied. Glykogen dagegen war stärker in den Eiern E vertreten als in E-, hatte aber nur wenig Einfluss auf den gesamt Kohlenhydratgehalt (GLY+GAL). Die Eier E hatten einen höheren Cholesteringehalt als E-, der allerdings so gering war, dass er nicht den Gesamtfettgehalt beeinflusste. Triglyceridspeicherung war unabhängig vom Nahrungsenergiegehalt. Damit ist auch der Energiegehalt der Reservestoffe der Eier unabhängig vom Energiegehalt der Nahrung. Das hatte Einfluss auf die geschlüpften Jungtiere (Tab. 9, 10), die sich auch nicht im Energiegehalt der Körperreservestoffe unterschieden, allerdings im Gewicht. Jungtiere von Eiern E, mit dem höheren Cholesterin- und Glykogengehalt, hattrn ebenfalls ein höheres Gewicht nach dem Schlüpfen.

Die italienischen Schnecken legten größere Eier, wenn sie mit energiearmer Nahrung E- und E gefüttert wurden. Die Trockenmasse war jedoch unabhängig von der Nahrung, so dass der Wassergehalt bei diesen Eiern auch größer war. Während der Fett- und der Glykogengehalt nicht mit dem Nahrungstyp variierten, ist der Galaktogengehalt bei den Eiern E- deutlich geringer als bei den Eiern E und E+. Letzteres beeinflusst den Energiegehalt der Reservestoffe, der in den Eiern E und E+ höher war und somit auch zu größeren Jungtieren mit höherem Energiegehalt führte.

Dichte: Bei geringerer Dichte legten die Albschnecken größere Eier mit einer größeren Trockenmasse, allerdings mit geringerem Glykogengehalt, der nur wenig Einfluss auf den Gesamtenergiegehalt der Reservestoffe hatte. Unterschiede im Fettgehalt wurden nicht beobachtet. Das komplette Ausbleiben an Jungtieren bei geringer Dichte ließ jedoch keinen Vergleich der Jungtiere zu.

Geographische Herkunft: Die Eigröße unterschied sich bei Albschnecken und italienischen Schnecken. Feucht- und Trockenmasse sowie Wassergehalt waren bei den italienischen Schnecken höher als bei den Albschnecken. Da der Gehalt an den verschiedenen Reservestoffen gleich war, hatten die italienischen Schnecken energiereichere Eier. Das komplette Ausbleiben an Jungtieren bei den Albschnecken in geringer Dichte ließ jedoch keinen Vergleich der Jungtiere zu.

Tab. 7. Reservestoffe und Energiegehalt in Schneckeneiern von *Helix pomatia* unterschiedlicher geographischer Herkunft, Dichte und Nahrung (E-: energiearm, E: mittlerer Energiegehalt, E+: energiereich). DM-Trockenmasse.

	ALB			**IT**		
	Geringe Dichte	**Hohe Dichte**		**Geringe Dichte**		
	E	**E-**	**E**	**E-**	**E**	**E+**
Frischmasse der Eier (mg)	73.73 ± 1.85	69.90 ± 1.80	65.90 ± 1.54	100.39 ± 3.72 [a]	100.01 ± 3.26 [a]	89.72 ± 3.09 [b]
Trockenmasse der Eier (mg)	20.73 ± 0.44	19.93 ± 0.40	17.76 ± 0.20	26.91 ± 0.92 [a]	25.21 ± 0.85 [a]	26.56 ± 0.76 [a]
Wassergehalt der Eier (mg/mg DM)	2.57 ± 0.07	2.48 ± 0.06	2.73 ± 0.10	2.74 ± 0.06 [a]	2.98 ± 0.07 [a]	2.39 ± 0.09 [b]
Galaktogengehalt (µg/mg DM)	26.15 ± 9.07	44.66 ± 12.56	51.80 ± 15.79	18.31 ± 4.88 [a]	53.03 ± 11.09 [ab]	81.55 ± 12.72 [b]
Glykogengehalt (µg/mg DM)	0.04 ± 0.004	0.04 ± 0.008	0.08 ± 0.01	0.03 ± 0.006 [a]	0.03 ± 0.01 [a]	0.02 ± 0.003 [a]
Totaler Fettgehalt (µg/mg DM)	4.37 ± 0.33	4.93 ± 1.34	3.96 ± 0.96	4.27 ± 0.40 [a]	4.43 ± 0.45 [a]	4.43 ± 0.38 [a]
Triglyceridgehalt (µg/mg DM)	0.03 ± 0.006	0.09 ± 0.03	0.03 ± 0.003			
Cholesteringehalt (µg/mg DM)	0.005 ± 0.002	0.002 ± 0.0003	0.008 ± 0.002			
Energiegehalt (J/Ei)	12.87 ± 3.19	19.15 ± 4.65	18.59 ± 4.46	12.97 ± 2.56 [a]	27.36 ± 5.10 [b]	41.86 ± 5.89 [b]

Tab. 8. Ergebnisse der statistischen Analysen (T-test, F-Test) der Reservestoffe und des Energiegehaltes in Schneckeneiern von Helix pomatia unterschiedlicher geographischer Herkunft, Dichte und Nahrung (E-: energiearm, E: mittlerer Energiegehalt, E+: energiereich). DM-Trockenmasse.

	Faktor Futtertyp		**Faktor Dichte**	**Faktor Herkunft**
	ALB – hohe Dichte	**IT- geringe Dichte**	**ALB - E**	**Geringe Dichte - E**
Frischmasse der Eier (mg)	T_{76} = 1.49, P = 0.14	$F_{2,54}$ =3.23, P = 0.047	**T_{72} = 3.21, P = 0.002**	**T_{60} = 7.57, P < 0.0001**
Trockenmasse der Eier (mg)	**$T_{60.96}$ = 4.87, P < 0.0001**	$F_{2,54}$ = 1.17, P =0.32	**$T_{52.80}$ = 6.17, P < 0.0001**	**$T_{33.96}$ = 4.68, P < 0.0001**
Wassergehalt der Eier (mg/mg DM)	**$T_{54.39}$ = 2.10, P = 0.040**	**$F_{2,54}$ = 16.01, P<0.0001**	T_{72} = 1.31, P = 0.19	**T_{60} = 3.82, P = 0.0003**
Galaktogengehalt (µg/mg DM)	T_{10} = 0.35, P = 0.73	**$F_{2,18}$ = 9.75, P = 0.001**	T_{10} = 1.41, P = 0.19	T_{11} = 1.83, P = 0.09
Glykogengehalt (µg/mg DM)	T_{10} = 2.78, P = 0.019	$F_{2,18}$ = 0.47, P = 0.63	**$T_{6.17}$ = 2.47, P = 0.047**	T_{11} = 0.91, P = 0.38
Totaler Fettgehalt (µg/mg DM)	T_{10} = 0.59, P = 0.57	$F_{2,18}$ = 0.05, P = 0.95	$T_{6.15}$ = 0.41, P = 0.70	T_{11} = 0.11, P = 0.92
Triglyceridgehalt (µg/mg DM)	$T_{5.13}$ = 2.04, P = 0.10		T_{10} = 0.13, P = 0.90	
Cholesteringehalt (µg/mg DM)	**$T_{5.30}$ = 3.31, P = 0.020**		T_{10} = 0.81, P = 0.44	$T_{3.04}$ = 1.78, P = 0.17
Energiegehalt (J/Ei)	T_{10} = 0.09, P = 0.93	**$F_{2,18}$ = 9.31, P = 0.002**	T_{10} = 1.36, P = 0.20	**T_{11} = 2.31, P = 0.040**

Tab. 9. Reservestoffe und Energiegehalt in frisch geschlüpften Jungtieren von *Helix pomatia* unterschiedlicher geographischer Herkunft, Dichte und Nahrung (E-: energiearm, E: mittlerer Energiegehalt, E+: energiereich). DM-Trockenmasse.

	ALB - hohe Dichte		**IT - geringe Dichte**		
	E-	**E**	**E-**	**E**	**E+**
Frischmasse der Jungtiere (mg)	72.00 ± 2.00	84.12 ± 3.64	91.00 ± 3.79 ^{a}	123.75 ± 8.44 ^{b}	115.45 ± 3.66 ^{b}
Trockenmasse der Jungtiere(mg)	21.38 ± 1.26	23.01 ± 1.14	26.03 ± 1.72 ^{a}	34.90 ± 1.74 ^{b}	31.29 ± 1.74 ^{ab}
Wassergehalt der Jungtiere (mg/mg DM)	2.49 ± 0.17	2.69 ± 0.09	2.56 ± 0.12 ^{a}	2.58 ± 0.23 ^{a}	2.79 ± 0.21 ^{a}
Galaktogengehalt(µg/mg DM)	15.31 ± 4.58	19.34 ± 2.71	15.63 ± 2.81 ^{a}	31.43 ± 5.17 ^{a}	31.44 ± 6.26 ^{a}
Glykogengehalt (µg/mg DM)	0.02 ± 0.006	0.02 ± 0.003	0.03 ± 0.007 ^{a}	0.07 ± 0.02 ^{a}	0.04 ± 0.009 ^{a}
Totaler Fettgehalt (µg/mg DM)	130.80 ± 7.51	104.03 ± 8.91	214.89 ± 23.31 ^{a}	265.01 ± 13.30 ^{a}	253.06 ± 17.63 ^{a}
Triglyceridgehalt (µg/mg DM)	0.88 ± 0.09	1.16 ± 0.31			
Cholesteringehalt (µg/mg DM)	2.41 ± 0.30	1.93 ± 0.27			
Energiegehalt (J/Jungtier)	114.70 ± 7.41	101.01 ± 8.71	225.16 ± 24.62 ^{a}	395.73 ± 5.45 ^{b}	322.67 ± 21.31 ^{b}

Tab. 10. Ergebnisse der statistischen Analysen (T-test, F-Test) der Reservestoffe und des Energiegehaltes frisch geschlüpften Jungtieren von Helix pomatia unterschiedlicher geographischer Herkunft, Dichte und Nahrung (E-: energiearm, E: mittlerer Energiegehalt, E+: energiereich). DM-Trockenmasse.

	Faktor Futtertyp	
	ALB - hohe Dichte	**IT – geringe Dichte**
Frischmasse der Jungtiere (mg)	**$T_{24.55}$ = 2.92, P = 0.007**	**$F_{2,26}$ = 10.47, P = 0.0005**
Trockenmasse der Jungtiere (mg)	T_{30} = 0.96, P = 0.34	**$F_{2,26}$ = 6.13, P = 0.007**
Wassergehalt der Jungtiere (mg/mg DM)	$T_{21.44}$ = 1.05, P = 0.30	$F_{2,26}$ = 0.48, P = 0.63
Galaktogengehalt(µg/mg DM)	T_{10} = 0.76, P = 0.47	**$F_{2,17}$ = 3.48, P = 0.05**
Glykogengehalt (µg/mg DM)	T_{10} = 0.76, P = 0.47	**$F_{2,17}$ = 3.44, P =0.06**
Totaler Fettgehalt (µg/mg DM)	T_{10} = 2.30, P = 0.045	$F_{2,16}$ = 1.93, P = 0.18
Triglyceridgehalt (µg/mg DM)	$T_{5.88}$ = 0.86, P = 0.42	
Cholesteringehalt (µg/mg DM)	T_{10} = 1.19, P = 0.26	
Energiegehalt (J/Jungtier)	T_{10} = 1.20, P = 0.26	**$F_{2,15}$ = 16.57, P = 0.0002**

Diskussion

In den vergangen Jahren wurden viele Studien zur Ernährung durchgeführt, mit dem Ziel die Schneckenzucht von *Cornu aspersum* zu verbessern (Dupont-Nivet *et al.* 1997, 1998, 2000; Madec *et al.* 2000). Diese Studie dagegen untersuchte den Einfluss der Nahrung auf das Wachstum und die Fortpflanzung von *Helix pomatia* und wies einige Abweichungen zu bereits gewonnenen Erkenntnissen auf. Ein bedeutendes Ergebnis ist der Unterschied von Wachstum, Überlebensrate und Fortpflanzung zwischen den Populationen. Auch wenn das Wachstum in den ersten acht Lebenswochen bei den Albschnecken schneller ist als bei den italienischen Schnecken und sich die Überlebensrate kaum unterscheidet, so zeigt sich im weiteren Verlauf, dass die italienischen Schnecken im ausgewachsenen Stadium größer sind und eine bessere Überlebensrate haben. Eine Überlebensrate von 20% für die Albschnecken beim Erreichen der geschlechtlichen Reife ist für eine Schneckenzucht nicht rentabel gegenüber 40% für die italienischen Schnecken. Da die italienischen Schnecken aus einer Schneckenzucht stammen, ist es möglich, dass Eigenschaften wie Wachstum, Größe und Überlebensfähigkeit ausgelesen und gezüchtet wurden, wie es bei *Cornu aspersum* der Fall ist (Dupont-Nivet *et al.* 1998, 2000).

Die Unterschiede waren besonders bei Fütterung mit fettreicher Nahrung markant. Bei den italienischen Schnecken hatte die fettreiche Nahrung keine Auswirkung auf das Wachstum, was den Ergebnissen von Wacker (2005) entspricht, wogegen bei den Albschnecken das Wachstum gestört wurde. Das Wachstum war schneller mit hoher Sterberate und die überlebenden Schnecken hatten eine geringere Größe bei Wachstumsende verglichen zu den Schnecken, die mit Basisfutter gefüttert wurden. Bei *Cornu aspersum* besteht die Trockenmasse an Muskelfleisch zu 10% aus Fetten (Gomot 1998) und wird durch fettreiche Fütterung erhöht (Milinsk *et al.* 2003). Allerdings ist diese Anreicherung von Fetten im Körper bei Zufütterung von Sojaöl geringer als bei Leinsamen. Da das fettreiche Futter in dieser Studie mit Sojaöl und Leinsamen versetzt wurde, wäre eine Anreicherung im Schneckenkörper sehr wahrscheinlich, außer die Population von der Schwäbischen Alb besitzt nicht die physiologischen Kapazitäten dies Fette zu extrahieren, zu verdauen und in Reserven anzulegen. Diese Reserven wären wichtig, um Überlebenschancen während der Überwinterung zu erhöhen.

Entsprechend Wackers (2005) Untersuchungen ist die Überlebensrate mit fettreicher Nahrung höher. Fette können als Reservestoffe angelegt werden (Borges *et al.* 2004), die dann auch während der Hibernation genutzt werden können. Obwohl die Überlebensrate für das E+-Futter in der ersten Hibernation sehr hoch war, sank sie während der Wachstumsphase nach der Hibernation und während der zweiten Hibernation extrem ab. Schnelleres Wachstum hat wahrscheinlich die Anlage von Reserven verringert, denn es besteht ein Kompromiss zwischen der Energiezuwendung für Wachstum und Reserveanlagen, die für lebenserhaltene Prozesse während des Wachstums und später für die Hibernation oder für die Fortpflanzung genutzt werden können (Karasov and Martinez del Rio 2007).

Die energiearme Nahrung ist fettreduziert und mit pflanzlichen Polymeren der Zellwände angereichert. Das Wachstum der Albschnecken mit dieser Fütterung verhält sich wie bei Albschnecken mit Basisfutter. Schnecken sind fähig Zellulose in verschiedene Zuckerkomponenten im Schlund zu zerlegen (Charrier & Rouland 1992), aber die Bakterienflora im Darm ist anschließend notwendig um die verbleibenden Polymere, wie Xylan, Pektin und Pullulan zu verdauen (Charrier et al. 2006). Die Sterberate während der Hibernation war allerdings ebenso hoch wie mit fettreichem Futter, was auf eine reduzierte Anlage an

Reservestoffen hindeutet und dieses Mal mit dem geringen Energiegehalt der Nahrung erklärt werden könnte. Auch wenn die Wachstumsrate geringer ist, so ist der Energiegehalt des Futters nicht ausreichend, um den Bedarf für Reserveanlagen zu decken.

Die Fütterung mit dem Basisfutter zeigte allerdings die beste Wachstumsrate, die zur höchsten Größe bei Wachstumsende mit der besten Überlebensrate führte. Zudem war hier auch sichtbar, dass eine geringe Haltungsdichte einen positiven Einfluss auf diese Parameter hatte. Selbst bei den E+ Albschnecken konnte die Endgröße bei geringer Dichte erhöht werden, was den positiven Einfluss auf das Wachstum bestätigt.

Die Unterschiede in der Fortpflanzung beruhten auf der geographischen Herkunft, wobei zwei verschiedene Fortpflanzungsstrategien zu erkennen waren, die bei *Cornu aspersum* auch innerhalb einer Population abhängig von der Fortpflanzungsperiode beschrieben wurden (Nicolai *et al*. *Ca*-I). Die italienischen Schnecken legten wenige, große Eier, die zu großen Jungtieren führen, wogegen die Albschnecken viele kleine Eier und kleine Jungtiere hervorbrachten. Die iatlienischen Schnecken produzierten Eier, die mehr Galaktogen, Trockenmasse und Wasser enthielten als Albschnecken. Dieser Trade-off wurde bereits von vielen Autoren beobachtet (z.B. Baur 1994, Madec *et al.* 1998, 2000, Baur 1993), wobei für die großen Eier ein höherer Energieeinsatz seitens des Muttertieres sowie besseres Wachstum und Überlebenschancen für die Jungtiere bescheinigt wird, was dann Auswirkungen auf die Populationsdynamik hat (Beckerman *et al.* 2002). Der Energiegehalt und die Größe der Jungtiere bestätigten diese Aussagen. Somit maximierten die italienischen Schnecken ihren Fortpflanzungserfolg mit energiereichem Futter durch höhere Energieinvestition in die Eier. Dies entspricht der „State-Dependant Theory“ (McNamara and Houston 1996) oder der ansteigenden Modelkurve der Fortpflanzungsinvestition als Funktion von Energiedisponibilität in der „Terminal Investment Theory“ (Fischer et al. 2009).

Die Albschnecken maximierten ihren Fortpflanzungserfolges durch eine höhere Anzahl an gelegten Eiern gleichen Energiegehaltes bei energiearmer Fütterung und investierten mehr Energie in das gesamte Gelege als die italienischen Schnecken. Das konnte auch bei *Cornu aspersum* beobachtet werden (Nicolai et al. *Ca*-II). Dies entspricht der „Optimal Offspring Theory“ (Smith and Fretwell 1974) und der abfallenden Modellkurve der Fortpflanzungsinvestition als Funktion von Energiedisponibilität in der „Terminal Investment Theory“ (Fischer et al. 2009). D.h. bei geringer Energiedisponibilität investieren Schnecken ein Maximum an Energiereserven in die Fortpflanzung durch Erhöhung der Eianzahl selbst mit dem Risiko einer hohen Sterblichkeit in der folgenden Hibernationsphase (Stelzer 2001).

Fette werden häufig als fortpflanzungsfördernd angesehen (Borges *et al.* 2004, Wacker & Von Elert 2003, Wacker 2005) und werden in der Fortpflanzungsperiode angereichert (Giokas *et al.* 2007). Mehrfach ungesättigte Fettsäuren agieren in der Regulation der Fortpflanzung und haben Auswirkung auf die Eigenschaften der Gelege (Wacker 2005), was auch die Ergebnisse dieser Studie bei den italienischen Schnecken belegten. Das mittlere und das fettreiche Futter bewirkten die Ablage von Eiern, die reich an Karbohydratreserven, besonders Galaktogen, waren und damit auch größere Jungtiere hervorbrachten. Die Größe der Eier innerhalb der italienischen Population war allerdings nicht hinweisend, denn die großen Eier der Nahrung E waren wasserreicher, aber nicht energiereicher als die kleinen Eier der Nahrung E+, und die Eier E- bei gleicher Größe wie Eier E hatten einen geringeren Karbohydratanteil und brachten demnach auch kleinere Jungtiere hervor. Dies entspricht nicht den Beobachtungen von Baur (1994) und

Madec *et al.* (1998), die eine positive Korrelation zwischen Ei- und Jungtiergröße bei *Arianta arbustorum* und *Cornu aspersum* beobachteten.

Die Fettanreicherung in der Nahrung führte nicht zu einer Fettanreicherung in den Eiern der Albschnecken im Gegensatz zu *Cornu aspersum* (Nicolai et al. *Ca*-II). Nur Glykogen und Cholesterin, wenn auch nur in geringen Mengen vorhanden, wurde in den Eiern E mit geringerer Trockenmasse gegenüber den Eiern E- angereichert, ohne jedoch den Energiegehalt zu beeinflussen. Cholesterin entspricht 86-92% von allen Sterolen in Schnecken (Voogt 1983; Zhu *et al.* 1994) und ist in die Hormonproduktion integriert (Flari and Edwards 2003). Schnecken können Cholesterin selber produzieren ausgehend von Desmosterol in der Nahrung (Zhu *et al.* 1994) oder von Azetat (Addink & Ververgaert 1963), das von Darmbakterien geliefert wird (Charrier *et al.* 2006). Bei *Cornu aspersum* wurde Cholesterin ebenfalls in Eiern angereichert, die kleiner und fettarmer waren, voraussichtlich um das Wachstum und die Entwicklung des Fötus zu fördern (Nicolai et al. Ca-III). Das entspricht unseren Beobachtungen, denn aus den Eiern E der Albschnecken schlüpften auch größere Jungtiere.

Die Haltungsmethode hatte ebenfalls Einfluss auf die Fortpflanzung. Die Eier besaßen bei hoher Haltungsdichte mehr Reservestoffe (Glykogen) und Jungtiere schlüpften nur aus Eiern bei hoher Haltungsdichte. Dies zeigte, dass neben der geographischen Herkunft und Futtertypen auch die Haltungsdichte einen wesentlichen Einfluss auf Wachstum und Fortpflanzung haben kann, wobei die Rolle der Haltungsdichte bei Wachstum und Fortpflanzung entgegengesetzte Wirkung hatte.

In der Schneckenzucht sollte daher auf eine extensive naturnahe Haltung geachtet werden und die Futterzusammensetzung auf Wachstumsphasen, Hibernation und Fortpflanzung entsprechend der Herkunft abgestimmt werden. Energiereiche Nahrung ist nicht vorteilhaft, da die wilden Albschnecken wahrscheinlich nicht die Fähigkeiten besitzen, diese Energie zu extrahieren. Da die Sterblichkeit in der Hibernation sehr hoch ist, sollten weitere Beobachtungen zur Kälteresistenz und den Einfluss von extremen Wetterbedingungen auf die Sterblichkeit gemacht werden.

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Treue Begleiter

Helix pomatia besitzt die Fähigkeit eine Reihe an Pflanzenfasern zu verdauen und verdankt diese einer vielfältigen Darmbakteriengemeinschaft, die bisher nur wenig erforscht wurde. Die energiearme Nahrung (E-), die für Fütterungstests genutzt wurde, ist reich an Zellwandbestandteilen, unter anderem Zellulose. Wachstum der Albschnecken mit dieser Fütterung verhalten sich wie bei Albschnecken mit Basisfutter (E), da Schnecken fähig sind, Zellulose in verschiedene Zuckerkomponenten im Schlund zu zerlegen (Charrier & Rouland 1992). Aber die Bakterienflora im Darm ist anschließend notwendig um die verbleibenden Polymere, wie Stärke, Xylan, Pektin und Pullulan zu verdauen (Charrier et al. 2006) (Tab. *hp*7).

Ab dem Schlüpfen der Jungtiere siedeln sich Bakterien im Darm der Schnecken an, die hauptsächlich aus dem Mineralboden stammen (Watkins & Simkiss 1990, Charrier *et al.* 1998). Während der Hibernationsphase wird eine permanente Flora unterstützt (Pereira & Breckenridge, 1981, Charrier, 1990) trotz dem Ausscheiden vorrübergehend mit der Nahrung aufgenommener Arten. Somit bleibt der Darm der Ort, in dem das Gefrieren der Schnecke initiiert wird, da sich unter den Bakterien Eisnukleationsagenzien befinden (Ansart et al. *Ca*-V).

