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ORIGINAL PAPER

A. Ansart · P. Vernon · J. Daguzan

Elements of cold hardiness in a littoral population of the land snail *Helix aspersa* (Gastropoda: Pulmonata)

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Abstract The land snail *Helix aspersa* can be considered partially tolerant to freezing, in the sense it can survive some ice formation within its body for a limited time, and possesses a limited ability to supercool. This study aimed at understanding what factors are responsible for the variation of the temperature of crystallization (*Tc*) in a littoral temperate population. The ability to supercool was maximal (ca. −5 °C) during dormancy periods (hibernation and aestivation) and minimal (ca. –3 °C) during spring and autumn, in relation with the decrease of water mass and the increase of osmolality. *Tc* decreased in October to remain stable through late autumn and winter; it increased quickly with the awakening of animals in April. Snails with an epiphragm had a significantly higher ability to supercool (ca. −4.8 °C) than snails which did not form an epiphagm (ca. −4.2 °C). The animals' size had a weak but significant influence on the realization of the *Tc*. It appeared that there was not a real cold-hardiness strategy in this population; rather a sum of parameters, varying in consequences of the external conditions and of the activity cycle, which are responsible for the enhancement of the supercooling ability during winter.

Keywords *Helix aspersa* · Land snail · Cold hardiness · Temperature of crystallization · Haemolymph osmolality

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A. Ansart (✉) · J. Daguzan
Université de Rennes 1,
UMR 6553 Ecobio, Equipe Ecophysiologie,
Bâtiment14, 263 Avenue du Général Leclerc,
CS 74205, 35042 Rennes Cedex, France
E-mail: armelle.ansart@univ-rennes1.fr
Tel.: +33-2-23236865
Fax: +33-2-23235054

P. Vernon
Université de Rennes 1,
UMR 6553 Ecobio, Station biologique,
35380 Paimpont, France

Abbreviations *DM* dry mass · *INAs* ice-nucleating agents · *Tc* temperature of crystallization · *WC* water content · *WM* water mass

Introduction

During cold periods, ectotherms are confronted with the problem of subzero temperatures. Classically, two main strategies are described to survive such environmental conditions (see reviews, e.g. Sømme 1982; Zachariassen 1985; Duman et al. 1991; Block 1995; Storey and Storey 1997; Ramløv 2000). Freezing-tolerant animals are able to survive freezing of their body fluids below their temperature of crystallization (*Tc*), the temperature at which the body fluids spontaneously freeze. In contrast, freezing-intolerant species cannot bear ice formation and survive subzero temperatures by extending their supercooling ability, i.e. by decreasing their temperature of crystallization. Different mechanisms are implicated in both strategies, including synthesis of cryoprotectants of a colligative type such as sugars and polyols, or non-colligative antifreeze proteins, and ice-nucleating agents (INAs), which can be alimentary particles, microorganisms, or proteins and lipoproteins. Although of a useful and convenient classification, in nature, all intermediary strategies between freezing tolerance and intolerance are encountered (Bale 1993, 1996; Sinclair 1999).

Although the literature on insect cold hardiness is extensive, few works are concerned with molluscs, particularly land snails. The species, *Arianta arbustorum*, had high *Tc* (>–10 °C) and was found to survive supercooling and brief ice formation in certain tissues (Stöver 1973). The *Tc* of *Anguispira alternata* ranged between ca. –7 °C in summer and ca. –15 °C in winter; this snail did not survive short exposure to temperatures below the *Tc* (Riddle 1981). Riddle and Miller (1988), comparing the hibernating behaviour of this species with two others, *Discus cronkhitei* and *Gastrocopta armifera*, demonstrated these three species to be freezing intolerant, with an extensive supercooling ability. Schmid

(1988) studied a small species *Vallonia perspectiva*, which had a low *T*c (ca. –15 °C) and was also intolerant to freezing.

The land snail *Helix aspersa* hibernates in Brittany from November to March. It hides in stonewall crevices or digs in the soil, forms an epiphragm covering the shell aperture, and enters a state of dormancy. During dormancy, its oxygen consumption and water loss are diminished (Riddle 1983), the heart rate is lowered (Bailey and Lazaridou-Dimitriadou 1991), and metabolism decreases to 5–30% of normal levels (Herreid 1977; Barnhart and MacMahon 1987).

