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Cold tolerance in terrestrial invertebrates inhabiting subterranean habitats

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Abstract: Most organisms are able to survive shorter or longer exposure to sub-zero temperatures. Hypothetically, troglaxenes characterized as not adapted, and troglaphiles as not completely adapted to thermally stable subterranean environment, have retained or partially retained their ability to withstand freezing, while most troglobionts have not. We tested this hypothesis experimentally on 37 species inhabiting caves in Slovenia, analyzing their lower lethal temperatures in summer and winter, or for one season, if the species was not present in caves during both seasons. Specimens were exposed for 12 hrs to 1°C-stepwise descending temperatures with 48 hr breaks. In general, the resistance to freezing was in agreement with the hypothesis, decreasing from troglaxenes over troglaphiles to troglobionts. However, weak resistance was preserved in nearly all troglobionts, which responded in two ways. One group, withstanding freezing to a limited degree, and increasing freezing tolerance in winter, belong to the troglobionts inhabiting the superficial subterranean habitats. The other group, which equally withstand freezing in summer and winter, inhabit deep subterranean or other thermally buffered subterranean habitats. Data on cold resistance can thus serve as an efficient additional measure of adaptation to particular hypogean environments.

Keywords: Cold resistance; Slovenia; troglaxenes; troglaphiles; troglobionts

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INTRODUCTION

Soon after the description of the leiodid beetle *Leptodirus hochenwartii* Schmidt, 1832 from the cave Postojnska jama—the first arthropod with conspicuous adaptation for living in hypogean habitats—intensive discussion began on the adaptation and ecology of animal species in the subterranean environment. Many ecological classifications of these organisms have been proposed, each of them being in particular restrained by the limited knowledge of species ecophysiology (Sket, 2008), and most of them burdened with the author's subjective judgments. Though not universally accepted (Desutter-Grancolas, 1999), the classification established by Schiner (1854) and Racovitza (1907) is sufficiently informative for many purposes in subterranean ecology and evolution (Boutin, 2004). It considers the three main ecological groups of animals in hypogean habitats: 1) Troglaxenes are the least adapted for living in the subterranean environment; they exhibit no

morphological adaptation and do not complete their life cycle there. 2) Troglaphiles alternate between the epigeal and hypogean habitats or live permanently in subterranean habitats; they show some moderate adaptation, such as partly reduced eyes and adaptations to compensate for the lack of visual orientation. Some among these do not complete their life cycle underground, while others do. 3) Troglobionts complete their life cycle in a hypogean environment, and most of them clearly show troglomorphic characteristics, like eyelessness and depigmentation. In contrast to the frequent preference for these three ecological groups, Novak et al. (2012) found that, on the one hand, troglaxenes and troglaphiles together represent a group of variously adapted species, rather than two ecologically clearly separated categories, and, on the other hand, troglobionts divide into two strictly separated subgroups.

Invertebrates are ectothermal and at their critical thermal minimum they enter chill-coma, where neuromuscular transmission and movement cease

(Bowler & Terblanche, 2008; MacMillan & Sinclair, 2011). After thawing, they reactivate from this reversible state, or—in cases where they have exceeded their lower thermal lethal minimum—die. During late spring and early summer frosts, such recoveries or deaths are also usual in some species which periodically inhabit caves (Novak et al., 2004).

One possible measure to determine cold resistance is the supercooling point (SCP), which is the lowest temperature an animal reaches before freezing (Lee, 2010); however, the ecological validity of the SCP may sometimes be ambiguous (Renault et al., 2002). Lower lethal temperatures (LLTs) are a more authentic measure of survival ability, usually expressed as LT_{50} , which is the measure of temperature at 50% mortality of individuals exposed to temperatures below 0°C (Leather et al., 1995; Watanabe, 2002).

Response to below-zero temperatures is one such additional measure that enables a comparative insight on the general scale. The scarcity of evidence about LLTs in terrestrial animal residents in caves (Sacharov, 1930; Kirchner, 1973; Pullin & Bale, 1986; Novak et al., 2004, 2007; Latella et al., 2008; Lencioni et al., 2010) does not provide consistent information on the topic, since these authors used a variety of experimental methods. Although not as stable as had previously been supposed, in the temperate zone, the temperature in the deep subterranean habitats like deep bedrock fissures and caves is close to the mean annual value in surface habitats (Luetscher & Jeannin, 2004; Culver & Pipan, 2009a, b). However, freezing is usually not expected in caves. Consequently, the highest tolerance to sub-zero temperatures is expected in the troglonenes and diminishes over the trogloniles to the troglobionts, which are adapted to the deep subterranean habitats. In practice, some aquatic (Hervant & Mathieu, 1997; Issartel, 2007; Colson-Proch et al., 2009) and terrestrial troglobionts (Peck, 1974; Latella et al., 2008; Lencioni et al., 2010) clearly show cold resistance. On the other hand, most Antarctic and Alpine species, which in their microhabitats are thermally buffered at above-zero temperatures, are intolerant to freezing (e.g., Zettel, 2000; Block, 2002; Elster & Benson 2004; Lipovšek et al., 2004; Novak et al., 2004).

