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Constraints on habitat possibilities: overwintering of a micro snail species facing climate change consequences in a harsh environment

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Original article

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The aim of this study was to establish the overwintering strategy and cold tolerance in *V. moulinsiana*. We observed a seasonal change in the SCP measurements, indicating that the species' preparation for winter results in a decrease in the SCP. Along with the results obtained from the frost survival experiment, this suggests the presence of a freezing avoidance strategy in this species. It overwinters in a buffered zone, separated from harsh conditions by a layer of leaf litter and snow, which protects it from temperature fluctuations and severe frost. The results of our research may indicate a possible poor resistance of these snails to snow-free conditions. Therefore, climate change-induced alterations could profoundly impact this species, especially considering the predicted disappearance of snow before the frost.

Key words: frost survival, freeze avoidant strategy, *Vertigo moulinsiana*, supercooling point.

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The body temperatures of ectotherms, such as molluscs, are similar to the microclimate temperature, and changes in the ambient temperature can dramatically affect their physiology. At sub-zero temperatures, when ectotherms are at risk of their body fluids freezing, their ability to survive such conditions is referred to as cold hardiness. Cold hardiness is commonly thought to be achieved by two alternative strategies: freeze avoidance and freeze tolerance (Salt 1961; Zachariassen 1985; Block 1990; Lee 1991), although mixed strategies also occur (Sinclair *et al.* 2015). In all three strategies, supercooling, i.e. the ability of the organism to maintain their body fluids in a liquid state below their freezing point, is critical. Freeze tolerant organisms will generally have a poor ability to supercool between -5°C and -10°C, which means that their tissues will freeze slowly and that there will be sufficient time for protection mechanisms to be invoked (e.g. the production of cryoprotectant substances that allow for the controlled propagation of ice within the body; Storey & Storey 1988). By contrast, freeze avoidant organisms, in which the ice formation in tissues is lethal, have an enhanced supercooling ability, often working in association with the synthesis of large amounts of antifreeze substances (Zachariassen 1985; Franks 2003; Ansart *et al.* 2014) or by adopting other freeze-avoidance tactics (e.g. cryoprotective dehydration; Holmstrup *et al.* 2002). Both alternatives – freeze avoidance and freeze tolerance – exist in molluscs, but land snails are generally freeze intolerant, as the freezing of their body fluids is lethal to these organisms (Ansart *et al*. 2001; Ansart & Vernon 2003; Ansart *et al.* 2014).

When exposed to frost, terrestrial molluscs often actively search for suitable shelters and relocate to unfrozen, protected habitats, which allows them to maintain high levels of humidity and avoid extreme temperatures (Ansart & Vernon 2003). Most land snails find favourable overwintering conditions by burying themselves in the soil (Pollard 1975; Attia 2004; Szybiak *et al.* 2004), which provides them with a buffer against temperature and moisture fluctuations. However, species that do not burrow can find shelter beneath a layer of leaf litter, remnants of vegetation or a layer of snow (Killeen 2003; Książkiewicz-Parulska *et al.* 2018; Lipińska *et al.* 2020). The importance of snow cover in the overwintering of land snails may become an issue when facing the consequences of climate change. Although temperatures are forecasted to rise, winter snow cover is expected to diminish before that time (Zhu *et al.* 2019), resulting in functionally colder winters for the species that rely on snow cover for overwintering. Therefore, it is crucial to understand their overwintering strategies, in order to predict the impact of snow disappearance on these organisms before the frost ceases to appear.

The aim of our study was to investigate the response of a representative species of terrestrial micro-snail to low temperatures. Desmoulin's whorl snail, *Vertigo moulinsiana*, is found exclusively in open wetlands where it experiences significant temperature fluctuations, both on a daily and a seasonal basis (Lipińska *et al.* 2020). However, the species demonstrates relatively high winter survival rates, ranging between 60 and 65% of individuals (Lipińska *et al*. 2020). During the winter, the species overwinters above ground, protected by a layer of leaf litter and snow cover.

We have established the overwintering and cold tolerance strategies in this species through the following: (1) investigating the severity of the frost exposure for individuals collected in winter, summer and autumn; (2) determining the supercooling points (SCPs) twice a year, in winter and summer; and (3) describing the overwintering microhabitat of this species. Due to the rarity of the species and its high sensitivity to habitat changes (Killeen 2003), the significance of the research topic is further amplified.

