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## **Is cold hardiness size-constrained?**

### **A comparative approach in land snails**

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## **Abstract**

Body water is a major element of the cold-hardiness strategies observed in ectothermic animals, in particular in freezing avoidant species for which body ice formation is lethal.

5 Here, we investigate the relationships, in terrestrial snails, between the temperature of crystallisation ( $T_c$ ) and body water (water mass and water content), shell shape, geographic and climatic distribution, taking into account phylogenetic inertia. Phylogenetic relationships among 31 species from 13 different families of terrestrial Gastropods were studied using 28S rRNA nuclear and COI mitochondrial sequence data, together with species-specific traits. Our  
10 results provide evidence for clear relationships between  $T_c$  and absolute / relative body water: smaller species with lower water content tended to be characterized by colder temperatures of crystallisation, although some exceptions were noticeable. Environmental conditions do not appear to affect  $T_c$  significantly, as well as shell shape which is however correlated with water content. This study confirmed that supercooling ability in land snails is size-  
15 constrained, with consequences on cold-hardiness strategies.

## **Keywords**

Terrestrial gastropods

20 Temperature of crystallisation

Body water

Phylogenetic relationships

PGLS

25

## **Introduction**

Temperature is the main environmental factor acting on physiological processes, shaping the geographic distribution of living organisms. Because extreme cold conditions directly threaten survival, one of the major goals of ectothermic species is to deal with minimal temperatures. Cold hardiness is thus a critical life history trait in the maintenance and development of a species in a cold-constrained habitat (Bale and Walters 2001; Moine et al 2002; Pither 2003; Bale and Hayward 2010; Chown et al 2010).

Cold hardiness is a widely studied aspect of ecophysiology, usually separating the species into two main categories: freezing avoidant and freezing tolerant species. What determines the cold tolerance strategy of a species is a combination of ecological (climate, microhabitat conditions; Kukul and Duman 1989; Costanzo et al 1998; Jing et Kang 2003), ontogenic (stage, mass; Vernon et al 1997; Grenot et al 2000; Jensen et al 2007), and phylogenetic factors (Voituron et al 2009; Nyamukondiwa et al 2011).

For both strategies, the supercooling ability, i.e. the ability to maintain body fluids at a liquid state below the freezing point, is a critical parameter. Freezing tolerant organisms generally will have a poor ability to supercool, between -5 and -10°C allowing slow freezing of tissues and thus sufficient time to implement protection mechanisms. On the contrary, in freezing avoidant organisms, for which ice formation in tissues is lethal, the supercooling ability will be enhanced, often in association with the synthesis of large amount of antifreeze substances (for more information on these strategies, see for example Ramløv 2000; Zachariassen and Kristiansen 2000; Block 2003).

The temperature at which a water solution spontaneously freezes (i.e. the temperature of crystallisation,  $T_c$ ) is determined by several factors: (i) the volume of the sample, the probability of nucleation being proportional to the number of water molecules (homogeneous

nucleation), (ii) the presence of active ice nucleating agents (= INA; gut bacteria, proteins or lipoproteins), causing heterogeneous nucleation, (iii) the presence of antifreeze substances as polyols and sugars with colligative action or thermal hysteresis proteins.

The relationship between  $T_c$  and water volume has been known for a long time. Bigg (1953) demonstrated that freezing temperature of pure water droplets is volume-dependent. Applied to animal ecology, this suggests that, in freezing avoidant species (i.e. in absence of active INA), a lower volume of body fluid is related to a more extended supercooling capacity (i.e. a lower  $T_c$ ). This has been proved successfully at both intra-specific (David and Vannier 1996; Costanzo et al 1997; Ansart and Vernon 2004) and inter-specific levels (Lee and Costanzo 1998; Zachariassen et al 2004). However, these last findings should be taken with caution because the evolutionary history of the analyzed species was not taken into account.

As demonstrated by Zachariassen et al (2004), the common idea that a larger volume of water also triggers a higher probability of heterogeneous nucleation has to be reconsidered: in their study, they showed that nucleation temperatures of freezing avoidant insect species were very close to that obtained from comparable pure water samples (Bigg curve, completed with data from MacKenzie 1977 and Wilson et al 2003), suggesting the absence of ice nucleating agents, even in the largest species.

Although they have not so far generated much interest in the cryobiologist community, terrestrial gastropods have a wide geographic distribution which justifies a thorough study of their ability to survive cold conditions and of the associated mechanisms. Furthermore, a better understanding of their cold-hardiness is expected to improve palaeoenvironmental interpretations of Quaternary glacial species assemblages. In north-western and central Europe, among the 350 snail species described, 35 are present over the Arctic Circle ( $66^{\circ}33'44''$ ) and 44 can be found at altitudes higher than 2000 m (Kerney et al 1983; Kerney and Cameron 1999). Related to their large habitat range is their ability to occupy buffered

microsites during cold season and to endure long periods of inactivity (Storey and Storey 1990, 2004; Bailey and Lazaridou-Dimitriadou 1991; Pakay et al 2002).

Information on cold hardiness strategies is only available for a few species of terrestrial gastropods (eight species of snails and ten of slugs) and their mechanisms are still poorly known (Storey et al 2007; Ansart et al 2010; Nicolai et al 2011; Slotsbo et al 2012; Košťál et al 2013). Although generalization is somewhat hazardous, smaller land snails (shell diameter up to *ca.* 15 mm) are typically freezing avoidant, although no active synthesis of antifreeze substances has been detected. On the contrary, larger species are considered partially freezing tolerant, i.e. able to survive short duration of freezing of their body tissues (see Ansart and Vernon 2003 for a review; Nicolai et al 2005). In the large snail *Cornu aspersum*, Ansart et al (2010) showed the presence of INA in the digestive tract, limiting the Tc of hibernating individuals. This coexistence of both strategies along a size gradient, which requires further confirmation, could reflect different advantages of each strategy as a function of the water volume, rather than an adaptation to environmental conditions. We hypothesize that, above a certain size (i.e. a certain water volume), freezing avoidance is not a viable strategy, because too many cryoprotective molecules would be required to maintain the body fluid in a liquid state. According to such a model, we should observe a switch in the Tc of species along the size gradient: small, freezing avoidant species with a Tc following the Bigg curve prediction, and large, partially freezing tolerant species for which the Tc is not dependent on the water volume.

The aim of this study is to determine which factors significantly influence the Tc of land snail species. We will consider both absolute and relative body water, i.e. water mass (WM) and water content (WC), which are both highly variable in land gastropods. The shell shape, from depressed to globular, from oblong to conic, can also play a role in water relationships with the environment (Goodfriend 1986) and consequently in cold tolerance. Previous studies on

*Helix pomatia* (Nicolai et al 2005) and *Cornu aspersum* (Ansart and Vernon 2004) showed significant Tc variations in snail populations living under different climates, but it was unclear if these variations were related to different size and body water mass and/or to adaptation to prevailing climatic conditions.

5 In this work, we present original data corresponding to standardized experimental conditions and we investigate relationships between the temperature of crystallisation and environmental, physical and physiological factors in 31 land snail species, taking into account phylogenetic inertia. Using this approach, we are able to partition the relative impact of evolutionary vs. ecological pressures on a life history trait having a key-role in species  
10 distribution.

## Material and methods

### *Animals collection, identification and rearing*

15 Individuals of 31 land snail species from 13 families were collected during their activity period (spring and early autumn 2010 and 2011) in different localities and on various habitats (Fig. 1, Table 1). Taxonomic identification was based on the Kerney and Cameron (1999) field guide and recent faunas from Gargominy and Ripken (2011) and Gargominy and  
20 Neubert (2011). For taxa particularly difficult to identify, we required help from Nicole Limondin-Lozouet (LGP, Meudon, France) and, for Clausiliids, from Olivier Gargominy (MNHN, Paris, France).