Previous work on the edible brown garden snail *H. aspersa* (Ansart et al. 2001a, 2001b) have shown that this animal possesses a limited ability to supercool, with a *T*c always above –10 °C. Although this species survives in its buffered environment mainly by a weak extension of its supercooling ability during winter, induced by decreasing photoperiods, to some extent it can also survive some ice formation in its body. Thus it may be considered a partially freezing-tolerant species, as defined by Sinclair (1999).

In this context, the cold hardiness strategy of snails from a littoral population was studied during a whole year, including the hibernation period, to determine how it varies as a function of *T*c, osmolality, time, age and epiphragm formation.

Materials and methods

Collection area and microhabitat conditions

Snails were collected at Le Conquet (Brittany, Western France), along an old stone wall, near the seashore. During summer, when air temperature is high or air humidity low, they enter a state of aestivation, remaining quiescent as long as the conditions are unfavourable for activity. Snails begin to hibernate approximately at the end of October, depending on the prevailing conditions, and awake in March–April. During winter, mean air temperature remains mild, ca. 8 °C, but it can drop to minimal values of –5 °C to –10 °C during the night (Meteo France). In the same area, Biannic (1995) registered the temperatures at soil level and in the overwintering site during the coldest winter day, using a thermocouple introduced in a stonewall crevice occupied by hibernating snails. Recordings were made every 3 h during 24 h. Temperatures attained –7.4 °C at soil level, and –2.4 °C in the crevice (Fig. 1). The rate of temperature change in the overwintering site was noticeably lower than at soil level, therefore the snails avoid extreme temperatures in their microhabitat, but have to survive temperatures below zero all day long.

Annual variation

During the year 1999–2000, 60 adult snails, distinguished by the existence of a reflected lip at the shell aperture, were collected each season: September (Autumn), December (Winter), April (Spring) and July (Summer). Half were used for measurement of *T*c and half for osmolality determination.

Winter variation

In parallel, during the winter (from November to March), the first 100 individuals found were collected each month, regardless of size

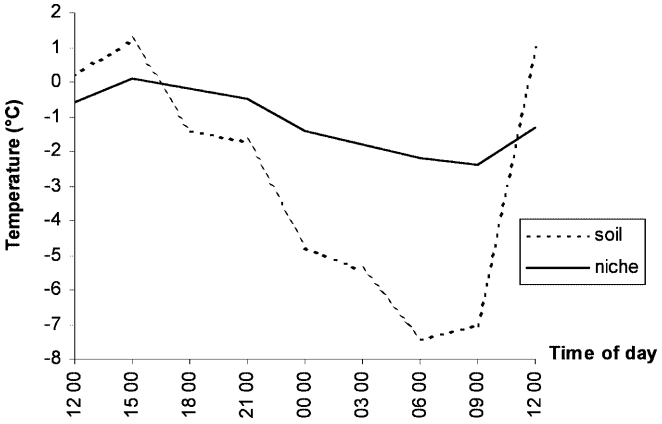

Fig. 1. Evolution of temperature at the soil level and in the overwintering niche during a cold night in winter in the study area (Biannic 1995)

(mature and immature) and activity state (epiphragmed or non-epiphragmed). Snails that possessed an epiphragm totally or partially covering the shell aperture were considered as hibernating; their *T*c was determined.

All snails (annual and winter variation) were sized with a calliper (0.1 mm) and weighed (0.001 g); their water content (WC), water mass (WM) and dry mass (DM) were determined gravimetrically.

Measurement of *T*c

Riddle (1981) measured the *T*c of the snail *Anguispira alternata* by inserting a thermocouple into the umbilicus and considered that it corresponded to a valid estimation of the body temperature. The umbilicus is not visible in *H. aspersa* so it was not possible to use this method. No exotherm could be recorded from thermocouples attached directly to the outside of the shell. Accordingly, a thermocouple (K-type) was inserted through a small hole (ca. 1 mm diameter), drilled in the shell, into contact with the tissues of the mantle of the pallial cavity (Schmid 1988; Biannic and Daguzan 1993; Biannic 1995). A rubber maintained the thermocouple and covered the hole, thus limiting the possible risks of inoculative freezing. This method generally did not injure the body tissues; when it did (haemolymph outflow), these snails were not used.

The thermocouple was connected to a thermometer, linked to a recorder, which could simultaneously measure seven temperatures. Snails were placed in a cryostat, in which the temperature was progressively lowered. The cooling rate was 0.5–1 °C/min as recommended by Salt (1961). Such a cooling rate is commonly used in cryobiology studies and is a basis for comparisons with other works about snails and, more generally, other invertebrate species. *T*c was recorded when the exothermic reaction due to ice formation appeared.