Materials and Methods

The species

Desmoulin's whorl snail *Vertigo moulinsiana* (Dupuy 1849) is a terrestrial micro snail (shell length ca 3 mm), identified as vulnerable throughout Europe (Killeen *et al.* 2012) and listed in Appendix II of the EU Habitats Directive. The biology of the species has been described by Pokryszko (1987; 1990) and Myzyk (2011). Individual snails have a mean life span of up to 15 months (Killeen 2003), and a typical population consists of 3 overlapping generations. The life history traits of the species were presented by Myzyk (2011): the adult mortality rate between consecutive months is from 10 to 15%. Each adult individual lays an average of 19 eggs during the laying season. The laying starts in May and ends in August, while the eggs hatch from June to September. On average, the young snails require 99 days from hatching to reach maturity: most do so in the following season, although 10-15% mature in the season of hatching, usually once the breeding period has ended. *Vertigo moulinsiana* inhabits open wetlands with high water and soil calcium levels (Killeen 2003; Nicolai et al. 2005; Lipińska *et al.* 2011). Within its habitat, the species may be found both in the plant litter and on plants (Lipińska *et al.* 2020). *Vertigo moulinsiana* snails live over a vertical range and can be found high up on the vegetation at certain times of the year (Książkiewicz-Parulska 2019). Significantly higher numbers of snails are present in the litter in spring, but in summer they occur more numerously on the plants, depending on the site (Jankowiak & Bernard 2013; Książkiewicz-Parulska 2019). Apart from seasonal differences, the heights at which the snails occur most likely depend on the local habitat and microclimatic conditions. With the onset of winter, *V. moulinsiana* descends to the ground or overwinters on sedge tussocks (Pokryszko 1990; Killeen 2003). As was already described, the adults of *V. moulinsiana* usually overwinter on plants, whereas the young snails do so in the litter (Książkiewicz-Parulska & Pawlak 2016; Książkiewicz-Parulska 2017; Książkiewicz-Parulska & Pawlak 2017; Książkiewicz-Parulska *et al.* 2018). However, in our study area there are no plants in the winter, only dried leaves and the stems of last year's vegetation.

Study area

The study area (6.1 ha) was located at the *V. moulinsiana* site, in the so-called Inland Delta of the River Nida (50°34′30″ N, 20°31′27″ E; for a more detailed description, see Lipińska *et al.* 2020). This area consists of a dense network of narrow and shallow (up to 1.5 m deep) erosion channels and old riverbeds, inhabited by communities of macrophytes and submerged communities. The snails were collected in moist areas with extensive patches of *Carex elata*.

Overwintering strategy in *V. moulinsiana* – SCP measurements

The measurements were performed in accordance with the recommended methodology (Sinclair *et al.* 2015). The supercooling point is usually measured on 20-30 individuals, which provides a robust sample size from which to assess the shape of the SCP distribution (Sinclair *et al.* 2015). The SCP was measured twice a year, according to the method used in previous work on land snails (Ansart *et al.* 2002). To obtain the SCP of the snail body fluids, 59 individuals (30 adults in June 2019; and 16 young and 13 adults in February 2020) were frozen. All the specimens used in the experiment were kept in a 5°C temperature for about 3 h, to prevent them from temperature shock and to check if they were alive (active during the experiment). After that, a thermocouple (K-type, probe diameter 0.5 mm) was inserted into each individual through the shell entrance. The snails were then placed in a freezer (Platilab 340 SV-3-STD; ALS Angelantoni Life Science deep freezer), the temperature of which was set to fall from +5°C to -20°C at a constant rate of 1°C/min, as was recommended in previous works involving SCP measurements (Salt 1961; Ansart *et al.* 2002; Sinclair *et al.* 2015). The temperature decrease was continuously recorded graphically, and the SCP was read off (± 0.1°C accuracy) from the falling temperature curve as the upward peak (rebound) due to the heat of crystallisation. After the rebound, the snails were warmed to room temperature to determine whether they had survived the measurement (according to the procedure for evaluating the cold tolerance strategy described by Sinclair *et al.* 2015). The temperature was raised at a rate of 1°C/min to prevent the heat wave effect and desiccation of the snails as a result of the sudden warming.