Species were all reared under standardised activity conditions (Incubator AquaLytic 186-4; 20°C, 16h:8h Light-Darkness photoperiod; *ad lib* food depending on species – cereal powder,  
25 litter, moss, snail eggs; moist synthetic foam as substrate) until the beginning of the

hibernation period (November). Over two weeks, they were gradually submitted to overwintering conditions (5°C, 8:16 LD photoperiod, neither food nor water provided), which were maintained constant for 4 months.

#### 5 *Water mass, water content and temperature of crystallisation*

Measurements were performed only on adult snails. Individuals were rapidly weighed (Sartorius CP224S  $\pm$  0.1 mg or Toledo XP2U  $\pm$  0.1  $\mu$ g, depending on species) before being equipped for Tc determination. A thermocouple (Testo 177-T4, thermocouple type K) was attached to each snail and animals were individually inserted into plastic tubes immersed in a cryostat (Huber Polystat CC3) filled with an antifreeze fluid maintained at 3°C. The temperature of the bath was then cooled at the rate of 0.5°C.min<sup>-1</sup>. The exotherm recorded by the thermocouple indicated spontaneous freezing of the animal, with Tc being defined as the lowest temperature at the start of the exotherm.

As very small species may become active very rapidly when manipulated, samples were maintained on ice once out of the incubator. On average, 20 seconds were necessary to correctly attach each snail to the thermocouple. Individuals displaying any sign of activity were discarded and excluded from further analysis.

After Tc measurement, snails were dehydrated in a 60°C oven for two days and weighted again to estimate their water mass (WM) and their water content (WC) expressed as WM divided by total mass. WM was log transformed to approach normal distribution. Depending on species, 5 to 30 individuals were retained for analyses.

*Shell volume/surface ratio*

We estimated for each species the mean surface- volume ratio (S/V) of the shell. For 5 to 10 individuals of each species, we measured the shell height (H), length (L), and width (W), as defined by Kosnik et al (2006). Depending on the individual size, measurements were either performed using a calliper or a binocular microscope and image treatment system (Image J). Estimation of the volume and surface of a gastropod shell is not a trivial question (e.g. Raup and Graus 1972). Some authors, following the suggestion of Powell and Stanton (1985), consider the formula for a cone as a good estimation of shell volume. However, this is based on only 3 globular marine species (*Thais haemastoma*, *Polinices duplicatus*, *Littorina ziczac*). To better approximate the shell volume, we directly estimated it for 8 of the biggest species (Co, Em, Ev, Hla, Ma, Pe, Tp, Zd) by weighing the volume of water displaced by the shell (with obstructed aperture) when placed in an hermetically closed recipient (Örstan 2011). The measurement was repeated for 10 individuals of each species. We then compared the volume obtained with those calculated with the formula of a cone and of an ellipsoid. In all cases, the best approximation was the arithmetic mean between the volume of a cone and the volume of an ellipsoid. Between both methods, direct measurement and calculation, we found a mean difference inferior to 7% (maximal difference: 13%) and a minimal correlation coefficient of 0.91 (p<0.001). Volume calculation could then be extrapolated to small species for which direct measurement is not possible.

The shell surface was evaluated as the arithmetic mean between the surface of a cone and the surface of an ellipsoid. The formula for the surface of an ellipsoid, being in reality of a high complexity, was approached thanks to the Knud Thomsen's formula:  $S_{\text{ellip}} = 4\pi[(H^pL^p+H^pW^p+L^pW^p)/3]^{1/p}$ , where  $p=1.6075$  (Klamkin 1971, 1976). The surface of a cone follows:  $S_{\text{cone}} = \pi(L/2)^2 + [\pi L/2 \cdot \sqrt{((L/2)^2+H^2)}]$ .

As S/V is highly related to the animal size, we used a corrected S/V,  $SV_{corr} = S/V \cdot (H+L+W)/3$ , allowing to consider varying shapes for comparable sizes.

### *Geographic and climatic data*

5

The distribution ranges of the different species were obtained from maps published in Kerney et al (1983) and completed in Kerney and Cameron (1999). The north-west European geographic area covered by this guide excludes Iberian Peninsula and Italia and extends eastward to include Hungary, Slovakia, Poland and Finland (Fig. 1). Distribution areas were scanned and marked on a projection map (WGS\_1984\_UTM\_Zone\_30N), using the software ArcInfo v.10.0. We extracted for each species the surface of the area occupied (with an error estimated at less than 3%), the minimal and maximal latitude and the minimal and maximal longitude ( $\pm 0.5^\circ$ ). Surface data were log-transformed prior to analyses.

10 For two species, for which maps were not available (*Cantareus apertus* and *Helix lucorum*) we compiled data from INPN (<http://inpn.mnhn.fr/>) and Fauna Europaea (<http://www.faunaeur.org/>), completed with personal observations.

We also compiled data on maximal altitude reached by species available on AnimalBase website (<http://www.animalbase.uni-goettingen.de/>).

15 Climatic data were obtained from the ECA&D website (European Climate Assessment & Dataset, <http://eca.knmi.nl/>; Klein Tank et al 2002). ECA&D provides freely available climatic information, compiling daily measurements over the 30 last years from 6596 meteorological stations throughout Europe and Mediterranean. We first recorded for each species 25 climatic indices (temperatures and cold indices). Extreme values of each index were retained only if they concerned at least 5% of the species distribution range.

Climatic and geographic informations were summarized by their factorial scores in a Principal Component Analysis, using the FactoMineR package in the R software (Lê et al 2008; Fig. 2). We retained the most pertinent variables (10 climatic indices and 6 geographic variables) based on their contribution to the variance of significant axes. The first one accounted for 71.9% of the total inertia and opposed at the left side Mediterranean and Atlantic species, with reduced distribution area and mild climate to ubiquitous and northern species at the right side. The second axis was less informative (13.6% of total variation): two variables, minimal latitude and minimal longitude, had an important contribution to its total variance (respectively 38.6% and 23.9%), separating species which were not found on western or southern limit of the study area (Co1, Pa, Cc) from the others. Species coordinates on the first axis were then used as a measure of their environmental conditions (ENV) and introduced in subsequent analyses. We also considered the minimal value of the mean daily temperature in December-January-February over the distribution range of each species for the last 30 years ( $ENV_{min}$ ) and the mean winter temperature at the collection site ( $ENV_{loc}$ ).  $ENV_{loc}$  was determined using data from the nearest MeteoFrance station, except for *Helix pomatia* collected in Germany and for which we considered data from the nearest ECA&D station; these data were available for various duration, from 2 to 20 years.

