More complex than expected: Cold hardiness and the concentration of cryoprotectants in overwintering larvae of five *Erebia* butterflies (Lepidoptera: Nymphalidae)

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Abstract. Understanding the factors restricting the distribution of some insect species to high altitudes is hindered by poor knowledge of temporal changes in their cold hardiness during overwintering. We studied overwintering larvae of five species of *Erebia* butterflies (Lepidoptera: Nymphalidae: Satyrinae) differing in altitudinal distribution: lowland *E. medusa*, submountain *E. aethiops*, subalpine *E. pronoe*, alpine *E. cassioides*, and subnivean *E. pluto*. We subjected them to three treatments, AutumnWarm (13/8°C), imitating conditions prior to overwintering; AutumnCold (5/0°C), imitating late autumn conditions; and WinterCold (5/0°C), differing from AutumnCold by a shorter photoperiod and longer exposure to zero temperatures. Supercooling points (SCP) did not differ between species in the AutumnWarm treatment, despite large differences in the concentrations of cryoprotectants (CrPC; lowest in *E. medusa* and *E. aethiops*). Lowland *E. medusa* was freeze-tolerant, the subalpine, alpine and subnivean species were freeze-avoidant, whereas submountain *E. aethiops* displayed a mixed strategy. SCPs diverged in the AutumnCold treatment: it increased in the lowland *E. medusa* (from –16.5 to –10.8°C) and reached the lowest value in *E. cassioides* (–21.7°C). In WinterCold, SCP increased in subalpine *E. pronoe* (from –16.1°C in AutumnWarm and –18.7°C in AutumnCold to –12.6°C). *E. medusa* decreased and *E. aethiops* increased their CrPCs between autumn and winter; the highest CrPC was recorded in subnivean *E. pluto*. CrPC did not correlate with SCP across species and treatments. Cryoprotectant profiles corroborated the difference between lowland and freeze-tolerant *E. medusa* and the three high altitude freeze-avoidant species, with *E. aethiops* in an intermediate position. Glycerol was surprisingly rare, trehalose was important in all species, and such rare compounds as monopalmitin and monostearin were abundantly present in *E. pronoe*, *E. cassioides* and *E. pluto*.

INTRODUCTION

Debates on the effects of climate change on biodiversity are much concerned with future fates of cold-adapted range restricted species, such as Lepidoptera in high altitude habitats within temperate zones, under a warming climate (Franco et al., 2006; Konvicka et al., 2014; Scalercio et al., 2014; Konvicka et al., 2016). In this context, it is notable how little is known regarding the mechanisms restricting cold-adapted species to their high altitude habitats. The mechanisms proposed so far include dependency on treeless habitats (and associated plants), which may shrink in extent with increasing timberline (Bila et al., 2016; Roland & Matter, 2016); dependency of overwintering immature stages on stable snow cover for winter insulation and hence threats due to less predictable snow (Vrba et al., 2012; Roland & Matter, 2016); the requirements of adults for thermal heterogeneity (Kleckova & Klecka, 2016; MacLean et al., 2016); or increased pressure from parasitoids or other natural enemies (cf. Pere et al., 2013). A wide variety of

potential factors, particularly those affecting subtler eco-physiological responses to climate, remain little-known and if any studies exist, they are usually limited to single species (Crozier, 2004; Davies et al., 2006; Atapour & Moharramipour, 2009; Vrba et al., 2014a).This is clearly not sufficient, given the diversity of alpine insects and remarkable differences, even among related species, in such parameters as supercooling ability(cf. Vrba et al., 2012, 2014b).

The butterfly genus *Erebia* Dalman, 1816 is an unusually species rich and predominately cold adapted Holarctic taxon with its centre of diversity in European mountains, mainly the Alps (Sonderegger, 2005). This grass-feeding genus originated in arid grasslands in current Central Asia, invaded current Europe between 23 and 17 Myr ago and diversified on emerging mountain grasslands (Pena et al., 2015). The high numbers of co-occurring species seems to be associated with its diversity of habitats associations (Kuras et al., 2000; Sonderegger, 2005), demography

structures (Kuras et al., 2003; Polic et al., 2014) and adults' thermal requirements (Kleckova et al., 2014). In addition, their current population genetic structure mirrors the rapid upslope and downslope movements during the Pleistocene climate fluctuations (Schmitt et al., 2006, 2016).