Overwintering strategy in *V. moulinsiana* – cold survival experiment

The snails were collected in the field on three occasions (June 2019, October 2019 and February 2020). The experiments were conducted on the same day as the snails were collected, or on the day after. The snails were stored and transported to the laboratory in plastic containers, together with moist litter collected at the same sampling site, at a temperature similar to the one measured in the field. Most of the snails collected in June were adults (98%); whereas in October and February, all the individuals collected were juveniles (100%).

Each of the June, October and February experiments was conducted in two trials: one of a 6 h duration and the other of a 24 h duration. The trials were conducted within a set range of temperatures: 20°C, 5°C, 0°C, -5°C, -10°C and -15°C, using climatic chambers (KK STD FIT), a refrigerator (CHL 3 PREM) and a laboratory freezer (ZLN 85 PREM; all manufactured by POL-EKO APARATURA). Thirty snails were exposed to each temperature (a total of 360 were used during each experiment). During the experiments, the snails were kept in cylindrical plastic containers with screw caps (6 containers per chamber, with 5 snails in each), in the dark, and with no litter or moisture added. At the beginning of each experiment, the chambers were switched on after the snails had been placed inside, with the temperature decreasing at a rate of ca 1°C/min (corresponding to the temperature drop in the SCP experiment). Following the experiment, the snails were wetted and warmed to room temperature at a rate of 1°C/min. A total of 1080 individuals were collected and used in the experiment.

Overwintering microhabitats

The study plot (20 x 20 m), straddling an area of stagnant water that supported mostly clumps of *Carex elata* (a total of 1305), was established. The study plot was surveyed in November 2008, the last month before the snow/ice cover appeared; and in May 2009, the first month after the snow/ice had melted. During each monthly survey, the whole of the study plot was meticulously searched for *V. moulinsiana* individuals: each sedge tussock was examined for 1 min (the method is described in detail in Lipińska & Ćmiel 2016; Lipińska *et al.* 2020). The number of individuals and the heights of their occurrence (above the ground) were recorded.

Microclimatic conditions at the wintering site

Miniature temperature and humidity data loggers (iButton DS1923-F5) were attached to two wooden frames installed in clumps of sedge in which the *V. moulinsiana* snails were found to be overwintering. The sensors were mounted vertically every 10 cm, at heights from 10 to 140 cm above the ground. Since the data was read off the sensors just once a month, only the data from those sensors operating continuously throughout the month was taken into account; while data from damaged sensors was not included in the database. The data was collected in November, December 2008 and January 2009. We chose to present the winter conditions of 2008/2009, as they represent the average winter conditions recorded since 1960. The mean number of days with snow cover between 1960 and 2010 was 46.6; while in the winter of 2008/2009, there were 43 days with snow cover (Czarnecka 2012).

Statistical analysis

The statistical analysis was performed using Dell Statistica 13.0 software.

A full factorial Generalized Linear Model (GLZ; logit, binomial) was constructed to test the influence of the temperature, trial duration and time of the snail collection on the snail survival rate. The GLZ scheme was as follows: snail dead/live (a binomial dependent variable); and categorical factors: trial duration, temperature, time (month) of collection, and the interactions between these factors (trial duration*temperature, trial duration*time of collection, temperature*time of collection and trial duration*temperature*time of collection).

To test the differences in the mean SCP between juvenile and adult snails and between seasons, a nested GLM was constructed (dependent variable: SCP; categorical variables: season, age class nested in a season). P-values for the differences between the age classes nested in the seasons were calculated using the Unequal N Tukey's HSD post-hoc test (sometimes referred to as the Tukey-Kramer Method).

The differences in the mean temperature between months and between the height above the ground in both the zone of snail occurrence (below 60 cm above the ground) and the zone of snail absence (above 60 cm above the ground) was tested using a full factorial ANOVA (dependent variable: temperature; categorical variables: month, height above the ground; with the interaction between the categorical factors: month*height above the ground). Because the temperature was measured every 10 cm, the variable height above the ground was treated as a categorical variable rather than a continuous variable.