### *Molecular data and tree construction*

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#### *DNA extraction, amplification and sequencing*

The protocol followed here has been described in Guiller et al (2001). Briefly, total genomic DNA was obtained from fresh material and genomic DNA was extracted using the chelex extraction protocol (Estoup et al 1996). We amplified fragments of approximately 433bp and 686bp for the 28S rRNA nuclear and COI mitochondrial genes respectively. The 28S (LSU)

fragment was amplified using primers 28SF (5'-AACGCAAATGGCGGCCTCGG-3') and 28SR (5'-AAGACGGGTCGGGTGGAATG-3') (Koene and Schulenburg, 2005). The COI region was amplified using FCOI (5'-ACTCAACGAATCATAAAGATATTGG-3') and RCOI (5'-TATACTTCAGGATGA

5 CCAAAAATCA-3') primers (Folmer et al 1994). Amplification of template DNA was carried out in 15 µl volumes with MyTaq Mix (2X) (Bioline, France), 0.20 µm each primer and 1.5 µl DNA (approx. 50ng). The PCR conditions were for 28S rRNA, 5 min at 95°C, followed by 35 cycles of 95°C (20 s), 62.5°C (30s), 72°C (1 min) and a final extension phase at 72°C for 10 min; for COI, an initial denaturation step of 94°C (5 min), followed by 35 cycles of 94°C (45 s), 52°C (45 s), 72°C (1 min) and a final extension phase at 72°C for 7 min. Amplification products were checked using 2% agarose gel stained with ethidium bromide. Double-strand sequences were obtained using an automated sequencer (Plate-forme de séquençage et génotypage OUEST-genopole®). We used sequences from the GenBank for *Pomatias elegans* (28S, accession n° AY014161) and the outgroup *Gibbula umbilicalis* (28S, 15 JN686190 and COI, JN686278).

### Sequence analysis

Mitochondrial sequences were aligned using the built-in assembly algorithm of the CODONCODE ALIGNER software (v3.5, CodonCode Corporation, Dedham, Massachusetts). For 28S rRNA gene, we manually adjusted the region based on the 28 rRNA sequence alignment of Euthyneuran gastropods published in Dayrat et al (2001). We removed a set of 212 sites (between the 426th to the 637<sup>th</sup> position) because the alignment was too ambiguous in that region. For the COI gene, we excluded the variable third codon position from the analysis to reduce the homoplastic effect of transitions on tree reconstruction, especially at

higher levels of divergence. New sequences produced for 28S and COI genes were submitted to GenBank (Table 2).

### Phylogenetic analysis

Phylogenetic relationships among individuals of different species were investigated using bayesian-based inference (BI) and maximum likelihood (ML) methods. The best fit model of nucleotide substitutions was selected prior to BI and ML analyses using the Akaike Information Criterion (AIC). The software MrAIC v1.4.2 (Nylander 2004) was used to

5 evaluate the fit of the data to 24 different models of nucleotide substitutions. The resulting best fit models were GTR+G and GTR+ $\Gamma$ +G for 28S and COI genes respectively (general time-reversible with six different rates for transitions and transversions, unequal base frequencies, a gamma distribution parameter  $\Gamma$  that describes rate variation across variable sites and a parameter  $I$  for invariable sites). Each model of nucleotide substitution was

10 incorporated in MRBAYES v3.1.1-p1 (Ronquist and Huelsenbeck 2003) and in PHYML v2.4.4 (Guindon and Gascuel 2003) for BI and ML analyses respectively. For ML analysis, the robustness of inferences was assessed by bootstrap resampling using 1000 repetitions. For Bayesian analyses, the posterior probabilities of trees and parameters were approximated with Markov Chain Monte Carlo (MCMC) and Metropolis coupling.

15 Single-gene analyses provided trees with good resolution but to improve the accuracy of phylogenetic inference and avoid topological variation found among trees constructed from each single gene, we analyzed genes simultaneously in concatenated 28S and COI sequences into a super-gene alignment of 881bp. For BI analysis, we ran two independent MCMC analyses with four chains each and a temperature set to 0.2. Each chain was run for

20 10,000,000 cycles with trees sampled every 100 generations. Posterior probabilities were obtained from the 50% majority rules consensus of trees sampled after discarding the trees

saved before chains reached apparent stationarity (i.e. a 'burn-in period' of 8,000 generations for both concatenated). The average standard deviation of split frequencies after 10,000,000 generations was below 0.01 (0.004423), indicating a very good convergence between the two runs.

5

### *Statistical analyses*

All analyses were implemented in the R software, v.2.14.0., using the Geiger (Harmon et al 2008), Caper (Orme et al 2012) and MuMIn (Bartoň 2009) packages.

10 Studying the relationships between traits needs to take into account the more or less common evolutionary history of species, i.e. the non-independence of data. Prior to conduct a phylogenetic comparative analysis (PCA), it is necessary to control for phylogenetic signal in data, unless classical cross-species statistics have to be applied. Another prerequisite to PCA is to control for the model of evolution of the data (Freckleton 2009).

15 As recommended by Revell (2010), we estimated for each variable and for each simple or multiple linear regression model, the Pagel's maximum likelihood  $\lambda$  which, scaling the internal branches of the tree, allows to have an estimation of the phylogenetic signal in dataset (0 = independence of data, i.e. "star" like tree; 1 = Brownian Motion (BM);  $0 < \lambda < 1$  = phylogenetic signal with an evolution model different of BM). Likelihood ratio tests permit to  
20 compare the calculated  $\lambda$  with the values 0 and 1 (Pagel 1999; Freckleton et al 2002). When adapted, the best evolution model was researched, based on the AIC value and comparison of likelihood with chi square tests (Nunn 2011).

Multiple phylogenetic correlations between explicative variables (logWM, WC,  $SV_{corr}$ , ENV variables) were tested with the Bonferroni correction for p.

To determine which factors explain changes in the mean Tc of species, we built linear models. Eight models (including complete model, K from 1 to 4) relating variables to Tc were examined, taking into account phylogenetic relationships between the 31 species. Models fit was estimated by corrected AIC (AIC<sub>c</sub>), the models with the minus AIC<sub>c</sub> and summing more than 90% of weights (i.e. relative fits) being retained.

## Results

WM, WC, Tc, SV<sub>corr</sub> (Table 2)

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The mean fresh mass of tested species ranged from 1.4 mg for *Columella edentula* to 33.5 g for *Helix lucorum*, corresponding to a WM of 0.6 mg and 22.1 g respectively, i.e. a variation factor of approximately 37,000 between the two extremes. Species WM appeared to be an excellent estimator of the total fresh mass and of the shell volume (PGLS, log-log correlations,  $r > 0.99$  and  $p < 0.001$ ). The WC varied between 28.45 gH<sub>2</sub>O.gFM<sup>-1</sup> for *Clausilia bidentata* and 68.77 gH<sub>2</sub>O.gFM<sup>-1</sup> for *Cantareus apertus*.

15

The minimal Tc was obtained for *C. edentula* with a mean of -16.8°C and the maximal for *H. lucorum* with -4.5 °C.

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SV<sub>corr</sub> was maximal for oblong and depressed shells and was minimal for conical and globular ones, varying between 7.5 and 11.1.

### *Environmental data*

As the variables ENV<sub>min</sub> and ENV<sub>loc</sub> were correlated with ENV (PGLS, respectively  $r = 0.97$ ,  $p < 0.001$  and  $r = 0.54$ ,  $p < 0.001$ ) and led exactly to the same conclusions, we considered only

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ENV in subsequent analyses. In the geographic limits of this study, *Ciliella ciliata* has the smallest range, with an area of *ca.* 27,500 km<sup>2</sup>; *Nesovitrea hammonis* and *Cochlicopa lubrica* could be found in the whole geographic area covered by this guide, except the centre of Iceland, with a distribution area of 3.37 million km<sup>2</sup>. Nine species were registered at altitudes above 2500 m (Aa, As; Cl, Cs, Em, Hli, Pa, Vc, Zd). The minimum daily temperature endured by the snails varied from -7.5°C for Mediterranean and Western littoral species (Co, Ev, Ca), with less than 70 of frost days per year, to -32.5°C for ubiquitous and Northern ones (11 species), with up to 220 frost days per year.