In this study, our aim was to establish to what extent the troglonene–troglonile–troglobiont model fits with the expected decreasing resistance to temperatures below 0°C in selected central European and Dinaric species inhabiting caves. For this purpose we measured their LLTs in winter and summer, to detect eventual seasonal differences in the response. We also hypothesized that, within these species, the range of response to under-zero temperatures is widest in troglonenes and diminish over trogloniles to troglobionts. Additionally, we tested whether, according to the LLTs, the presence of two troglobiont groups (shallow and deep *sensu* Novak et al., 2012) could be detected.

MATERIALS AND METHODS

Sample collection

Specimens of a representative series of 37 species divided among troglonenes, trogloniles and troglobionts (Table 1) were selected for measurement of their LLTs. They were collected from 14 caves and abandoned mines (in the following text: caves) in Slovenia at altitudes of 365–760 m. Besides the two gastropod species, all the others were arthropods. The specimens were collected during two critical periods: in summer or in winter, or in both seasons if specimens were present in caves. In *Amilenus* and both *Gyas* species, individuals of various stages were present in summer (adults) and winter (nymphs), while adults, older and the younger larvae of both *Troglophilus* species occurred synchronically in caves. In principle, five individuals of each stage and sex—depending on the species—of non-protected species, and three individuals each of the protected species were collected for the investigation. For a few species, we used a smaller number of specimens due to collecting difficulties.

In 54 ecologically investigated caves in Slovenia measured air temperature profiles (details in Novak, 2005; Novak et al., 2004, 2012) were used to characterize habitat temperatures of the species studied. For the habitats for each species, we calculated the mean, minimal and maximal temperatures (T_{mean} , T_{min} and T_{max}) to compare with the LLTs.

Laboratory analyses

The following protocol was arranged to measure LLTs and simulate repeated frosts in natural habitat. The LLT measurements were carried out in a precise thermostatic cooling chamber THK/V1-2020 (Elpromer, Slovenia) with a temperature adjustment of $\pm 0.1^\circ\text{C}$, and a cooling/warming movement of $\sim 10^\circ\text{C}/\text{hr}$. Measurements started at -2.0°C and were stopped at -12.0°C . Experimental individuals were placed in groups of up to five specimens—depending on their size and number—on a piece of wet paper in 2 dL vessels moistened every 5th day to prevent desiccation. After a two-day acclimation in a refrigerator at 6.0°C , the vessels were placed in the cooling chamber. The specimens were exposed to a particular temperature for 12 hrs, afterwards kept for 48 hrs in the refrigerator at 6.0°C and then exposed to a temperature 1.0°C lower. Specimens' condition was checked after 24 hrs at 6.0°C , when the eventual dead ones were removed. The procedure was repeated until all the specimens had died.

Statistical analysis

Specimens differing by sex and stage were first tested using the Mann-Whitney U test with a Bonferroni correction for two comparisons, and in *Troglophilus neglectus* where adults, older and younger larvae co-occurred, for six comparisons to analyze whether they differ in LLTs either in summer or winter (in the following: *s* and *w*). There were no significant differences in any experimental species (adjusted *p* value for sex: $p > 0.025$, for *T. neglectus*: $p > 0.008$)

Table 1. Experimental species to determine LLTs. Biospeological categories: x troglaxene, f troglophile, t troglobiont. w winter.