Results

Overwintering in *V. moulinsiana* – SCP measurements

The basic statistics for the SCP in the *V. moulinsiana* snails collected in June 2019 and February 2020 are presented in Table 1. The differences in the SCP between seasons were significant (nested GLM; df=1; F=27.0; p<0.0001), but the difference between the age classes was not (nested GLM; df=1; F=0.22; p=0.6392). However, the post-hoc test highlighted significant differences in the SCP between the adult snails collected in summer, and both the adult and juvenile snails collected in winter (Unequal N Tukey's HSD; p=0.0058 and p=0.0005, respectively; Fig. 1A). None of the *V. moulinsiana* snails survived the SCP measurements.

Overwintering in *V. moulinsiana* – cold survival experiment

The full factorial GLZ (logit, binomial) showed that the influence of trial duration, and all the tested interactions (trial duration*temperature, trial duration*time of collection, temperature*time of collection and trial duration*temperature*time of collection) were not significant, but that both the temperature and time of collection were significant (Tab. 2). The probability of a snail's survival (from ca 0.8 to 1.0) was the highest at temperatures higher than or equal to 0°C; whereas below 0°C, the lower the temperature, the lower the probability of a snail's survival (Fig. 1B). The highest overall probability of survival was recorded in the February sample of snails, with the lowest recorded in those collected in October (Fig. 1B). The probability of survival of the snails at -5°C was the highest in those collected in

Table 1

Basic statistics of the Supercooling Point measured in summer 2019 and winter 2020 in *V. moulinsiana* snails

Season	Age class	N	Mean [°C]	Standard Deviation	Median [°C]	Minimum [°C]	Maximum [°C]	Range [°C]
Summer	Adults	30	-7.1	1.9	-9.6	-10.8	-3.4	7.4
Winter	Adults	13	-9.7	1.8	-9.8	-12.7	-6.9	5.8
	Juveniles	16	-10.0	2.5	-7.3	-15.0	-6.3	8.7
	Adults + juveniles	29	-9.9	2.2	-9.6	-15.0	-6.3	8.7

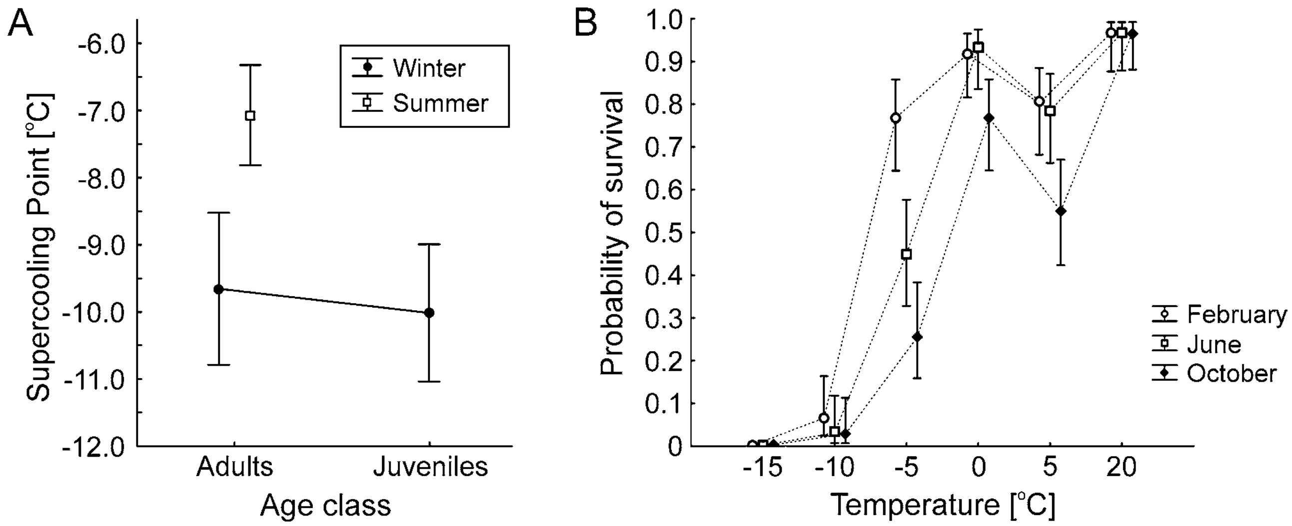

Fig. 1. A – Differences in mean Supercooling Points (SCPs) between juvenile and adult snails collected in winter and summer. Whiskers denote 95% confidence intervals of means. B – results of the Generalized Linear Model (GLZ; logit, binomial) showing differences in *V. moulinsiana* survival between temperatures during each cold survival experiment trial conducted in February, June and October.