#### 10 *Phylogenetic data and tree*

The topology of the BI and ML trees based on combined 28S and COI sequences were consistently similar. However, since bootstrap values did not strongly support ML tree, we considered and showed the BI tree based on combined 28S and COI genes. The corresponding topology showed a first genetic gap between the Caenogastropoda *Pomatias elegans* and all the other species, belonging to the Heterobranchia clade. A second genetic gap separated both Vertiginidae from other Stylommatophoran species, splitted into clades A and B (Fig. 3). The A clade, including only Sigmurethra species, differentiated the Clausiliidae from the Helicoidea, strongly supporting the monophyly of Helicidae and Hygromiidae *s.l.* However, the tree resolution did not allow to totally resolve phylogenetic relationships among Helicidae and polytomies appeared. The B clade consisted of both Sigmurethra and Orthurethra taxa and included families that were well supported (Oxychilidae, Pupillidae and Enidae). The lack of monophyly of the Orthurethra was due to the position of Vertiginidae and Oxychilidae, which were however well supported (credibility value of respectively 100% and 85%).

### *Phylogenetic signal and mode of evolution*

5 For the variables Tc, logWM, WC and SV<sub>corr</sub>, the Pagel's  $\lambda$  ranged from 0.96 to 1; it was significantly different from 0, indicating phylogenetic signal for data and did not significantly deviate from 1, indicating a BM model of evolution. For ENV,  $\lambda$  was equal to 0.785 and was neither different from 0, nor from, precluding any statement.

Evolution models incorporating  $\lambda$  scaling always fitted better to the data than simple BM ( $\chi^2$ ,  
10  $p < 0.005$ , between log likelihood obtained for both models). To deal with polytomies in the tree topology, we performed PGLS method (Grafen 1989) to analyse dataset. Diagnostic tools revealed no trend between residual values and fitted values and no outlier, demonstrating their adequate application.

### 15 *Correlations between variables and model selection*

Taking into account phylogenetic relationships between species, the correlation between variables logWM and WC was not significant. However, as  $p$  was near the significance threshold ( $p = 0.015$  for  $\alpha = 0.0083$ ) and as both variables are partially redundant, we performed  
20 subsequent analyses with the residuals values of the cross-species regression  $WC = f(\log WM)$  ( $r^2 = 0.36$ ,  $df = 29$ ,  $p < 0.001$ ), called WC<sub>res</sub> and ranging from -18.51 to 11.77 gH<sub>2</sub>O.gFM<sup>-1</sup>.

The only significant phylogenetically-corrected correlation was between WC<sub>res</sub> and SV<sub>corr</sub> (Table 3). Land snails with oblong or depressed shell (i.e. with the highest SV<sub>corr</sub>) had the lowest WC<sub>res</sub> in opposition to species with globular shell (Fig. 4).

The best-fit models, i.e. with the lowest AIC<sub>c</sub> and cumulated weight of 93.7% (Table 4), took into account logWM and WC<sub>res</sub>, which were the only significant predictors of the mean Tc (Fig. 5). ENV and SV<sub>corr</sub> did not contribute to Tc variation. LogWM and WC<sub>res</sub> explained as much as ca. 57% of the total variance of Tc, the contribution of logWM (estimated coefficients) being always higher than that of WC<sub>res</sub>.

Tc values predicted by logWM and WC<sub>res</sub> variations (slope coefficients considered were arithmetic means of those of retained models) were compared with observed values (Fig. 5): nine of the 31 species had a Tc differing less than two degrees of what was expected from models and 10 were between two and three degrees. For only two species (Pe, Nh), the observed Tc were more than two degrees higher than expected values, whereas three (Aa, Cs, Hla) had a Tc more than six degrees below that expected.

## Discussion

One frequent weakness of comparative analyses compiling large datasets is that they rely on bibliographic sources that often involve missing values, various ways of data collection and precision, as well as uncertain phylogeny (Freckleton 2009). The main limit of studies based on complete experimental data is the low number of species included into the analysis, generally under 30 (see for example: Cruz et al 2001 – 19 species; Moran 2004 – 17 species; Nyamukondiwa et al 2011 - 18 species; Strachan et al 2011 – 25 species; Anker and Baeza 2012 – 18 species). A noticeable exception is the recent work of Kellermann et al (2012) reporting data on desiccation and cold resistance for more than 90 *Drosophila* species, in relation with their geographic distribution. This thorough analysis revealed a moderate-to-strong phylogenetic inertia for cold resistance traits. Here, we were able to obtain complete

data for 31 land snail species, a number sufficient to detect phylogenetic dependence of data when present (Freckleton et al 2002; Blomberg et al 2003).

We were indeed able to detect a strong phylogenetic signal for physical and physiological variables, which are known to be largely conserved through evolution, in contrast to

5 environmental data, for which it was not possible to determine phylogenetic dependence or independence (Blomberg et al 2003).

Phylogenetic multiple regressions showed that the water mass and the water content were both independent predictors of the mean temperature of crystallisation of snail species during hibernation, explaining together at least 55% of the variation, whereas no effect of

10 environmental variables and of shell shape was detected. Strachan et al (2011) established a relationship between the water mass and the supercooling ability of first larval instars of freezing intolerant *Drosophila* species, but they did not detect any phylogenetic signal.

Nevertheless, it is worth noting that their study was conducted on a single genus, with a variation of the mean fresh mass and of Tc of respectively *ca.* x200 and x2. Our study

15 considered 13 families from different clades, with a very important variation factor in fresh mass (*ca.* x37,000) and a variation factor of Tc of only *ca.* x4, that could highlight the necessity to consider a large range of variation in studied traits for such experiments.

Even if no ENV effect appeared in analyses, our study does not allow to conclude to the absence of adaptive strategy in cold hardiness under different climatic conditions. At a larger

20 scale (366 species of north-west European land snails), Hausdorf (2003) observed a decrease of body size with latitude, but this effect disappeared when phylogenetic relationships were taken into account, as it was mainly due to the predominance of small bodied clades at higher latitudes and some large bodied clades as Helicoidea at lower latitudes. This would lead to conclude that the phylogenetic constraint on the body size, i.e. on the water volume, is the

25 main factor of species distribution and can be related to their supercooling ability. Thus, only

the smallest species with low  $T_c$  could persist in the coldest areas. Further investigations on species cold-hardiness are needed to test this hypothesis, as well as more precise data on conditions prevailing in overwintering microsites.

5 The snail shell shape is the result of many contradictory or overlapping causes with no clear pattern. In particular, relationships between environmental conditions, as climatic ones, and height, whorl number or aperture size are poorly understood (Goodfriend 1986). To Goodfriend (1983), more elongated shells with smaller aperture would allow snails to retract more deeply and would limit water loss during inactive period. On the contrary, Machin (1967) showed that a significant water loss occurred through the shell in dormant snails, 10 animals with a high surface-volume ratio shell being more sensitive to desiccation. In this study,  $SV_{corr}$ , reflecting the shell shape, had no effect on  $T_c$ , but we found a significant (phylogenetically-corrected) correlation between the water content of hibernating snails and their  $SV_{corr}$ . Snails with globular shells had a higher water content during overwintering than oblong, conical or depressed ones, what would support Machin's hypothesis (1967). Globular 15 shells would be beneficial to face dehydration in high temperature conditions but could not contribute to decrease the  $T_c$  in winter conditions. As globular shells are mainly found in one of the considered clades (Helicoidea), which also contains the biggest species, it remains difficult to disentangle the different effects.