Haemolymph osmolality

Haemolymph was collected by heart puncture through a small hole in the shell; the liquid obtained was centrifuged at 10,000 *g* for 5 min to remove particulate matter (MacNabb 1985; Ramløv 1999). Samples were stored at –20 °C until osmolality was measured with a vapour pressure osmometer (5500 Wescor). Two measurements were taken per individual to obtain an average value.

WC, WM and dry mass

After experimentation, snail shells were removed rapidly; the shell and soft parts were placed for 48 h at 90 °C in a drying oven to

determine directly the DM and shell mass. WM corresponds to the difference: total fresh mass−(DM + shell mass). WC is expressed as the ratio: (WM/DM).

Results

Annual variation of *T*c

Despite a narrow annual range of *T*c and a relatively high inter-individual variation, an overall significant difference appears between the values obtained for the four seasons (ANOVA, $F_{(120;\ 3)} = 9.94$, $P < 0.0001$; Fig. 2). The *T*c is maximal and identical in spring (–2.7 ± 1.4 °C, $n = 30$) and autumn (–2.9 ± 1.7 °C, $n = 28$, Fisher test, $P = 0.722$). It is minimal and does not differ in aestivating (–4.6 ± 2.3 °C, $n = 36$) and hibernating (–4.8 ± 2.3 °C, $n = 30$) snails (Fisher test, $P = 0.582$).

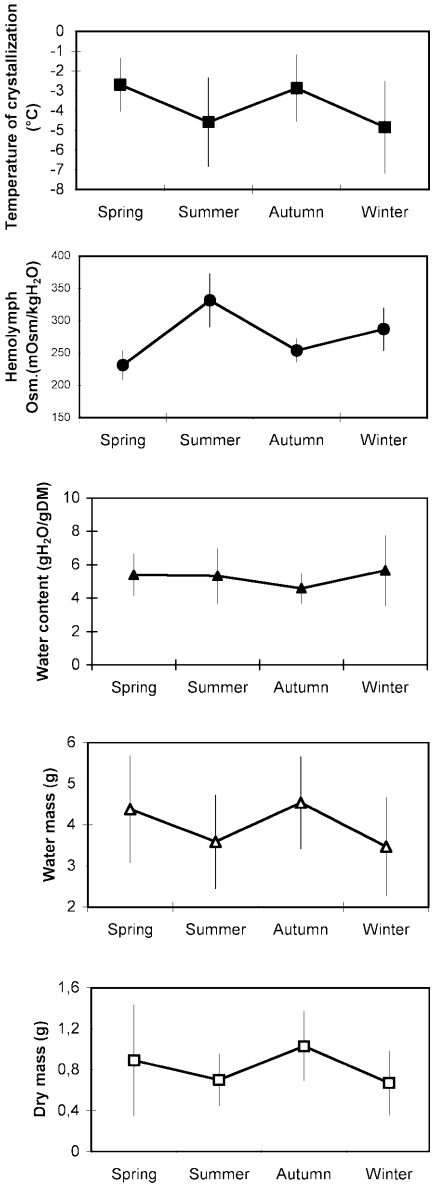

Fig. 2. Annual variation of different parameters in the adult land snail *Helix aspersa*. $n = 28$–36 for the temperature of crystallization (*T*c) and the haemolymph osmolality. $n = 53$–66 for water content (WC), water mass (WM) and dry mass (DM). *Vertical bars* indicate the SD

There is a low variability in WC, although a significant difference between groups is detected by ANOVA ($F_{(235;\ 3)} = 4.48$, $P = 0.004$). The value obtained in autumn (4.58 ± 0.88 g H_2O/g DM, $n = 53$) is significantly lower than the WC measured for the three other seasons, between which no difference is detected (spring: 5.39 ± 1.25 g H_2O/g DM, $n = 60$; summer: 5.33 ± 1.65 g H_2O/g DM, $n = 66$; winter: 5.58 ± 2.03 g H_2O/g DM, $n = 60$).