Table 2

Results of the full factorial Generalized Linear Model (GLZ; logit, binomial) showing the effect of trial duration, temperature and time of collection on snail survival, including interactions between predictors on the *Vertigo moulinsiana* survival. Df – degrees of freedom, W – Wald statistic

Effect	df	W	p
Intercept	1	0.1	0.8126
Trial duration	1	0.3	0.5706
Temperature	5	280.0	<0.0001
Month (time of collection)	2	8.2	0.0169
Trial duration*temperature	5	2.5	0.7792
Trial duration*Month (time of collection)	2	0.2	0.8879
Temperature*Month (time of collection)	10	13.5	0.1984
Trial duration*Temperature*Month (time of collection)	10	8.4	0.5878

February (Fig. 1B). The probability of survival of the individuals kept at -10°C was very low (close or equal to 0), and none of the snails exposed to -15°C survived.

Overwintering microhabitats

In November, the snails were found at an average height of 35 cm (minimum=30 cm; maximum=40 cm; SD=4.5) above the ground. Shortly after the ice had melted, the snails were found low down, just above ground level (mean=5 cm, minimum=0 cm, maximum 25 cm; SD=5.7). The mean height of the vegetation was 100 cm (minimum=60 cm, maximum=140 cm; SD=15.2) in May, and was 80 cm (minimum=9 cm, maximum=110 cm, SD=11.3) in November.

Microclimatic conditions at the wintering site

Air temperature increases with a decreasing height above the ground, i.e. the closer to the ground, the higher the temperature. This was manifested in both the monthly averages, medians and minimum values of the air temperature in December, but only in the minimum values in November. Unfortunately, the data for January was incomplete, because the data loggers, mounted at heights from 10 to 40 cm above the ground, were damaged by ice (a layer ca. 40 cm thick) covering the study area. The lowest temperature in the analysed period – 21.22°C – was recorded in January at a height of 130 cm above the soil surface (Tab. 3)

Table 3

Basic statistics for air temperature in November, December 2008 and January 2009. Data for January are incomplete, because data loggers were damaged by the ice layer covering the study area

Month	Height [cm]	N	Air temperature [°C]				
			Mean	Median	Minimum	Maximum	Std. Dev.
November	140	864	4.22	4.4	-7.99	17.74	5.47
	130	864	4.16	4.33	-8.11	18.58	5.52
	120	864	4.14	4.37	-8.18	17.85	5.54
	110	864	4.12	4.31	-8.28	18.61	5.56
	100	864	4.08	4.36	-7.86	18.92	5.56
	90	864	3.98	4.21	-7.85	17.63	5.49
	80	864	3.93	4.22	-6.76	15.74	5.24
	70	864	3.84	3.93	-6.36	15.26	5.04
	60	864	3.85	3.85	-6.04	14.79	4.86
	50	1152	4.35	4.74	-5.91	21.86	5
	40	1440	4.19	4.57	-6.61	19.32	4.7
	30	1440	4.03	4.37	-5.91	14.91	4.29
	20	1440	3.98	4.16	-5.62	12.5	3.83
	10	1440	4.09	4.81	-2.09	10.77	2.84
December	140	960	-0.13	-0.23	-11.72	11.21	4.78
	130	960	-0.16	-0.29	-11.78	11.19	4.76
	120	960	-0.23	-0.32	-11.85	11.14	4.77
	110	960	-0.3	-0.35	-11.83	11.84	4.78
	100	960	-0.31	-0.39	-11.78	11.52	4.67
	90	960	-0.42	-0.53	-11.76	10.74	4.54
	80	960	-0.44	-0.48	-11.28	10.18	4.31
	70	960	-0.48	-0.57	-10.16	9.86	4
	60	960	-0.38	-0.48	-9.56	9.15	3.7
	50	852	0.74	0.21	-7.55	12.15	3.81
	40	744	1.58	2.53	-7.05	12	4.04
	30	744	1.53	2.61	-7.04	11.84	3.9
	20	744	1.69	2.65	-6.94	11.56	3.58
	10	744	2.34	3.06	-3.98	8.08	2.62
January	140	1344	-3.52	-2.12	-21	8.51	5.45
	130	1344	-3.56	-2.11	-21.22	8.81	5.45
	120	1344	-3.58	-2.19	-21.02	8.44	5.48
	110	1344	-3.54	-2.12	-21.07	9.14	5.49
	100	1344	-3.55	-2.18	-21.07	9.46	5.47
	90	1344	-3.62	-2.29	-20.9	8.68	5.46
	80	1344	-3.61	-2.42	-21.21	7.9	5.26
	70	1344	-3.55	-2.46	-19.37	6.53	4.89
	60	1344	-3.38	-2.27	-18.56	6.02	4.54
	50	672	-2.77	-1.9	-13.63	4.31	3.65
	40	0	–	–	–	–	–
	30	0	–	–	–	–	–
	20	0	–	–	–	–	–
	10	0	–	–	–	–	–