We hypothesized that small land snail species would have a low  $T_c$  depending on the body 20 water volume, no INA being active, and that large species would exhibit poor supercooling ability independently of water mass and content variations (Ansart and Vernon 2003; Nicolai et al 2005). However, we did not detect any switch in  $T_c$  values depending on species size, but a linear regression between  $T_c$  and body fluid variables. Moreover, it is worth noting that in our models, the mean estimated coefficient for  $\log WM$  predictor (2.62) is not significantly 25 different of that of the Bigg curve, predicting the  $T_c$  as a function of pure water mass

variation ( $T_c = 2.25 \log WM - 24$ ; slope comparison: ANOVA, interaction term,  $F=3.50$ ,  $p=0.07$ ; see Zachariassen et al 2000). Our results also demonstrate the necessity to take into account the hydration state of tissues in predictions, as it reflects the amount of freezable water. Only two species had a high  $T_c$  considering their size: the Oxychilidae *Nesovitrea hammonis* and the Pomatiidae *Pomatias elegans*. The first remained active for several weeks before ceasing activity when placed under hibernation condition, probably retaining more INA than species being dormant for a longer time; the second is a Caenogastropoda, some intertidal close-related species having some freezing tolerance level (Sinclair et al 2004). Although no outlier was found in the selected predicting models, some  $T_c$  observations were significantly lower than values predicted from multiple regressions, with differences reaching ca. 7 degrees for *Arianta arbustorum* and 6 degrees for *Helicigona lapicida* and *Cepaea sylvatica*. All three species are Helicidae (i.e. large snails) which can be encountered at high altitude (above 2000 m) and also at high latitude for *A. arbustorum*. Such finding suggests the existence of adaptive mechanisms in these species, as synthesis of cryoprotectants, and would deserve further investigations. Our study did not allow to determine the presence of INA in large snail species contrary to small species, as we hypothesized, but it also does not prove their absence as their action can be masked by body fluid parameters: for such species, it could be useless to eliminate or inactivate such INA during cold periods.

This study aimed to address the two following questions: does the temperature of crystallisation of a species depend on its size, i.e. its body water, and are nucleating agents more active in large species? Although experimental approach is a way to guarantee data consistency, it can force the expression of studied traits. The necessity of rearing animals for several months in standardised conditions can also introduce a bias in the sampled species, since some species did not survive laboratory conditions.

In spite of these caveats, results are consistent with the hypothesis that Tc is size-constrained in land snails, large species with high water mass and water content exhibiting a lower ability to supercool. Although we were not able to detect the presence of active INA in large species, our observations suggest freezing avoidance to be more common in small species (as  
5 confirmed by existing studies and particularly the recent work of Košťál et al 2013); in the majority of large snail species, Tc depends on body fluid parameters, making the freezing avoidance strategy more difficult to adopt and leading to partial freezing tolerance as shown in *Helix pomatia* and *Cornu aspersum* (Nicolai et al 2005; Ansart et al 2001). This work confirms the strong impact of evolutionary pressures on life-history traits as cold hardiness in  
10 ectotherms.

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## Figure captions

**Figure 1.** Distribution area covered by the Kerney et al (1983) and Kerney and Cameron (1999) guides. Collection sites (number from 1 to 6) of the land snail species are indicated with reference to  
5 Table 1. 1: Marais Poitevin; 2: Mercantour; 3: Bretagne; 4: Rhine Valley; 5: Pyrénées; 6: Corse (modified from Kerney et al 1983)

**Figure 2.** Species factorial scores on the first two Principal Component Analysis axes with inset  
10 showing the plot of the ‘environmental’ (10 climatic and 6 geographic variables) vectors on the same plane.

**Figure 3.** Fifty percent majority-rule consensus phylogram from the BI analysis of 28S and COI sequences of 31 land snail species. The tree is rooted using *Gibbula umbilicalis* as outgroup. Branches without posterior probability values (values in italics) are supported by less than 50% of the  
15 sampled trees. 28S and COI GenBank accession numbers are given in Table 2. Scaled photographs of the species shells are presented.

**Figure 4.** Correlation between residual water content ( $WC_{res}$ ) and mean  $SV_{corr}$  in 31 land snail species. Dotted line: cross-species correlation; black line: correlation corrected for phylogeny. Shell  
20 form : += globular,  $\Delta$ =depressed,  $\times$ =oblong,  $\circ$ =conical.

**Figure 5.** Temperature of crystallisation ( $T_c$ ) as a function of the log of water mass (logWM) and the residual water content ( $WC_{res}$ ) in land snails.

**Table 1**

Position in the Gastropod classification (following Bouchet and Rocroi, 2005) of the 31 north-west European land snail species studied. The code used in subsequent figures for each species is indicated, as well as the collection site with reference to Fig. 1, and general distribution (Kerney et al., 1983; Kerney and Cameron, 1999).

Clade	Super-family	Family	Sub-family	Species	Code	Collection site	Distribution
<b>CAENOGASTROPODA</b>							
<b>LITTORINIMORPHA</b>							
	LITTORINOIDEA	Pomatiidae		<i>Pomatias elegans</i>	Pe	1	Mediterranean and Western Europe
<b>HETEROBRANCHIA</b>							
<b>STYLOMMATOPHORA</b>							
ORTHURETHRA							
	COCHLICOPIDEA	Cochlicopidae		<i>Cochlicopa lubrica</i>	Cl	2	Holarctic
	PUPILLOIDEA	Pupillidae		<i>Pupilla muscorum</i>	Pm	1	Holarctic
				<i>Pupilla alpicola</i>	Pa	2	Alps
		Chondrinidae		<i>Abida secale</i>	As	2	Western Europe and Alps
		Valloniidae		<i>Vallonia costata</i>	Vc	2	Holarctic
		Vertiginidae		<i>Columella columella</i>	Col	2	Arctic and Alps
				<i>Columella edentula</i>	Ce	2	Palaearctic
	ENOIDEA	Enidae		<i>Ena montana</i>	Em	2	Central Europe and Alps
				<i>Zebina detrita</i>	Zd	2	Southern Europe
SIGMURETHRA							
	CLAUSILIOIDEA	Clausiliidae		<i>Macrogastra attenuata</i>	Ma	2	Central and Western Europe, Alps
				<i>Clausilia bidentata</i>	Cb	1	Northern and Western Europe
	PUNCTOIDEA	Discidae		<i>Discus rotundatus</i>	Dr	3	Central and Western Europe
	GASTRODONTOIDEA	Oxychilidae		<i>Oxychilus draparnaudi</i>	Od	3	Mediterranean and Western Europe
				<i>Nesovitrea hammonis</i>	Nh	2	Holarctic
	HELICOIDEA	Helicidae	Helicinae	<i>Helix pomatia</i>	Hp	4	Central and Southern Europe
				<i>Helix lucorum</i>	Hl	5	Mediterranean and Central Europe
				<i>Cornu aspersum</i>	Cas	3	Mediterranean and Western Europe
				<i>Cantareus apertus</i>	Ca	6	Mediterranean
				<i>Cepaea nemoralis</i>	Cn	1	Western Europe
				<i>Cepaea hortensis</i>	Ch	3	Central and Western Europe
				<i>Cepaea sylvatica</i>	Cs	2	Alps
				<i>Eobania vermiculata</i>	Ev	6	Mediterranean
				<i>Theba pisana</i>	Tp	1	Mediterranean and Atlantic
			Ariantinae	<i>Arianta arbustorum</i>	Aa	2	Central and Western Europe
				<i>Helicigona lapicida</i>	Hla	5	Central and Western Europe
		Cochlicellidae		<i>Cochlicella acuta</i>	Co	1	Mediterranean and Atlantic
		Hygromiidae	Hygromiinae	<i>Hygromia limbata</i>	Hli	5	South-Western Europe
				<i>Trochulus hispidus</i>	Th	2	Europe
			Ciliellinae	<i>Ciliella ciliata</i>	Cc	2	Alps
			Geomitrinae	<i>Candidula unifasciata</i>	Cu	2	Central Europe

**Table 2**

Means and standard errors of fresh mass (FM), water mass (WM), water content (WC), temperature of crystallisation (Tc) and corrected surface-volume ratio (SV<sub>corr</sub>) of the 31 land snail species ranged by increasing mass. For SV<sub>corr</sub>, *n* is comprised between 5 and 10; the general shell form, as defined by Falkner et al. (2002) is given: C=conical, D=depressed, O=oblong, G=globular. GenBank accession numbers are indicated. \* Sequence imported from GenBank.