Higher taxon	Family	Species
Gastropoda	Helicidae	1 x <i>Chilostoma (Josephinella) lefeburi</i> (A. Ferrussac 1821) 2 x <i>Faustina illyrica</i> (Stabile 1864)
Oniscoidea	Trichoniscidae	3 t <i>Androniscus stygius</i> (Nemec 1897) 4 t <i>Titanethes albus</i> (C. Koch 1841)
Araneae	Agelenidae	5 x <i>Malthonica silvestris</i> (L. Koch 1872)
	Linyphiidae	6 f <i>Troglohyphantes diabolicus</i> (Deeleman Reinhold 1978)
	Nesticidae	7 x <i>Nesticus cellulanus</i> (Clerck 1757)
	Tetragnathidae	8 f <i>Meta menardi</i> (Latreille 1804) 9 x <i>Metellina merianae</i> (Scopoli 1763)
Opiliones	Sclerosomatidae	10 f <i>Amilenus aurantiacus</i> (Simon 1881) 11 x <i>Gyas annulatus</i> (Olivier 1791) 12 x <i>Gyas titanus</i> (Simon 1879) 13 x <i>Leiobunum rupestre</i> (Herbst 1799)
	Ischyropsalididae	14 t <i>Ischyropsalis hadzii</i> Roewer 1950
	Nemastomatidae	15 x <i>Paranemastoma bicuspidatum</i> (C. L. Koch 1835) 16 x <i>Paranemastoma quadripunctatum</i> (Perty 1833)
Microcoryphia	Machilidae	17 x <i>Trigoniophthalmus alternatus</i> (Silvestri 1904)
Orthoptera	Rhaphidophoridae	18 f <i>Troglophilus cavicola</i> (Kollar 1833), w: adults + larvae 19 f <i>Troglophilus neglectus</i> (Krauss 1879), w: adults + larvae
Lepidoptera	Geometridae	20 x <i>Triphosa dubitata</i> (Linnaeus 1758)
	Noctuidae	21 x <i>Scoliopteryx libatrix</i> (Linnaeus 1758)
	Nymphalidae	22 x <i>Aglais io</i> (Linnaeus 1758)
Coleoptera	Carabidae	23 t <i>Anophthalmus hitleri</i> (Scheibl 1937)
		24 f <i>Laemostenus (Antisphodrus) schreibersii</i> (Küster 1846)
	Leiodidae	25 t <i>Aphaobiella tiscincensis</i> (Pretner 1956)
		26 t <i>Aphaobius milleri alphonisi</i> (Müller 1914)
		27 x <i>Catops tristis</i> (Panzer 1794)
		28 t <i>Ceuthmonocharis pusillus</i> (Jeannel 1924)
		29 t <i>Ceuthmonocharis robici robici</i> (Ganglbauer 1899)
		30 x <i>Choleva sturmi</i> (Brisout 1863)
		31 t <i>Leptodirus hochenwartii</i> (Schmidt 1832)
Hymenoptera	Ichneumonidae	32 x <i>Diphyus quadripunctorius</i> (Müller 1776)
Diptera	Culicidae	33 x <i>Culex pipiens</i> (Linnaeus 1758)
	Heleomyzidae	34 x <i>Heleomyza (Heleomyza) cf. captiosa</i> (Gorodkov 1961)
	Limoniidae	35 x <i>Chionea (Sphaeconophilus) austriaca</i> (Christian 1980) 36 x <i>Limonia nubeculosa</i> (Meigen 1804)
	Mycetophilidae	37 f <i>Speoptea leptogaster</i> (Winnertz 1863), larvae

in either *s* or *w* season, allowing the combining of all specimens of a species irrespectively of sex and developmental stage in further analyses. For the analysis of trends in cold resistance, the species mean seasonal LLTs were calculated.

For 28 species with both the *s* and the *w* LLT measurements, a normalized plot of the *s* vs. the *w* LLTs was used for graphical analysis of species pre-classified within troglaxenes, trogliphiles and troglobionts (Table 2). Seasonal differences among categories in their mean LLTs were tested separately for *s* and *w* using analysis of variance (ANOVA), followed by a post-hoc mean separation by the Tukey Honest Significant Difference (HSD) test for unequal sample sizes. The effect size was calculated using the formula $r = Z/\sqrt{N}$; where $Z = \text{Kolmogorov-Smirnov } Z$ and $\sqrt{N} = \text{square root of the sample size}$ (Field, 2009). To demonstrate general trends in the response differences

among troglaxenes, trogliphiles and troglobionts, we calculated the integrated distributional curves of the *s* and *w* LLTs for each category. To evaluate the possible presence of two categories of troglobionts (Novak et al. 2012), if such two groups exist also according to their *s* and *w* LLTs, we grouped *Ischyropsalis hadzii*, *Anophthalmus hitleri*, *Ceuthmonocharis pusillus*, and *C. robici robici* as previously recognized shallow subgroup and *Androniscus stygius*, *Titanethes albus*, *Aphaobiella tiscincensis*, and *Leptodirus hochenwartii* as deep subgroup for further detailed comparison. The agreement of the LLTs with air T_{mean} , T_{min} and T_{max} at the settled sites within the 54 caves was tested using the correlation analysis. The data analysis was carried out with the statistical software SPSS 21.0.

RESULTS

Among the 37 species under study, 28 were collected from caves and tested for LLTs in *s* and *w*, five only in *s* and four only in *w* (Table 2).

Seasonal differences in LLTs

Seasonal differences between *s* and *w* LLTs of individual species varied from non-significant to highly significant (Table 2). Most species could equally withstand *s* and *w* LLTs, while a few were more resistant to *s* than to *w* freezing, and the reverse for a few other species. On average, the LLTs differed by less than 1°C between the seasons. Only a few taxa exhibited $\geq 1^\circ\text{C}$ difference between the *s* and the *w* LLTs (Table 3). Within the groups, the *s* and *w* LLTs did not significantly differ in troglaxenes, but differed significantly in trogliphiles (Mann-Whitney U test, $U=1269.5$, $p=0.001$) and troglobionts (Mann-Whitney U test, $U=842.5$, $p<0.001$). Their corresponding effect sizes were 0.045, 0.289 and 0.363, respectively, i.e., increasing from non-existent in troglaxenes over small in trogliphiles to medium in troglobionts.

