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Supercooling ability is surprisingly invariable in eggs of the land snail *Cantareus aspersus* ☆

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Abstract

From an ontogenetic point of view, invertebrate eggs are generally the most freezing intolerant stage of a species. Development state, water content and acclimation may affect their supercooling ability. In this study, we measured fresh mass, water content and temperature of crystallisation (T_c) of eggs of the edible land snail *Cantareus aspersus*, depending on its form (“*aspersa*” *vs.* “*maxima*”), incubation temperature (20, 12 and 7 °C) and physiological age (as part of the complete development). We also tested their tolerance to freezing. Despite a high number of individual observations ($n = 759$) and significant differences of fresh mass and water content between both subspecies, no effect of origin, incubation temperature or development state has been found in this study. T_c remained constant whatever the condition, with an overall mean of -5.40 ± 0.24 °C (mean ± SD). We suggest that fresh mass is important, a high water content and a constantly wet surface confer to land snail eggs a poor ability to supercool. Moreover, the presence of ice nucleating agents at the egg surface (microorganisms present in the soil, calcium carbonate crystals of the egg shell) might induce freezing. Thus, considering the present results, to delay hatching by cryopreservation of eggs does not seem possible.

Keywords: Land snail; Egg; Cold hardiness; Temperature of crystallisation; Water content; Ice nucleating agent

Invertebrate eggs are generally small, subspherical, smooth structures containing a high concentration of organic compounds such as sugars, and are exempt of nucleating agents inducing the formation of ice crystals [1,21,22]. For these reasons, invertebrate eggs frequently exhibit a stronger ability to supercool than pupae, larvae or adults [3,4,6,33,34] but are highly susceptible to freezing. Insects which over winter as eggs can supercool to temperatures as low as −30 to −50 °C [30,37], while eggs of species emerging before winter also exhibit high supercooling abilities, from −25 to −40 °C. So far, no freezing tolerant eggs of insects or other terrestrial arthropods have been found, however many insects overwinter in the egg stage, attaining cold hardiness by diapause or acclimation at prechilling temperature [35].

Although Carrillo and Cannon [3] found no variation of temperature of crystallisation (T_c) in 1- and 4-day-old eggs of the Indian meal moth *Plodia interpunctella* (Lepidoptera: Pyralidae), Vernon et al. [34] demonstrated a variation in supercooling ability of the eggs of *Osmoderma eremita* (Coleoptera: Cetoniidae) related to embryogenesis and associated with an important gain of water. In the same way, Jing and Kang [19] found that in the locust *Locusta migratoria* (Orthoptera: Acrididae), the egg supercooling point increased gradually from freshly laid egg to old egg; eggs prior to hatching always had a much higher supercooling point. They also pointed to differences among populations, northern populations being more cold-hardy than southern ones, and to the positive effect of acclimation to low temperature on cold hardiness. Geographical

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differences also exist in cold hardiness of eggs of the yellow spotted longicorn *Psacothea hilaris* (Coleoptera: Cerambycidae) [29]. Strathdee et al. [31] demonstrated an effect of seasonal changes and acclimation on supercooling ability of aphid eggs. Cryprotectants such as polyols [30,35] and sugars [2] have been shown to play important roles in eggs ability to withstand low temperature stress. In the Arctic stonefly *Arcynopteryx compacta* (Plecoptera: Perlodidae), extensive water loss has been shown to promote supercooling and survival of eggs enclosed into ice [12].

Although terrestrial molluscs can be found in cold environments [10,11], the cold tolerance of their eggs has received even less attention from cryobiologists than eggs of terrestrial insects.

The land snail *Cantareus aspersus* (formerly *Helix aspersa*) is widespread in Western Europe (from northern Africa to northern England), which has been colonized probably during the Holocene by the anthropochorous form *C. aspersus* subsp. "*aspersa*". A strong morphological polymorphism can be observed in North Africa, where its spatial structure has led to the recognition of several forms [15]. One of these forms, namely the giant *C. aspersus* subsp. "*maxima*", and *Ca* "*aspersa*" are reared in Europe for economic (*i.e.* gastronomic) purposes.

These hermaphroditic snails reproduce with reciprocal fecundation; a few days after coupling, they lay between 100 and 200 eggs, digging a little hole in the soil. The eggs form a mass protected from desiccation by mucus. They need almost two weeks at 20 °C to hatch. The embryo and albumen are enveloped into a calcified shell, kept humid by external mucus [20]. Galactogen is the main carbohydrate resource of terrestrial snail eggs, reaching 36% of their dry weight. During embryogenesis, 46% to 78% of this reserve is depleted [13].

To plan their production, snails' farmers would desire to stop embryo development and stock eggs at low temperature. However, no data are available on cold hardiness in terrestrial gastropod eggs. In this study, we investigate the effect of three factors, origin, incubation temperature and development time on supercooling ability of *C. aspersus* eggs.

Material and methods

Adults

Ca "*aspersa*" and *Ca* "*maxima*" adults used in this experiment originated from the same farm (L'Escargotier, Corps-Nuds, France). As indicated by its name, the "*maxima*" form is larger (Table 1); it also possesses a dark mantle edge, whereas this is clear in *Ca* "*aspersa*".

Snails were placed under controlled conditions defined as optimal for breeding activity of *C. aspersus*: Light–Darkness (L–D)16 h:8 h, 20 °C and 80% rH [9]. For each subspecies, four cages with 10 individuals were prepared. The cage bottom was covered with a layer of moist synthetic foam. Animals were fed *ad libitum* with industrial powder (Aliments Berton, Le Boupère, France), and two laying jars filled with commercial sterilized compost were placed in each box.

Table 1
Some life history features of both *Cantareus aspersus* forms (after [26])

	Ca "*aspersa*"	*Ca* "*maxima*"
Adult shell diameter (mm)	25–35	40–45
Adult fresh mass (g)	10–15	25–40
Life span (year)	3–4	10–12

Each day, the laying jars were inspected for clutches of eggs and dead snails removed. Boxes were washed and food replaced once a week.

Clutches

For each cage, the first clutch containing more than 160 eggs was preserved, assuring a different parental origin for each one and avoiding individual effect. Four clutches from *Ca* "*aspersa*" and four from *Ca* "*maxima*" were placed in Petri dishes on wet absorbing paper. Each clutch was divided into three parts, the first was held at 20 °C, the second at 12 °C and the third at 7 °C after two days at 12 °C to avoid a thermal shock.

Just after egg-laying (Week 0), 10 eggs were used for estimating fresh mass, temperature of crystallisation (T_c) and dry mass, and 5 were used for freezing survival assay.

These measurements were also performed at 20 °C on week 1, and on weeks 1, 2, 3, 4 at 12 and 7 °C (Table 2).

Subsequent clutches were divided equally between the three incubation temperatures. Each one was assessed for number of eggs and time to hatching.

Temperature of crystallisation (T_c) and mass data

The fresh mass (FM) of eggs was measured with a microbalance (Sartorius CP 224 S, ±0.1 mg) before determination

Table 2
Physiological age of the snails according to the experimental design

Origin	Incubation temperature (°C)	Number of eggs/clutch	Time after laying	Physiological age*
Ca "*aspersa*" and *Ca* "*maxima*"	20	30	*W*0	0.08
			*W*1	0.64
	12	60	*W*1	0.27
			*W*2	0.51
			*W*3	0.75
			*W*4	0.99
	7	60	*W*1	0.05
			*W*2	0.09
			*W*3	0.13
			*W*4	0.17

W, week, *W*0, 1 day after laying. Four clutches were used for each geographical origin and equally separated between incubation temperature conditions.

* As part of full development.