Die Darmbakterienflora erscheint wichtig, um Verdauungsprozesse und den Zugang zu essentiellen Nährstoffen zu schaffen. Doch hat die Dynamik der Bakteriengemeinschaft auch Einfluss auf die Überwinterungsphase und eventuell Überlebenschancen?

Hypothesen dieser Studie:

- Die Darmbakterienflora setzt sich teilweise aus vorübergehenden mit der Nahrung aufgenommenen Arten zusammen, so dass zwischen geographisch unterschiedlichen Schneckenpopulationen auch Unterschiede in der Bakteriengemeinschaft auftreten müssten
- Die permanent vorhandenen Bakterienarten im Darm sind schneckenspezifisch und müssten daher auch während der Hibernation in beiden Populationen auftreten.
- Das Einsetzen der Hibernation ist vom gesundheitlichen Zustand der Schnecke abhängig und kann in Verbindung mit der Bakterienflora stehen
- Eisnukleationsaktive Bakterien gehören zu einer permanenten Darmflora

Tab. *hp7*. Bisher bekannte Bakterienarten im Darm von *Helix pomatia* und ihre fermentativen Fähigkeiten, die bisher getestet wurden (Charrier et al. 2006).

	BAKTERIENARTEN	DIGESTIONSKAPAZITÄTEN
Helix pomatia **(Schweden)**	*Buttiauxella agrestis*	Melibiose, Chitin, Arabinogalaktan
	Citrobacter gillenii	Cellobiose, Maltose, Glycerol
	Kluyvera intermedia [1]	Fructose, Galaktose, Glukose, L-Arabinose, Mannose, Sucrose, Xylose, Polyole, Pektin
	Lactococcus lactis	Cellobiose, Maltose, Fructose, Mannose
Helix pomatia **(Frankreich)**	*Lactococcus lactis*	
	Obesumbacterium proteus	Fructose, Galaktose, Glukose, L-Arabinose, Mannose, Xylose, Mannitol
	Enterobacter amnigenus	Cellobiose, Maltose, Melibiose, Raffinose
	Enterococcus raffinosus	Fructose, Galaktose, Glukose, L-Arabinose,
	Enterococcus malodoratus	Mannose, Sucrose, Xylose, Arabinogalaktan, Polyols

[1] Diese Art hat im anaeroben Kulturmilieu eine Eisnukleationsaktivität von -9°C (Nicolai et al. 2005).

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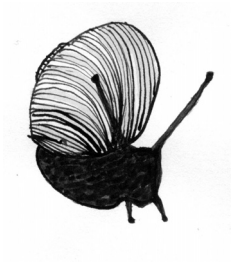

Seasonal dynamic of the bacterial community structure in the compartmentalized gut of three populations of the land snail *Helix pomatia*

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Reference: Nicolai et al. *Hp*-I

Abstract: Terrestrial gastropods have an extraordinary efficiency in plant fibre digestion and may owe it to a rich microbial community. However the seasonal dynamic of bacterial community structure in respect to environmental and trophic conditions in the snail habitat, as well as the meaning for cold hardiness remains unclear. Therefore we investigated the bacterial gut community structure of endangered land snail species *Helix pomatia* originating from three populations differing in climatic and trophic conditions. DNA was extracted from different digestive tract compartments of snails in activity and hibernation, and, additionally, of snails at hibernation beginning and of starved but not hibernating snails in one population. The fingerprinting analysis using PCR-DGGE followed by NMDS showed differences in the bacterial community structure between populations and the seasonal physiological state. Most of the sequenced DGGE fragments represented Gamma- and Alphaproteobacteria, that were common to all populations, while Mollicutes were only found in IT and RHE population and Betaproteobacteria were only present in ALB population. Some allochthonous strains might be transiently abundant in the intestinal tract of foraging snails in respect to food type available in the snail's habitat. Some strains were permanently present in the gut after expulsion of the gut content at hibernation beginning and during hibernation and could be considered as autochthonous. The persistence of the ice-nucleating bacteria *Xanthomonas campestris* in hibernating snails was consistent with the low body supercooling ability observed in some helicid species. The anatomic compartmentalization of the intestinal tract is underlined by changing physicochemical conditions which was reflected by the bacterial community structure. Further studies should therefore focus on the relation between environmental bacteria and gut bacteria in order to better understand the habitat quality needed to sustain this endangered species.

Keywords: PCR-DGGE, Proteobacteria, Firmicutes, Mollicutes, Hibernation, *Xanthomonas campestris*, Ice nucleating activity

Introduction

Terrestrial gastropods have an extraordinary efficiency in plant fibre digestion of 60-80% (Davidson, 1976, Charrier & Daguzan, 1980) and may owe it to a rich microbial community (Myers & Northcote, 1959, Strasdine & Whitaker, 1963, Charrier, 1990). The digestive tract harbours bacteria with metal chelating (Simkiss, 1985) and fermentative activity (Charrier, *et al.*, 1998, Charrier, *et al.*, 2006) particularly on chitin (Jeuniaux, 1955, Charrier, *et al.*, 2006) and soluble cellulose (Lesel, *et al.*, 1990, Simkiss & Watkins, 1990). Chitin, an fungal component, could serve as readily available nitrogen source (Speiser, 2001), and lactate and acetate provided by bacteria could be used by the snails as energy resource (Charrier, *et al.*, 2006) or as precursors in biosynthesis process, like cholesterol *de novo* biosynthesis (Addink & Ververgaert, 1963). Land snails might foster organic matter degradation by bacteria, especially in the distal intestine and the digestive gland, where endogenous enzymatic activity is low (Charrier & Rouland, 1992, Flari & Charrier, 1992). The compartmentalization of the digestive tract of helicid snails is underlined by a pH gradient from acidic in the crop to neutral or alkaline in the distal intestine, the whole gut being anoxic (Charrier & Brune, 2003). The variety of physicochemical conditions in the gut might trigger spatial structuration of microbial communities, like emphasized in termites (Schmitt-Wagner, *et al.*, 2003).

In earlier studies on cultivable intestinal bacteria of *Helix pomatia* and *Cornu aspersum* (Charrier, *et al.*, 1998, Charrier, *et al.*, 2006, Kiebre-Toe et al. 2003) facultative anaerobes species appeared to be dominant: *Citrobacter* sp., *Enterobacter* sp., and *Klebsiella* sp., which is in accordance with the existence of microoxic regions in the vicinity of the apical epithelium (Charrier & Brune, 2003). Most of the cultivated strains from Enterobacteriaceae as well as *Lactococcus* sp. and *Clostridium* sp. were also found in environmental samples and food products (Olsson, *et al.*, 2004, Charrier, *et al.*, 2006). Therefore, the bacterial flora of Helicidae intestine seems to be mainly exogenous, ingested with food and faeces as well as by scraping the soil, the later being especially the case in newly hatched snails (Charrier, 1990, Watkins & Simkiss, 1990). However, since Enterobacteriaceae and Enterococcaceae are generally assigned to enteric environments, snail gut was suggested being the natural niche for *Buttiauxella* sp. and *Kluyvera* sp. (Muller, *et al.*, 1996). The persisting bacterial community during dormancy was supposed to be composed of mainly autochthonous species of the intestine (Charrier, *et al.*, 2006).

Snails preserve bacterial flora partially during hibernation through continuous secretions of mucus in the distal intestine and rectum (Pereira & Breckenridge, 1981, Charrier, 1990). Gut bacteria might present ice nucleating activity reducing cold hardiness in partial freezing tolerant snail species like *Cornu aspersum* (Ansart et al. *Ca-V*, Ansart et al. 2001, Ansart el. 2005, Kiebre-Toe et al. 2003) and *Helix pomatia* (Nicolai et al. 2005). The tested bacteria in the study of Ansart et al. (*Ca-V*) and Nicolai et al. (2005) revealed a week ice nucleating activity in the given culture conditions of one bacterial strain persisting during hibernation, but further studies are necessary to identify ice nucleating active bacteria in snails. By contrast, in *Anguispira alternata* gut clearance at the initiation of hibernation contribute to a great supercooling ability (-16°C) (Riddle 1981), and in freezing intolerant insect species the elimination of gut bacteria was largely studied (e. g. Cannon and Block 1988, Zachariassen 1980). Moreover, sensitivity of microbiota to climate conditions was suggested to influence the density of colonisation in the intestine of Swedish *Helix pomatia* (Charrier, *et al.*, 2006), because bacterial strains deploy different strategies of resistance to climatic variations (Balows, *et al.*, 2005, Foucaud-Scheunemann & Poquet, 2003).

Functional studies described extensively the importance of bacterial gut flora for the snail's digestion and nutrient providing, but the seasonal dynamic of bacterial community structure in respect to environmental and trophic conditions in the habitat, as well as the meaning for cold hardiness remains unclear. Therefore, we focused in this study on *Helix pomatia* which has a large latitudal (Greece to Sweden) and longitudal (France to Poland) distribution in Europe

(Nietzke 1970), exposing individuals to a wide range of different altitude, habitats, climatic situations and annual variations. Moreover, *Helix pomatia* is an endangered species suffering from habitat destruction (hedges and skirts of the forest) by intensive land use and accumulation of toxic substances in the soil (Dallinger et al. 2001). It has a long life cycle reaching maturity only after 3-4 years (Nietzke 1970). The species is protected in Europe since 2002 by the appendix III of the Bern convention and standing to benefit from a general collection interdiction in Germany since 2005 (§42 Abs. 1 Nr.1 BNatSchG). Protection measures in Germany include promoting snail farming and lowering habitat disturbance, both requiring studies about crucial life stages in order to limit mortality in the species artificial and natural habitat, respectively. Mortality during hibernation seems to be the key factor of population dynamic in Helicidae of Europe (Cain 1983, Peake 1978), and non hibernating snails (without epiphragm) were reported in natural conditions (Nietzke 1970, Kilias 1980), as well as in farm conditions (personal communication) raising some concerns.

We chose three geographically distinct populations of *Helix pomatia* to investigate the bacterial community structure in activity and hibernation in order to better understand the origin of bacterial flora and its influence on cold hardiness processes. We hypothesise (i) that the initiation of hibernation accompanied by gut clearance might provoke changes in the bacterial community structure in the intestinal tract of *Helix pomatia* and be related to cold hardiness, and (ii) that the bacterial community structure of active snails differs between populations of different geographic origins according to habitat and climate differences, but an endogenous flora persists during hibernation whichever population origin.

We chose the DGGE fingerprinting method to analyze the bacterial composition in the intestine and in the digestive gland of *Helix pomatia* to get a view about the community composition of dominant strains (Claesson*, et al.*, 2009). A comparative study of different fingerprinting methods had shown, that DGGE gives the clearest discrimination among microbiota communities (Okubo & Sugiyama, 2009), and was applied to a wide range of questions in the context of community structure and dynamic (e. g. Brunvold et al. 2007, Sandaa et al. 1999, 2003, Befring Hovda et al. 2007, Knapp et al. 2009a,b, Okubo & Sugiyama, 2009).

Material and Methods

SAMPLING OF *HELIX POMATIA* AND HIBERNATION ACCLIMATISATION

In September 2006 snails were collected (authorisation §2 Abs. 2 BNatSchG from September 18th 2005) in three geographically distant forest habitats (Figure 1).

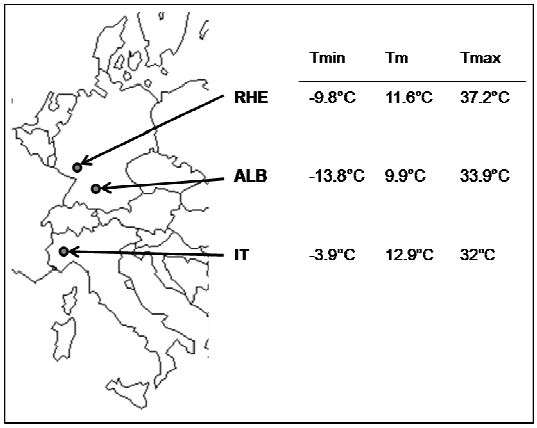

Figure 1. Location of collecation areas of different populations of *Helix pomatia* with climatic data for sampling year (http://www.ilmeteo.it and http://www.dwd.de). ALB Schwäbische Alb in Germany, RHE: Rhein valley in Germany, and IT: Cherasco, Piemont in Italy). Tmin – minimal temperature, Tm – mean temperature, Tmax – maximal temperature.

Population IT: collected in the forest (dominated by *Carpinus betulus* and *Quercus robur* with ground vegetation dominated by *Stellaria holostea*, *Hedera helix*, *Rubus fruticosus* and *Lonicera periclymenum*) near Cherasco, region of Piemont in Italy (44° 38' 60N, 7° 50' 60E, 200 m a.s.l.). Population RHE: collected in the forest (dominated by *Carpinus betulus* and *Robinia pseudoacacia* with ground vegetation dominated by *Urtica dioica, Impatiens non-tangere, Geranium robertianum*, *Galeopsis tetrahit*, *Ranumculus ficaria*, *Chelidonium majus,* and *Fragaria vesca*) near Grünstadt-Asselheim, Rhein valley in Germany (49° 33' 14N, 8° 9' 46E, 150 m a.s.l.). Population ALB collected in the forest (dominated by *Carpinus betulus* and *Alnus incana* with ground vegetation dominated by *Urtica dioica, Fumaria officinalis*, *Impatiens non-tangere, Geranium robertianum*, *Viola reichenbachiana, Anemone nemoros, Dentaria bulbifera*) near Weiler-Indelhausen, mountains Schwäbische Alb in Germany (48° 18' 7N, 9° 29' 47E, 800 m a.s.l.). The climate is characterized by a gradient of the annual minimal and mean temperature (Figure 1).

5 snails of each population were starved for one day in order to empty the gut and directly stored at -80°C (active snails: sample A). Because it is difficult to collect snails in hibernation conditions (frozen soil, unknown location of burial), 5 further snails of each population were progressively transferred to the laboratory and acclimated to hibernation condition (temperature 5 ± 1°C, relative humidity: 50%, 24h darkness) over two weeks without feeding. By the end of the second week snails obturated the peristom of their body shell with a hard calcified epiphragm, thereby initiating hibernation. After three months of hibernation snails were stored at -80°C (hibernating snails: sample: H). In the RHE population we collected 20 snails that we transferred to hibernation conditions and stored five of them at -80°C right after epiphragm finalization (hibernation beginning sample: Hb) and five of them without epiphragm, but inactive for 2 weeks in hibernation conditions with a deeply withdrawn body in the shell cavity (non hibernating sample: nH). Such non hibernating snails could generally be observed in natural conditions (Nietzke 1970, Kilias 1980) and occurred also in the ALB and IT sample (2 and 1 snails, respectively). These snails do not produce an epiphragm and initiate hibernation, which is usually accompanied by the rapid death in early winter.

DNA EXTRACTION, PCR-DGGE AND SEQUENCING

All snails were surface-sterilized with ethanol (70%) and dissected on a sterile workbench. The digestive tract was extracted and separated in three parts: the proximal intestine (PI), the distal intestine (DI) and the digestive gland (DG). For DNA extraction we pooled tissues of the 5 individuals of each sample and homogenized the pool using sterile Circonium beads in a bead-beating step (2 min, 30 Hz, Retsch™ MM2000, USA). After addition of 650 µl CTAB 2% (in 1 M TrisHCl pH 8, 5 M NaCl and 0.5 M EDTA) and 20 µl Proteinase K (20 mg/ml), followed by bead-beating, the homogenate was incubated for 1 h at 65°C (Polystat, Bioblock Scientific, France) and then centrifuged (14000 g, 2 min, 4°C, Sigma® 3K 12, Fisher Bioblock Scientific, France). To the supernatant were added 600µl of phenol:chlorophorm:isoamylalkohol (25:24:1, v/v) and slightly shaken for 5 min. After centrifugation (14000g, 4min, 4°C), the supernatant was mixed with 600 µl chloroform and centrifuged again (14000g, 10min, 4°C). The supernatant was then added to a twofold volume of isopropanol in order to precipitate the DNA. After 1.5 h of incubation at -20°C and a centrifugation (14000 g, 30 min, 4°C), DNA was washed with 500 µl cooled ethanol (70%) and centrifuged again (14000g, 15min, 4°C). Extracted DNA was then dried at ambient temperature for 30-40 min before adding TE buffer (10 mM Tris pH 7.6, 1 mM EDTA pH 8) and purifying twice using the Kit MicroSpin S-400 HR (Bioscience, UK) according to the manufacturer's protocol. DNA yield and quality was assessed by concentration measurements using NanoDrop® (NanoDrop Technologies, DE, USA), and subsequently a twofold dilution was performed.

Extracted DNA was amplified in a PCR thermocycler (Mastercycler personal Eppendorf®, USA) with a general primer, the 16S rDNA primer set 518-r (ATT-ACC-GCG-GCT-GCT-GG) and the GC-clump primer gc-338-f (CGC-CCG-CCG-CGC-GCG-GGC-GGC-

GGC-GCG-GGG-GCA-CGG-GGG-GAC-TCC-TAC-GGG-AGG-CAG-CAG). Each PCR mixture of 25 µl contained one unit of DNA polymerase-dNTP mix puReTaq Ready-to-go PCR bead (illustra™, GE Healthcare, UK), 0.1 µg of extracted DNA, and 0.25 µM of each primer. The PCR included an initial 5 min denaturation at 94°C and was followed by 35 thermal cycles of 0.5 min at 94°C, 0.75 min at 55°C and 1.5 min at 72°C. Amplification was completed with a final extension step at 72°C for 20 min. PCR products were verified by electrophoresis 2% (w/v) agarose gels (55 min, 100 V) and ethidium bromide staining (10 $mg.ml^{-1}$) using the molecular marker Ladder (Invitrogen, France).

For DGGE (Dcode System, BioRad, Canada), 2 µl PCR product was loaded onto 8% (w/v) polyacrylamide gels with denaturing gradient of 40-55% (100% denaturant according to 7 M urea plus 40% formamide in 50X TAE buffer) and was run 15 h at 75 V and 60°C in 0.5X TAE buffer (20 mM Tris, 10 mM Acetate, 0.5 mM EDTA pH 7.6). After electrophoresis, the gel was stained with ethidium bromide (10 $mg.ml^{-1}$) and scanned using UV (Transilluminator HOOD, BioRad, Canada with camera Canon CCIR). DGGE banding patterns were normalized and analyzed using the software Quantity One (BioRad, Canada).

Selected DNA bands (2 bands per line on the gel) were excised from the gel and incubated 12 h in 25 µl TE buffer at 5°C. In order to purify the excised DNA bands, 2 µl of eluted DNA in sterile TE buffer were used for PCR with the same primer set and the same cycle, followed by DGGE in the same conditions as described above. Excision of the selected bands, PCR and DGGE on the excised bands were repeated again before a final twofold PCR on the purified excised DNA bands. The PCR amplicons were used for sequencing (COGENICS, Takeley, UK).

DATA ANALYSIS

In order to detect differences of band patterns between population samples, the nonmetric multidimensional scaling method (NMDS) with Jaccard similarity coefficient was applied to presence/absence data determined for each band and based on the normalized minimal threshold density (Legendre & Legendre, 1998, Legendre & Legendre, 2007, Ramette, 2007). The stress coefficient indicates the badness-of-fit, this is the quality of the NMDS ($S < 0.20$: acceptable). Distances between points (corresponding to each gut compartment) were calculated with the equation $d_{ij} = ((x_i-x_j)^2 + (y_i-y_j)^2)^{0.5}$, where d_{ij} is the distances between two points i and j, and x and y are the scores on the first and the second axis, respectively. According to Okubo & Sugiyama (2009), the Kruskal-Wallis test was used on scores of the first and second axes and on distances to analyse bacterial community differences. All analyses were conducted with the software R 2.8.0 (R Core Team, 2008).

All sequences were aligned with the software BioEdit Sequence Alignment Editor7.0.9.0 (Hall, 1999) using ClustalW (Thompson, *et al.*, 1994) and compared to those found in the GenBank database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Only results with the highest E-value have been retained and phylogenetic affiliation was analysed with the Neighbour Joining method using the software MEGA 4.1 (Tamura, *et al.*, 2007). The consistency of the unrooted tree was validated by bootstrapping (n = 1000). The taxonomic hierarchy of the sequences was determined using the RDP classifier tool (http://rdp.cme.msu.edu/index.jsp, (Wang, *et al.*, 2007)) with a bootstrap cutoff of 50% since bands were < 250 pb (Claesson, *et al.*, 2009).

Results

BACTERIAL GUT COMMUNITY STRUCTURE AS A FUNCTION OF SEASONAL CONDITIONS AND GEOGRAPHIC ORIGIN

The DGGE profiles allowed us to gain insights into the structural composition of bacteria community in the compartmentalized gut of the land snail *H. pomatia*. In general, band number in gut compartments of population samples reached from 4 to 16 bands (Figure 2). Bacterial gut communities were also clearly discriminated on the first axis of NMDS with an acceptable stress

coefficient (Figure 3). Furthermore, the bacterial communities of the three studied populations were not only separated in activity conditions (Kruskal-Wallis test, $H_2 = 7.2$, $P = 0.027$), but also in the hibernation conditions (Kruskal-Wallis test, $H_2 = 6.49$, $P = 0.039$), on the first axis.

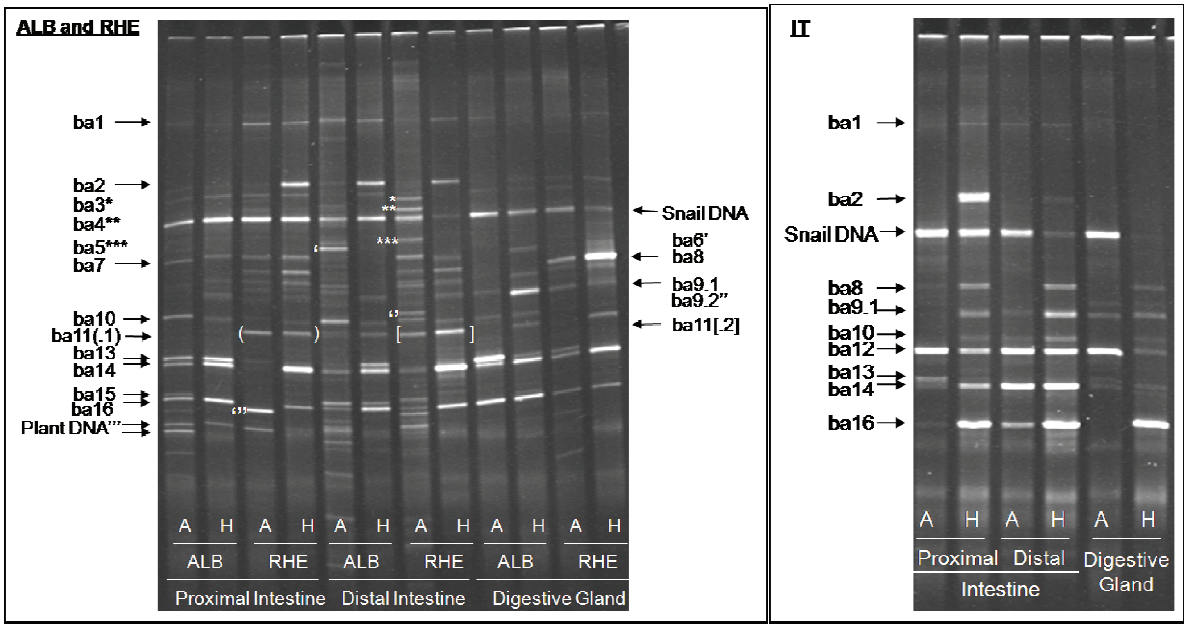

Figure 2. Fingerprints of the bacterial community in each gut compartment of different populations (Schwäbische Alb in Germany: ALB, Rhein valley in Germany: RHE, and Italy: IT) of *Helix pomatia* in different conditions (activity: A, and hibernation: H). Two bands were sequenced in those lines of 16S rDNA bands, which are indicated by ba1 to ba16. Several bands revealed to be not of bacterial origin after alignment in GenBank (snail and plant DNA).