Differences in LLTs between the ecological groups

The LLT range was the widest for the troglaxenes (*s*: -2--12°C; *w*: -2--10), and narrowed in the trogliphiles (*s*: -2--9; *w*: -2--9) and even more so in the troglobionts (*s*: -2--5; *w*: -2--6). The resistance to freezing decreased from troglaxenes over trogliphiles to troglobionts (Fig. 1). We obtained a statistically significant overall F test from the ANOVA for the mean LLTs in *s* ($F_{2,30}=6.97$, $p=0.003$). Posteriori testing showed significant differences between troglaxenes and troglobionts (HSD for unequal N, $p=0.017$). Normalized results of the LLTs are shown in Fig. 2 together with the best fits of error function (Gauss error function) to the data.

Subgroups in troglobionts

All troglobionts under study exposed a certain degree of freezing tolerance, i.e., they withstood temperatures below 2°C. A t test showed no significant difference between the *s* and the *w* LLTs in troglaxenes and trogliphiles, while troglobionts demonstrated significantly lower LLTs in *w* ($t=4.26$, $df=106$, $p<0.001$). The troglobionts classified in the shallow

Table 2. Descriptive statistics of LLTs and ambient air T in 37 terrestrial invertebrate species inhabiting caves. Current numbers before species correspond to those in Table 1 and Fig. 1. Ns, Nw number of individuals in summer and winter; s-LLTs, w-LLTs summer and winter LLTs, and Mann-Whitney U test significance of s-LLTs vs. w-LLTs (ns non significant, * <0.05 , ** <0.01 , *** <0.001 , - analysis could not be performed); Ts caves and Tw caves summer and winter temperature in cave sections where species occurred.