Species	<i>n</i>	FM (mg)	WM (mg)	WC (gH <sub>2</sub> O.gFM <sup>-1</sup> )	Tc (°C)	SV <sub>corr</sub>	Genbank Acc. Nos. 28S	Genbank Acc. Nos. COI
<i>Columella edentula</i>	(9)	1.36 0.39	0.64 0.24	50.22 12.87	-16.81 1.89	7.94 C	JX911259	JX911290
<i>Columella columella</i>	(20)	2.33 0.55	1.08 0.31	42.09 7.98	-13.82 2.18	8.60 C	JX911264	JX911295
<i>Vallonia costata</i>	(21)	2.42 0.21	1.13 0.17	46.77 4.91	-15.69 1.11	9.29 D	JX911281	JX911312
<i>Pupilla muscorum</i>	(25)	5.50 1.01	2.67 0.67	44.04 6.71	-14.81 2.09	7.75 C	JX911278	JX911309
<i>Pupilla alpicola</i>	(24)	5.91 0.96	2.85 0.50	46.13 5.96	-15.18 1.98	7.42 C	JX911276	JX911307
<i>Nesovitreia hammonis</i>	(8)	11.05 1.13	5.44 0.85	49.07 4.34	-8.73 1.56	8.60 D	JX911277	JX911308
<i>Cochlicopa lubrica</i>	(26)	14.95 2.72	8.08 1.97	55.43 6.64	-14.02 1.72	8.30 O	JX911261	JX911292
<i>Abida secale</i>	(14)	20.72 1.74	8.26 1.76	38.96 7.91	-14.36 2.46	9.02 O	JX911254	JX911285
<i>Candidula unifasciata</i>	(9)	31.70 13.09	16.27 7.52	50.18 6.42	-14.02 1.66	7.85 G	JX911266	JX911297
<i>Clausilia bidentata</i>	(17)	39.50 6.69	10.86 2.02	28.45 7.85	-16.14 1.14	11.0 O	JX911257	JX911288
<i>Discus rotundatus</i>	(29)	64.62 8.29	27.37 4.49	42.03 4.12	-14.16 1.63	9.06 D	JX911267	JX911298
<i>Trochulus hispidus</i>	(7)	69.88 15.55	43.21 11.03	49.77 10.33	-12.11 1.69	8.15 D	JX911279	JX911310
<i>Macrogastra attenuata</i>	(30)	78.99 8.55	30.14 5.42	35.82 6.4	-14.60 0.89	10.58 O	JX911274	JX911305
<i>Ciliella ciliata</i>	(11)	99.64 24.56	59.18 15.16	59.12 2.83	-6.84 1.40	8.12 D	JX911258	JX911289
<i>Cochlicella acuta</i>	(18)	102.98 14.61	49.62 13.85	47.42 8.13	-10.46 2.15	9.04 O	JX911263	JX911294
<i>Ena montana</i>	(29)	202.87 36.68	103.14 27.70	48.88 6.12	-8.37 2.26	8.38 O	JX911268	JX911299
<i>Pomatias elegans</i>	(29)	359.15 69.90	168.24 33.57	47.09 5.37	-5.53 0.66	7.48 G	AY01416161*	JX911283
<i>Oxychilus draparnaudi</i>	(10)	378.14 82.18	230.32 49.15	61.15 4.34	-5.21 0.92	8.85 D	JX911275	JX911306
<i>Hygromia limbata</i>	(8)	579.59 158.92	320.75 109.72	54.42 7.85	-6.98 1.35	7.69 G	JX911272	JX911303
<i>Zebrina detrita</i>	(22)	606.24 139.11	335.97 135.85	52.43 16.72	-10.55 1.92	8.28 O	JX911282	JX911313
<i>Helicigona lapicida</i>	(17)	651.42 80.22	306.19 61.57	47.05 7.46	-14.57 2.45	9.46 D	JX911270	JX911301
<i>Theba pisana</i>	(30)	998.23 185.44	628.73 156.30	62.39 7.39	-7.27 1.84	7.57 G	JX911280	JX911311
<i>Arianta arbustorum</i>	(15)	1666.33 435.94	867.73 297.65	51.08 8.35	-14.59 1.55	7.75 G	JX911253	JX911284
<i>Cepaea hortensis</i>	(30)	1796.15 267.01	1086.70 212.74	60.08 6.50	-8.44 2.26	7.60 G	JX911260	JX911291
<i>Cepaea sylvatica</i>	(12)	2055.50 324.93	1156.17 195.01	56.39 4.78	-11.88 2.26	7.71 G	JX911265	JX911296
<i>Cantareus apertus</i>	(5)	2819.40 235.77	1938.00 150.01	68.77 1.17	-5.72 0.54	7.79 G	JX911255	JX911286
<i>Cepaea nemoralis</i>	(20)	3052.85 689.98	1694.70 392.20	55.69 5.56	-10.21 1.79	7.71 G	JX911262	JX911293
<i>Eobania vermiculata</i>	(8)	4205.25 646.64	2251.25 338.07	53.73 4.62	-7.86 0.51	7.89 G	JX911269	JX911300
<i>Cornu aspersum</i>	(30)	4549.18 1374.59	2966.01 967.29	64.90 5.35	-5.42 1.97	7.63 G	JX911256	JX911287
<i>Helix pomatia</i>	(30)	18406.17 2605.40	11885.35 1824.61	64.28 3.59	-6.17 2.86	7.48 G	JX911273	JX911304
<i>Helix lucorum</i>	(13)	34069.78 4556.17	17546.44 3602.08	51.34 5.6	-4.45 2.07	7.81 G	JX911271	JX911302

**Table 3**

Matrix of phylogenetic correlations between explicative variables. The correlation coefficients  $r$  are indicated above diagonal,  $p$  values are indicated below it. Taking into account Bonferroni correction, significativity is considered for  $p < 0.0083$ . Df=29.

	<b>WC<sub>res</sub></b>	logWM	<b>SV<sub>corr</sub></b>	ENV
<b>WC<sub>res</sub></b>	-	0.0	<b>0.54</b>	0.0
LogWM	<i>NS</i>	-	0.0	0.36
<b>SV<sub>corr</sub></b>	<b>0.001</b>	<i>NS</i>	-	0.0
ENV	<i>NS</i>	<i>NS</i>	<i>NS</i>	-

**Table 4**

Best-supported models (total weight=93.7%) for predicting Tc in land snails (n = 31species). LL: log likelihood of the model, AICc: Akaike's Information Criterion corrected for the number of parameters in the model,  $\Delta$ AICc: difference in AICc between a model and the best-fitting one,  $\lambda$ : Pagel's maximum likelihood, S.E.: Standard Error of the estimated coefficient for each predictor.