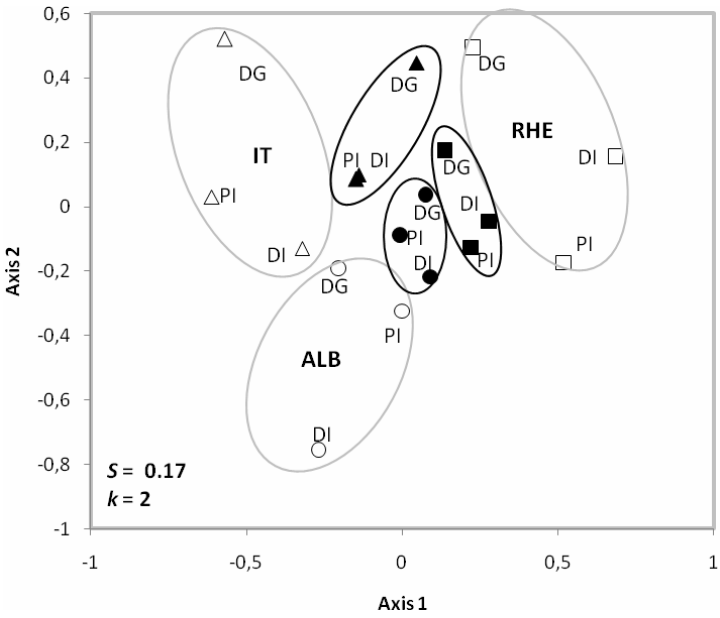

Figure 3. NMDS plot of bacterial DGGE fingerprinting patterns in different populations (Schwäbische Alb in Germany - ALB: circles, Rhein valley in Germany - RHE: squares, and Italy - IT: triangles) and different conditions (activity - A: open symbols, hibernation - H: black symbols) based on extracted 16S rDNA from the content of different compartments of the digestive tract (proximal intestine: PI, distal intestine: DI, digestive gland: DG) in the land snail *Helix pomatia*. *S* indicates the stress and *k* the total number of axes used in the analysis. Kruskal-Wallis test of scores between samples on the 1st axis: $H = 15.50$, $DF = 5$, $N = 18$, $P = 0.0084$, and on the 2nd axis: $H = 8.81$, $DF = 5$, $N = 18$, $P = 0.12$.

The densest bands could be successfully purified and sequenced, corresponding to ba1 - ba16 (Figure 2). Several bands appeared not to be of bacterial origin after alignment in the GenBank database (snail and plant DNA) and were discarded from further community or taxonomic analysis. Most of the excised 16S rDNA sequences were closely related to sequences from Pseudomonadaceae, Enterobacteriaceae, Xanthomonadaceae and Sphingomonadaceae with a similarity ≥ 98% (Figure 4).

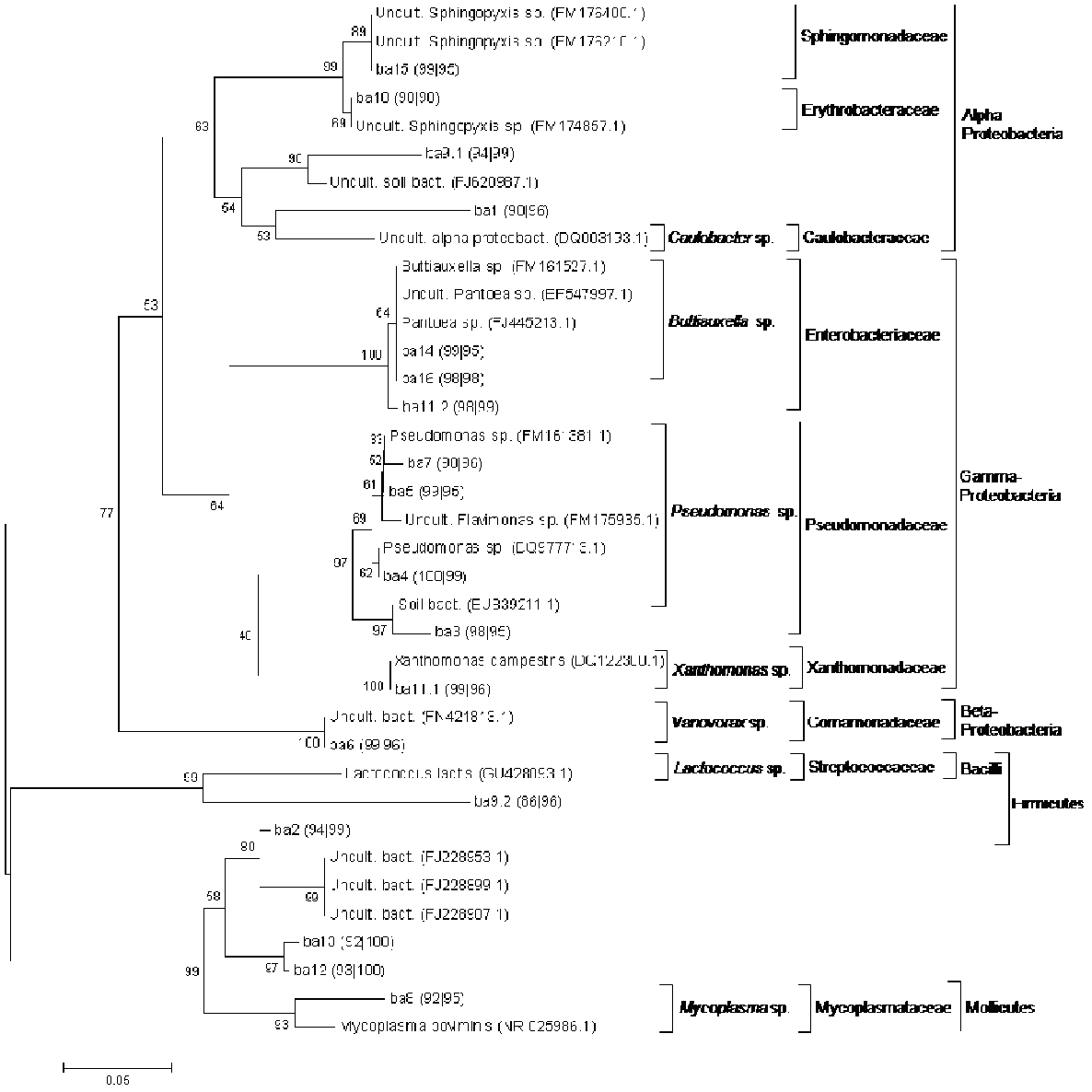

Figure 4. Neighbour joining tree of 16S rDNA gene sequences (*N* = 18) extracted from gut of *Helix pomatia* depicting the phylogenetic relationships of DGGE band sequences (ba1 – ba16, see figure 2) with closest related sequences from the GenBank. The scale bar represents 5% divergence, and bootstrap values (in %) from 1000 resamplings are shown at the nodes. Values in brackets, following sequences from this study, give the percentage of similarity and query coverage to the closest related sequence derived from GenBank database. Sequences from the GenBank database are followed by their accession number. Taxonomic hierarchy was determined with the RDP classifier tool.

The similarity of ba9.2 with *Lactococcus lactis* was only 86% and of ba8 with *Mycoplasma bovirhinsis* only 92%, as well as the similarity of some bands (ba1, ba2, ba9.1,

ba12, ba13) with sequences of unknown bacteria or other DGGE bands ranged from 90 to 94%. The taxonomic affiliation of unknown GenBank sequences and bands could be determined with the RDP classifier tool, revealing an affiliation to the genus of *Variovorax* sp. for ba6, to the class Alphaproteobacteria for ba1 and ba9.1, and to the Phylum Firmicutes for ba2. The bands ba12 and ba13 could not be classified due to poor sequencing quality. The closest relatives in the GenBank database were of the genus *Pantoea* sp. for ba11.2, ba14, and ba16, *Flavimona*s sp. for ba5, and *Sphingopyxis* sp. for ba15, while the same bands and sequences from the GenBank were classified to the genus *Buttiauxella* sp., the genus *Pseudomonas* sp., and the family Erythrobacteraceae, respectively, by the RDP classifier tool. The closest relatives from the alignment in GenBank database for ba1 and ba9 were assigned to the genus *Caulobacter* sp. and to the class of Alphaproteobacteria, respectively.

Gammaproteobacteria and Alphaproteobacteria were the most abundant classes in snail guts of all populations (Figure 5). However, among Alphaproteobacteria IT population lacked Pseudomonadaceae, and Xanthomonadaceae were only found in RHE population. Betaproteobacteria (Comamonadaceae) were only present in ALB population in activity condition, and Mollicutes were specific of RHE and IT population. One phylotype of Firmicutes appeared only in hibernation conditions of all populations, the other only in the RHE population, in activity.

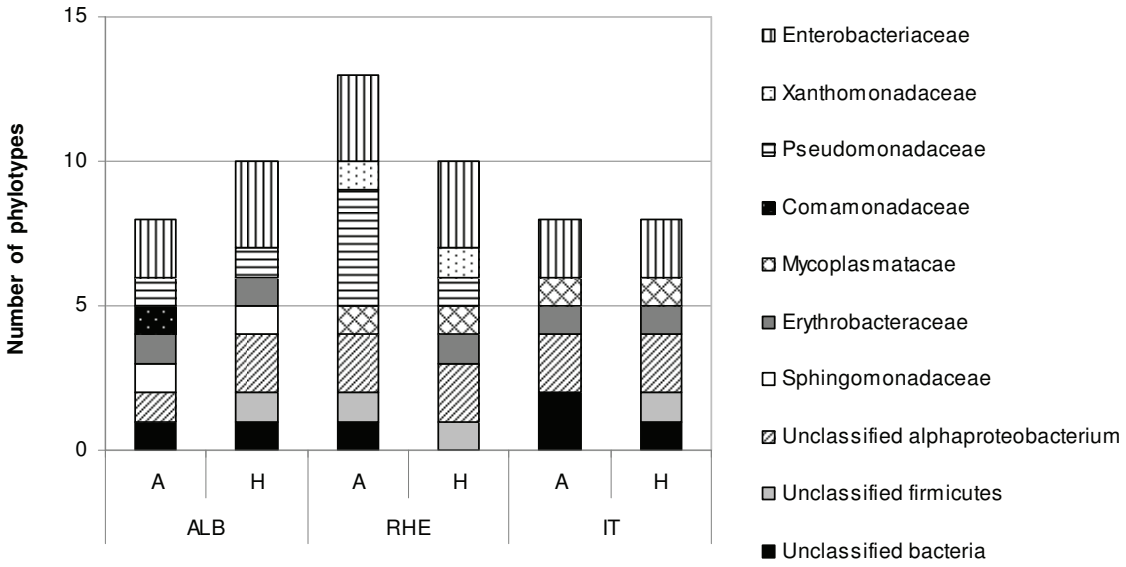

Figure 5. Relative abundance of each taxon at the class-level within gut content of population samples (Schwäbische Alb in Germany: ALB, Rhein valley in Germany: RHE, and Italy: IT) of *Helix pomatia* in different conditions (activity: A, and hibernation: H). N is the number of analysed phylotypes per population sample.

BACTERIAL COMMUNITY STRUCTURE IN THE COMPARTMENTALIZED GUT FOR DIFFERENT PHYSIOLOGICAL STATES

In the DGGE profile of RHE snails at the beginning of hibernation and in non hibernating snails (Figure 6) we could identify the same sequences of 16S rDNA bands by alignment with the sequences of the DGGE in figure 2. The band patterns of DGGE fingerprinting of all physiological states in RHE population showed a significant discrimination of bacterial community structure on the second axis in the NMDS plot with an acceptable stress coefficient (Figure 7). Non hibernating snails showed the most dissimilar bacterial community structure.

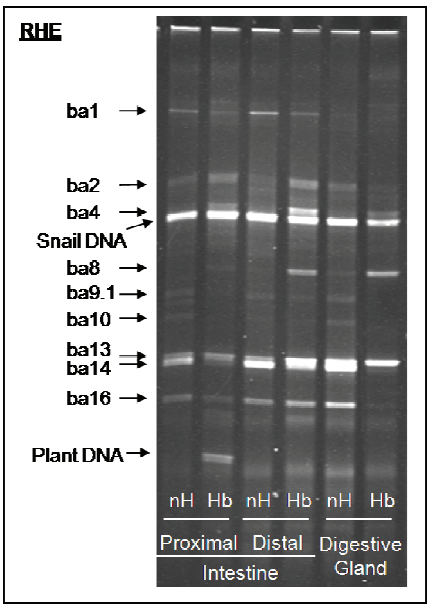

Figure 6. Fingerprints of the bacterial community in each gut compartment of the RHE population (Rhein valley in Germany) of *Helix pomatia* in different physiological states (hibernation beginning: Hb, non hibernating individuals: nH). Two bands were sequenced in those lines of 16S rDNA bands, which are indicated by ba1 to ba16. Several bands revealed to be not of bacterial nature after alignment in GenBank (snail and plant DNA).

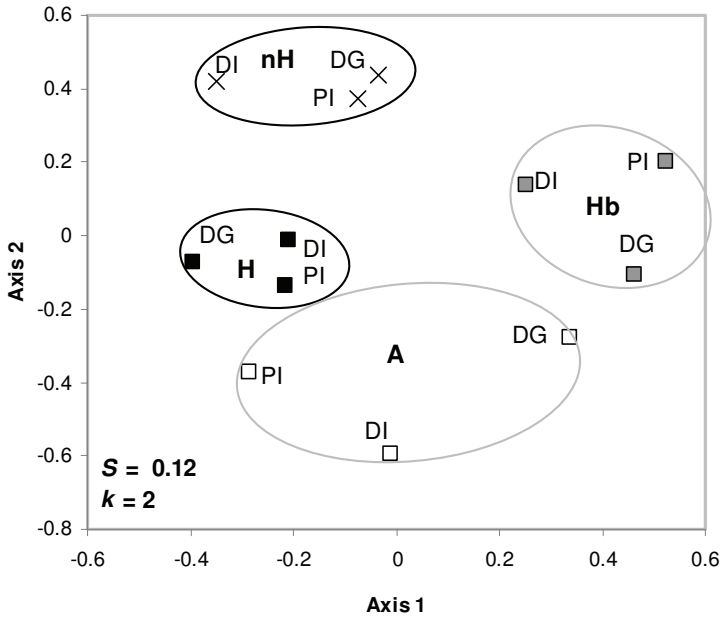

Figure 7. NMDS plot of bacterial DGGE fingerprinting patterns in the RHE population (Rhein valley in Germany) in different physiological states (activity - A: open symbols, hibernation beginning - Hb: grey symbols, hibernation - H: black symbols, non hibernating individuals – nH: crosses) based on extracted 16S rDNA from the content of different compartments of the digestive tract (proximal intestine: PI, distal intestine: DI, digestive gland: DG) in the land snail *Helix pomatia*. *S* indicates the stress and *k* the total number of axes used in the analysis. Kruskal-Wallis test of scores between samples on the 1st axis: H = 6.90, DF = 3, N = 12, P = 0.075, and on the 2nd axis: H = 9.67, DF = 3, N = 12, P = 0.022.

As already observable in the NMDS plot of the three populations (Figure 3), the different compartments of the digestive tract were well distanced from each other in activity on the NMDS plot, but approached each other in hibernation (A *versus* H, Kruskal-Wallis test, H_1 =

7.75, *DF* = 1, *N* = 18, *P* = 0.005). In the NMDS plot of the RHE population (Figure 7), hibernating and non hibernating snails of the RHE population showed comparable distances between gut compartments (H *versus* nH, Kruskal-Wallis test, *H* = 0.43, *DF* = 1, *N* = 6, *P* = 0.51), whereas a distance gradient is observable from active snails *via* hibernation beginning to hibernating snails on the NMDS plot (A *versus* Hb *versus* H, Kruskal-Wallis test, *H* = 7.26, *DF* = 2, *N* = 9, *P* = 0.027). Moreover, in terms of bacterial rDNA band number (Figure 2, 6), the distal intestine seemed to be rich in different bacteria species in active and hibernating snails as well as in snails at hibernation beginning (*N* = 12, 10, 6 bands, respectively) compared to the proximal intestine (*N* = 5, 8, 4 bands, respectively) and the digestive gland (*N* = 4, 5, 3 bands, respectively). In contrast, the non hibernating snails had a richer bacterial community in the proximal intestine (*N* = 8 bands), compared to the distal intestine (*N* = 5 bands) and the digestive gland (*N* = 6 bands). In terms of abundance of analysed phylotypes (Figure 8), unclassified bacteria were restricted to the digestive gland in activity, but became abundant in all gut compartments within non hibernating snails as well as in snails at hibernation beginning. Firmicutes, usually present in proximal and distal intestine in both samples, hibernation and hibernation beginning, were present in proximal intestine and digestive gland in non hibernating snails as well as in distal intestine of active snails. Mollicutes were abundant in all three gut compartments in active snails, but were restricted to distal intestine and digestive gland at hibernation beginning before colonising again the proximal intestine during hibernation.

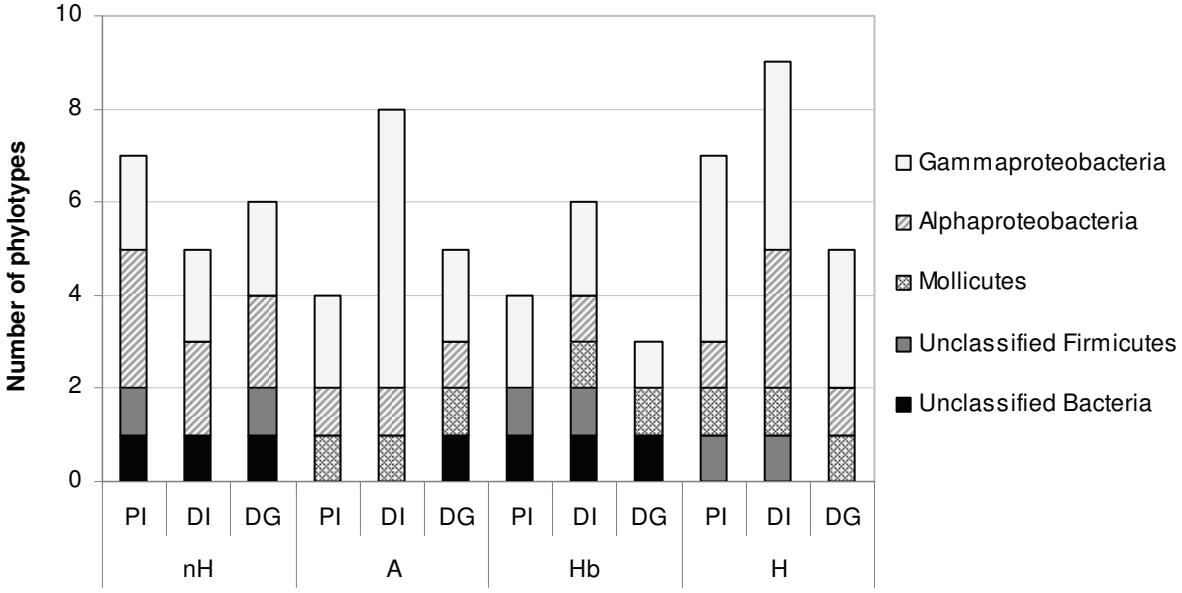

Figure 8. Relative abundance of each taxon at the class-level within gut content of RHE population (Rhein valley in Germany) of *Helix pomatia* in different physiological states (activity - A, hibernation beginning - Hb, hibernation - H, non hibernating individuals – nH). N is the number of analysed phylotypes per population sample.

Discussion

The DGGE fingerprinting technique with the NMDS analysis revealed, that the intestine of the land snail *Helix pomatia* harboured a rich bacterial flora which seemed to be specific of each population when snails are in activity. Among the most abundant rDNA fragments we sequenced Pseudomonadaceae sp. and Enterobacteriaceae that dominated the intestinal and digestive gland community of all populations. According to the work of Charrier et al. (2006)

and Kiebre-Toe et al. (2003), *Pseudomonas* sp., *Pantoe*a sp. and *Buttiauxella* sp. were the most commun strains in the snail's intestine. However, Pseudomonadacea were absent in the IT population and rare in ALB population. Besides these Gammaproteobacteria, alphaproteobacteria were abundant. While Sphingomonadaceae were only found in ALB snails, Erythrobacteraceae were present in all populations. From alignment with sequences in GenBank we could deduce that all these strains come from the environment (Boyd*, et al.*, 2007, Austin*, et al.*, 2009, Delmotte*, et al.*, 2009), and were probably ingested with food or by soil scraping according to earlier studies (Charrier, 1990, Watkins & Simkiss, 1990).

Because of the exeogenous origin of the major community members, micro-environmental conditions like the soil composition, the vegetal food source and climatic factors could influence the bacterial community structure of each population. For instance, the Betaproteobacterium present in ALB populations was identified as *Variovorax* sp. previously found in an environmental sample of Switzerland (Delmotte*, et al.*, 2009). *Variovorax* sp. is a ubiquitous aerobic soil bacterium frequently associated with important biodegradative processes. The organic matter content in the sandy clay soil with pH 5.8 of the ALB habitat leveled 14.63% whereas only 4.85% were recorded in the sandy loam soil with pH 7.2 in the RHE habitat (personnel observation). Such soil difference influences the biodegradative activity (Lavelle & Spain 2005) and might be related to the abundance of *Variovorax* sp. in the snail habitat. Mollicutes, only present in RHE and IT populations, are facultative anaerobes and inhabit plant or mucous animal surfaces (Hayflick and Chanock 1965). In fact *Mycoplasma* sp. needs cholesterol for growth (Hayflick and Chanock 1965), which is synthesized *de novo* in land snails (Addink and Ververgaert 1963, Nicolai et al. *Ca*-III) and which occurred at higher level in body fluids of *Helix pomatia* in warm climate (Nicolai et al. *Hp*-III) because of its function in thermal adaptation through membrane-stabilizing effects (Robertson and Hazel 1997, Sperfeld and Wacker 2009).

One strain of unclassified Firmicutes (ba2) appeared among dominant bacteria only in hibernation of all populations, whose closest relatives were sequences obtained from gut content of a Planorbid snail (unpublished, Acession numbers FJ228953, FJ228899, FJ228907). Charrier et al. (2006) postulated that bacterial strains isolated from snails in a starved state, like aestivation or hibernation, correspond to eurythermic strains which could be reasonably considered as autochthonous of the snail gut.

The differences in the bacterial community structure between snails in activity and snails in hibernation were striking on the NMDS plot. Whereas the snail populations in activity harboured well discriminated bacterial communities, the snails in hibernation showed approaching community structures without losing in diversity of phylotypes. Gut clearance and physicochemical changes attended by the initiation of hibernation might induce the restructuration of the bacterial community. Some strains could disappear or being reduced, while other not dominant strains during activity, gain in space and become important during hibernation. In fact, during hibernation a high abundance of bacteria were observed in the snail gut (Jeuniaux, 1955, Charrier, 1990). Enzymatic activity that depends on the physiological state, e.g. carbohydrase activity (Flari & Charrier, 1992), could also be linked to changing microbial community structure. Some cellulases and chitinases were suggested to be of bacterial origin (Myers & Northcote, 1959, Strasdine & Whitaker, 1963, Charrier, 1990).