Species	Ns/Nw	s-LLTs Mean \pm Sd Min – Max	Ts caves Mean Min – Max	w-LLTs Mean \pm Sd Min – Max	Tw caves Mean Min – Max	Species	Ns/Nw	s-LLTs Mean \pm Sd Min – Max	Ts caves Mean Min – Max	w-LLTs Mean \pm Sd Min – Max	Tw caves Mean Min – Max
1 <i>Chilostoma lefeburi</i>	5 / 5 *	-3.6 \pm 0.5 -4 – -3	16.4 15.1 – 17.6	-4.8 \pm 0.4 -5 – -4	10.0 9.5 – 10.7	20 <i>Triphosa dubitata</i>	10 / 10 **	-8.4 \pm 2.2 -11 – -5	11.8 7.0 – 21.5	-4.5 \pm 0.5 -5 – -4	2.3 -4.8 – 9.1
2 <i>Faustina illyrica</i>	5 / 5 ns	-4.2 \pm 0.4 -5 – -4	13.7 8.5 – 22.0	-4.2 \pm 0.4 -5 – -4	2.6 -3.6 – 9.6	21 <i>Scoliopteryx libatrix</i>	1 / 10 -	-8	11.6 10.3 – 13.6	-5.7 \pm 0.7 -7 – -5	3.4 -3.2 – 11.4
3 <i>Andronuscus stygius</i>	10 / 10 ns	-3.5 \pm 0.5 -4 – -3	9.0 9.0 – 9.1	-3.8 \pm 0.4 -4 – -3	8.5 8.5 – 8.5	22 <i>Aglais io</i>	2 / 3 ns	-9.0 \pm 0.0 -9 – -9	11.6 8.7 – 14.4	-9.0 \pm 1.0 -10 – -8	5.2 5.2 – 5.2
4 <i>Titanethes albus</i>	10 / 10 ns	-2.5 \pm 0.7 -4 – -2	8.6 8.5 – 8.8	-2.6 \pm 0.7 -4 – -2	9.0 9.0 – 9.1	23 <i>Anophthalmus hitleri</i>	2 / 2 -	-3.0 \pm 0.0 -3 – -3	8.3 8.1 – 9.0	-4.5 \pm 0.7 -5 – -4	8.2 8.1 – 8.2
5 <i>Malthonica silvestris</i>	6 / 4 ns	-6.0 \pm 2.2 -8 – -4	11.7 10.9 – 13.5	-6.0 \pm 2.3 -8 – -4	2.2 0.6 – 3.6	24 <i>Laemostenus schreibersii</i>	3 / 8 ns	-4.7 \pm 0.6 -5 – -4	10.6 5.7 – 18.3	-4.9 \pm 0.4 -5 – -4	6.9 5.2 – 9.0
6 <i>Troglohyphantes diabolicus</i>	7 / 4 ns	-7.6 \pm 1.6 -9 – -5	10.0 8.2 – 21.3	-7.8 \pm 1.3 -9 – -6	5.7 -1.0 – 8.0	25 <i>Aphaobiella tisnicensis</i>	6 / 6 ns	-3.0 \pm 0.0 -3 – -3	9.8 9.8 – 9.9	-3.2 \pm 0.4 -4 – -3	8.2 8.0 – 9.0
7 <i>Nesticus cellulanus</i>	10 / 10 ns	-7.3 \pm 1.3 -9 – -6	10.6 8.1 – 14.8	-7.0 \pm 1.2 -9 – -6	6.2 3.1 – 9.6	26 <i>Aphaobius milleri alphonsi</i>	- / 4 -	-	-	-3.8 \pm 0.5 -4 – -3	7.5 6.1 – 9.1
8 <i>Meta menardi</i>	10 / 10 ns	-3.4 \pm 1.9 -8 – -2	12.6 6.0 – 22.0	-4.5 \pm 1.5 -8 – -3	4.4 -4.5 – 11.4	27 <i>Catops tristis</i>	4 / - -	-6.0 \pm 0.8 -7 – -5	13.9 6.9 – 17.1	-	-
9 <i>Metellina merianae</i>	10 / 10 ns	-6.6 \pm 1.7 -8 – -3	12.5 7.2 – 22.0	-7.6 \pm 1.2 -9 – -6	2.4 -3.6 – 11.4	28 <i>Ceuthmonocharis pusillus</i>	6 / 6 **	-3.8 \pm 0.4 -4 – -3	13.3 12.2 – 15.5	-5.2 \pm 0.4 -6 – -5	9.8 9.8 – 9.9
10 <i>Amilenus aurantiacus</i>	10 / 10 ns	-4.4 \pm 0.5 -5 – -4	14.7 7.2 – 21.5	-4.8 \pm 0.4 -5 – -4	5.5 -3.4 – 11.4	29 <i>C. robici robici</i>	6 / 6 **	-3.2 \pm 0.4 -4 – -3	7.9 5.7 – 14.8	-4.8 \pm 0.4 -5 – -4	10.2 9.8 – 10.5
11 <i>G. annulatus</i>	10 / 10 ***	-4.7 \pm 0.5 -5 – -4	10.8 10.8 – 10.8	-2.6 \pm 0.5 -3 – -2	5.7 2.1 – 8.4	30 <i>Choleva sturmi</i>	- / 2 -	-	-	-4.0 \pm 0.0 -4 – -4	7.3 7.3 – 7.7
12 <i>Gyas titanus</i>	10 / 10 ns	-3.4 \pm 0.5 -4 – -3	10.4 10.4 – 10.4	-2.9 \pm 0.7 -4 – -2	2.0 -3.5 – 6.5	31 <i>Leptodirus hochenwartii</i>	6 / 6 ns	-3.8 \pm 1.0 -5 – -3	8.6 8.5 – 8.8	-4.3 \pm 0.5 -5 – -4	9.0 9.0 – 9.1
13 <i>Leiobunum rupestre</i>	10 / - -	-3.4 \pm 0.5 -4 – -3	17.3 12.9 – 21.5	-	-	32 <i>Diphyus quadripunctorius</i>	- / 2 -	-	-	-7.5 \pm 0.7 -8 – -7	5.9 2.6 – 8.4
14 <i>Ischyropsalis hadzii</i>	6 / 6 **	-2.0 \pm 0.0 -2 – -2	8.7 8.1 – 9.7	-3.5 \pm 0.5 -4 – -3	8.6 8.6 – 8.7	33 <i>Culex pipiens</i>	2 / 5 ns	-5.5 \pm 0.7 -6 – -5	15.7 8.5 – 21.5	-5.0 \pm 0.0 -5 – -5	4.1 -2.5 – 9.9
15 <i>Paranemastoma bicuspidatum</i>	10 / - -	-3.8 \pm 0.4 -4 – -3	9.2 7.7 – 12.2	-	-	34 <i>Heleomyza cf. captiosa</i>	5 / - -	-10.4 \pm 1.8 -12 – -8	8.3 8.0 – 8.4	-	5.2 1.8 – 8.1
16 <i>P. quadripunctatum</i>	10 / 9 ***	-4.3 \pm 0.5 -5 – -4	11.2 9.8 – 12.5	-7.4 \pm 0.7 -8 – -6	-	35 <i>Chionea austriaca</i>	- / 1 -	-	-	-7	5.3 1.6 – 8.9
17 <i>Trigoniophthalmus alternatus</i>	10 / 10 ***	-2.7 \pm 0.5 -3 – -2	8.1 8.0 – 8.2	-4.0 \pm 0.0 -4 – -4	4.1 -3.6 – 9.6	36 <i>Limonia nubeculosa</i>	10 / - -	-4.7 \pm 0.5 -5 – -4	10.9 5.8 – 22.0	-	-
18 <i>Troglophilus cavicola</i>	10 / 20 **	-3.2 \pm 0.4 -4 – -3	12.3 7.6 – 21.5	-4.2 \pm 0.6 -6 – -4	6.9 0.1 – 11.4	37 <i>Speolepta leptogaster</i>	9 / 6 **	-4.1 \pm 0.6 -5 – -3	11.1 7.1 – 20.4	-2.0 \pm 0.0 -2 – -2	3.1 -3.5 – 8.4
19 <i>T. neglectus</i>	10 / 20 *	-3.4 \pm 0.5 -4 – -3	13.5 8.4 – 22.0	-4.6 \pm 0.8 -6 – -4	5.8 -2.5 – 10.5						