Survival during winter is important in determining an insects' abundance and requires major restructuring of physiological functions: an individual interrupts its development and shifts to maintenance processes and to tissue and cell protection (Lee, 1991) In numerous temperate zone species, this involves diapause (Denlinger, 1991), a process initiated long before the onset of cold temperatures, in late summer or early autumn, and entering this phase depends on appropriate reading of external, typically photoperiodic, clues (Kostal et al., 2014). Some species, however, just increase their cryoprotectant content while not undergoing true diapause (Pullin & Bale, 1989c).

There are two major strategies for surviving winter frosts (Sinclair et al., 2015), both of which are reported for species of *Erebia* (cf. Vrba et al., 2012). Freeze-tolerant insects allow for the freezing of extracellular body liquids, whereas freeze-avoidant species decrease the freezing temperature of body fluids so that they do not freeze; some insects may display mixed strategies, depending on presence or absence of external ice (Sinclair et al., 2015). Survival at low temperatures is facilitated by synthesis of polyols, low-molecular sugars and alcohols that decrease the temperature of crystallization of body fluids (= supercooling point, SCP), and contribute to the protection of cell membranes and proteins (Ramlov, 2000). Examples of cryoprotective polyols in Lepidoptera include glycerol, sorbitol, glucose, fructose and trehalose (Pullin & Bale, 1989b, c; Vrba et al., 2014a; Williams et al., 2014). There is only fragmentary information on how individual polyols contribute to cold hardiness, how they are connected with the two strategies, how their content varies during winter and how it varies among related species. It seems that usually, one or two compounds found in high concentrations colligatively modify the supercooling point, whereas several rarer compounds contribute non-colligatively to stabilizing macromolecules and biomembranes (Storey & Storey, 1991; Kostal et al., 2001).

Comparison of cold hardiness in four *Erebia* species whose overwintering larvae were acclimated in constant conditions (Vrba et al., 2012) revealed freeze-tolerance in a lowland species, *E. medusa* (Denis & Schiffermüller, 1775), and freeze-avoidance in three species from mountain and alpine environments: *E. sudetica* (Staudinger, 1861), *E. epiphron* (Knoch, 1783) and *E. tyndarus* (Esper, 1781).These four species respond counter-intuitively to low temperatures: the lowland species is more cold hardy (surviving –21°C) than its mountain or alpine congeners (Vrba et al., 2012). It is not known, however, how cold hardiness in *Erebia* spp. is connected to polyol profiles, or how the profiles change depending on external conditions.

Here, we study the overwintering of *Erebia* larvae in more detail. As in Vrba et al. (2012) we compare several species differing in altitudinal distribution and their habitats, but contrary to the earlier study, we simulated the thermal conditions the larvae most likely experience in nature: moderately low temperatures prior to freezing, autumn conditions close to freezing, and mid-winter conditions. We asked how these conditions are mirrored in the SCP, assayed the larval polyol profiles and compared the situations within and across species. We hypothesized that (1) species whose larvae experience unpredictable snow cover will be more cold-hardy than those experiencing more predictable insulating snow cover in their natural habitats; (2) cold hardiness will be highest under simulated winter conditions; (3) polyol content will negatively correlate with the super cooling point; and (4a) polyol profiles will be similar in closely related species or, alternatively (4b) species experiencing similar thermal conditions will be more similar in their polyol profiles.

MATERIAL AND METHODS

Species studied

All the species of *Erebia* studied are univoltine, i.e. they have a single generation per year. They all feed on grasses and overwinter as larvae, low in grass tussocks, attached by their legs to the bases of grass stems (Sonderegger, 2005). In the high altitude species, prolonged two-year (semivoltine) development may occur, varying among sites and years (Kleckova et al., 2015; Zakharova & Tatarinov, 2016). The five species studied form an altitudinal cline, as far as their maxima and optima are concerned. *E. medusa* (Denis & Schiffermüller, 1775) is a lowland species. It mainly occurs at low altitudes, but can ascend to ca 2000 m a.s.l. in the Alps. Its distribution ranges from Central France to Northern China. It inhabits late-successional grasslands, e.g. abandoned meadows and pastures, forest-steppes, grassy woodland clearings and margins (Schmitt, 2002; Stuhldreher & Fartmann, 2015). Larvae overwinter in the third instar (Sonderegger, 2005).