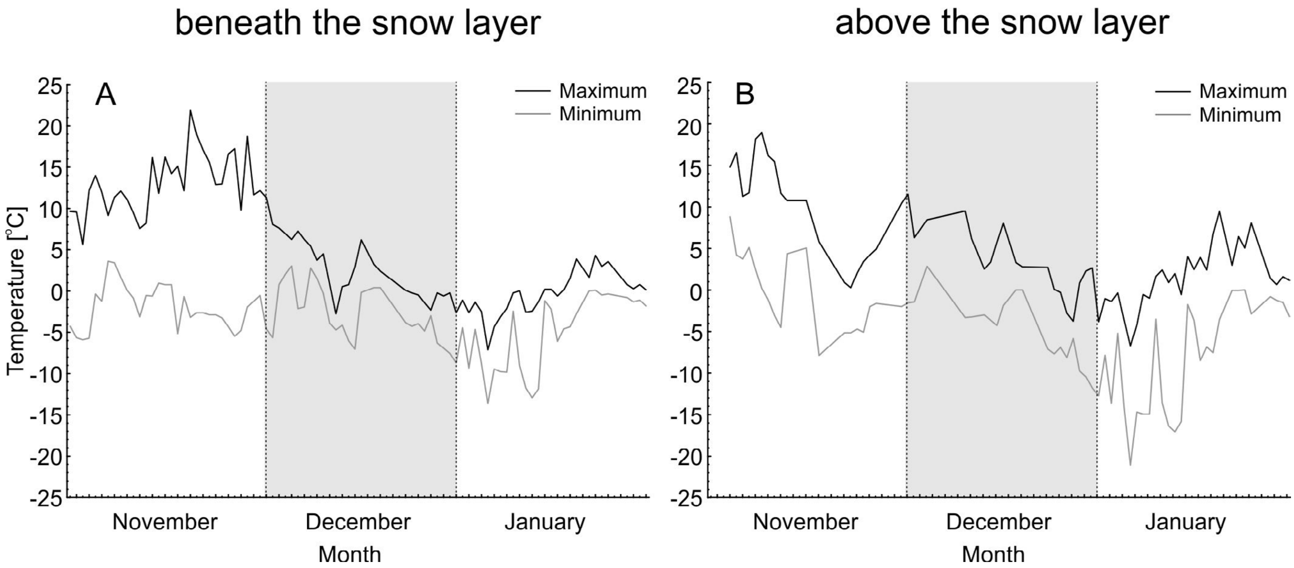

Fig. 2. Air temperature amplitudes in the period from November 2008 to January 2009, at a height of 20 cm (A) and 100 cm (B) above ground level.

The maximum temperatures dropped with a decreasing height, but only as far as the 60 cm level. In November and December, the highest maximum temperatures were measured at a height of 50 cm (Tab. 3); while the highest temperature, 21.86°C, was recorded in November, 50 cm above the ground.