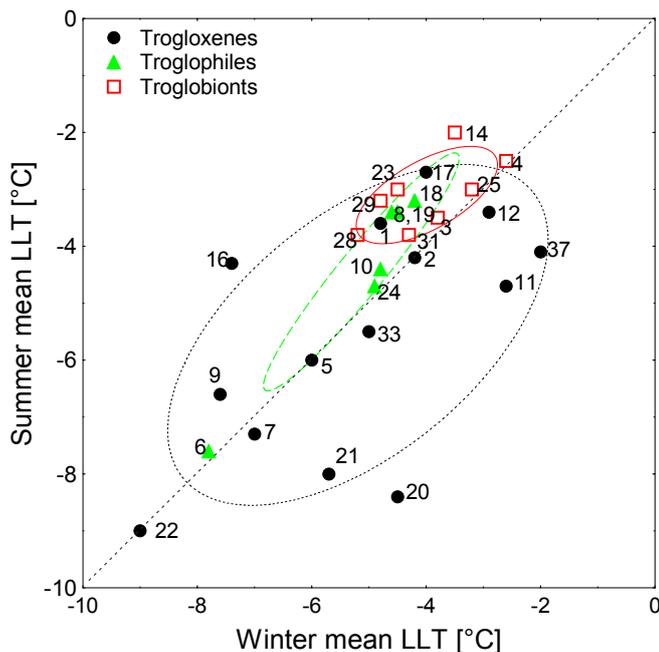


Fig. 1. Summer and winter mean LLTs in 28 species (current numbers as in Table 2) with both summer and winter data. The dotted line represents the balance axis between the summer and winter LLTs.

subgroup, represented by *Ischyropsalis hadzii*, *Anophthalmus hitleri*, *Ceuthmonocharis pusillus* and *C. robici robici*, significantly enhanced their resistance to sub-zero temperatures in winter, lowering their *w* LLTs at an average of 1.5°C with respect to the *s* LLTs, while *Andronuscus stygius*, *Titanethes albus*, *Aphaobiella tisnicensis* and *Leptodirus hochenwartii*, classified in the deep subgroup endured -2.0--3.8°C in summer and -3.2--5.2°C in winter, and the representatives of the deep troglobiont subgroup withstood -2.5--3.8°C and -2.6--4.3°C, respectively. Between shallow and deep subgroup there was no statistical difference in *s* LLTs, while these differences were significant for *w* LLTs (Mann-Whitney U test, $Z=3.55$, $p<0.001$).

Correspondence between the LLTs and caves temperatures

In all the species under study the LLTs were lower than T_{min} in the cave placements where the specimens occurred. There were no correlations between the LLTs and T_{min} , T_{mean} and T_{max} and the placement of these specimens in the caves.

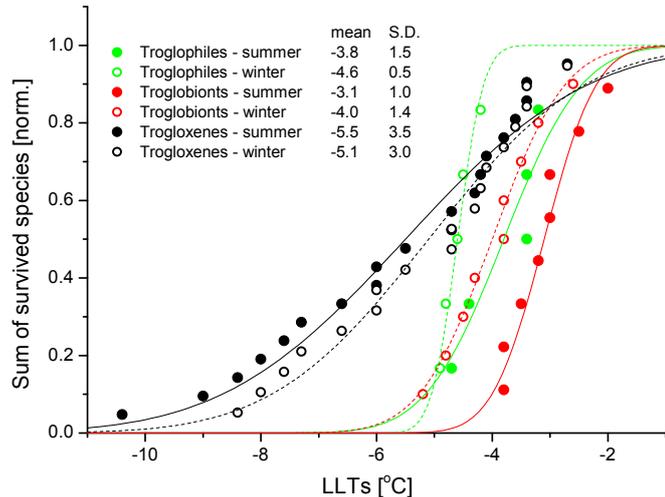


Fig. 2. Normalized distribution curves of LLTs in troglonexes, troglophiles and troglobionts exposed to 12-hr freezing intervals. The curves represent best fits to the error function to the data.

DISCUSSION

Multiple freeze-thaw cycles in a single winter are common in surface habitats in temperate latitudes and may present significant challenges to survival in insect species (Marshall & Sinclair, 2011). While specimens in deep caves can effectively avoid extreme values, such conditions are expected widely in ice and other cold caves, the entrance cave sections and bedrock fissure systems connected with the surface and the SSHs (Gers, 1998; Růžička, 1999; Culver & Pipan, 2009a, b; Růžička et al., 2013). Most frequently subfreezing conditions occur in winter conditions without snow, which is often caused by wind, especially the strong north-east wind, the bora (burja), with gusts of over $200 \text{ km}\cdot\text{hr}^{-1}$ (WineAndWeather, 2011) in the Dinaric region. The methodology used in our investigation simulated the repeated half-day frosts which occasionally affect the habitats of many species under study.