In insects some intestinal bacteria strains attracted much attention in studies about freezing resistance. *Enterobacter agglomerans* and *Enterobacter taylorae* was found in insect guts during activity, initiating ice formation in the insect's body between 0 and -5°C (Lee*, et al.*, 1991). Before winter these strains were actively expulsed from the gut, thereby enhancing the supercooling ability of the insect. These ice-nucleating bacteria owe the capacity of freezing initiation at temperatures between –10 and 0°C to the presence of specific proteins on their cell surfaces, like proved in *Pseudomonas fluorescens* (Pseudomonadaceae, Corotto*, et al.*, 1986) and *Erwinia herbicola* (Enterobacteriaceae, Warren & Corotto, 1989). *Pseudomonas syringae* was identified as INA responsible for freeze injuries in plants (Lindow*, et al.*, 1982), and

Xanthomonas campestris showed also INA activity (Kim*, et al.*, 1987). In earlier studies on freezing resistance in *Helix pomatia* and *Cornu aspersum* the intestinal content showed ice nucleating activity, which could be inhibited by antibiotics (Ansart*, et al.*, 2002) and heat (Ansart et al. *Ca-V*). *Kluyvera* sp was identified acting around –9°C, but persisted in snail gut during hibernation (Nicolai et al. 2005). The possible presence of *Xanthomonas campestris* in the snail gut in activity as well as in hibernation in the RHE population is consistent with the supercooling ability of –2°C in activity and –6°C in hibernation of this population (Nicolai, *et al., Hp*-II).

The digestive tract of *Helix pomatia* is anatomically as well as physicochemically compartmentalized (Charrier & Brune, 2003) which was also reflected by the bacterial community structure in this study. The passage of the gut content through the intestinal tract could transiently lead to an enrichment of minor allochthonous bacteria populations when the compartment provides favourable environmental conditions (Schmitt-Wagner*, et al.*, 2003). This was visible in the proximal intestine and digestive gland in activity of the RHE population. At hibernation beginning snails expulse the gut content thereby eliminating some phylotypes, like Mollicutes and Alphaproteobacteria from the proximal intestine and the later also from the digestive gland. At this moment unclassified bacteria became abundant in the proximal and distal intestine, but were reduced during hibernation. In contrast to nutrient providing by the snail through foraging, the mucus secretion in the gut during hibernation (Pereira & Breckenridge, 1981, Charrier, 1990) supports only a permanent community (Charrier*, et al.*, 2006) that colonise nearly equally all compartments of the intestinal tract in our study.

The community structure within the intestinal tract of non hibernating snails was close to that in hibernating snails on the NMDS plot, but the diversity of phylotypes resembles to the community of snails at hibernation beginning. However the complete lost of Mollicutes from the whole intestinal tract in non hibernating snails was striking. However, it remains unclear if the bacterial community reflects poor body conditions or if pathogen strains were present.

Despite the method of PCR-DGGE was shown to be adequate to detect and compare differences in microbial community structures (Okubo & Sugiyama, 2009), we could not find the same diversity described by Charrier et al. (2006). DGGE is limited by the fact that profiles show only the most dominant phylotypes in the investigated samples (Claesson*, et al.*, 2009). Furthermore, some bands from different positions in one column of the DGGE gel were shown to contain the same sequence: ba14 and ba11.2 were assigned to Pantoea sp. and ba2, ba12, ba13 were assigned to the same sequences of uncultured bacteria isolated from the gut of planorbid snails. Bacteria can feature up to 15 operons, with less than 1% nucleotide difference, in their genome (Acinas*, et al.*, 2004). These small divergences are enough to lead to multiple bands on the DGGE. Such sequence variations could also be introduced artificially as a consequence of PCR amplification (Speksnijder*, et al.*, 2001). Another critical issue is the specificity of the primer used which could reduce considerably the number of revealed bacteria phylotypes, as observed earlier for some other primer sets (Malin & Illmer, 2008). In fact we excised some important bands from the DGGE gel that were assigned to snail or plant DNA.

From DGGE and NMDS results obtained in this study we conclude that *Helix pomatia* harbours permanently a rich gut microbial community. Transiently allochthonous bacterial populations might colonise the gut of foraging snails in respect to the geographical origin of the snail population and reflect climatic and trophic conditions of the snail's habitat. Changes in the community structure in the different gut compartments are attended by changes in the physiological state and could be vital for maintenance of organism functioning. Further studies should therefore focus on the relation between environmental bacteria and gut bacteria in order to better understand the habitat quality needed to sustain this endangered species.

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Nur keine kalten Füβe bekommen

Eine hohe Sterberate während der Hibernation wurde besonders mit energiearmem und energiereichem Futter beobachtet, was auf einen Mangel an Energiereserven zurückzuführen sein kann. Dies ist entweder durch den Energiegehalt des Futters begründet oder durch die schnelle Wachstumsrate mit energiereichem Futter, so dass mehr Energie in Wachstum als in den Aufbau von Reserven für die Hibernation investiert wurde.

Zum Einsetzen der Hibernation müssen physiologische Prozesse aktiviert werden, die die Kälteresistenz erhöhen, da *Helix pomatia* nur kurze Zeit das Gefrieren der Körperflüssigkeiten überlebt (Nicolai et al. 2005). Trotz dem Ausscheiden von vorrübergehend mit der Nahrung aufgenommener Bakterienarten aus dem Darm, wird das Verbleiben einer permanenten Flora während der Hibernationsphase von der Schnecke unterstützt (Pereira & Breckenridge, 1981, Charrier, 1990, Nicolai et al. *Hp*-I). Somit bleibt der Darm der Ort, in dem das Gefrieren der Schnecke initiiert wird, da sich unter den Bakterien Eisnukleationsagenzien befinden (Ansart et al. *Ca*-V, Nicolai et al. *Hp*-I).

Um die Kälteresistenz zu erhöhen, müssen daher Mechanismen wie Wasserverlust zur Anreicherung von Frostschutzmitteln (Polyole und Zucker) einsetzen (Storey 1997, Ramløv 2000, Holmstrup & Zachariassen 1996) oder Frostschutzproteine produziert werden (Zachariassen 1985), die den Gefrierpunkt der Körperflüssigkeiten herabsenken (Abb. *hp*15). Da *Helix pomatia* unterschiedlicher geographischer Herkunft durch die Regulierung des Wasserverlustes die Kälteresistenz nur in begrenzter Form an regionale Klimabedingungen anpassen kann (Nicolai et al. 2005), ist besonders der Aspekt der Frostschutzmittel interessant.

Hypothesen dieser Studie:

- *Helix pomatia* produziert ein multiples System an Frostschutzmitteln.
- Die Kälteresistenz ist an das regionale Klima einer Population angepasst.

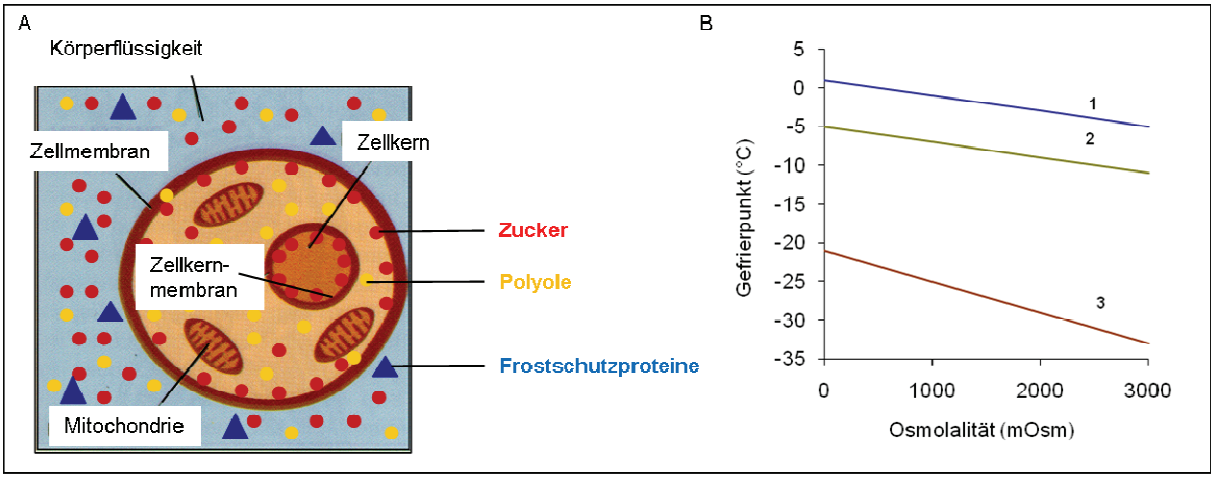

Abb. *hp*15. Unterkühlung der Körperflüssigkeit durch die Anreicherung von Zuckern und Polyolen sowie durch die Produktion von Frostschutzproteinen (A). Fusionspunkt einer Glycerollösung (1), Gefrierpunkt mit Nukleationsagenzien (2), Gefrierpunkt ohne Nukleationsagenzien (3). (Block und Young 1979, Zachariassen und Hammel 1976).

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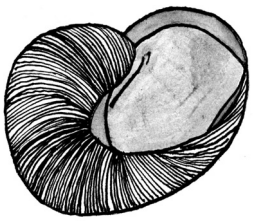

Accumulation of multiple cryoprotectants in the haemolymph of two populations in the land snail *Helix pomatia*

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Reference: Nicolai et al. *Hp*-II

Abstract: Ectothermic species, like land snails, have to face subzero temperatures in winter to which they respond usually with a state of inactivity. Hibernation involves specific physiological processes, like metabolic depression, water loss and cryoprotection. *Helix pomatia* is a widely distributed species in Europe which encounters a wide range of climatic conditions. However, it is still unknown how haemolymph composition of metabolites could contribute to cold hardiness in hibernation in this endangered land snail. Therefore, we investigated by UPLC and GC-MS techniques the metabolite composition of haemolymph in activity and hibernation of a mountain population (ALB) and a valley population (RHE) in Germany. We used a linear and a multivariate model to relate metabolite composition to body conditions and cold hardiness. The amount of amino acids depended on body dry mass in activity. In the ALB population dry mass and amino acid content was higher than in the RHE population. Galactose was the most abundant component in haemolymph and abundant in activity, besides glucose. Both saccharides might indicate glycolytic activity providing energy for locomotion and foraging. Differences between populations in amino acid and saccharide composition might therefore reflect differences in microhabitat conditions, like climate and vegetal food. In hibernation Asp, Glu, Gly, hydroxy-Pro, glycerol and triglycerides were importantly accumulated in both populations. Their content was correlated to the decrease of body supercooling point und body water mass. Therefore these metabolites might be accumulated through the increase of haemolymph concentration due to body water loss, and they may act as cryoprotectants in *Helix pomatia* enhancing cold hardiness during hibernation. Further studies should focus on the influence of environmental factors on seasonal changes in haemolymph composition and the meaning of these physiological processes for snail survival in a circannual rhythm.

Keywords: Amino acids, Cold hardiness, Galactose, Gastropods, GC-MS, Hibernation, Polyols, Triglycerides, UPLC

Introduction

Among the numerous environmental constraints, ectothermic species, like land snails, have to face subzero temperatures in winter which limits their geographic distribution (David et al. 1996). The response to harsh climatic conditions in winter is usually a state of inactivity, hibernation, in a temperature and humidity buffered refuge. The body is fully withdrawn, and the shell is obturated by an epiphragm (Lind 1988, Ansart et al. 2002). The initiation of hibernation involves physiological adjustments to enhance cold hardiness.

In dormancy some general physiological and biochemical adjustments could be observed: (i) metabolic depression regulated with specific proteins (Brooks & Storey 1995), (ii) lower heart rate and body mass decrease due to consumption of energy stores (Rizatti & Romero 2001), and (iii) water loss (Arad 2001, Riddle 1981).

Some physiological mechanism specific to hibernation can contribute to a higher supercooling ability thereby enhancing survival probability in land snails that implement cold hardiness strategies ranging from freezing intolerant to partially freezing tolerant (Ansart et al. 2001, Nicolai et al. 2005). Generally, the reduction of the volume of freezeable water enhances supercooling ability, because it is more unlikely that an ice embryo will spontaneously form and that the body fluid will contain an active ice nucleator (Lee & Costanzo 1998, Vali 1995). Water loss coupled with gut clearance at the initiation of hibernation might contribute to a great supercooling ability (-16°C) in *Anguispira alternata* (Riddle 1981), through the expulsion of ice nucleating agents from the intestinal tract (Lee et al. 1995).

Cryoprotectants have different functions in cold hardiness: depressing the body supercooling point by the increase of osmolarity of body fluids (Storey 1997, Ramløv 2000) and protecting cells from lethal intracellular freezing (Costanzo et al. 1993) or dehydration (Meryman 1971), but also as energy reserve available upon arousal (Lewis et al. 2004, Driedzic and Ewart 2004). Their accumulation often results from body dehydration (Holmstrup & Zachariassen 1996) or from activation of metabolic pathways producing e.g. 100 times higher sorbitol levels in winter compared to summer in freshwater snails (Tsvetkov & Konichev 2009). The most important cryoprotectors largely studied in insects and some ectothermic reptiles are polyhydric alcohols, like sorbitol, mannitol and glycerol, and saccharides, like glucose, fructose and trehalose (Block 1995, Storey 1997). In the freshwater snail *Lymnea stagnalis* and in the oligoptera *Hemideina maori*, free amino acids have been described acting as cryoprotectant (Karanova & Gakhova 2007, Sømme 1967, Ramløv 1999). In fact Mohammed et al. (2007) have shown that amino acids can exhibit similar cryoprotection as trehalose, and Khlifaoui et al. (2005) and Kundu et al. (2001) demonstrated that amino acids improved the cryoprotective action of glycerol. Lipids also appear acting as cryoprotectants often cited in relation with thermoregulation of membrane fluidity at subzero-temperatures. In termites, trehalose and unsaturated lipids increase during winter suggesting a role as cryoprotectants (Lacey et al. 2010). In the soil arthropod *Orchesella cinta,* the appearance of mainly unsaturated fatty acid in membranes and in storage lipids (triglycerides) was due to cold acclimatisation (Dooremalen and Ellers 2010). Furthermore, in presence of cholesterol, freezing temperature of water bound to the phospholipidic bilayer was depressed (Lee et al. 2008).

However until now, cryoprotectants in land snails have been poorly studied. That's why we investigated metabolite composition of the haemolymph in relation to cold hardiness in two geographically distinct populations of *Helix pomatia*. This species has a large latitudal (Greece

to Sweden) and longitudal (France to Poland) distribution in Europe (Nietzke 1970), exposing individuals to a wide range of climatic situations and annual variations. *Helix pomatia* initiate hibernation between August and October (Lind 1988) by burrowing into the soil and forming an epiphragm. The species is partially freezing tolerant with seasonal variations in supercooling ability. Mean supercooling point levels -2°C in activity, but reaches -4.5 to -6°C in hibernation due to water loss and an increase in haemolymph osmolality (Nicolai et al. 2005). *Helix pomatia* survives freezing only for several hours. Mortality during hibernation seems to be the key factor in population dynamic of Helicidae in Europe (Cain 1983, Peake 1978).

In this study we advanced the following hypotheses:

(1) Since Nowakowska et al (2006) detected an increase of glycerol in cold acclimated individuals of *Helix pomatia*, the accumulation of cryoprotectants during hibernation through body water loss could contribute to cold hardiness in this species. Besides glycerol, *Helix pomatia* might accumulate polyols and saccharides as well as free amino acids and lipids (triglycerides and cholesterol) for cryoprotection during hibernation, compared to the physiological state of activity. The role of free amino acids and lipids in cryoprotection of land snails has not been studied until now.

(2) In an earlier study on *Helix pomatia*, a lower supercooling ability distinguished a Swedish from a French population, which was explained by differential water loss and haemolymph osmolality (Nicolai et al. 2005). In the oligochete, *Enchytraeus albidus*, such geographic differences in cold-hardiness have been attributed to a differential adjustment of the body glucose content in a German *versus* Greenland population (Slotsbo et al. 2008). Therefore, cold hardiness strategies in *Helix pomatia* might depend on geographic origin, and metabolite composition in the haemolymph might represent physiological adaptations to habitat and microclimatic conditions.

Material and Methods

BIOLOGICAL MATERIAL

In September 2006 snails were collected in two different locations of different altitudes in Germany (authorisation §2 Abs. 2 BNatSchG from September 18th 2005). Population RHE: collected in the forest (dominated by *Carpinus betulus* and *Robinia pseudoacacia* with ground vegetation dominated by *Urtica dioica, Impatiens non-tangere, Geranium robertianum*, *Galeopsis tetrahit*, *Ranumculus ficaria*, *Chelidonium majus,* and *Fragaria vesca*) near Grünstadt-Asselheim, Rhein valley in Germany (49° 33’ 14N, 8° 9’ 46E, 150 m a.s.l.). Population ALB collected in the forest (dominated by *Carpinus betulus* and *Alnus incana* with ground vegetation dominated by *Urtica dioica, Fumaria officinalis*, *Impatiens non-tangere, Geranium robertianum*, *Viola reichenbachiana, Anemone nemoros, Dentaria bulbifera*) near Weiler-Indelhausen, mountains Schwäbische Alb in Germany (48° 18’ 7N, 9° 29’ 47E, 800 m a.s.l.). The climate is characterized by different minimal and maximal temperatures in winter and summer as well as by precipitation height in summer (Figure 1). In the Rhein valley compared to the mountains Schwäbische Alb, the climate is warmer with a mean year temperature of 11.6°C *versus* 9.9°C, respectively, and drier in summer (May to August) with a mean precipitation of 73.7 mm *versus* 83.45 mm, respectively, but more humid the remaining months of the year (59.5 mm *versus* 51.3 mm, respectively).

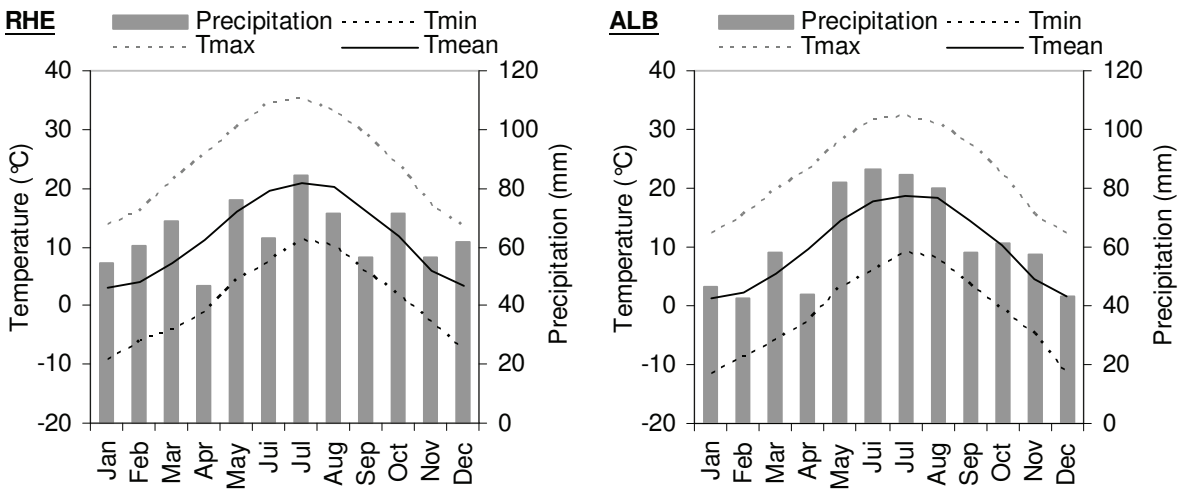

Figure 1. Climate in the Rhein valley (RHE) and in the region Schwäbische Alb (ALB), Germany, represented as mean of the period 1999-2008. Tmin, Tmax, and Tmean – minimal, maximal, and mean air temperature (2 m above soil surface), respectively. (http://www.dwd.de, station Karlsruhe and Stuttgart, respectively).

8 snails of each population were used immediately (active snails: sample A) for measurement of the supercooling point, the body dry mass and water mass, as well as for haemolymphe extraction. Because snails are hard to collect in hibernation conditions (frozen soil, unknown location of burial) 12 additional snails of each population were progressively transferred to hibernation condition (temperature 5 ± 1°C, relative humidity: 50%, 24h darkness) over two weeks without feeding in the laboratory. By the end of the second week snails obturated the peristom of their body shell with a hard calcified epiphragm, thereby initiating hibernation. After three months of hibernation 9 snails were used for the same procedures (hibernating snails: sample H). The final number of snails used in experimentation was $N_{ALB\text{-}A}$ = $N_{ALB\text{-}H}$ = 8, $N_{RHE\text{-}H}$ = 9, $N_{RHE\text{-}A}$ = 7, because some snails died during hibernation or shad to be discarded from further analysis because haemolymph extraction failed.

BODY SUPERCOOLING POINT (BSP) DETERMINATION

As described by Nicolai et al. (2005) a hole of 1 mm diameter was drilled by a trepan (S2000, Georg Schick Dental GmbH, Schemmerhofen, Germany) in the snail shell above the pallial cavity. A thermocouple (K-type: Chromel-Alumel, Fischer Scientific, France) was inserted through the shell hole into contact with the mantle tissues. In the case of injury of the body tissues (hemolymph outflow) snails were discarded from further analysis. The thermocouple was connected by a wire to an electronic multichannel thermometer (Tempscan C8600, Comarck Electronics Ltd., Hertfordshire, UK) in order to simultaneously measure temperatures of several snails. Measurements were done every second and transmitted to a printer (HP deskjet 500, Hewlett-Packard Company, Palo Alto, CA, USA). Snails were placed in a plastic box (5 x 5 x 5 cm) which was placed in a thermostat (MT 3, Lauda GmbH & Co. KG, Lauda-Königshofen, Germany) filled with silicone oil (Kryo 51, Lauda GmbH & Co. KG, Lauda-Königshofen, Germany) and cooled by a through-flow cooler (DLK 10, Lauda GmbH & Co. KG, Lauda-Königshofen, Germany). Their body temperature was first acclimated to 5°C.

Then the temperature in the thermostat was decreased at a cooling rate of 0.6°C.min^{-1}. This cooling rate lies in between 0.5 to 1°C.min^{-1}, commonly used in cryobiology and recommended by Salt (1966), which enables comparisons with other studies on cold-hardiness in invertebrates. The BSP was recorded at the start of the exotherm (release of heat) due to crystallization of supercooled body fluids (Lee 1989). The lowest temperature in the thermostat could be –12°C which is well below earlier recorded BSP in Helix pomatia (-9°C, Nicolai et al. 2005). The snails were taken out of the thermostat right after reaching BSP.

HAEMOLYMPH EXTRACTION AND SUPERCOOLING POINT (HSP) MEASUREMENT

A hole of 2-3 mm diameter was drilled by the trepan in the snail shell above the pericardiac cavity. Up to 250 µl of haemolymph could be collected by heart puncturing and was centrifuged at 10,000 g, 4°C for 5 min to eliminate shell and tissue particles (Ramløv 1999). Samples were stored at –80°C until metabolites analysis. The measurement of the HSP was performed using the freezing droplet method (Vali 1971, 1995). Five droplets of 5 µl per individual haemolymph sample were placed onto the surface of aluminum pans (Fisher Scientific, France). The pans floated on the silicone oil bath of a cryostat (Polystat CC3, Huber) cooled from 5°C to –15°C at a cooling rate of 0.6°C per minute. One thermocouple, connected to the electronic thermometer, was fixed on one aluminum pan to follow the temperature changes on the pan surface. The freezing of the droplet (becomes opaque) was observed visually, the HSP was recorded for each droplet and mean HSP was calculated for each haemolymph sample.

METABOLITES DETERMINATION

For one extraction protocol we took 100 µl of haemolymph per individual from each sample of the ALB and the RHE population. For UPLC and GC-MS analysis we used the protocol of Renault et al. (2010) and lipid analysis was done according to the protocol of Hervant et al. (1999). All metabolite levels were expressed in mass per volume of haemolymph, using molecular mass for conversion if necessary.

For both UPLC and GC-MS analysis, 100 µl of haemolymph was suspended in 600 µl of methanol and agitated at 1500 rpm for 15 min. Subsequently, 300 µl of chloroform were added and samples were agitated for five additional minutes. Finally, 600 µl of ultra-pure water were added, samples were then vigorously vortexed and centrifuged at 4000 g for 10 min. 700 µl of upper phase (methanol-water phase) per samples were transferred to a clean micro tube and dried in vacuum (miVac DNA concentrator, Genevac, Suffolk, UK).