E. aethiops (Esper, 1777) is a submountain species. Its Eurosiberian distribution ranges from France to Western Siberia. It inhabits sparse woodlands, grassy forest clearings and margins (e.g., Slamova et al., 2013) from sea level to about 1500 m a.s.l. in the Alps. It overwinters as a second-instar larva (Sonderegger, 2005).

E. pronoe (Esper, 1780) is a subalpine species. This European species inhabits most of the continent's high mountains, except in Scandinavia and mountains surrounding the Mediterranean basin. It prefers mountain grasslands (Kleckova et al., 2014) from about 900–2800 m a.s.l. It overwinters as small, first instar larvae (Sonderegger, 2005).

E. cassioides (Reiner & Hochenwarth, 1792) is an alpine species. Distributed in the Eastern Alps and Apennines at altitudes of about 1600–2600 m. It is a member of the taxonomically difficult *E. tyndarus* complex, in which populations from the Western Alps and Balkan peninsula were recently considered to be separate species (Schmitt et al., 2016). The habitats are sparsely vegetated rocky substrates (Kleckova et al., 2014). Larvae overwinter in the first or second instar (Sonderegger, 2005).

E. pluto (De Prunner, 1798) is a subnivean species. European populations, occur in the Alps and central Apennines. It uses the most extreme habitats, sparsely vegetated unstable screes in alpine to subnivean zones (1800–3000 m a.s.l.). Larval development lasts two years, the larvae overwinter either in the second or the last (fifth) instar (Sonderegger, 2005).

Rearing of larvae and experimental treatments

For all species, we collected inseminated females in their natural localities (*E. medusa*: May 2015, Český Krumlov, Czech Republic, 48°50′N, 14°19′E, 570 m a.s.l.; *E. aethiops*: August 2015, Au, Tirol, Austria, 47°06′N, 10°57′E, 1200 m a.s.l.; *E. pronoe*: August 2015, Pfafflar, Tirol, Austria, 47°17′N, 10°39′E, 2000 m a.s.l.; *E. cassioides*: August 2015, Hochgurgl, Tirol, Austria, 46°54′N, 11°03′E, 2200 m a.s.l.; *E. pluto*: July 2015, Rettenbachgletscher, Tirol, Austria, 46°56′N, 10°55′E, 2900 m a.s.l.).

The females were kept in plastic boxes, in groups of up to 5 individuals, containing a selection of grasses from the original localities. The eggs were allowed to hatch, the newly hatched larvae were placed into cooled incubators (Sanyo MIR-153), where the outdoor natural photoperiod was maintained by adjusting manually on a weekly basis. Newly hatched larvae of all the species studied were reared under a summer temperature regime (20°C day / 10°C night) until they reached their respective overwintering instars in September. Duration of this summer regime thus depended on species' phenology (larval hatching – *E. medusa*: mid-June, *E. pluto*: early August, *E. aethiops*, *E. pronoe*, *E. cassioides*: mid-August).

In mid-September, larvae of each species were divided into two groups, which were subsequently reared under two different thermoperiodic regimes: *warm* treatment (13°C day / 8°C night) and *cold* treatment (5°C day / 0°C night), with the photoperiod following outdoor conditions. For SCP identification and cryoprotectant assays, we sampled 16 and 10 larvae, respectively, at two different phases of overwintering: *autumn* (end of October, i.e. after 45 days exposure: AutumnCold and AutumnWarm treatments) and *winter* (mid-January, i.e. 4 months: WinterCold treatment only). Samples for the SCP and cryoprotectant assays were always taken on the same day for a single species.

Cold hardiness measurements

The SCP was measured individually using a PICO recorder with hand-made type K thermocouples (Hanson & Venette, 2013), which were attached to the body of the experimental caterpillars in a syringe (Brunnhofer et al., 1991) using zincoxide thermoconductive paste. Starting at 5°C, the larvae were gradually cooled in a Calex desktop freezer with a manually controlled cooling rate of 1°C per minute [considered fast enough for this experiment and still biologically realistic (Nedved et al., 1995)] by gradual submerging the syringe with a caterpillar into a box containing aluminium marbles. After the exotherm was detected, the larva was kept in the cooling device until its body temperature decreased again to the value of the crystallization temperature and then removed and warmed up at room temperature.