Cold resistance varies between and within species and depends on many factors, such as the developmental stage and age of an individual, its genetic potential, the season, the chill-coma temperature and the duration of exposure to low temperatures, nutrition, cold hardiness dynamics itself, diapause dynamics, photoperiodism, exposure to air currents, cryo-protective dehydration, the presence of nucleation agents and nucleation mechanisms, intracellular changes—such as mitochondrial disintegration, refreezing, experimental conditions etc., all these within the wide range of cryo-protection plasticity (Storey & Storey, 1988; Leather et al., 1995; Sømme, 1999; Worland et al.,

2000; Block, 2002; Block & Zettel, 2003; Ansart & Vernon, 2003; Sinclair et al., 2003; Hodkova & Hodek, 2004; Danks, 2006; Lagerspetz & Vainio, 2006; Hawes & Bale, 2007; Bowler & Terblanche, 2008; MacMillan & Sinclair, 2011; Vesala & Hoikkala, 2011). The stepwise 1°C -graded arrangement of our experiment does not allow precise determination of the LLTs; the real values may be, on average, about 0.5°C higher. On the other hand, although frequent freezing and rethawing is not painful to insects, it does cause permanent injuries, which in turn lower their ability to sustain low temperatures (Marshall & Sinclair, 2011). Like SCPs, LLTs also depend on the duration of exposure to low temperatures; after long-term exposure, the organisms may die from the exhaustion of energy reserves (Renault et al., 2002). In our experimental species with low LLTs—mostly troglonexes—these were probably underestimated because of many repeated refreezing and rethawing during the experimental procedure (cf. Marshall & Sinclair, 2011). *Gyas annulatus* represents a special case among troglonexes, showing no freezing tolerance in winter, which is the consequence of overwintering in water current-close, thermostated overwintering habitats (Novak et al., 2004). *Speolepta leptogaster* among troglophiles and *Ischyropsalis hadzii* among troglobionts are further cases of seasonal intolerance.

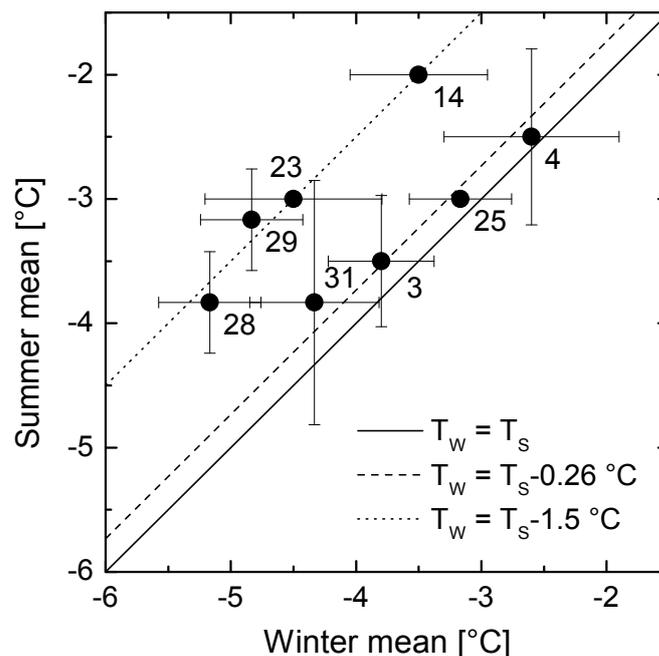


Fig. 3. Summer and winter mean LLTs of troglobionts (species numbers as in Table 2). Species 14, 23, 28, 29 belong to the shallow subgroup, species 3, 4, 25, 31 belong to deep subgroup. The solid line represents the balance axis between the summer and winter LLTs and dotted lines are guide to the eye for winter mean LLTs (T_w), which are lower than summer mean LLT (T_s) for 0.26°C in the deep subgroup or 1.5°C , respectively.

Table 3. Taxa exhibiting $\geq 1^\circ\text{C}$ difference between the average summer and winter LLTs. The differences vs. the opposite season are in $^\circ\text{C}$ in brackets. Numbers in front of the names as in Table 2.