For free amino acids analysis, dry residues were suspended in 50 µl of ultra-pure water and 5 µl of the resulting extract were sampled for amino acids derivatization according to the AccQ•Tag Ultra Derivitization Kit protocol (Waters Corporation, Milford, MA). Amino acids were analyzed using an Acquity UPLC® system (Waters Corporation, Milford, MA) by injecting 1 µl of the derivatization mix onto an Acquity UPLC® BEH C18 1.7 µm 2.1 x 100 mm column heated at 55°C. Amino acids were eluted at 0.7 ml.min^{-1} flow with a mix of 10-fold diluted AccQ•Tag Ultra Eluent (A; Waters Corporation, Milford, MA) and acetonitrile (B) according to the following gradient : initial, 99.9 % A ; 0.54 min, 99.9 % A ; 6.50 min, 90.9 % A; 8.50 min, 78.8 % A; 8.90 min, 40.4 % A; 9.50 min, 40.4 % A; 9.60 min, 99.9 % A; 10.10 min, 99.9 % A. Derivatized amino acids were detected at 260 nm using a photo diode array detector. Amount of amino acids were obtained using standard solutions of all aminoacids.

For GC-MS analysis, the remaining 40 µl of extract were dried in vacuum and then suspended in 630 µl of pure water in order to obtain the initial concentration. Subsequently, 200 µl of this extract were transferred to GC-MS glass vials and dried in vacuum. Dried samples were dissolved in 50 µl of freshly prepared methoxyamine hydrochloride solution in pyridine (20 mg/ml) and agitated for 90 min at 30°C. Afterwards, 50 µl of N-methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA; Sigma, #394866) were added and derivatization was conducted at 37°C for 30 min under agitation. Incubation took place at room temperature over-night before injection. GC-MS system consisted of a TriPlus autosampler, a Trace GC Ultra chromatograph and a Trace DSQII quadrupole mass spectrometer (Thermo Fischer Scientific Inc, Waltham, MA). 1 µl of the derivatization mix was injected onto a Thermo TR-5MS 30 m x 0.25 mm ID x 0.25 µm column under the following heating protocol: 70°C for 4 min, increase of temperature at 5°C.min^{-1} until 310°C, 310°C for 5 min. Metabolites were eluted at 1 ml.min^{-1} flow with Helium and injected at 250°C in the mass spectrometer at a speed flow of 25 ml.min^{-1} and a transfer line temperature of 250°C. Chromatograms were deconvoluted using the AMDIS software v2.65 (http://chemdata.nist.gov/mass-spc/amdis/) associated with NIST libraries. Amount of metabolites were obtained using standard solutions. In order to quantify the content of glucose and galactose that was not analyzed by GC-MS procedure because of its high abundance, we used the Enzyplus® EZS 784+ Lactose/D-Galactose Kit (BIOCONTROL, Bellevue, WA, USA) on 48 µl of the extract solution and the Enzyplus® EZS 784+ D-Glucose Kit (BIOCONTROL, Bellevue, WA, USA) on 100 µl of the extract solution.

For each lipid analysis (triglycerides and cholesterol), 100 µl of haemolymph was suspended in 1.5 ml methanol-chloroform solution (methanol:chloroform 1:2 v/v), followed by a 12 h incubation at –20°C. Phase separation was obtained by adding 300 µl KCl (2 g.l^{-1}) and heating for five minutes at 40°C. The inferior phase (chloroform phase) was separated in two aliquots of 400 µl. Both aliquots were dried under nitrogen flow at 30°C for 15 min before suspension in 300 µl solution BSA (3% w/v, fatty acid free) - Triton (0.2% v/v) for triglyceride analysis and in 10 µl Ethanol (95°) for cholesterol analysis. The content of triglycerides and cholesterol was determined by measuring the absorbance with a microplate spectrophotometer (VERSAmax™ microplate reader, Sunnyvale, CA, USA), using the Triglyceride Assay Kit (Cayman Chemicals, Ann Arbor, Michigan, USA) and the Cholesterol RTU™ kit (Biomerieux, France).

MEASUREMENT OF BODY MASS

Frozen snails were weighed (Analytic A200S, Sartorius AG, Göttingen, Germany), to determine their body mass (BM) before removing the shell which was weighed separately (SM). To determine the dry mass of flesh (DM), the soft body parts were frozen and lyophilized (Trivac, Oerlikon Leybold Vacuum GmbH, Köln, Germany). The water mass (WM) of a snail was calculated by the following equation: WM = BM - (DM + SM).

DATA ANALYSIS

Statistical analyses were carried out using “R” (R Core Team 2007). After snail collection we analyzed individual body size in the constituted samples using ANOVA. In statistical analysis of cold hardiness traits (BSP, HSP, DM, WM), we tested the effects of population origin and physiological state as well as the covariate body mass using generalized linear model procedures (GLM) with Gaussian distribution and identity link function (Nelder

and Weddenburn 1972). After a first analysis, models were simplified by backward elimination of non significant effects from the full model (Crawley 2007). Tukey HSD served as post hoc test. We performed Pearson correlations between body supercooling point and dry mass as well as water mass on the whole dataset.

Metabolite contents in haemolymph, except for rarely (in only one or two individuals of a sample) detected metabolites, were analyzed using GLM procedures with gamma distribution and power (-1) link function. After a first analysis, models were simplified by backward elimination of non significant effects from the full model (Crawley 2007). We performed Kendall correlations with body supercooling point, dry mass and water mass on the whole dataset. Redundancy analysis (RDA) was conducted on metabolite content as response variables and biological parameters as explanatory variables. This analysis shows how much of the variance in one set of variables can be explained by the other. The inter-correlation coefficient between variables retained for this type of analysis was $R < 0.90$. Distance matrix was calculated by the Jaccard method and cluster analysis was performed with the Ward method. In order to test the validity of explanatory variables we performed a Monte-Carlo test with 1000 permutations. Finally, representation shows the vector plot of metabolites, the correlation plot of biological variables and the position of each individual on two retained canonical axes in respect to their metabolite composition and biological parameters.

Results

Water mass and dry mass were well described by the model (high deviance reduction, Table 1) and decreased significantly during hibernation in the ALB population, but not in the RHE population (Figure 2).

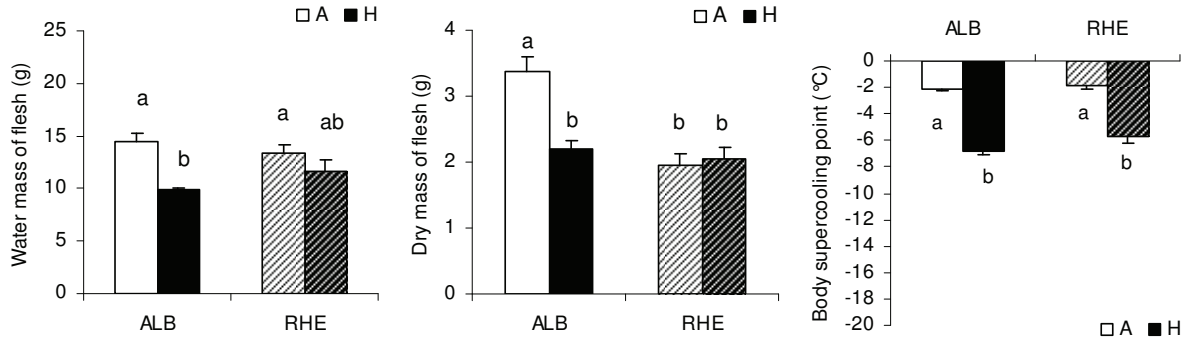

Figure 2. Water mass, dry mass and body supercooling point (M±SE) in *Helix pomatia* in activity (A) and hibernation (H) from two populations: mountains Schwäbische Alb (ALB) and Rhein Valley (RHE), in Germany. Significant differences of the final model of GLM (Table 3) and Tukey HSD ($P < 0.05$) are indicated by letters. Error bars denote standard errors. $N_{ALB\text{-}A} = N_{ALB\text{-}H} = 8$, $N_{RHE\text{-}H} = 9$, $N_{RHE\text{-}A} = 7$.

The factor origin and the interaction term were significant for dry mass, indicating that ALB snails had generally a higher dry mass which they lost during hibernation whereas RHE snails kept their low amount of dry mass. These changes were not due to body size because samples of collected snails did not differ in mean body size (BS = 38.01 ± 0.08 mm, ANOVA, origin: $F = 0.02$, $P = 0.89$, state: $F = 0.04$, $P = 0.84$, interaction: $F = 0.05$, $P = 0.82$, $N_{ALB\text{-}A} =$

$N_{ALB\text{-}H}$ = 8, $N_{RHE\text{-}H}$ = 9, $N_{RHE\text{-}A}$ = 7). The body supercooling point was only influenced by the state, which explained all the 84.11% of the deviance reduction (Figure 2, Table 1). It decreased during hibernation in both populations and did not covariate with body mass. Body supercooling point was correlated to water mass (Pearson, R = 0.49, DF = 30, P = 0.002) on the whole dataset, but not to dry mass (Pearson, R = 0.27, DF = 30, P =0.07). In fact, water mass showed a similar pattern with Body supercooling point, as it did differ between states, but not between populations. Haemolymph supercooling point lay well below the body supercooling point and did neither differ between populations nor between states (HSP = -14.71 ± 0.17°C, GLM, P > 0.05, N = 32).

Table 1. Summary of the analysis of deviances on cold hardiness traits observed in two populations, Schwäbische Alb (ALB) and Rhein Valley (RHE), of *Helix pomatia* in hibernation (H) and activity (A). We indicated the explained deviances for the final linear model, followed by degrees of freedom (*DF*), *F*- and *P*- values. $N_{ALB\text{-}A}$ = $N_{ALB\text{-}H}$ = 8, $N_{RHE\text{-}H}$ = 9, $N_{RHE\text{-}A}$ = 7.

	Total deviance reduction (%)	**Model term**	**Explained deviance (%)**	***DF***	***F***	***P***
Water mass of flesh (WM)	90.79	origin				ns
		state	35.07	29	100.21	<0.0001
		O x S				ns
		BM	64.93	28	185.50	<0.0001
Dry mass of flesh (DM)	84.39	origin	32.43	30	47.33	<0.0001
		state	15.53	29	22.67	<0.0001
		O x S	6.86	28	10.01	<0.0001
		BM	45.17	27	65.92	0.0038
Body supercooling point (BSP)	84.11	origin				ns
		state	100	30	158.85	<0.0001
		O x S				ns
		BM				ns

The techniques used for metabolite analysis allowed to identify and to quantify a large number of metabolites (Figure 3, Table 2). Gluconic and succinic acid (carboxyl acids), putrescine (polyamine) as well as maltose and mannobiose (saccharides) occurred only in one or two individual samples were thus discarded from linear modeling. Except for Ser, Gln, β-Ala, myo-inositol, β-D-methylglucopyranoside (β-D-MGP), hydroxy-Pro, triglycerides and cholesterol, metabolite content varied between population origins and generally showed higher contents in ALB snails than in RHE snails. Only Asp, Glu, Gly, galactose, glucose and glycerol were also significantly influenced by the physiological state. Hydroxy-Pro and triglycerides were solely influenced by the state. Tyr, Pro and α-Ala showed besides the significant effect of origin also a significant interaction, while cholesterol had only a significant interaction term. Among these metabolites Asp, Glu, Gly, hydroxy-Pro glycerol and triglycerides increased, while galactose decreased in both populations during hibernation. Glucose decreased in ALB snails during hibernation and increased slightly in RHE snails, while α-Ala, Tyr and Pro staid constant in the ALB population and increased during hibernation in the RHE population.

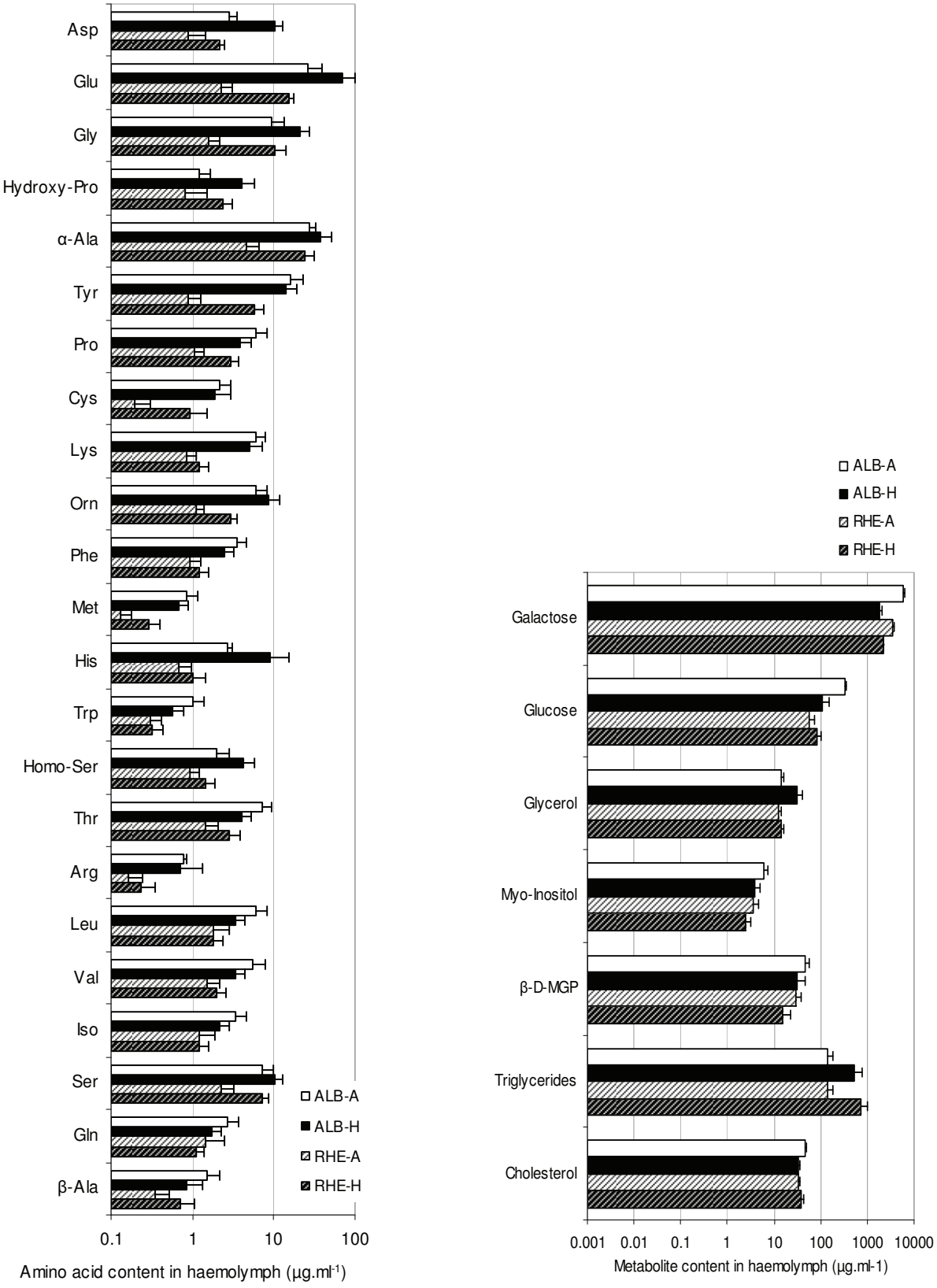

Figure 3. Metabolite content in haemolymph in *Helix pomatia* in activity (A) and hibernation (H) from two populations: Schwäbische Alb (ALB) and Rhein Valley (RHE), in Germany. Error bars denote standard errors. Note log scale. $N_{ALB-A} = N_{ALB-H} = 8$, $N_{RHE-H} = 9$, $N_{RHE-A} = 7$.

Table 2. Results of the GLM and Kendall's correlation performed on metabolite contents in haemolymphe of *Helix pomatia* from two populations, mountains Schwäbische Alb (ALB) and Rhein Valley (RHE), and in two states, hibernation (H) and activity (A). *F* and *P* are indicated for the final model. $N_{ALB\text{-}A} = N_{ALB\text{-}H} = 8$, $N_{RHE\text{-}H} = 9$, $N_{RHE\text{-}A} = 7$. For abbreviations see table 1.

	GLM						Correlation					
	Origin		State		O x S		BSP		WM		DM	
	F	*P*	*F*	*P*	*F*	*P*	*τ*	*P*	*τ*	*P*	*τ*	*P*
Asp	19.39	0.0001	11.43	0.002		ns	-0.32	0.005	-0.32	0.005		ns
Glu	16.94	0.0003	5.66	0.024	8.22	0.008	-0.35	0.003	-0.33	0.004		ns
Gly	4.82	0.037	6.03	0.021	6.03	0.021	-0.46	0.0001	-0.38	0.001		ns
Hydroxy-Pro		ns	4.96	0.034		ns	-0.26	0.018	-0.22	0.041		ns
α-Ala	4.82	0.037		ns	7.61	0.010	-0.27	0.015		ns		ns
Tyr	11.98	0.002		ns	8.90	0.006		ns		ns		ns
Pro	5.93	0.022		ns	4.89	0.035		ns		ns	0.25	0.020
Cys	4.77	0.037		ns		ns		ns		ns	0.30	0.009
Lys	24.34	<0.0001		ns		ns		ns		ns	0.35	0.003
Orn	13.10	0.001		ns		ns		ns		ns	0.29	0.011
Phe	10.59	0.003		ns		ns		ns		ns	0.311	0.006
Met	9.32	0.005		ns		ns		ns		ns	0.28	0.012
His	8.21	0.008		ns		ns		ns		ns	0.30	0.007
Trp	7.02	0.013		ns		ns		ns		ns	0.35	0.003
Homo-Ser	6.49	0.016		ns		ns		ns	0.21	0.046		ns
Thr	6.44	0.017		ns		ns		ns		ns	0.38	0.001
Arg	6.19	0.019		ns		ns		ns		ns	0.26	0.019
Leu	5.96	0.021		ns		ns		ns		ns	0.29	0.009
Val	5.97	0.021		ns		ns		ns		ns	0.29	0.009
Iso	4.51	0.042		ns		ns		ns		ns	0.28	0.012
Ser		ns		ns		ns	-0.29	0.010		ns		ns
Gln		ns		ns		ns				ns	0.26	0.018
β-Ala		ns		ns		ns		ns		ns		ns
Galactose	12.59	0.001	86.07	<0.0001	8.93	0.006	0.46	0.0001	0.36	0.002	0.33	0.004
Glucose	16.94	0.0003	5.66	0.024	8.22	0.008		ns	0.26	0.019	0.53	<0.0001
Glycerol	6.33	0.018	6.43	0.017		ns	-0.33	0.004	-0.29	0.010		ns
Myo-Inositol		ns		ns		ns		ns		ns		ns
β-D-MGP		ns		ns		ns		ns		ns		ns
Triglycerides		ns	12.39	0.001		ns	-0.23	0.03		ns		ns
Cholesterol		ns		ns	4.98	0.03		ns		ns		ns

Some metabolite contents were negatively correlated to both body supercooling point and water mass, except α-Ala, Ser and triglycerides that were not related to water mass (Table 2). Metabolites that were not negatively correlated to the body supercooling point were positively correlated to dry mass, except Galactose which was positively correlated to both dry mass and the body supercooling point. Tyr, Homo-Ser, β-Ala, Myo-Inositol, β -D-MGP and cholesterol were not correlated to any of both parameters.

RDA analysis showed that metabolite composition could be well explained by biological parameters and related to origin and physiological state (Figure 4). During activity ALB and RHE population were well separated in respect to their metabolite composition and placed on the second and first axis, respectively. The first axis was mainly negatively correlated to dry mass ($R = -0.47$), while the second axis was mainly positively correlated to body supercooling point and water mass (both $R = 0.71$) as well as to dry mass ($R = 0.79$). Both populations had a similar metabolite composition during hibernation and were placed in one single cluster on the lower part of the second axis.

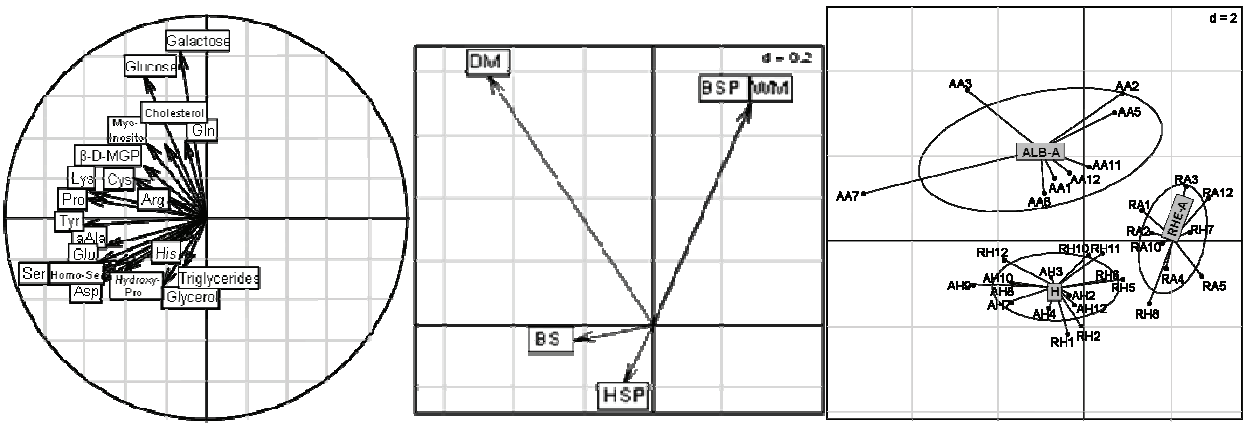

Figure 4. Results of RDA analysis on metabolite content in haemolymph and on biological parameters (correlation plot) of *Helix pomatia* in activity (A) and in hibernation (H) from two populations: mountains Schwäbische Alb (ALB) and Rhein Valley (RHE), in Germany. Monte-Carlo test (1000 permutations): P = 0.01. The part of eigenvalue of constrained and unconstrained axes were 67.49% and 32.51%, respectively. RDA was constructed on the first two constrained axes with the part of eigenvalues of 49.42% and 35.76%, respectively. DM – body dry mass, WM – body water mass, BSP – body supercooling point, HSP – haemolymph supercooling point, BS – body size.

Discussion

Metabolite content in haemolymph reflected very well the physiological state and body conditions in two populations of *Helix pomatia*. In the active state the metabolite composition was mainly related to dry mass, which differentiated both populations. Individuals from the RHE population had a lower dry mass and lower metabolite content in haemolymph while body size did not differ from those in the ALB population. Therefore the amount of metabolites in haemolymph might correspond to the body condition, which might be related to climatic and trophic factor of the geographic origin. Climatic conditions differ in both populations, and the warmer and drier climate in summer as well as the more humid climate in winter in the RHE region might be in relation to seasonal variations of dry mass and water mass.

Galactose and glucose mainly defined the active state in the ALB population (RDA plot), indicating a high demand of energy throughout the body, e. g. for foraging, locomotion and maintenance. The high quantity of galactose was striking and highlights the accumulation of galactogen in the albumen gland during reproduction (Tompa 1984) and the subsequent allocation to eggs (Nicolai et al. *Ca*-II), suggesting that galactose is the major component for energy liberating glycolysis in *Helix pomatia*. The reason, why galactose content exceeds glucose content, could be explained by diet choices. Galactans are incorporated in hemicelluloses polymers, like arabinogalactans and galactomannans, found abundantly in leguminous plants (Flari and Charrier 1992, Mora 1992).