Cryoprotectant analysis

The cryoprotectant concentration (CrPC) assays followed procedures described in (Kostal & Simek, 1995; Kostal et al., 2007). The larvae were weighed, stored frozen at –80°C, then thawed, homogenized in 70% ethanol and the samples centrifuged. The supernatant was purified using a hexane treatment and after drying in a hydrogen stream derivatized with O-methylhydroxylamine (oximation, 80°C for 30 min) and trimethylsilylimidazol (silylation, 80°C for 30 min). After derivatization and re-extraction into isooctane, 1 µl aliquots were injected using an AOC-20s autosampler into a gas chromatograph Shimadzu GC-2014 equipped with a flame ionization detector and controlled by GC Solution software (all from Shimadzu, Japan). The concentrations of low-molecular mass sugars and polyols (putative cryoprotectants) were determined after separation on a DB-1MS capillary column

(29 m × 0.25 mm, 0.25 µm film thickness from Agilent Technologies, USA). Hydrogen carrier gas flow rate was 1.25 mL/min. The injector and detector temperatures were 270 and 320°C, respectively. The GC oven temperature program started at 140°C, held for 0 min, programmed at 10°C/min ramp to 200°C, held 2 min, programmed at 7°C/min to 235°C, held 0 min, 50°C/min to 280°C and held 6 min. Gas chromatography coupled to mass spectrometry (Trace Ultra gas chromatograph with programmed temperature vapourizing injector and GC/MS DSQ quadrupole instrument with electron and chemical ionization, both from Thermo Fisher Scientific, USA) and a capillary column DB 1-MS (30 m × 0.25 mm, 0.25 µm film thickness from Agilent Technologies, USA) were used for polyol identification by comparing the spectra to those of authentic standards. Helium carrier gas flow rate was 1.1 mL/min, 1 µl injected, injector temperature 180°C, MS ion source and transfer line temperatures were set to 220 and 280°C, respectively. The oven temperature program was the following: initial temperature 110°C, held for 0 min, 10°C/min ramp to 182°C, held 0 min, 2°C/min ramp to 196°C, held 0 min, 35°C/min ramp to 300°C and held 2.5 min. The chemicals used were purchased from Sigma-Aldrich Co. Carbohydrate System Check Mix from RESTEK, USA, was used as a QC control for checking polyols quantification. Concentrations were expressed as µg/mg fresh body mass.

Statistical analysis

We originally intended to subject all five species to all three treatments and thus to attain a balanced experimental design. However, we did not obtain sufficient numbers of larvae of *E. cassioides* and *E. pluto*. Therefore, only three species (*E. aethiops*, *E. medusa*, *E. pronoe*) were subjected to all three treatments; *E. cassioides* was subjected to AutumnCold and WinterCold treatments, and *E. pluto* to AutumnCold treatment only (cf. Fig. 1). This unbalanced design required re-calculating some of the below analyses separately within species and treatments.

SCPs and CrPC s were compared separately for AutumnCold (all five species), WinterCold (four species) and AutumnWarm (three species) using one-way ANOVAs. Factorial ANOVAs were used to compare SCPs from the two cold treatments, AutumnCold and WinterCold (four species, less *E. pluto*) and all three treatments (three species: *E. aethiops*, *E. medusa* and *E. pronoe*). We also correlated average SCP and CrPC values across all species and treatments, expecting that low SCP would be associated with high CrPC.

For analyses of cryoprotectant profiles, we used a multivariate ordination technique, the redundancy analysis (RDA) computed in CANOCO 5 (Ter Braak & Smilauer, 2013). RDA relates the composition of samples, here, the specific cryoprotectant concentrations measured per individual, to predictors characterizing the samples (i.e., species identity, treatment) and tests the significance of the ordination using Monte Carlo tests. We used non-transformed data and 999 permutations for each Monte Carlo test.