The lowest T	Troglonexes	Troglophiles	Troglobionts
in summer	11 <i>Gyas annulatus</i> (-2.1) 20 <i>Triphosa dubitata</i> (-3.9) 21 <i>Scoliopteryx libatrix</i> (-2.3)	37 <i>Speolepta leptogaster</i> (-1.9)	
in winter	9 <i>Metellina merianae</i> (-1.0) 16 <i>Paranemastoma quadripunctatum</i> (-3.1) 17 <i>Trigoniophthalmus alternatus</i> (-1.3)	8 <i>Meta menardi</i> (-1.1) 18 <i>Troglophilus cavicola</i> (-1.0) 19 <i>Troglophilus neglectus</i> (-1.2)	14 <i>Ischyropsalis hadzii</i> (-1.5) 23 <i>Anophthalmus hitleri</i> (-1.5) 28 <i>Ceuthmonocharis pusillus</i> (-1.4) 29 <i>C. robici robici</i> (-1.6)

Despite these problems, the overall responses of the 37 selected central European and Dinaric troglonemes, troglonemes and troglonemes are comparable on the general level: lower a species sub-zero LLT, desto greater variability in individual LLTs. The species under study represent the expected general response to freezing: there is a clear decline in their LLTs from troglonemes, to troglonemes and troglonemes. This is congruent with the general hypothesis of their increasing adaptation to the habitat.

Three troglonemic species deserve comment. The spider *Troglodyphantes diabolicus* is relatively common in the investigated caves (Novak et al., 2012), where individuals usually settle in wall fissures and recesses of up to 10 cm in diameter, and similar sized sites between stones and pits in the clay. For this reason, they frequently make webs over the pitfall trap orifices (authors and Slavko Polak's unpublished data). It has also been reported from small mammal burrows (Deeleman Reinhold, 1978) and water drips, which probably originate in vertical bedrock fissures (Pipan et al., 2008). This species thus prefers narrow subsurface habitats rather than caves, which is in agreement with its relatively high level of freezing resistance throughout the year, comparable to that for the troglonemes. A second troglonemic spider species, *Meta menardi*, is better adapted to the hypogean habitat than the similarly sized trogloneme *Metellina merianae* (Novak et al., 2010) and its average LLT fits into the trogloneme range, and a few individuals showed the relatively high freezing resistance of -8°C . Such tolerance has been previously reported by Turquin (1971). Being also reported from screes (Růžička, 1999; Růžička et al., 2013) we support Růžička's suggestion that *M. menardi* is among species showing an intermediate stage in adaptation to the hypogean habitat.

In contrast, *Speolepta leptogaster* larvae show no cold resistance in winter. They mostly settle on ceiling and upper walls in caves emitting warm air. Such cave ceiling and blow holes, inaccessible to humans, form another thermally stable subterranean microhabitat, where temperatures generally do not fall below 0°C during winter. Like *G. annulatus*, *S. leptogaster* show moderate cold resistance in summer. Nearly all troglonemes under study showed a weak tolerance to below-zero temperatures. This is in accordance with Peck's (1974) and Leinconi et al. (2010) findings in other troglonemes. The investigated troglonemes responded in two ways and can be strictly differentiated with this respect. The species of the shallow trogloneme subgroup—*Ischyropsalis hadzii*, *Ceuthmonocharis pusillus*, *C. robici robici*, and *Anophthalmus hitleri*—were moderately resistant to freezing, enduring 1.5°C lower mean temperatures, in average, in winter than in summer. This seasonality clearly indicates their response to the winter external conditions which temporarily disturb their habitats, such as SSHs, by winter frosts.

The deep trogloneme subgroup—consisting of *Androniscus stygius*, *Titanethes albus*, *Aphaobiella tisnicensis* and *Leptodirus hochenwartii*—showed weak and nearly equal freezing resistance throughout the

year. This subgroup appears to have adapted to the deep subterranean and any other subterranean habitat where freezing does not occur. Species carrying out its amphibious way of life, like *Titanethes albus*, known to submerge in water for long periods (Sket, 1986; Vittori et al., 2012), may avoid freezing in this way. Besides, species preferring the terrestrial phreatic environment («milieu phreatique terrestre» *sensu* Jeannel, 1926), like *Aphaobiella tisnicensis*, are thermally buffered at above-zero temperatures (Novak et al., 2012). Such intolerance to freezing in terrestrial animals has been reported also from other close-to-water subsurface habitats, e.g., beside glaciers (Zettel, 2000), and between pebbles and stones near water streams (Novak et al., 2004).

CONCLUSIONS

Our study reveals that in central European and Dinaric troglonemes, troglonemes and troglonemes, resistance to temperatures below 0°C generally decreases in accordance with their increasing adaptation to the hypogean habitat. Troglonemes are most diverse in tolerance to sub-zero temperatures, from non-existent to high. Troglonemes are in between troglonemes and troglonemes with this respect. Most troglonemes show moderate resistance to freezing, and are divided into the two identified ecological subgroups. Species of the first subgroup stand sub-zero temperatures significantly better in winter than in summer; this indicates that they most likely—at least temporary in winter—experience freezing in their preferred habitats. The second trogloneme subgroup responds more or less equally in summer and winter, which is considered a residual evolutionary tolerance. Both such responses appear also in troglonemes.

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