In contrast, the WM follows the same pattern as the *T*c (ANOVA, $F_{(235;\ 3)} = 12.53$, $P < 0.0001$), being higher during activity (4.38 ± 1.30 g, $n = 60$ in spring and 4.54 ± 1.12 g, $n = 53$ in autumn, Fisher test, $P = 0.489$) and lower during inactivity phases (3.59 ± 1.14 g, $n = 66$ in summer, and 3.44 ± 1.22 g, $n = 60$ in winter, Fisher test, $P = 0.507$). The *T*c is significantly correlated to the WM ($r = 0.34$, $n = 124$, $P = 0.0001$, Fig. 3), whereas it is independent of the WC ($r = 0.13$, $n = 124$, $P = 0.144$).

The DM also follows this variation pattern (ANOVA, $F_{(235;\ 3)} = 11.57$, $P < 0.0001$), showing that energy reserves are accumulated during activity (DM = 0.89 ± 0.54 g, $n = 60$, in spring and 1.03 ± 0.34 g, $n = 53$, in autumn, Fisher test, $P = 0.053$) and consumed during dormancy (DM = 0.70 ± 0.25 g, $n = 66$, in summer and 0.67 ± 0.31 g, $n = 60$, in winter, Fisher test, $P = 0.323$).

Haemolymph osmolality shows an annual evolution inverse to that of other factors, from 231.1 ± 23.1 mosmol/kg H_2O in spring ($n = 30$) to 331.5 ± 41.2 mosmol/kg H_2O in summer ($n = 30$) (ANOVA, $F_{(111;\ 3)} = 59.78$, $P < 0.0001$). It is significantly negatively correlated to the water mass ($r = 0.41$, $n = 115$, $P < 0.0001$, Fig. 4), indicating that osmolality is a function of the water mass, i.e. the solutes present in the haemolymph become more or less concentrated. There is no correlation between osmolality and WC ($r = 0.08$, $n = 115$, $P = 0.41$). However, during summer, osmolality (331.5 ± 41.2 mosmol/kg H_2O, $n = 30$) is significantly higher than during winter

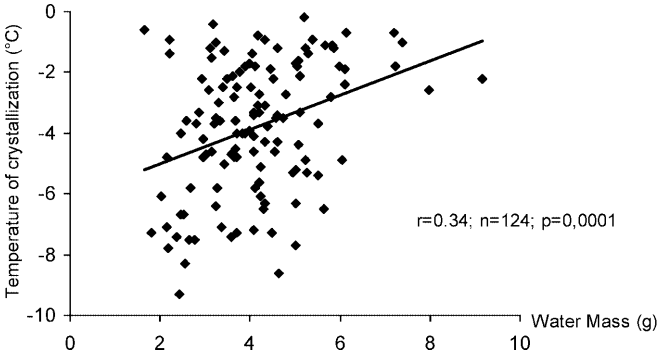

Fig. 3. Relationship between the *T*c and the WM in adult *H. aspersa*

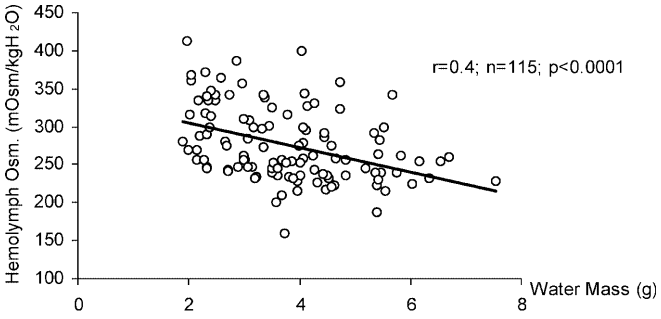

Fig. 4. Relationship between the haemolymph osmolality and WM in adult *H. aspersa*

(287.0 ± 33.0 mosmol/kgH_2O, $n = 30$; Fisher test, $P < 0.0001$), whereas WC, WM and *T*c are identical.

Variation of the *T*c from October to April

When the *T*c was measured every month from October to April (Fig. 5), a significant difference was found (ANOVA, $F_{(262;\ 6)} = 5.66$, $P < 0.0001$). At the beginning of October, before the onset of hibernation, the *T*c was –3.6 ± 1.6 °C ($n = 30$). It decreased to –4.7 ± 1.6 °C ($n = 40$) at the end of the month and then remained stable through late autumn and winter, with a minimum of –4.9 ± 2.3 °C ($n = 40$) in January and –4.9 ± 2.0 °C ($n = 35$) in February. It then increased to a maximal value of –2.9 ± 1.9 °C ($n = 30$) when snails awoke in April.