	$r^2$ adj	LL	AICc	$\Delta$ AICc	$\lambda$	predictors	estim. coeff.	S.E.	$p$	weight (%)
<b>Tc~logWM + WC<sub>res</sub> + ENV</b>	0.599	-69.12	147.12	<b>0</b>	0.797	(intercept)	-12.48	2.16	<0.001	37.3
						<b>logWM</b>	2.37	0.52	<0.001	
						<b>WC<sub>res</sub></b>	0.19	0.06	<0.01	
<b>Tc~logWM + WC<sub>res</sub></b>	0.574	-70.43	147.29	<b>0.17</b>	0.839	(intercept)	-13.00	2.32	<0.001	34.3
						<b>logWM</b>	2.80	0.47	<0.001	
						<b>WC<sub>res</sub></b>	0.19	0.06	<0.01	
<b>Tc~logWM + WC<sub>res</sub> + SV<sub>corr</sub></b>	0.561	-70.31	149.52	<b>2.40</b>	0.844	(intercept)	-15.65	6.27	<0.05	11.3
						<b>logWM</b>	2.88	0.51	<0.001	
						<b>WC<sub>res</sub></b>	0.21	0.08	<0.05	
<b>Tc~logWM + WC<sub>res</sub> + SV<sub>corr</sub> + ENV</b>	0.585	-69.03	149.61	<b>2.49</b>	0.801	(intercept)	-14.59	6.13	<0.05	10.8
						<b>logWM</b>	2.44	0.55	<0.001	
						<b>WC<sub>res</sub></b>	0.21	0.08	<0.05	

**Figure 1**

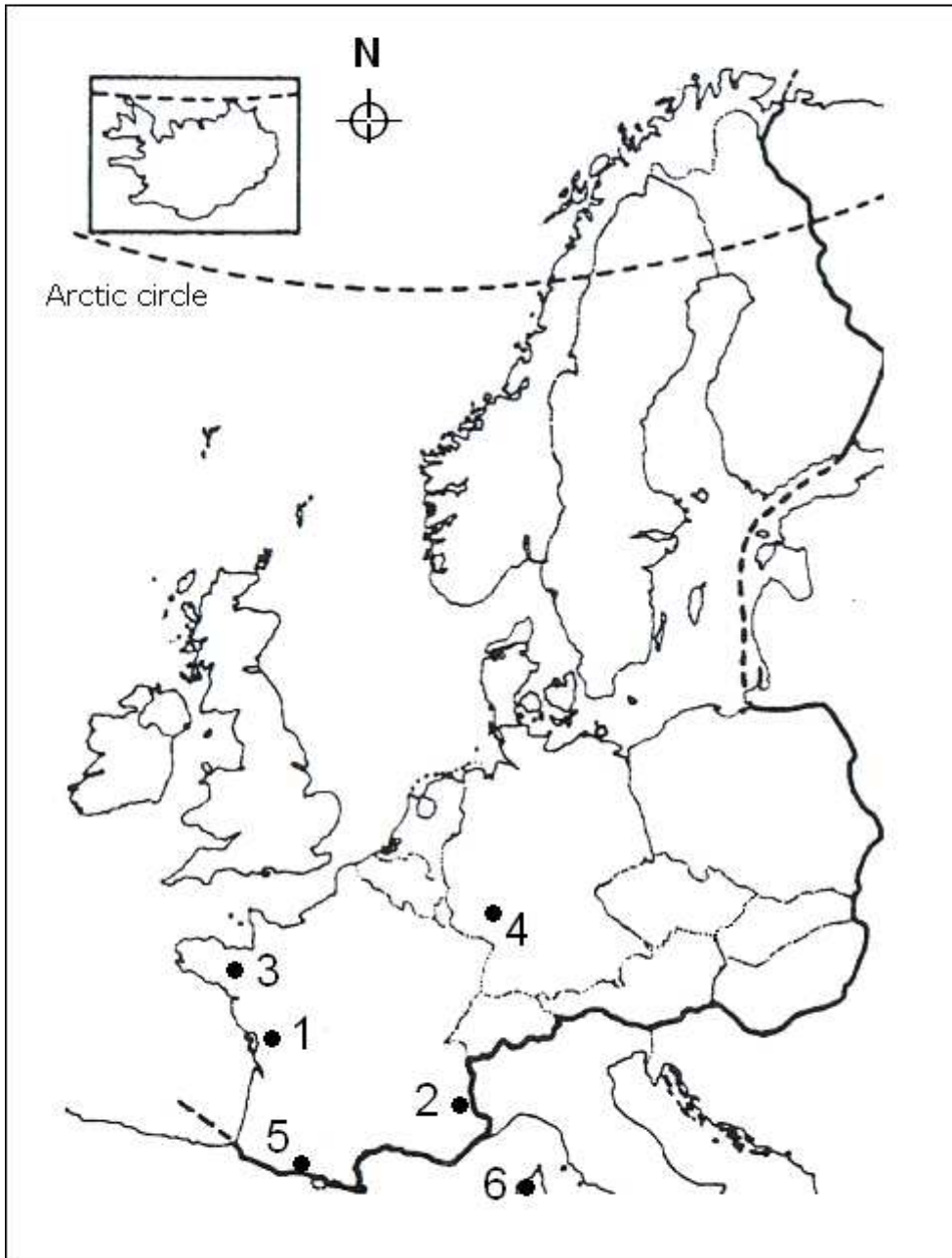


Figure 2

*Ansart, Guiller, Moine, Martin and Madec – Cold hardiness is size-constrained in land snails*