The transition to the hibernation state involves loss of water and dry mass in the ALB population while water loss in the RHE population was not significant and dry mass loss was negligible. Finally, after three months of hibernation snails of both populations did neither differ in these biological traits nor in metabolite composition of their haemolymph. Asp, Glu, Gly, hydroxy-Pro, glycerol and triglycerides defined the hibernation state. The accumulation of these metabolites could either reflect (i) the synthesis or accumulation of cryoprotectants (Storey 1997, Tsvetkov & Konichev 2009), (ii) a mechanism of osmoregulation (Wieser & Schuster (1974), (iii) the consumption of stored energy (Rees & Hand 1993) or (iv) the synthesis of specific

energy stores usable rapidly upon arousal (Lewis et al. 2004, Driedzic and Ewart 2004, van der Horst et al. 1974, Michaelidis et al. 2008).

In an earlier study of *Helix pomatia* the higher osmolarity of haemolymph due to water loss, reducing the haemolymph volume by a factor of 4 (Burton 1964), could be related to higher supercooling ability (Nicolai et al. 2005). Wieser & Schuster (1975) found in the haemolymph of inactive *Helix pomatia* a high amount of energetic amino acids, like Ser, Gly, Asp, Ala, Thr, and Gln, but could not relate it to osmoregulation, because of the compensation by other amino acids. Nevertheless, in our study several amino acids, like Asp, Glu, Gly, α-Ala, hydroxy-Pro, and Ser, might contribute to enhanced supercooling ability since they are negatively correlated to body supercooling point and assuming that the composition of body fluids is close to that of haemolymph in *Helix pomatia* (Wieser & Schuster 1975). Although haemomymphe supercooling point staid constant and well below the body supercooling point in both physiological states, these amino acids might act as cryoprotectants in organs counteracting ice nucleating activity of some molecules, e. g. lipoproteins (Aunaas 1982) or crystals of minerals (Mugnano et al. 1996). Karanova & Gakhova (2007) suggested the role as cryoprotectants of some upregulated amino acids, Ala, His, Glu, Gly, and Ser, in the freshwater snail *Lymnea stagnalis* at near-zero temperatures. In the orthoptere *Hemideina maori* Ala and Pro were described as cryoprotectants (Sømme 1967, Ramløv 1999). However, Slotsbo et al. (2008) did not consider the low accumulation of Pro, Gln and Ala in the oligochete, *Enchytraeus albidus*, for cryoprotection. Especially in the RHE population content of the mentioned amino acid as well as of α-Ala, Tyr and Pro increased considerably, twice to seven times, during hibernation. This is consistent with the fact that RHE snails reached the same supercooling ability as ALB snails which could therefore be related to (i) the same water mass and (ii) approaching amino acid composition.

Proteins could be consumed during dormancy, when polysaccharide reserves are depleted (Rees & Hand 1993). Nitrogen excretion, as a result of protein catabolism, involves the production of urea (Charrier & Daguzan 1980). However, in an earlier study on *Helix pomatia*, Pro and Arg were completely absent though they are products of the urea cycle (Wieser & Schuster 1975). The urea cycle in *Achatina fulica* needs the activation of Gln synthetase, Orn transcarbomylase, argino-succinate synthase and arginase (Hiong et al. 2005). Among the amino acids involved in the urea cycle only Pro was significantly increased during hibernation in the RHE population. Therefore, we cannot suggest that amino acid composition in hibernation reflects protein catabolism.

Glycerol was accumulated in hibernation in both populations, which is consistent with the observation in cold acclimated individuals of *H. pomatia* (Nowakowska et al. 2006). In the work of Nowakowska et al. (2006) glycerol accumulation was neither related to glycogen catabolism nor to water loss. By contrast in *Cornu aspersum* glycerol content did not change by cold acclimation (Ansart et al. 2008) and in *Anguispira alternata* no glycerol, sorbitol, and mannitol were found in the body during hibernation (Riddle 1981). Seasonal changes in glycerol content were more pronounced in the ALB population than in the RHE population and were correlated to water loss, according to the observation of Nicolai et al. (2005). Glycerol might not exclusively act as cryprotectant, but might also serve as energy reserve usable upon arousal (Lewis et al. 2004).

In the freeze tolerant oligochete, *Enchytraeus albidus*, glucose accumulated twice to three times in dormancy and was therefore qualified as cryoprotectant (Slotsbo et al. 2008). Although, in *Cornu aspersum* the glucose accumulation in cold acclimated individuals at twice the level as

in warm acclimated individuals the authors avoided such a conclusion (Ansart et al. 2008). We support their view, because in the RHE population glucose content was only slightly increased during hibernation (58.2 $\mu g.ml^{-1}$) compared to activity (81.8 $\mu g.ml^{-1}$) while decreasing during hibernation in the ALB population.

Triglycerides accumulation during hibernation was correlated to the decrease of body supercooling point. The content was three times (ALB) to five times (RHE) higher in hibernation and could be related to cryoprotection, according to observations about unsaturated lipids in termites (Lacey et al. 2010). Triglycerides serve as precursors for other lipids, like phospholipids in membranes. The fatty acid composition of triglycerides and subsequently of phospholipids ensures the fluidity of lipid stores and membranes, respectively, at low temperature regimes in the soil arthropod *Orchesella cinta* (Dooremalen and Ellers 2010) and in the oligochete *Dendrobaena octaedra* (Overgaard et al. 2009). Besides cryoprotection, triglycerides might also serve as energy reserve for reproduction upon arousal like in *Helix lucorum* (Michaelidis et al. 2008). In *Megalobulimus oblongus* lipids were not accumulated prior to hibernation (Roselis et al. 1994), but produced during hibernation from lactate in *Helix lucorum* (Michaelidis et al. 2008). Galactose and glucose could also be transformed in triglycerides (Arakelova et al. 2004), which would be consistent with the observed decrease of these metabolites during hibernation.

By contrast, van der Horst (1974) observed reduced lipid synthesis in hibernation of *Cepea nemoralis* and suggested that thermoregulation of membranes has not a high importance during hibernation which would be consistent with the unchanged cholesterol content in both populations. Although cholesterol depresses freezing temperature of membranes (Lee et al. 2008), it is usually associated with thermo-regulation of membrane stability in adaptation to high temperatures (Crocket 1998, Robertson & Hazel 1997). In *Daphnia* sp. the demand of dietary cholesterol increases as ambient temperature increases (Sperfeld & Wacker 2009).

In conclusion, *Helix pomatia* adjusted their physiological processes to hibernation conditions through the accumulation of several cryoprotectants. In activity metabolite compositions of haemolymph differed greatly between populations, probably due to different body conditions and to differences in microclimatic and trophic factors of habitat. Therefore, further studies should focus on the influence of microclimatic factors on seasonal changes in haemolymph composition and the meaning of these physiological processes for snail survival in a circannual rhythm.

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Manche mögen's nicht heiß

Um die Kälteresistenz zu erhöhen, reichert *Helix pomatia* zweier geographisch unterschiedlicher Populationen Frostschutzmittel in der Körperflüssigkeit an: Glycerol, Aminosäuren und Triglyceride. Die Konzentrationserhöhung dieser Substanzen ging mit dem Wasserverlust und dem Absinken des Gefrierpunktes einher (Nicolai et al. *Hp*-II).

Unter anderem können auch warme Wetterbedingungen zu einer Ruhephase, der Ästivation führen, in welcher der Wasserverlust das größte Problem darstellt (Withers et al. 1997, Arad 2001). Obwohl *Helix pomatia* nur kurze Ästivationsphasen im Sommer einlegt, die nur wenige Tage dauern, kann die Art mit Extremsituationen, wie anhaltende Trockenheit und Hitze, konfrontiert sein. In diesem Falle setzen ähnliche Mechanismen wie in der Hibernation zur Regulierung der Dehydrierung ein (Abb. *hp*16) (Block 1996).

Helix pomatia beginnt generell von August bis Oktober, die Hibernation zu initiieren und erwacht erst im April bei ausreichend Regen (Lind 1988). Die Ästivation setzt in der Regel bei längerer Trockenheit ein. Es erscheint, als ob Umweltindikatoren *Helix pomatia* zum Einsetzen der Ruhezustände und zum Erwachen veranlassen, wie es bereits bei *Cornu aspersum* und *Helix lucorum* beobachtet wurde (Ansart et al. 2001, Lazaridou-Dimitriadou und Saunders 1986).

Entsprechend der Umwelteinflüsse im Verlauf eines Jahres müssten die physiologischen Prozesse von *Helix pomatia* Hibernations-, Ästivations- und Aktivitätsphasen markieren, um die Überlebenschancen entsprechend saisonalen Wetterbedingungen zu erhöhen.

Hypothesen dieser Studie:

- Im Verlauf eines Jahres sind Hibernations-, Ästivations- und Aktivitätsphasen bei *Helix pomatia* durch unterschiedliche biochemische Zusammensetzungen der Haemolymphe zu unterscheiden.
- Hibernation und Ästivation werden durch bestimmte Umweltfaktoren initiiert
- In der Ästivation sind ähnliche physiologische Mechanismen wie in der Hibernation zu erkennen.

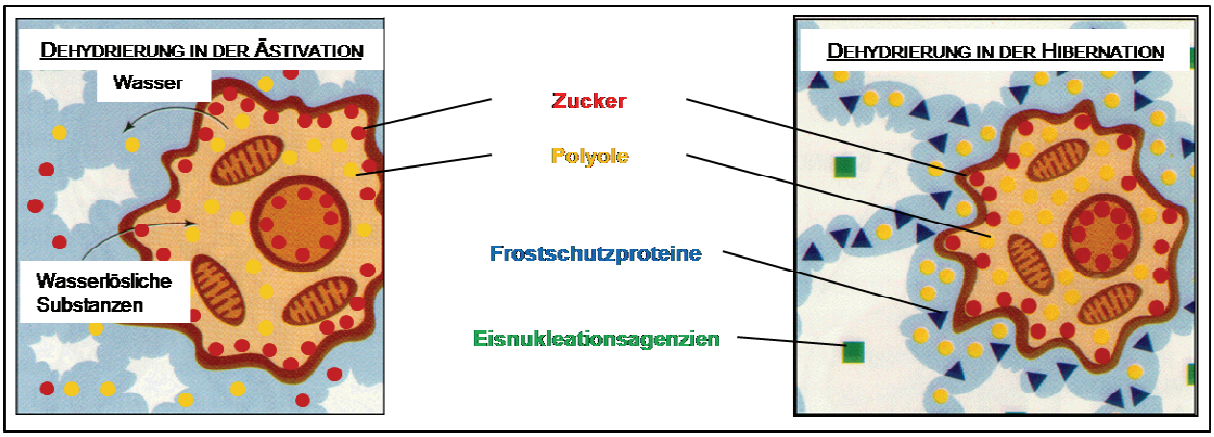

Abb. *hp*16. Dehydrierung in der Ästivation und in der Hibernation. Eisnukleationsagenzien können mit der Eisbildung die Dehydrierung der Zelle noch erhöhen, da Wasser in den Eiskristallen gebunden ist (Block 1996).

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Adjustment of metabolite composition in the haemolymph to micro-environmental variations in the land snail *Helix pomatia*

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Reference: Nicolai et al. *Hp*-III

Abstract: In temperate regions, land snails are subject to subzero temperatures in winter and hot temperatures, often associated with drought, in summer. The response to these environmental factors is usually a state of inactivity, hibernation and aestivation, respectively, in a temperature and humidity buffered refuge. Both dormancy states involve physiological adjustments to resist cold or heat stress. We investigated in this study on the endangered species *Helix pomatia* how environmental factors in the microhabitat and body condition influence the metabolite composition of haemolymph. We used UPLC and GC-MS techniques and analyzed annual biochemical variations in a multivariate model. Hibernation and activity months differed in metabolite composition, however physiological states were blurred, especially by June and March that tended to the opposite physiological state. Snails used photoperiod as cue for seasonal climatic variations in order to initiate a physiological state. They were also highly sensible to temperature variations, therefore permanently adjusting their physiological processes. In general, amino acid content was low in hibernation months probably due to metabolic depression. High galactose levels gave evidences for the persistence of metabolic activity with energy expenditure during hibernation. Triglycerides were accumulated prior to hibernation and might act as cryoprotectants or energy reserves upon arousal. In March snails activated physiological processes related to arousal, in October after the formation of epiphragm they might prepare for cryoprotection. In June snails had the highest galactose content in the year which coincides with reproductive period. April was exceptionally hot and dry showing some evidences for heat stress responses, like the protection of cells from dehydration by polyols and saccharides, the membrane stabilization by cholesterol and enhanced metabolism using the anaerobic succinic acid pathway to sustain costly stress responses. Mortality was high in April. Further studies should focus on the limit of the capacity to physiologically adjust to environmental variations.

Keywords: Cholesterol, Galactose, Gastropods, GC-MS, Heat resistance, Hibernation, Polyols, Triglycerides, UPLC

Introduction

Land snails are subject to annual cycles of activity and dormancy in relation to seasonal changes in temperature and humidity, and therefore should be adapted with a range of behavioral and physiological mechanisms that will ensure their survival under their specific microhabitat conditions. In temperate regions, these ectothermic animals with continuously moist tegument encounter two opposite extreme climatic situations during the year: subzero temperatures in winter and hot temperatures, often associated with drought, in summer. The response to these environmental factors is usually a state of inactivity, hibernation and aestivation, respectively, in a temperature and humidity buffered refuge. In both dormancy states the body is fully withdrawn, and the shell is obturated by an epiphragm (Lind 1988; Ansart et al. 2002).

Both dormancy states share some physiological and biochemical adjustments. Metabolic depression, regulated by specific proteins (Brooks & Storey 1995), involves the suppression of protein and glycogen synthesis (Ramnanan et al. 2007). The rate of lipid synthesis is reduced in *Cepea nemoralis* (Van der Horst et al. 1974) as well as glycolytic activity in *Helix lucorum* (Michaelidis et al. 2008), whereas in *Helix pomatia* a high glycolytic activity is conserved (Wieser & Wright 1978). Moreover heart beat and oxygen consumption is lowered and body dry mass decreases due to consumption of energy stores (Rizzatti & Romero 2001). In *Oreohelix* sp. polysaccharides are first catabolised followed by proteins (Rees & Hand 1993). In this case, urea accumulation as a result of protein catabolism (Charrier & Daguzan 1980) was observed to limit dormancy duration due to an increase of mortality at higher urea levels.

In hibernation, the reduction of the volume of freezeable water, accompanied by an increase in osmolarity of body fluids, enhances cold hardiness (Vali 1995; Holmstrup & Zachariassen 1996; Lee & Costanzo 1998; Nicolai et al. 2005). This means that cryoprotectants, polyols and saccharides, are accumulated in body fluids (Storey 1997; Ramlov 2000) and protect cells from lethal intracellular freezing (Costanzo et al. 1993). Moreover antifreeze proteins stabilize the supercooling state (Zachariassen 1985) and cholesterol protect cell membranes from freezing (Lee et al. 2008). Triglycerides and their fatty acid composition ensure fluidity of lipid body stores and cell membranes (Overgaard et al. 2009; van Dooremalen & Ellers 2010). In *Helix pomatia* triglycerides are accumulated during hibernation (Nicolai et al. *Hp*-II).

In aestivation responses to heat and drought involve the production of heat shock proteins (Ramnanan et al. 2009) and the increase of antioxidant defense (MacDonald & Storey 2006). In *Helix pomatia*, the antioxidant defense system is very efficient with a high activity of antioxidant enzymes and a high level of reduced glutathione throughout aestivation (Nowakowska et al. 2009). Water loss can be regulated by some snails, like *Rhagada tescorum* (Withers et al. 1997), to withstand up to 12 months of dormancy, whereas *Theba pisana* is subject to dehydration, accompanied by an increase in osmotic concentration and mechanisms of osmoregulation (Arad 2001). In this case, polyols and saccharides act as cell protectants through interactions with membrane macromolecules and immobilization of cytoplasme (Meryman 1971; Alpert 2006). Moreover, cholesterol ensures thermo-stabilization of membranes (Hazel 1995, Crocket 1998) when a high amount is available (Sperfeld & Wacker 2009), which could be given by a constant lipid metabolism like observed in *Oreohelix* sp (Rees & Hand 1993; Storey 2002).

Seasonal changes of environmental factors that are used as cues by land snails to initiate dormancy states have been intensively studied. In *Cornu aspersum*, hibernation is mainly triggered by photoperiod (Ansart et al. 2001b; Ansart et al. 2002). Temperature and moisture might play a role for the initiation of the active state in *Helix pomatia* (Hernadi et al. 2008;

Tischler 1974), and the circannual rhythm might define arousal period (Bailey 1981). Aestivation is controlled by a decrease of humidity or by the combined effect of increasing temperature and increasing photoperiod in *Helix lucorum* (Lazaridou-Dimitriadou & Saunders 1986).

However until now, the seasonal variation of heatprotectants or cryoprotectants as a function of seasonal changes in environmental factors has been poorly studied in land snails. That's why we investigated metabolite composition of the haemolymph in relation to microclimatic conditions in the habitat of *Helix pomatia* during one year. *Helix pomatia* has a large latitudal (Greece to Sweden) and longitudal (France to Poland) distribution in Europe (Nietzke 1970), exposing individuals to a wide range of climatic situations and annual variations. *Helix pomatia* has only short aestivation periods in summer where it attaches to bushes or stones by mucous secretion (Lind 1988). The species exhibit a long hibernation period beginning sometimes in August and lasting until April where it burrows into the soil and closes its shell by a heavily calcified epiphragm (Lind 1988). It is a partially freezing tolerant species (Ansart et al. 2001a; Nicolai et al. 2005), which enhances cold hardiness by water loss and accumulation of amino acids, glycerol and triglycerides (Nicolai et al. *Hp*-II), but survives only for several hours to freezing. Mortality during hibernation seems to be the key factor in Helicidae populations of Europe (Cain 1983; Peake 1978).

The purpose of this study was to explore how the different physiological states in a circannual rhythm of a land snail are characterized by physiological changes reflected in metabolite composition. We hypothesized that seasonal microclimatic variations in the snail's habitat provoke changes in metabolite composition in relation to cold or heat resistance, thereby defining the physiological state of activity, hibernation or aestivation. Therefore, we investigated metabolite composition as responses to environmental factors and biological parameters, and observed snail behavior and mortality during one year.

Material and Methods

BIOLOGICAL MATERIAL

In September 2006 adult snails were collected in the forest near Weiler-Indelhausen, mountains Schwäbische Alb in Germany (48° 18' 7N, 9° 29' 47E, 800 m a.s.l.). Prevailing trees are *Carpinus betulus* and *Alnus incana* with ground vegetation dominated by *Urtica dioica, Fumaria officinalis*, *Impatiens nou-tangere, Geranium robertianum*, *Viola reichenbachiana, Anemone nemorosa, Dentaria bulbifera*).

120 snails were put in a 2 x 2 m outdoor cage and 26 in a mesocosmos outdoor cage (1 x 1 x 0.6 m) in the Experimental Farm Tachenhausen of the University of Economics and Environment Nürtingen, located near Oberboihingen, mountains Schwäbische Alb in Germany (9° 23' 37'' E, 48° 38' 54'' N, 350m a.s.l.). In both cages grew wild plants, like *Taraxacum* sp., *Ranunculu*s sp., *Trifolium* sp., *Mentha arvensis* and *Geranium robertianum,* and they were supplemented with planted cultured plants, like *Brassica napus*, *Spinacia oleracea*, *Beta vulgaris*, *Lactuca sativa*, *Brassica rapa* and *Raphanus sativus*. Several refuge possibilities were installed, like stones, moos, branches and pieces of wood. Snails were fed *ad libitum* twice a week with fresh plant material: *Brassica napus*, *Helianthus annuus*, *Helianthus tuberosus*, *Pisum sativum*, *Medicago sativa*, *Brassica rapa*, *Raphanus sativus*, *Phaseolus* sp. and *Trifolium pratense*. Snails were exposed to all weather conditions, but protected from sun by trees (*Quercus* sp.) in the afternoon. From October 2006 until February 2008 snails were weighed and

observed for their activity and mortality every week. In May 2007, 16 snails from the outdoor cage were reintroduced in the mesocosmos because of the high mortality during winter and spring. Beginning in February 2007, 6 snails were sacrificed each month over one year from the outdoor cage for measurement of the supercooling point, the body dry mass and water mass, as well as for haemolymph extraction. Snails collected from April to October had no epiphragm, while snails collected from November to March were hibernating snails with epiphragm. In October 2007, five snails with epiphragm were additionally collected for analysis, and we distinguished them in the following by sample Oct+ for snails with epiphragm and sample Oct- for snails without epiphragm. Microclimate data were recorded near the cages: minimal (min), maximal (max) and mean (m) temperature at 20 cm above the soil surface (TSF, in °C); precipitation (precip, in mm); minimal (min), maximal (max) and mean (m) relative humidity at 20 cm above the soil surface (RH, in %), as well as minimal (min) and maximal (max) soil temperature at -20 cm (TS, in °C).

BODY SUPERCOOLING POINT (BSP) DETERMINATION

As described by Nicolai et al. (2005) a hole of 1 mm diameter was drilled by a trepan (S2000, Georg Schick Dental GmbH, Schemmerhofen, Germany) in the snail shell above the pallial cavity. A thermocouple (K-type: Chromel-Alumel, Fischer Scientific, France) was inserted through the shell hole into contact with the mantle tissues. In the case of injury of the body tissues (hemolymph outflow) snails were discarded from further analysis. The thermocouple was connected by a wire to an electronic multichannel thermometer (Tempscan C8600, Comarck Electronics Ltd., Hertfordshire, UK) in order to simultaneously measure temperatures of several snails. Measurements were done every second and transmitted to a printer (HP deskjet 500, Hewlett-Packard Company, Palo Alto, CA, USA). Snails were placed in a plastic box (5 x 5 x 5 cm) which was placed in a thermostat (MT 3, Lauda GmbH & Co. KG, Lauda-Königshofen, Germany) filled with silicone oil (Kryo 51, Lauda GmbH & Co. KG, Lauda-Königshofen, Germany) and cooled by a through-flow cooler (DLK 10, Lauda GmbH & Co. KG, Lauda-Königshofen, Germany). Their body temperature was first acclimated to 5°C. Then the temperature in the thermostat was decreased at a cooling rate of 0.6°C.min^{-1}. This cooling rate lies in between 0.5 to 1°C.min^{-1}, commonly used in cryobiology and recommended by (Salt 1966), which enables comparisons with other studies on cold-hardiness in invertebrates. The BSP was recorded at the start of the exotherm (release of heat) due to crystallization of supercooled body fluids (Lee 1989). The lowest temperature in the thermostat could be –12°C which is well below earlier recorded BSP in Helix pomatia (-9°C, Nicolai et al. 2005). The snails were taken out of the thermostat right after reaching BSP.

HAEMOLYMPH EXTRACTION AND SUPERCOOLING POINT (HSP) MEASUREMENT

A hole of 2-3 mm diameter was drilled by the trepan in the snail shell above the pericardiac cavity. Up to 250 µl of haemolymph could be collected by heart puncturing and was centrifuged at 10,000 g, 4°C for 5 min to eliminate shell and tissue particles (Ramlov 1999). Samples were stored at –80°C until metabolites analysis. The measurement of the (HSP) was performed using the freezing droplet method (Vali 1971; Vali 1995). Five droplets of 5 µl per individual haemolymph sample were placed onto the surface of aluminum pans (Fisher Scientific, France). The pans floated on the silicone oil bath of a cryostat (Polystat CC3, Huber) cooled from 5°C to –15°C at a cooling rate of 0.6°C per minute. One thermocouple, connected to the electronic thermometer, was fixed on one aluminum pan to follow the temperature changes

on the pan surface. The freezing of the droplet (becomes opaque) was observed de visu, the HSP was recorded for each droplet and mean HSP was calculated for each haemolymph sample.

METABOLITE DETERMINATION

We pooled haemolymph from six individuals collected each month and made two repetitions of each analysis per month. For UPLC and GC-MS analysis we used the protocol of (Renault et al. 2010) and lipid analysis was done according to the protocol of (Hervant et al. 1999). All metabolite levels were expressed in mass per volume of haemolymph, using molecular mass for conversion if necessary.