Influence of the epiphragm

During the course of winter, the percentage of epiphragmed snails increased from none in October to a maximum of 87% at the end of January. It then decreased to only 19% in late February and to 6% in April. If all values are considered, whatever the size of the snail or the month of measurement, snails with epiphragms had a *T*c significantly lower than those which are not epiphragmed (–4.8 ± 2.1 °C, $n = 115$, vs. –4.2 ± 1.9 °C, $n = 154$, *t*-test, $P < 0.02$). The WM of epiphragmed individuals (1.56 ± 1.44 g, $n = 115$) and non-epiphragmed snails (1.31 ± 1.39 g, $n = 154$) did not differ significantly (*t*-test, $P = 0.151$), as well as the WC (6.50 ± 1.50 g H_2O/g DM vs. 6.18 ± 1.48 g H_2O/g DM, respectively; *t*-test, $P = 0.088$). Nevertheless, this absence of difference is confounded by the fact that snails which form an epiphragm are significantly bigger than those which do not (19.0 ± 6.2 mm, $n = 115$, vs. 17.2 ± 5.9 mm, $n = 154$, *t*-test, $P = 0.016$).

Influence of body size

From October to April, immature snails have a *T*c significantly lower (–4.6 ± 1.9 °C, $n = 227$) than that of the adults (–3.8 ± 2.0 °C, $n = 52$, *t*-test, $P < 0.005$). If all values are considered (Fig. 6), there is an overall positive correlation between the supercooling ability and the individual size ($r = 0.21$, $n = 269$, $P < 0.001$).

Larger snails also have a higher WM ($r = 0.94$, $n = 269$, $P < 0.0001$) and WC ($r = 0.22$, $n = 269$, $P = 0.0003$), so the effect of size on the *T*c could be mainly triggered by the greater quantity of water in the body. Moreover, the correlation is significant but weak ($r = 0.24$, $n = 269$, $P < 0.0001$), between WM and *T*c, as well as between WC and *T*c ($r = 0.15$, $P = 0.018$, $n = 269$), the opposite of the result obtained with annual data.

Discussion

Annual variation

The supercooling ability of the studied population is weak, always above –10 °C, and with little annual variation. The *T*c is lowest during dormancy periods in summer and winter, and is highest during active periods, in spring and autumn. In the snail *Arianta arbustorum*, exhibiting *T*c in the same range, Stöver (1973) did not

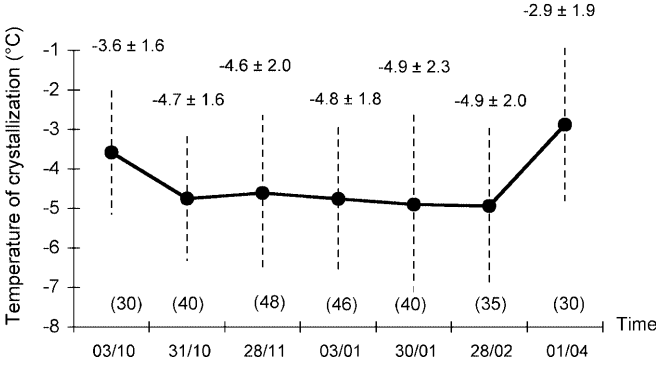

Fig. 5. Variation of the *T*c from entry into hibernation to awakening, in a population of *H.aspersa*, independently of individual size. Means ± SD are noted. *n* is indicated in *parentheses*

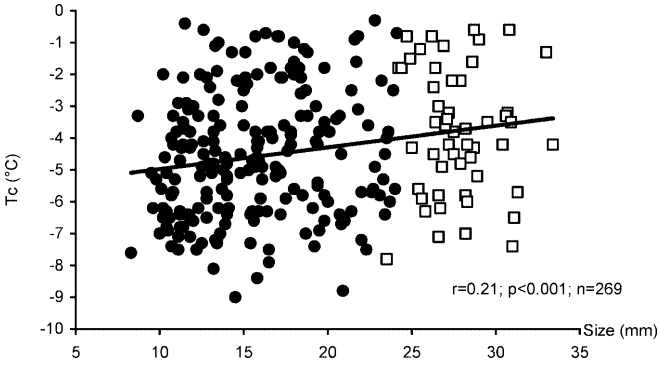

Fig. 6. Relationship between the *T*c and individual size. Adults are represented with *white squares* and juveniles with *black circles*. r^2, *P* and *n* are indicated on the graph

find any difference between the *T*c of winter and summer animals.