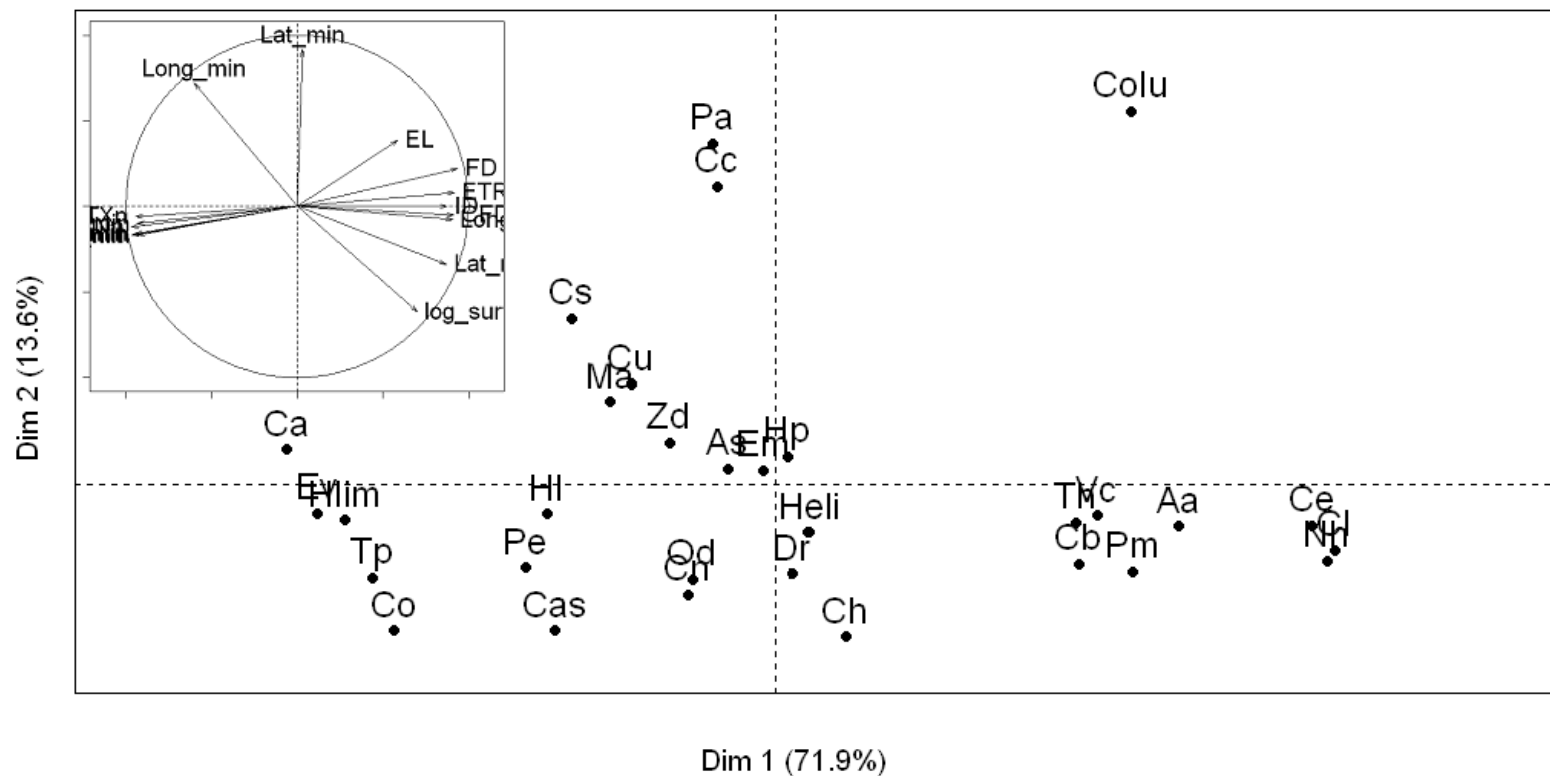


Figure 3

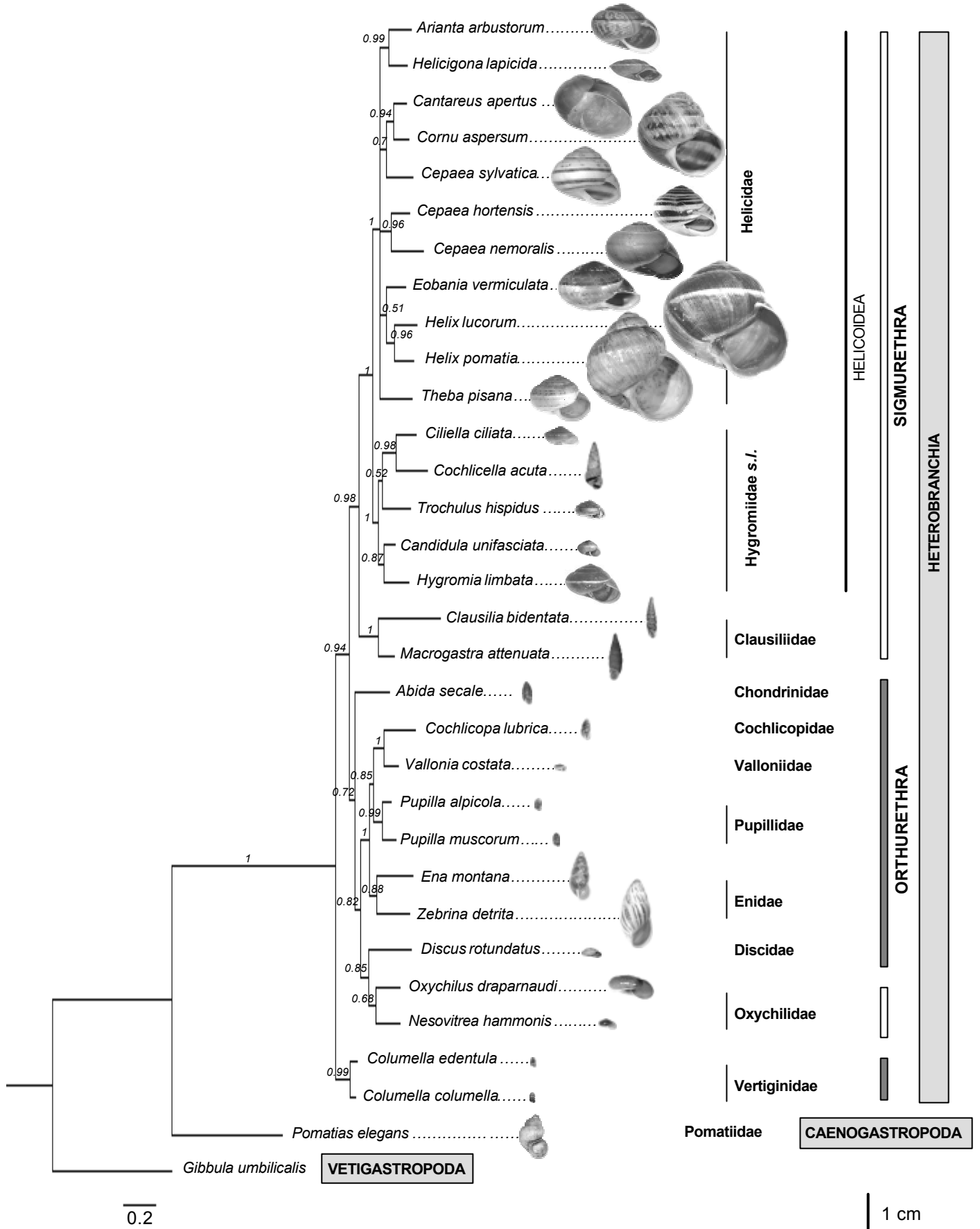


Figure 4

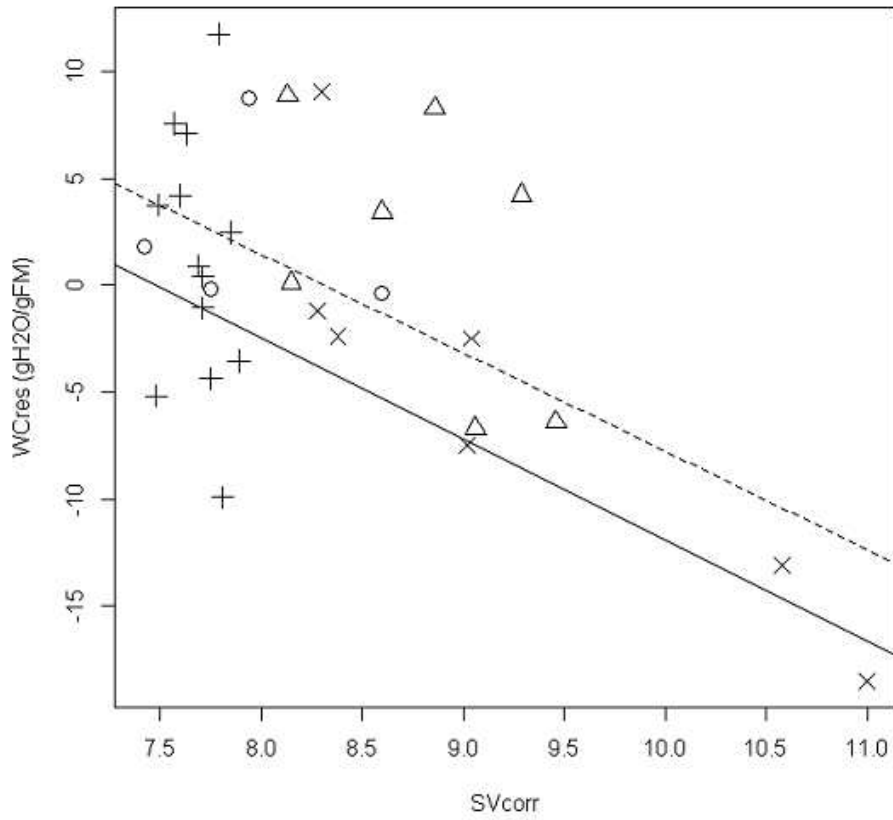


Figure 5