For both UPLC and GC-MS analysis, 100 µl of haemolymph was suspended in 600 µl of methanol and agitated at 1500 rpm for 15 min. Subsequently, 300 µl of chloroform were added and samples were agitated for five additional minutes. Finally, 600 µl of ultra-pure water were added, samples were then vigorously vortexed and centrifuged at 4000 g for 10 min. 700 µl of upper phase (methanol-water phase) per samples were transferred to a clean micro tube and dried in vacuum (miVac DNA concentrator, Genevac, Suffolk, UK).

For amino acids analysis, dry residues were suspended in 50 µl of ultra-pure water and 5 µl of the resulting extract were sampled for amino acids derivatization according to the AccQ•Tag Ultra Derivitization Kit protocol (Waters Corporation, Milford, MA). Amino acids were analyzed using an Acquity UPLC® system (Waters Corporation, Milford, MA) by injecting 1 µl of the derivatization mix onto an Acquity UPLC® BEH C18 1.7 µm 2.1 x 100 mm column heated at 55°C. Amino acids were eluted at 0.7 $ml.min^{-1}$ flow with a mix of 10-fold diluted AccQ•Tag Ultra Eluent (A; Waters Corporation, Milford, MA) and acetonitrile (B) according to the following gradient : initial, 99.9 % A ; 0.54 min, 99.9 % A ; 6.50 min, 90.9 % A; 8.50 min, 78.8 % A; 8.90 min, 40.4 % A; 9.50 min, 40.4 % A; 9.60 min, 99.9 % A; 10.10 min, 99.9 % A. Derivatized amino acids were detected at 260 nm using a photo diode array detector. Amount of amino acids were obtained using standard solutions of all amino acids.

For GC-MS analysis, the remaining 40 µl of extract were dried in vacuum and then suspended in 630 µl of pure water in order to obtain the initial concentration. Subsequently, 200 µl of this extract were transferred to GC-MS glass vials and dried in vacuum. Dried samples were dissolved in 50 µl of freshly prepared methoxyamine hydrochloride solution in pyridine (20 mg/ml) and agitated for 90 min at 30°C. Afterwards, 50 µl of N-methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA; Sigma, #394866) were added and derivatization was conducted at 37°C for 30 min under agitation. Incubation took place at room temperature over-night before injection. GC-MS system consisted of a TriPlus autosampler, a Trace GC Ultra chromatograph and a Trace DSQII quadrupole mass spectrometer (Thermo Fischer Scientific Inc, Waltham, MA). 1 µl of the derivatization mix was injected onto a Thermo TR-5MS 30 m x 0.25 mm ID x 0.25 µm column under the following heating protocol: 70°C for 4 min, increase of temperature at $5°C.min^{-1}$ until 310°C, 310°C for 5 min. Metabolites were eluted at 1 $ml.min^{-1}$ flow with Helium and injected at 250°C in the mass spectrometer at a speed flow of 25 $ml.min^{-1}$ and a transfer line temperature of 250°C. Chromatograms were deconvoluted using the AMDIS software v2.65 (http://chemdata.nist.gov/mass-spc/amdis/) associated with NIST libraries. Amount of metabolites were obtained using standard solutions. In order to quantify the content of glucose and galactose that was not analyzed by GC-MS procedure because of its high abundance we used the Enzyplus® EZS 784+ Lactose/D-Galactose Kit (BIOCONTROL, Bellevue, WA, USA) on 48 µl of the extract solution and the Enzyplus® EZS 784+ D-Glucose Kit (BIOCONTROL, Bellevue, WA, USA) on 100 µl of the extract solution.

For each lipid analysis (triglycerides and cholesterol), 100 µl of haemolymph was suspended in 1.5 ml methanol-chloroform solution (methanol:chloroform 1:2 v/v), followed by a 12 h incubation at –20°C. Phase separation was obtained by adding 300 µl KCl (2 $g.l^{-1}$) and heating for five minutes at 40°C. The inferior phase (chloroform phase) was separated in two aliquots of 400 µl. Both aliquots were dried under nitrogen flow at 30°C for 15 min before suspension in 300 µl solution BSA (3% w/v, fatty acid free) - Triton (0.2% v/v) for triglyceride analysis and in 10 µl Ethanol (95°) for cholesterol analysis. The content of triglycerides and cholesterol was determined by measuring the absorbance with a micro plate spectrophotometer (VERSAmax™ microplate reader, Sunnyvale, CA, USA), using the Triglyceride Assay Kit (Cayman Chemicals, Ann Arbor, Michigan, USA) and the Cholesterol RTU™ kit (Biomerieux, France), respectively, as well as a calibration curve elaborated with standard solutions.

BODY MASS MEASUREMENT

Frozen snails were weighed (Analytic A200S, Sartorius AG, Göttingen, Germany), to determine their body mass (BM) before removing the shell which was weighed separately (SM). To determine the dry mass of flesh (DM), the soft body parts were frozen and lyophilized (Trivac, Oerlikon Leybold Vacuum GmbH, Köln, Germany). The water mass (WM) of a snail was calculated by the following equation: WM = BM - (DM + SM).

DATA ANALYSIS

Statistical analyses were carried out using "R" (R Core Team 2008; Team 2007). ANOVA was performed on monthly data of supercooling points, dry mass and water mass. Pearson method was used for correlation between individual supercooling point and dry mass as well as water mass. For weekly measured body masses we used an analysis of variances for repeated measurements (rmANOVA).

Redundancy analysis (RDA) was conducted on metabolite content as response variables as well as environmental and biological parameters as explanatory variables. This analysis shows how much of the variance in one set of variables can be explained by the other. The inter-correlation coefficient between variables retained for this type of analysis was $R <$ 0.90. Distance matrix was calculated by the Jaccard method and cluster analysis was performed with the Ward method. In order to test the validity of explanatory variables we performed a Monte-Carlo test with 1000 permutations. Finally, representation shows the vector plot of metabolites, the correlation plot of biological variables and the position of each individual on two retained canonical axes in respect to their metabolite composition as well as environmental and biological parameters.

Environmental variables used in the RDA were relevant to snail activity which means that we used temperature on the soil surface for months in activity without epiphragm (April - October-) and soil temperature at -20 cm for months of hibernation with epiphragm (October+ - March). Therefore we called this variable: temperature of the snail habitat (TSH). We presume a very quick adjustment of physiological processes to environmental factors, so that we used only data recorded during 5 days before snail collection in the RDA analysis. Mean photoperiod per day (PPm) for this period was calculated using the equation:

$$PPm = (\sum_n (275.5 * \sin (0.0172 * (d_n - 80)) + 734.5)) / n$$

where d is the number of days elapsed since January 1st and n the number of all consecutive days.

Results

The highest temperature fluctuations were recorded during summer months at the soil surface, while during months of snail hibernation the temperatures in the soil were buffered (Figure 1A). However, high temperature variations during winter without snow were poorly buffered (November, December, February, and March) compared to the month with snow (January 2007). In contrast to climate data of April from the last 10 years (Precip = 40 mm, Tm = 10°C, http://www.dwd.de, station Stuttgart) April was exceptionally dry and hot with great temperature fluctuations during the day. Therefore we could observe an unusual aestivation period in April (Figure 1B). In contrast to October 2006 hibernation period started early in October 2007 with appearance of 90% hibernating snails by the beginning of the month (Figure 1B). In general snails were active from April until October and inactive from October until March.

Body mass per body size describes the body condition and varied between months and between individuals in the mesocosmos (October 2006-May 2007 rmANOVA, between months: $DF = 7$, $F = 17.76$, $P < 0.0001$, between individuals: $DF = 8$, $F = 39.32$, $P<0.0001$; May 2007-January 2008, rmANOVA, between months: $DF = 8$, $F = 26.13$, $P < 0.0001$, between individuals: $DF = 10$, $F = 27.43$, $P<0.0001$) (Figure 1C). During hibernation variations were less important and we recorded a mortality of 25% at the end of hibernation in March 2007. During the dry period of April, body conditions dropped down which was accompanied by a high mortality reaching a rate of 75% at the beginning of May 2007. During the period of activity which corresponded to summer months with high precipitation snail body conditions improved. The best body condition was recorded in June and July which coincided with reproduction period.

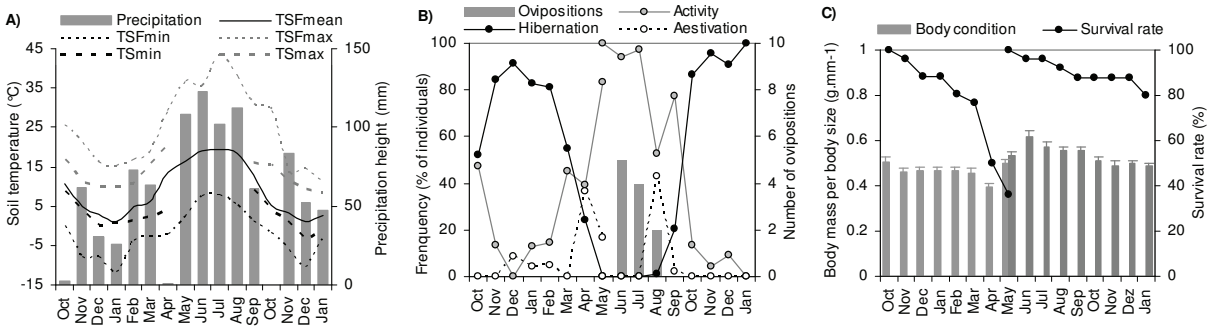

Figure 1. Microclimate (A), behavioral (B) and biological parameters (C) in the mesocosmos with *Helix pomatia* from October 2006 until January 2008. Note that there was only one week of snow in January 2007. In May 2007 new snails were reintroduced because of the high mortality. $N = 26$ in October 2006 and May 2007. TSF- temperature at the soil surface (+20cm), TS- temperature in soil (-20 cm).

To analyze the variation of body mass and supercooling point during the year we collected snails with constant body size (38.33 ± 0.27 mm, ANOVA, $F = 1.57$, $DF = 12$, $P = 0.12$, $N = 76$). Body mass however fluctuated during the year (ANOVA, $F = 7.59$, $DF = 12$, $P < 0.0001$, $N = 76$) as well as dry mass (ANOVA, $F = 5.11$, $DF = 12$, $P < 0.0001$, $N = 76$) and water mass (ANOVA, $F = 9.82$, $DF = 12$, $P < 0.0001$, $N = 76$) (Figure 2A). Therefore we could observe a high positive correlation of body mass with dry mass (Pearson, $R = 0.95$, $DF = 11$, $P < 0.0001$, $N = 13$) and water mass (Pearson, $R = 0.98$, $DF = 11$, $P < 0.0001$, $N = 13$). Body

supercooling point differed between months (ANOVA, $F = 7.08$, $DF = 12$, $P < 0.0001$, $N = 76$), but not haemolymph supercooling point (ANOVA, $F = 1.57$, $DF = 12$, $P = 0.13$, $N = 65$) (Figure 2B). However the mean body supercooling point per month was neither correlated to mean dry mass (Pearson, $R = 0.24$, $DF = 11$, $P = 0.21$, $N = 13$) nor to mean water mass (Pearson, $R = 0.40$, $DF = 13$, $P = 0.088$, $N = 13$).

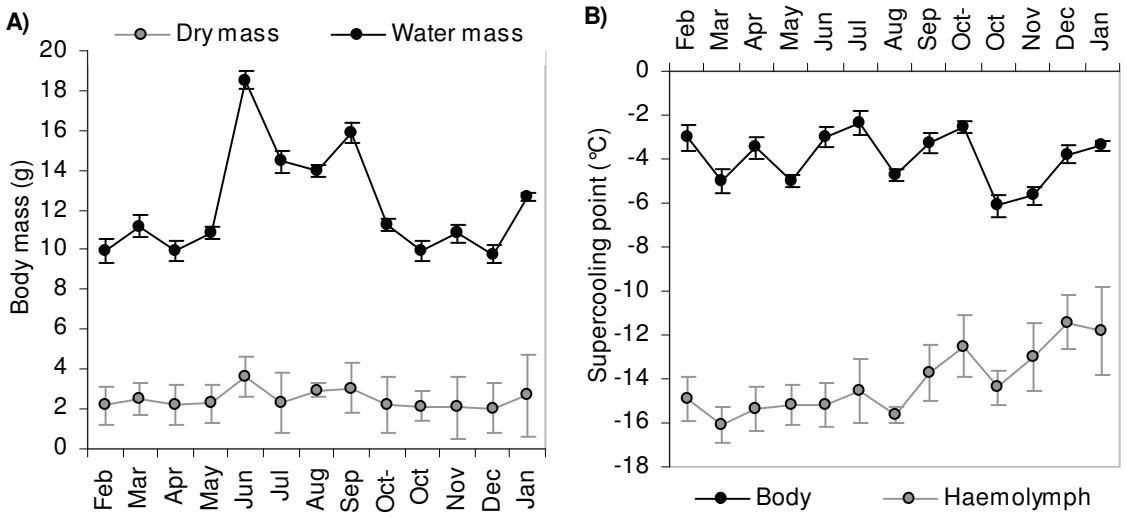

Figure 2. Body mass (A) and supercooling point (B) of *Helix pomatia* collected each month in the outdoor cages. $N = 76$. Observations took place from February 2007 until January 2008.

The annual variation of the metabolite content is represented in the table of the Appendix allowing give an overview on general amounts of metabolites in the haemolymph. Amino acids were present at low levels, while saccharides represented the main components of haemolymph throughout the year. In spite of the striking increase in content of succinic acid, myo—inositol, maltose, β-D-Methylglycopyranoside (β-D-MGP), triglycerides in some months, the RDA analysis (Figure 3) permit to emphasize the inter-relation of the metabolite composition with biological parameters and microclimate data. On the RDA plot the metabolite composition of haemolymph showed a separation of hibernation months and activity months on the first axis, however with overlapping of both clusters especially due to June and March (Figure 3). Mean photoperiod (PPm) which is highly correlated to maximal temperature in the snail habitat (TSHmax) (Pearson, $R = 0.98$, $DF = 11$, $P < 0.0001$, $N = 13$) and to mean temperature in the snail habitat (TSHm) (Pearson, $R = 0.94$, $DF = 13$, $P < 0.0001$) explained mainly the first axis ($R = -0.69$) followed by minimal temperature in the snail habitat (TSHmin) ($R = -0.66$). Mean relative humidity (RHm) was correlated to the second axis ($R = 0.81$) and might explain the low position of April on the second axis. Months of activity were characterized by a high content in amino acids while months of hibernation showed mainly an increase in triglycerides and galactose. Striking was the position of Oct+ (October sample of snails with epiphragm) with high saccharide content, as well as the position of April characterized by high contents of glycerol, myo-inositol, maltose, succinic acid, and cholesterol. β-D-MGP was also accumulated in April and highly correlated to myo-inositol (R = 0.98). That's why it did not appear in the RDA analysis.

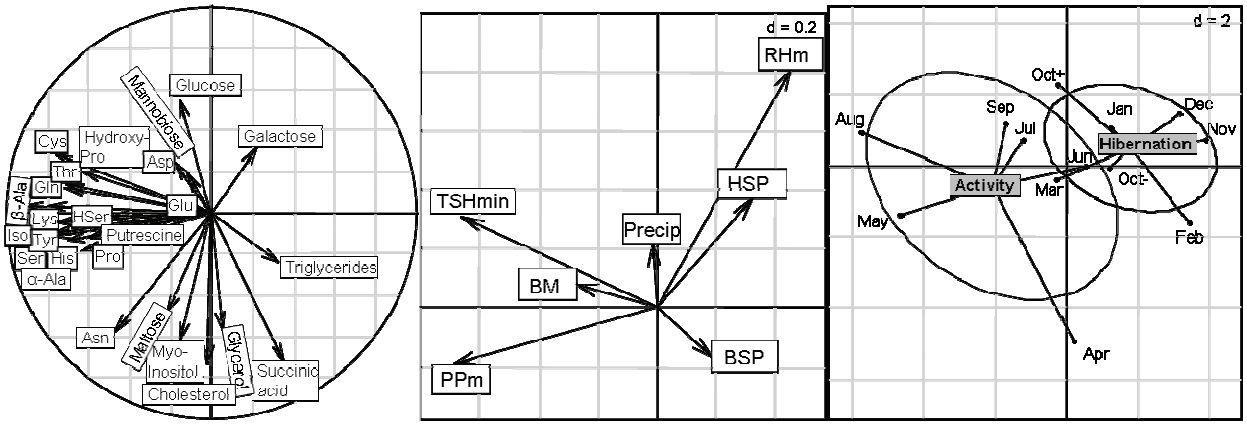

Figure 3. Results of RDA analysis on metabolite content in haemolymph of *Helix pomatia* and environmental data (correlation plot) associated to biological paramaters. Observations were done from February 2007 until January 2008. Monte-Carlo test (1000 permutations): $P = 0.017$. Eigenvalues of constrained and unconstrained axes were 84.71% and 15.29%, respectively. RDA was constructed on the first two constrained axes with the part of eigenvalues of 41.91% and 17.01%, respectively. PPm – mean photoperiod, TSH – minimal temperature of the snail habitat, RHm – mean relative humidity, BM – body mass, BSP – body supercooling point, HSP – haemolymph supercooling point.

Discussion

Metabolite content in haemolymph reflects greatly the physiological state and body conditions during the year. *Helix pomatia* in a natural population was observed to initiate hibernation from August until October when precipitation was high (Lind 1988). Arousal from hibernation in this population also depended on precipitations, but occurred not before mid-April in four successive study years (Lind 1988). Consistently, we observed an activity period from April until October (Oct-) and a hibernation period from October (Oct+) until March. The metabolite composition in haemolymph differed between the hibernation period and the activity period, despite some overlap of clusters on the RDA plot. Nevertheless a clear aestivation period could not be distinguished, according to observations of Lind (1988).

In an earlier study on *Helix pomatia* in constant laboratory conditions some amino acids were supposed to act as cryoprotectants because they were correlated to body supercooling point (Nicolei et al. unpublished). Those that were not correlated to body supercooling point were correlated to body dry mass. The active state in this study was characterized by an increase in amino acid content, but could neither be related to dry mass nor to body supercooling point. Cryoprotectants are accumulated through body dehydration in *Helix pomatia* (Holmstrup & Zachariassen 1996; Nicolai et al. 2005) and depress the body supercooling point by the increase of osmolarity of body fluids (Storey 1997; Ramlov 2000). Under natural conditions in the mesocosmos water mass and body supercooling point variations were high and not correlated. Therefore we could conclude that amino acids did not act as cryoprotectants. The decrease of amino acids in hibernation might indicate metabolic depression (Brooks & Storey 1995; Ramnanan et al. 2007).

In hibernation months only the increase of triglycerides and galactose content might have contributed to a significant change in metabolite composition besides the decrease in amino acid content. Both could have a function in cryoprotection, like observed for lipids in termites (Lacey et al. 2010) and in *Helix pomatia* (Nicolai et al. *Hp*-II), and for saccharides in the oligochete,

Enchytraeus albidus (Slotsbo et al. 2008). However, galactose is the main saccharide in *Helix pomatia* and is usually highly present in active state (Appendix, Nicolai et al. *Hp*-II), like glucose in *Lymnea stagnalis* (Karanova & Gakhova 2007). Therefore, the high content of galactose in winter would rather indicate the maintenance of an important glycolytic activity and energy expenditure in hibernation like proposed by (Wieser & Wright 1978). The reason, why galactose content exceeds glucose content, could be explained by diet choices. Galactans are incorporated in hemicelluloses polymers, like arabinogalactans and galactomannans, found abundantly in leguminous plants (Flari and Charrier 1992, Mora 1992).

Triglycerides showed the closest relation to body supercooling point and therefore to cryoprotection, but they could also have the function as energy reserve, because they seemed to be accumulated before hibernation initiation (Oct-, Appendix). Their decrease during the first months of hibernation could be due to catabolism for energy production or to transformation in phospholipids and integration in membranes to ensure membrane fluidity at low temperatures (Overgaard et al. 2009; van Dooremalen & Ellers 2010). In *Helix lucorum* lipids are produced during hibernation from lactate (Michaelidis et al. 2008) and serve as energy reserve for reproduction upon arousal, since authors supposed that lipids were not accumulated prior to hibernation like in *Megalobulimus oblongus* (Roselis et al. 1994). The increase of triglycerides in January and February in *Helix pomatia* could also indicate a synthesis during hibernation, like suggested in *Helix lucorom* (Michaelidis et al. 2008) in order to constitute an energy reserve for arousal.

Photoperiod (highly correlated to maximal temperature) and minimal recorded temperatures during day were mainly related to the differentiation of both physiological states. This is consistent with works about *Cornu aspersum* (Ansart et al. 2001b; Ansart et al. 2002) and *Helix pomatia* (Hernadi et al. 2008; Tischler 1974), where hibernation was triggered by photoperiod and temperature, and arousal was triggered by temperature and soil moisture, respectively. The minimal occurring temperature per day was also as important as mean photoperiod and might generate short term adjustments of physiological mechanisms. This point may be particularly important, since body supercooling point and body mass were highly variable throughout the year and could not be related to haemolymph composition. It seems that snails did not adopt a clear and constant strategy to enhance cold hardiness during winter. In *Helix pomatia* cold hardiness includes body water loss resulting in the increase of haemolymph osmolality and therefore the accumulation of cryoprotectants (Nicolai et al. 2005). The sensibility to temperature variations in the microhabitat could have induced individuals to adapt their physiological mechanism to micro changes even in hibernation. Temperature in soil during recent winters is less buffered because of the prevalent absence of snow in Germany. Daily activations or inactivations of specific physiological processes might be responsible for blurring a clear separation of cold hardiness processes in hibernation and glycolytic activity to sustain locomotion, foraging and reproduction in activity. Especially, during hibernation fast physiological changes could lead to high mortality when energy reserves are limited.

The position of June on the RDA plot (Figure 3) shows that snails in this month had similar metabolite composition with snails in hibernation. This could be due to physiological changes during reproduction period. (Borges et al. 2004) and (Giokas et al. 2007) suggested the use of lipids as energy source during reproduction. Since triglycerides are also accumulated in hibernation (Nicolai et al. *Hp*-II), snails in June and during hibernation might have similar physiological processes involved in lipid production. Moreover, snails in June had the highest

content in galactose which coincides with high reproductive activity and highlighting the allocation of galactogen to eggs as principal egg component (Tompa 1984).

The position of March on the RDA plot (Figure 3) showed that snails in this month had similar metabolite composition with snails in activity. The arousal of snails involves some physiological processes that need the activation of metabolic pathways (Hernadi et al. 2008; Nowakowska et al. 2009) and might bring snails in an active state well before they push off the epiphragm.

High relative humidity was related to the formation of an epiphragm in October (Oct+), consistently to observations of Lind (1988). After the formation of epiphragm, saccharides (glucose, galactose, mannobiose) were the main components in haemolymph, indicating either high glycolytic activity associated to the initiation of hibernation (Wieser & Wright 1978) or the transport of saccharides to cells in apprehension of cryoprotection (Costanzo et al. 1993) and water loss (Meryman 1971).

Low relative humidity explained in our study the metabolite composition of haemolymph in April, when snails had an exceptional aestivation period. Generally, a long resting period per day occurs in July and August under dry conditions (Lind 1988), which can be prolonged to aestivation period for several days with attachment to trees and bushes when morning dew is not sufficient for daily activation, like observed in *Theba pisana* (Arad 2001). Polyols (glycerol and myo-inositol), saccharides (maltose and β-D-MGP), succinic acid, and cholesterol accumulated in haemolymph. *Helix pomatia* might have several heat and dehydration stress responses, like the synthesis of antioxidants (Nowakowska et al. 2009) or of heat shock proteins observed in *Cantareus apertus* (Reuner et al. 2008).