It has been previously shown for the same population (Ansart et al. 2001b) that low temperature acclimation has no effect on supercooling ability, whereas declining photoperiods, which have been shown to be responsible for the induction of hibernation (Jeppesen and Nygard 1976; Jeppesen 1977), trigger a decrease of the *T*c. The fact that summer *T*c is equivalent to winter *T*c has probably no adaptive value and is a collateral of the inactive state.

Hibernation and aestivation in land snails are not considered as equivalent processes (Herreid 1977; Bailey 1981; Iglesias et al. 1996); although the term dormancy is generally used to indicate both states, some authors consider hibernation close to a diapause, because it has an obligatory aspect, and aestivation as a simple quiescence (Abeloos 1965; Bailey 1981; Biannic 1995). However, this depends mostly on the species and region studied (Iglesias et al. 1996).

In the present study, *T*c, WC and WM are equivalent in summer and winter, and osmolality is significantly lower in winter than in summer. Other mechanisms must be implicated in the low winter *T*c. There can be a reinforced protection against inoculative freezing, or a diminution of the INAs in hibernating snails. It has been shown (Ansart et al. 2002) that starvation and antibiotics result in a better supercooling ability in adult *H. aspersa*. In hibernating snails of the study population, which are inactive for longer periods than those aestivating and which stop feeding several weeks before hibernation (Jeppesen and Nygard 1976), the quantity of INAs in the digestive tract may be less important and could explain the differences obtained between summer and winter animals.

Colligative cryoprotectants (sugars, polyols) are not implicated in the decrease of *T*c during winter. Synthesis of these low molecular weight cryoprotectants, common in insect cold-hardiness strategy, triggers an increase in the haemolymph osmolality in addition to that due to WC decrease (Zachariassen 1985). In our study, the haemolymph osmolality is correlated with WM: the enhancement measured during winter is only due to dehydration. Moreover, preliminary results (A. Ansart, unpublished data) have shown that no enhancement of the osmolality after the onset of freezing can be detected, contrary to some earthworms and frogs, which synthesize cryoprotectants only once ice begins to form in the body (Holmstrup et al. 1999; Storey and Storey 1985). In molluscs, it seems that this type of cryoprotectant is rarely used. In the existing studies, sugars or polyols have not been detected in hibernating or cold-acclimated individuals (Kanwisher 1966; Riddle 1981; Biannic 1995). Moreover, studies on energetic consumption during hibernation in land snails showed that glucose is the main fuel used and that its concentration in haemolymph is largely decreased during dormancy periods: ca. 27 mg % during active periods, 11 mg % in summer and 8 mg % in winter (Goddard and Martin 1966). Glycerol was found to be correlated with freezing tolerance in the intertidal mollusc *Melampus bidentatus*, however, its concentration was much lower than that found generally in insects, and a colligative action was unlikely (Loomis 1985).

Variation from October to April

The *T*c of the study population decreased when winter conditions drew near, were lowest during the coldest months and then increased to a maximal value in spring, when the inactive period was terminated. This pattern of supercooling ability, even if weak, is typical of animals intolerant to freezing, which extend their supercooling ability during winter to avoid ice formation in their body fluids (e.g. Block 1995). Nevertheless, it has been previously shown (Ansart et al. 2001a) that *H. aspersa* can be considered a partially freezing-tolerant species (see Sinclair 1999), i.e. it can survive some ice formation in its body for a limited time (at –5 °C: 73–90% survival for 4 h, 0–33% for 8 h; at –10 °C: 53–73% after 4 h, 0% after 8 h; Ansart 2001; Ansart et al. 2001a) but dies when the ice formation process has attained a lethal level (between 40% and 60% of the body water turned into ice, Ansart et al. 2001a).

H. aspersa seems to survive cold temperature during winter, mainly by extension of its supercooling ability, but when the *T*c is reached, it can survive some supplementary hours in the partially frozen state. Long-term experiments (Ansart 2001) have shown that young and adult snails exposed to a freezing event (90–120 min at –10 °C) during hibernation are able to grow and reproduce normally after awakening. Thus, this tolerance to partial freezing can have an adaptive value for survival.