Above the zone of the snail occurrence (60-140 cm) in the winter months, differences in the mean temperatures were statistically significant only between months (full factorial ANOVA; F=5279.1; p<0.0001). Neither the differences in the mean temperatures measured at successive heights above ground level, nor the interaction between the month and height factors were statistically significant (full factorial ANOVA; height: F=0.90; p=0.5180; month*height: F=0.4, p=0.9874). In each of the winter month of measurements, the temperature readings were significantly higher at 50 cm than at other heights (full factorial ANOVA; F=6.0; p<0.0001).

Within the snail occurrence zone (0-50 cm), there were statistically significant differences in the average temperatures between the November and December measurements (full factorial ANOVA; F=695.0; p<0.0001), both at successive heights above the ground (full factorial ANOVA; F=5.0; p=0.002) and in the interaction between the months and height above the ground (full factorial ANOVA; F=4.9; p=0.002). Beneath the snow level (Fig. 2A), smaller temperature fluctuations were observed compared to those in the zone above the snow (Fig. 2B). Above the snow, lower minima and higher maxima were recorded than in the level below (Fig. 2).

Discussion

Our results indicate that *V. moulinsiana* applies mechanisms for winter preparation and employs a freezing avoidance strategy. The presence of mechanisms for winter preparation could be observed in the cold survival experiment results, where the snails' survival rate decreased with a falling temperature, and at -5°C the survival rate of the snails collected in February was significantly higher than in those collected in June and October. What we can assume is that the snails collected in February were already overwintering, so they were ready and physiologically accustomed to low temperatures; moreover, they had already survived earlier frosts. In fact, they were naturally pre-selected, because weaker, unprepared and non-adapted individuals would most likely have been eliminated earlier during the winter. It is also possible that the survival rate of the October sample was the lowest because most of them were freshly-hatched individuals that had yet to experience low temperatures, as the first frosts would be lethal to snails unprepared for, or not so well accustomed to low temperatures. Nevertheless, the results revealed no significant differences in the SCP between adults and juveniles. Moreover, the higher SCPs obtained in the snails collected in summer confirms the physiological mechanisms found in animals preparing for winter on the basis of seasonal changes in their body water content, as the reduction in total body water increases the cryoprotectant concentrations (Ansart & Vernon 2003; Nicolai *et al.* 2005).

The SCP measurements confirmed the presence of a freezing avoidance strategy in this species. The SCPs were significantly lower in the snails collected in winter (ranging from -6.3 to -15°C) than in those collected in summer (ranging from -3.4 to -10.8°C). Moreover, none of the snails survived the cold tolerance experiment at -15°C, while only a few of those collected in February survived at -10°C, which is in accord with the mean SCP obtained in February for the juveniles used in both experiments (-10.0°C). This is another confirmation of the presence of a freezing avoidance strategy in this species, with lethal effects caused by the formation of ice crystals in its tissues (Sinclair *et al.* 2015).

The wide range of mean SCP measurements (-7.1°C in summer, - 9.9°C in winter) suggests that within this temperature interval, there may be a wider range of phenotypic possibilities with regards to cold tolerance among individuals, reflecting a significant differentiation in the reaction norms. A wide range of reaction norms should develop in species inhabiting more diverse but less stable environments. Under severe conditions, the ranges of the species' responses will therefore be narrower than in the lowlands, where the conditions are milder but the habitats are more diverse (Futuyma *et al.* 2008). However, it was not possible to ascertain whether the observed variability in the SCP is the result of genetic or environmental differences.

A comparison of the *V. moulinsiana* SCP with the SCPs of two other Vertiginidae species may be helpful in revealing whether the geographic latitude and associated climatic conditions does indeed have significance for the overwintering strategies of these species. *Columella edentula* (SCP: -16.81°C; Ansart *et al.* 2014) and *Columella columella* (SCP: -13.82°C; Ansart *et al.* 2014) are similar to *V. moulinsiana* (SCP: -7.1°C (summer measurement), -9.9°C (winter measurement)) in terms of their body size (adult shell length: *V. moulinsiana* 2.7 mm, *C. edentula* ca 3 mm and *C. columella* ca 3.5 mm), and all three inhabit very moist to wet habitats on calcareous substrates. The most likely reason for the higher SCP of *V. moulinsiana* is its adaptation to low temperatures. It is an Atlantic-Mediterranean species distributed from Ireland to Russia and southwards to North Africa (Killeen 2003), while *C. edentula* is a Euro-Siberian species that extends as far as the Caucasus and central Asia (Wiktor 2004). The range of *C. columella*, an Arctic-Alpine species (Pokryszko 2004), includes northern Europe, the mountains of central Europe, Siberia, Crimea (Yajla), Armenia and the mountains of the Middle East, as well as North America.