Polyols are byproducts of carbohydrate metabolism (Driedzic & Ewart 2004) and indicate a stress response in a freshwater snail (Tsvetkov & Konichev 2009). They could be a response to high temperatures and act with saccharides in cell stabilization, comparable to the cryoprotecting function (Storey 1997). In fact saccharides were observed protect cells by immobilization of the cytoplasme and by interaction with macromolecules to stabilize membranes at high temperatures (Alpert 2006). They could also act as osmoregulateurs to avoid dehydration (Meryman 1971). An accumulation of maltose was also related to cryoprotection in freshwater snails (Karanova & Gakhova 2007). Block (1996) raised the question about differences in physiological adjustments to drought and cold, since cryoprotectant accumulation often goes along with water loss. The accumulation of polyols and saccharides during April in *Helix pomatia* might therefore be the result of water loss and serve as heat protection against cell injuries and dehydration.

Thermo-regulation of membranes is often associated to a high demand in cholesterol which stabilizes membranes and ensures its fluidity (Robertson & Hazel 1997; Sperfeld & Wacker 2009). Cholesterol is not ingested with food, but has to be synthesized *de novo* from precursors (Addink & Ververgaert 1963; Flari & Edwards 2003). The high abundance of succinic acid indicated high anaerobic glycolytic activity with depletion of body polysaccharides stores in order to sustain energy production (Barnes et al. 1993). The succinic acid pathway is an alternative pathway to the lactate pathway (anaerobic glycolyse generating 2 ATP) evolved in mollusks inhabiting anoxic habitats or exhibiting long periods of dormancy in order to generate 3 ATP per input of glucose. The produced energy could be needed in costly stress responses (Davis & Schreck 1997). Succinic acid may be further metabolized in fatty acids, and pyruvate, another product from succinic acid pathway, may be metabolized to lactate, acetate, alanine and ethanol (Barnes et al. 1993). Except ethanol, these metabolites are used in lipid production (triglycerides and cholesterol) in land snails (Addink & Ververgaert 1963, Michaelidis et al.

2008). The depletion of body energy stores immediately after hibernation to sustain energy and cholesterol production might be a reason for high mortality during April.

In conclusion, the initiation and maintenance of dormancy states in *Helix pomatia* were accompanied by physiological adjustments involving water loss regulation, cryoprotectant or heatprotectant accumulation. Even though snails use photoperiod as cue for seasonal environmental changes, adjustments to short term variations in temperature seemed to be frequent. Temperature and humidity buffered refuges are therefore crucial for maintenance of sustainable physiological states. Further studies should focus on the limit of this capacity to adjust physiological processes to environmental variations in this endangered land snail.

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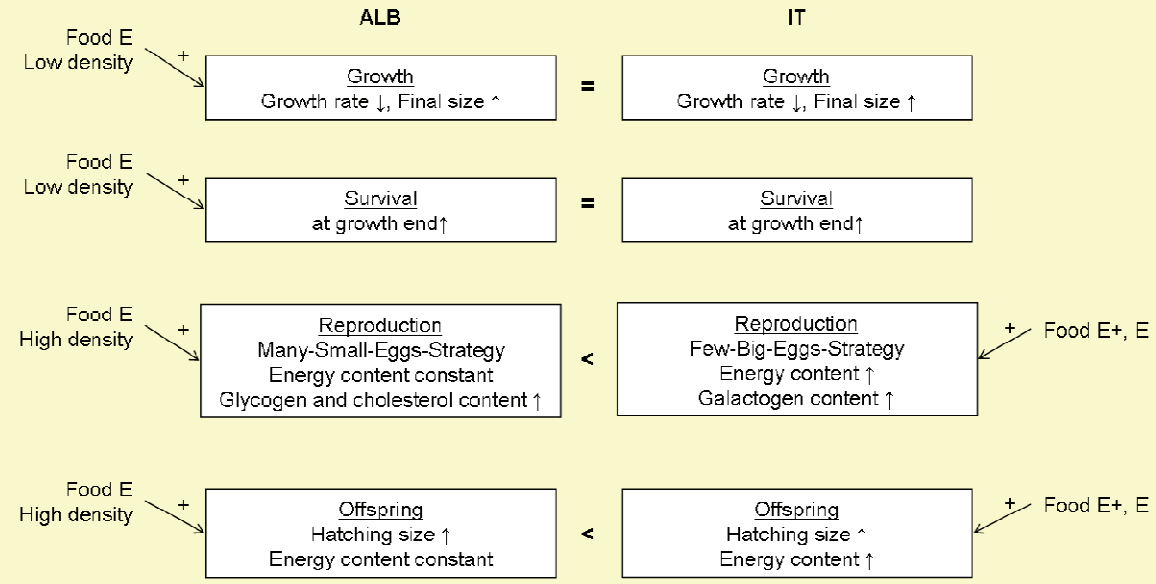
Appendix

Metabolite content (in µg.ml^{-1}) in haemolymph of *Helix pomatia* collected each month in the outdoor cage from February 2007 until January 2008. Average of two replicate measurements from six pooled individual's haemolymph. Apr – Oct-: snails without epiphragm, Oct+ - Mar: snails with epiphragm.

	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct-|Oct+	Nov	Dec	Jan
Hydroxy-Pro	0.54	5.84	1.63	14.21	0.90	6.92	5.35	5.33	1.81|13.90	0.74	2.40	0.46
His	1.20	2.59	2.66	7.74	7.47	2.88	5.17	5.11	1.85|1.27	0.97	1.20	1.32
Asn	0.00	0.26	0.52	0.58	0.21	0.47	0.11	0.16	0.00|0.00	0.13	0.00	0.14
Ser	5.09	8.20	10.51	8.99	8.71	12.48	11.73	11.68	6.68|8.73	3.87	5.53	4.68
Gln	1.42	8.39	1.81	10.67	5.36	17.67	12.04	11.97	0.52|1.09	1.34	1.83	0.71
Arg	0.71	0.33	5.65	1.20	0.92	1.35	2.38	2.40	0.94|1.24	0.22	0.30	4.54
Gly	10.65	26.07	8.16	8.16	4.58	3.13	9.97	9.92	14.11|6.01	8.61	15.56	7.55
HSer	1.19	3.14	2.04	1.93	1.15	0.94	2.66	2.63	2.37|2.31	0.75	1.82	0.89
Asp	0.80	9.57	3.54	4.57	2.69	2.13	7.14	7.09	3.62|2.07	6.15	5.76	8.83
Glu	5.55	74.45	18.64	20.22	10.92	6.92	26.50	26.34	24.77|13.20	23.41	33.02	15.61
β-Ala	0.26	0.72	0.66	2.09	0.73	0.65	1.64	1.59	1.59|0.93	0.25	0.52	0.38
Thr	2.83	3.12	2.61	6.06	3.76	2.75	7.33	7.29	5.69|2.59	2.59	2.78	3.21
α-Ala	45.92	42.29	41.94	42.12	22.76	39.09	62.36	62.02	49.53|34.73	25.54	29.58	35.44
Pro	4.00	3.12	6.82	5.66	4.53	5.26	9.23	9.35	5.15|2.66	3.65	3.66	2.08
Orn	3.33	4.82	4.59	8.46	4.17	5.72	9.51	9.43	4.55|2.96	3.01	3.89	4.12
Cys	0.00	0.60	1.77	4.40	3.07	4.15	7.52	7.24	3.36|2.52	1.83	1.76	1.12
Lys	2.29	3.29	3.03	6.23	2.99	4.22	6.87	6.81	2.85|3.27	1.07	1.72	1.51
Tyr	1.92	14.08	8.15	13.75	4.30	3.19	13.69	12.91	9.00|9.41	3.63	4.00	2.85
Met	0.34	0.25	0.60	1.04	0.65	0.67	0.96	0.95	0.41|0.46	0.31	0.31	0.18
Val	3.38	3.44	4.48	6.96	5.55	4.68	8.81	8.74	4.54|3.18	1.64	2.40	2.65
Iso	2.09	1.92	2.06	3.51	3.02	1.85	3.61	3.60	2.61|1.82	0.79	1.19	1.40
Leu	3.26	3.05	3.59	7.35	5.52	4.33	6.99	6.94	4.15|3.11	1.47	2.02	2.29
Phe	1.40	2.27	1.62	4.34	2.65	2.92	5.35	5.32	2.62|1.28	1.04	1.18	0.80
Trp	0.27	0.34	0.40	1.36	1.41	0.47	1.11	1.11	0.49|0.29	0.27	0.39	0.22
Succinic acid	8237.12	1112.31	7491.55	0.00	0.00	234.48	0.00	0.00	4784.47|855.70	0.00	18.31	850.60
Putrescine	2.14	16.53	9.99	20.13	1.13	1.26	16.83	7.20	16.24|14.73	3.72	5.03	0.00
Myo-Inositol	2.76	2.79	25.44	5.41	4.93	5.73	8.05	2.67	4.88|7.45	1.32	1.76	13.46
Glycerol	34.24	41.67	47.20	32.44	29.74	22.50	28.70	22.96	48.54|32.02	21.75	35.15	12.93
β-D-MGP	36.43	23.57	649.77	82.91	66.13	111.09	159.55	27.93	111.67|126.28	12.48	19.77	225.17
Maltose	0.83	0.22	15.47	3.62	7.61	2.31	8.57	0.22	13.08|5.88	0.00	0.26	4.96
Galactose	2820.16	1920.11	3900.22	2880.16	6420.36	4620.26	3780.21	5040.28	3420.19|3900.22	5760.32	3900.22	5640.31
Glucose	243.55	109.15	87.48	58.59	273.41	324.86	374.90	306.26	284.63|273.56	208.09	106.63	299.45
Mannobiose	0.00	0.00	0.00	39.50	0.00	0.00	149.03	0.00	457.47|221.55	0.00	0.00	0.00
Triglycerides	889.66	554.48	281.38	281.38	564.83	43.45	163.45	111.72	1005.52|115.86	163.45	53.79	817.24
Cholesterol	8.49	7.37	8.49	7.00	9.99	4.87	3.75	2.37	4.12|1.25	2.25	3.62	2.12

Translation: The observations that were made on *Helix pomatia* of the Swabian Alp population and the Italian population in the experimental snail farm in Tachenhausen (Box *hp*3) showed that the Swabian Alp population had a lower fitness with food that is rich in lipids or plant cell wall polymers. The basic food usually used in snail farming had a positive influence on growth, survival and reproduction, whereas the density of individuals in the cages had showed no clear advantage or disadvantage. Italian snails show close results to those obtained for growth and survival with the Swabian Alp population, but food had no influence. The reproduction however was positively influenced by the basic food and the energy rich food. Galaktogen was allocated to eggs and offspring which may result in higher offspring survival (Baur 1994, Madec *et al.* 1998) and therefore higher fitness for Italian snails fed with these types of food. It is possible that the Italian snails were already adapted to farm conditions and selected for size and reproductive success like in *Cornu aspersum* (Dupont-Nivet *et al.* 1998, 2000).
Because of the high mortality during growth and the subsequently restraint possibility of statistical analysis, the results about reproduction could only be used with diffidence and mainly as information for snail farming. However, it makes no sense to continue investigate food conditions since the mortality was mainly observed during hibernation. A well balanced food would therefore give good results for establishing a farm population with acceptable reproduction quota when the mortality during hibernation could be reduced.
During hibernation *Helix pomatia* adjusted its physiological processes to enhance cold hardiness. The intestine was emptied and gut bacteria were expulsed. The shell aperture was closed with a calcified epiphragm in order to avoid high dehydration. A permanent gut flora was maintained, which could potentially initiate the crystallization of body fluids at near zero temperatures. The decrease of the body supercooling point from -2°C in summer to -6°C in winter is only small and mainly due to water loss and subsequently accumulation of cryoprotectants in haemolymph and body fluids. *Helix pomatia* accumulates glycerol, some amino acids and triglycerides in two geographically and climatically distinct populations. The regulation of water loss and of the intensity of cryoprotectant accumulation could then represent adaptations to regional climate (Nicolai *et al.* 2005).
Hibernation and activity were influenced by environmental factors like photoperiod and minimal temperature. Because of the variability of temperature in the snail's microhabitat, physiological processes have to be constantly readjusted. When the refuge does not sufficiently buffer temperature variations, snails show not a clear physiological strategy, which could be the reason for winter mortality. On the other hand extreme climatic situation during the year could provoke high mortality, like the absence of precipitations and high temperatures. The aestivation is then characterized by the accumulation of polyols and cholesterol as heatprotectants accompanied by high metabolic activity to produce them.
The well elaborated microhabitat conditions in a snail farm could therefore be crucial for snail survival. Refuge possibilities, like loose soil, stones, mosses, leafs, branches and bushes, might be extremely important to give snails the possibility to avoid temperature variations and enhance survival probability.

Box *hp*3. Diet treatment influence in *Helix pomatia*



Die Beobachtungen, die im Versuchsgarten Tachenhausen an Albschnecken und italienischen Schnecken der Art *Helix pomatia* (Box *hp*3) durchgeführt wurden, zeigen deutlich, dass Albschnecken mit fettreichem oder auch mit pflanzenfaserreichem Futter ihre Fitness reduzieren. Das Basisfutter dagegen, das üblicherweise in der Schneckenhaltung genutzt wird, hatte einen positiven Einfluss auf Wachstum, Überleben und Fortpflanzung, wobei die Haltungsdichte nicht eindeutig Vorteile oder Nachteile aufwies. Italienische Schnecken ähneltenn in Wachstum und Überleben den Albschnecken, wobei keines der Futter einen negativen oder positiven Effekt hatte. Die Fortpflanzung dagegen war positiv vom energiereichen und vom Basisfutter beeinflusst, besonders durch die Anreicherung von Galaktogen in Eiern und Jungtieren. Dies deutet auf höhere Überlebenschancen der italienischen Jungtiere hin (Baur 1994, Madec *et al.* 1998) und damit auch auf eine bessere Fitness. Da die italienischen Schnecken aus einer Schneckenzucht stammen, ist es möglich, dass Eigenschaften wie Wachstum, Größe und Überlebensfähigkeit ausgelesen und gezüchtet wurden, wie es bei *Cornu aspersum* der Fall ist (Dupont-Nivet *et al.* 1998, 2000).

Aufgrund der hohen allgemeinen Sterberate während der Tests sind die Ergebnisse zur Fortpflanzung nicht statistisch korrekt unterlegt und daher eher informativ für die Schneckenzucht zu nutzen. Es macht jedoch keinen Sinn, noch weiter nach einer Verbesserung der Fortpflanzung durch die Fütterung zu suchen, da die Sterberate besonders in der Hibernationsphase anstieg. Eine ausgeglichene Fütterung bei mittlere Haltungsdichte dürfte zu einem guten Erhalt der Zuchtpopulation mit marktwirtschaftlichen Vermehrungsquoten führen, wenn die Hibernationsterblichkeit verringert werden kann.

Während der Hibernation paβte *Helix pomatia* ihre physiologischen Prozesse so an, dass die Kältersistenz erhöht wurde. Der Verdauungstrakt wurd geleert, wobei Darmbakterien ausgeschieden wurden, und das Gehäuse verschlossen wurde, um den Wasserverlust zu regulieren. Eine permanente Darmflora blieb jedoch zurück, die potentiell die Formation von Eis im Körper bei 0°C-nahen Temperaturen bewirken könnte. Das Absinken der Gefriertemperatur bei Schnecken im Winter war auch nur gering (von -2°C im Sommer auf -6°C im Winter) und wahrscheinlich durch den Wasserverlust und damit einhergehende Anreicherung von Frostschutzsubstanzen in den Körperflüssigkeiten zu begründen. *Helix pomatia* reicherte Glycerol, einige Aminosäuren und Triglyceride als Frostschutzmittel an. Dies scheint universell zu sein, da es bei zwei geographisch und klimatisch unterschiedlichen Populationen nachgewiesen wurde, wobei der Wasserverlust und damit die Konzentration der angereicherten Stoffe reguliert werden kann und wahrscheinlich zur Anpassung an regionale Klimabedingungen dient (Nicolai et al. 2005).

Hibernation und Aktivität wurden von Umweltfaktoren beeinflusst, d.h. von Tageslänge und minimaler Temperatur. Aufgrund der Variabilität der Temperatur im Mikrohabitat wurden physiologische Prozesse von Aktivität und Hibernation immer wieder angepasst. Solange der Unterschlupf die täglichen Temperaturunterschiede nicht abfangen kann, ist eine konstante physiologische Strategie nicht erkennbar. Dies könnte der Grund für eine hohe Sterberate im Winter sein. Andererseits waren extreme Wettersituationen im Laufe eines Jahres zeitweise für

eine hohe Sterberate verantwortlich, z. B. das Ausbleiben von Niederschlägen und hohe Temperaturen. Die einsetztende Ästivationsphase war dann durch eine Anreicherung von Polyolen und Cholesterin als Wärmeschutzmittel und durch eine hohe metabolische Aktivität zur Herstellung dieser charakterisiert.

Das Anlegen eines optimalen Habitats innerhalb der Schneckenzuchtanlage erscheint auschlaggebend. Unterschlupfmöglichkeiten, wie lockere Erde, Moos, Steine, Laubhaufen, Zweige und Büsche, sind extrem wichtig, um den Schnecken die Möglichkeit zu geben Temperaturunterschieden auszuweichen und somit Überlebenschancen zu erhöhen.

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PERSPECTIVES

Personal projects

The competences that I have acquired during my thesis allow me to consider some further investigations in relation to the evolution of life history traits in terrestrial pulmonates.

1. Since semelparous snails and many slugs lay eggs that have to overcome the cold season, an inter-specific approach of cold hardiness of eggs could give some insights about evolution of egg size, egg content and egg-laying behavior. Climatic and trophic conditions in the habitat may contribute to particular egg structures enhancing cold hardiness or may lead to behavioral adjustments in oviposition in order to maximize reproductive success.
2. In the domain of conservation biology the knowledge of habitat requirements and physiological limits of adaptation to stochastic environment could be useful in the elaboration of protection measures. For instance in the land snail *Tyrrhenaria ceratina*, an endangered snail of Corsica, the colonization of restored biotopes requires a wide range of management measures that are based on knowledge about dietary demands, seasonal behavior and physiological adjustments to extreme situations.
3. Invasive species that are harmful to biomass productivity in agricultural ecosystems force to biological pest control. Physiological limits to thermal stress and dehydration, nutritional value of culture plants and the physico-chemical conditions in the soil may influence the success of biological pest control for slugs.

Therefore, my future professional projects include the application of technical knowledge acquired during this doctoral work as well as advanced training in bio-statistical modeling in these research fields.

L'impact du régime alimentaire sur la reproduction et les processus physiologiques chez les escargots terrestres *Cornu aspersum* et *Helix pomatia*

L'acquisition et l'allocation de ressources sont des tâches fondamentales chez tous les animaux. Les organismes rencontrent souvent de l'hétérogénéité dans l'espace et dans le temps et devront adapter leur traits d'histoire de vie, décrits comme un ensemble héritable de règles qui déterminent l'allocation vers la croissance ou la mise en réserve pour la survie *versus* la reproduction. Chez *Cornu aspersum,* la stratégie de reproduction dépend des contraintes saisonnières et l'investissement dans la reproduction est influencé par l'énergie disponible dans l'environnement. Des nutriments présents dans l'alimentation, notamment des lipides, sont déposés dans les œufs, et une source mixte de calcium a pour effet que la coque des œufs est plus épaisse. Ceci pourrait augmenter la probabilité de survie des juvéniles. Des bactéries présentes dans l'alimentation et dans la terre pourraient être responsables d'une diminution de la capacité de surfusion, et elles pourraient persister dans l'intestin pendant l'hibernation. Chez *Helix pomatia*, qui est utilisé dans des élevages biologiques afin de maintenir cette espèce menacée comme spécialité régionale sous le nom Albschneck®, le succès reproducteur est faible même avec une alimentation riche en énergie, comparativement à une population en provenance d'un élevage intensif Italien. *Helix pomatia* préserve une riche communauté bactérienne dans l'intestin qui pourrait contenir des bactéries nucléantes, mais accumule aussi des cryoprotecteurs, comme des acides aminés, des triglycérides et du glycérol, pour améliorer la résistance au froid pendant l'hiver. Toutefois, la mortalité pourrait être liée à un ajustement perpétuel des processus physiologiques aux variations importantes de la température et aux situations climatiques extrêmes ainsi qu'à une allocation limitée vers des processus de survie. Des mesures de protection devront donc inclure la préservation de refuges dans l'habitat naturel et artificiel.

The impact of diet treatment on reproduction and thermo-physiological processes in the land snails *Cornu aspersum* and *Helix pomatia*

Resource acquisition and allocation is a basic task of all animals. Organisms often encounter environmental heterogeneity in space and time, and may adapt their life histories that have been described as heritable set of rules determining allocation to growth or storage for survival *versus* reproduction. In *Cornu aspersum* reproductive strategy depended on seasonal time constraints and investment in reproduction was influenced by the energy available in the environment. Nutrients available in food, like lipids, were deposited in eggs, and a mixed calcium source affected the thickness of egg shell. This might enhance offspring survival. Bacteria in food or soil could be responsible for low supercooling ability and might persist during hibernation in the gut. In *Helix pomatia* that is used recently in low input snail farms in Germany to maintain this endangered species as a regional specialty under the name Albschneck®, showed low reproductive success even with high energy food compared to an Italian farm population. *Helix pomatia* preserved a rich bacterial community in the gut probably with ice nucleating bacteria and accumulated cryprotectants, like amino acids, triglycerides and glycerol, to enhance cold hardiness during hibernation. However mortality could be related to perpetual physiological adjustments to high temperature variations and extreme weather situations like dryness, but also be due to limited resource allocation to survival maintaining processes. Protection measures of this species should therefore include the preservation of refuge possibilities in the natural or artificial habitat.

Der Einfluss der Ernährung auf die Fortpflanzung und auf thermo-physiologische Prozesse bei den Landschnecken *Cornu aspersum* und *Helix pomatia*

Die Aufnahme und Zuteilung von Ressourcen ist eine Grundaufgabe aller Tiere. Organismen treffen oftmals auf Umweltheterogenität in Ort und Zeit und müssen ihre Merkmale der Lebensgeschichte anpassen. Diese werden als vererbbare Regeln beschrieben, die die Ressourcenzuteilung zu Wachstum oder Speicherung *versus* Fortpflanzung festlegen. Bei *Cornu aspersum* hingen die Fortpflanzungsstrategien von der Saison ab, und Fortpflanzungsinvestment war von verfügbarer Energie in der Umwelt beeinflusst. In der Nahrung vorhandene Nährstoffe, wie Fette, wurden für die Eibildung genutzt, und eine gemischte Kalziumquelle im Futter hatte Einfluss auf die Dicke der Eischale. Dies könnte die Überlebenschancen des Nachwuchses erhöhen. Bakterien in der Nahrung könnten dagegen für eine geringere Unterkühlungsfähigkeit im Winter verantwortlich sein und im Winter im Darm verbleiben. Bei *Helix pomatia*, die seit einigen Jahren in extensiven Schneckenzuchtanlagen gehalten wird, um die Art als regionale Spezialität unter dem Namen Albschneck® zu erhalten, wurde ein geringerer Fortpflanzungserfolg verzeichnet, selbst mit energiereichem Futter, verglichen zu einer italienischen Zuchtpopulation. *Helix pomatia* behält während der Hibernation eine reiche Bakterienflora mit wahrscheinlich eiserzeugenden Bakterien im Darm und akkumuliert Frostschutzmittel, wie Aminosäuren, Triglyceride und Glycerol, um die Kälteresistenz zu erhöhen. Trotzdem kann die hohe Sterberate mit den ständigen physiologischen Anpassungen an hohe Temperaturvariationen und extremen Wetterbedingungen, wie z. B. Trockenheit, zusammenhängen, aber auch mit einer zu geringen Ressourcenzuteilung für lebenserhaltende Prozesse. Artenschutzmaβnahmen sollten daher den Erhalt von vielfältigen Unterschlupfmöglichkeiten im natürlichen und künstlichen Lebensraum fördern