The presence of the epiphragm (partial or total) indicates the hibernating state (Iglesias et al. 1996). Epiphragmed snails are bigger and have a lower *T*c than that of non-epiphragmed individuals for similar WC and WM. Previous work by Biannic (1995) pointed to a differential ability to hibernation between young and adult snails; according to the author, below a certain size (19 mm), snails were not able to hibernate. However, we found very young snails (< 10 mm) with an epiphragm. The WM and WC being size-dependent, epiphragmed snails are proportionally less hydrated, which contributes to the enhancement of their supercooling ability.

Moreover, the epiphragm itself constitutes a physical barrier to inoculative freezing (Riddle 1983). Inoculative freezing is triggered at temperatures above the normal *T*c by contact with ice crystals or organic detritus present in the immediate environment of the animal. It can be an important cause of winter mortality in certain species, like some acari (Burks et al. 1996) or the beetle *Leptinotarsa decemlineata* (Costanzo et al. 1997). In *H. aspersa*, the presence of the epiphragm added to the shell, and the fact that snails are often strongly attached to the rocky substrate, assures that supercooling is effi-

cient, and that they will effectively freeze at the *T*c, not above; active snails, however, because of their slimy nature, are particularly sensitive to inoculative freezing.

Size is an important factor in the determination of the individual *T*c: smaller snails have a higher *T*c. Lee and Costanzo (1998) have pointed to the relationship between *T*c and size at the inter-specific level, suggesting that very small animals (arachnids and insects) possess an extensive supercooling ability, whereas bigger animals like reptiles and amphibians are mostly freezing tolerant with high *T*c, probably as a function of their larger water volume. Species with greater water volume have a higher probability of the spontaneous formation of an ice embryo in their body and a higher probability of possessing particularly active INAs. In our study, this relationship also seems to exist at the intra-specific level.

Ontogenic differences can appear in the development of the cold hardiness strategy. David and Vannier (1996, 1997) found in different millipede species a size-dependent *T*c, with smaller animals exhibiting a higher supercooling ability. Moreover, smaller animals cool more quickly and thus, as lethal ice quantity is more rapidly attained, are usually less tolerant to exposure to low temperature (Loomis 1991). Thus, a more extended supercooling ability of young snails can improve their survival during winter, as their resistance to freezing will be less important than that of adults.

WC is commonly used to express the variation of the hydric compartment. However, it is by definition also dependent on DM. In *H. aspersa*, which alternates between periods of activity and periods of dormancy, the DM is mostly variable and these changes can in some way mask the variations of water quantity. For example, in the present study, WC is minimal in autumn, when animals feed and stock reserves for future hibernation, whereas water volume is maximal. The correlation between WM and size is very strong ($r = 0.94$). Moreover, WM is well correlated with the *T*c for both series of measurements ($r = 0.34$ and $r = 0.24$), whereas correlation is weakly significant for WC in one case ($r = 0.15$, $P = 0.02$, hibernation variation) and not in the other. In the study of cold hardiness, WM may be a better indicator for this species, for which the DM is highly variable, than WC. This is understandable because, as mentioned above, the probability of crystallization is enhanced with a bigger water volume (Lee and Costanzo 1998).

The *T*c is an important descriptor in the cold hardiness strategy of an animal. However, it is not sufficient, and the temperature at which the animal will die is also important. Lethal temperature can be above, at, or below the Tc. *H. aspersa* did not show any prefreeze mortality and it can survive for a few hours below its *T*c, before lethal ice content is reached, depending on exposure temperature. *T*c therefore seems to be a good indicator of when *H. aspersa* dies, i.e. few hours after the onset of ice formation.

This study describes the variations of several parameters playing a role in the cold-hardiness strategy of a population. It seems that there is not a real overwintering strategy to face subzero temperatures in this population. Rather, there is a sum of parameters varying in consequences of the external conditions and of the activity cycle, which are responsible for a slight enhancement of the supercooling ability during winter, probably improving the survival of individuals. Behavioural adaptation (choice of a buffered hibernation site), concentration of haemolymph solutes due to the diminution of the hydric compartment, prevention of inoculative freezing by the shell and the epiphragm and diminution of the INAs quantity by starvation all contribute to the improved supercooling ability in winter.

The study population is not exposed to severe cold stress, however, *H. aspersa* is also found in cold places such as mountain environments. We can wonder which of these adaptations will be reinforced in populations inhabiting colder environments. Will they improve their behaviour to avoid cold, extend their ability to supercool, or enhance their tolerance to freezing, and by which means?

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