The habitat conditions are quite harsh in the habitats of *V. moulinsiana*. There is high humidity along with significant temperature fluctuations. Low temperatures (below 0°C) may persist for longer than they did during our experiment. The maximum duration of temperatures between -5 and -15°C recorded at our study site was 40 h; whereas for temperatures between -15 and -20°C it was 10 h (Lipińska *et al.* 2020). Therefore, how is it possible that in the experiment, the snails did not survive in the temperatures that naturally occur in their habitat? Our results indicated the existence of a buffer zone during the winter, containing a thick layer of withered and decaying plant matter, mulch and snow. The level of 50 cm above the ground appeared to mark the upper boundary of that zone, below which it was significantly warmer than above. Moreover, not only the minimum temperatures were higher in this zone than above it, but also the maximum temperatures in the zone did not reach values as high as those recorded above the zone. Consequently, a more constant temperature was maintained there, which may also be of great importance for wintering snails, as they are then not subjected to constant changes as a result of strongly fluctuating temperatures. This phenomenon of snow insulating wintering organisms being sheltered from low temperatures has previously been described by many authors; for example, Andrewartha & Birch (1954), Merriam *et al.* (1983), Malyshev & Henry (2012). Moreover, in the microclimate beneath the snow, temperatures between 0°C and 2°C may persist even when the air temperatures above the snow are from -4 to -22°C lower (Pearce 2001).

At this point, it is impossible to state whether the *V. moulinsiana* snails actively search for appropriate overwintering habitats, or whether they overwinter in the place where winter finds them. However, this species can climb on to plants and can be found at various heights above the ground during the seasons. In spring, these snails occur low down, on the previous year's decayed stems and the leaves of monocotyledons (Killeen *et al.* 2012); but in summer and autumn, they have been found to climb to heights of 30-50 cm (Pokryszko 1990), 1 m (Myzyk 2011), more than 2 m (Killeen 2003) and even several metres above the ground (Cameron *et al.* 2003). This is consistent with our study, as the measurements made in November showed that the snails occurred at an average height of 35 cm. In May, however, just after the ice cover had melted, the snails were found no higher up than 5 cm. It is quite conceivable that the plant shoots had been damaged by frost (Pearce 2001; Malyshev & Henry 2012) and that the snails had dropped to the ground together with the vegetation. However, active searching for wintering sites is normal in snails, especially those with a freeze avoidance strategy (Ansart & Vernon 2003). In particular, terrestrial molluscs frequently exposed to freezing often move to unfrozen, buffered habitats (Ansart & Vernon 2003).

Cold hardiness is a basic factor regulating the species' ranges. Moreover, recent rapid climate changes may be causing these distributions to shift (Futuyma *et al.* 2008). In its early stages, climate change will most likely result in snowless but cold winters (Zhu *et al.* 2019). The results of the low-temperature survival experiment highlighted the poor resistance of these snails to snow-free conditions. On the other hand, both in the frost survival experiment and the SCP measurements, a portion of the population exhibited increased frost resistance, especially during the winter measurements. This gives us hope for the adaptation and survival of this species in the face of ongoing climate changes. However, it should be borne in mind that data collected in the laboratory may not fully reflect the natural conditions.

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Author contributions

A.M.L., P.O. and A.M.Ć. designed the study. A.L., A.M.Ć. and M.G-S. collected the data. A.M.L. and M.G-S. conducted the laboratory experiments. A.M.L. and A.M.Ć. wrote the main manuscript text. A.M.Ć. conducted the statistical analysis, and prepared Tables and Figures. All of the authors reviewed the manuscript.

Conflict of interest

The authors declare no competing interests.

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