We ran the RDAs first separately within treatments, i.e. with five species for AutumnCold, four species for WinterCold and three species for AutumnWarm. These analyses visualized and statistically tested the differences among species subjected to these three treatments. Subsequently, for the four species for which at least two experimental treatments were available, we ran separate single-species analyses to visualize the differences in cryoprotectant profiles between treatments and to directly test for treatment effects.
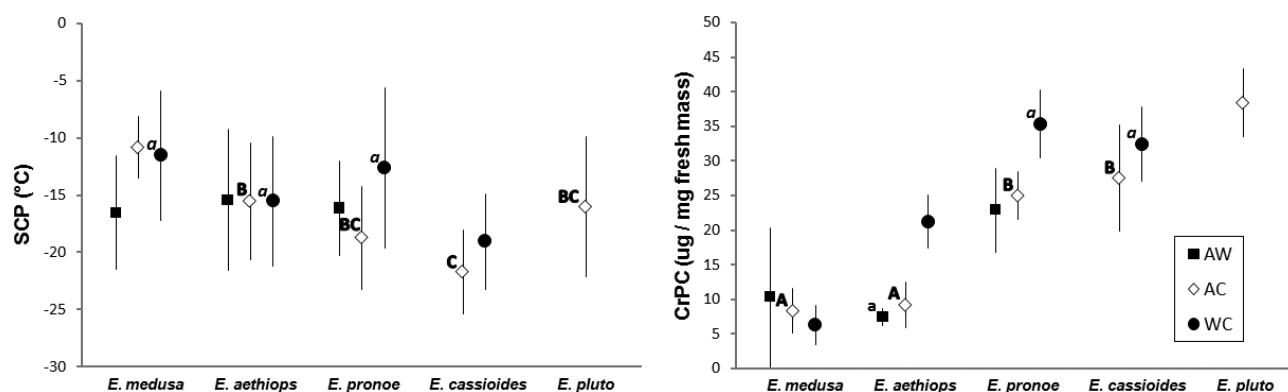
Fig. 1. Interspecific differences (means ± standard deviations) in the super cooling points (SCP – Plot A) and total cryoprotectant concentrations (CrPC – Plot B) in overwintering larvae of *Erebia* butterflies subjected to one of three treatments (AW – AutumnWarm, AC – AutumnCold, WC – WinterCold). Significance of the differences were assessed using one-way ANOVAs within individual treatments. Plot A – AW: $F_{(2,45 \, df)} = 0.16$, NS; AC: $F_{(4,75df)} = 12.60$, $P < 0.0001$; WC: $F_{(3,48 \, df)} = 5.10$, $P < 0.0001$. Plot B – AW: $F_{(2,26 \, df)} = 35.94$, $P < 0.0001$; AC: $F_{(4,45 \, df)} = 70.6$, $P < 0.0001$; WC: $F_{(3,36 \, df)} = 93.2$, $P < 0.0001$. The letters accompanying the means refer to results of Tukey's HSD tests.

RESULTS

Supercooling points relative to the concentrations of cryoprotectants

All larvae of *E. medusa* survived SCP measurement and had an obligate freeze-tolerant strategy. In the three high altitude species (*E. pronoe*, *E. cassioides*, *E. pluto*), no larva survived the measurements. A complex pattern appeared in *E. aethiops*, where in all three treatments, a minority of individuals (AutumnWarm: 3, AutumnCold: 5, WinterCold: 3; N = 16 in all cases) survived the freezing of their body fluids and the SCPs of surviving vs. non-surviving individuals differed markedly (AutumnWarm: –8.5 ± 2.10[SD]°C vs. –17.0 ± 5.68°C, AutumnCold: –10.1 ± 2.43°C vs. –18.0 ± 3.92°C, WinterCold: –10.3 ± 2.16°C vs. –16.7 ± 5.58 °C).

In the AutumnWarm treatment, which simulates conditions prior to first freezing, *E. aethiops*, *E. medusa* and *E. pronoe* did not differ in SCP, which were ≈ –16°C, despite significant differences in CrPC, which were much lower in *E. medusa* and *E. aethiops* than in *E. pronoe* (Fig. 1). In the AutumnCold treatment, which simulates conditions before the onset of very low temperatures, SCPs were highly significantly different. It was highest in the lowland *E. medusa*; followed by similar values in the submountain *E. aethiops*, subalpine *E. pronoe* and subnivean *E. pluto*, and lowest in the alpine *E. cassioides*. The pattern was only partly mirrored by the CrPCs, which were low for the two low altitude species (*E. medusa*, *E. aethiops*), followed by the subalpine *E. pronoe* and alpine *E. cassioides*, and highest in the subnivean *E. pluto*. In the WinterCold treatment, which simulates winter conditions, SCP was lower in the alpine *E. cassioides* than in lowland *E. medusa*, submountain *E. aethiops* and the subalpine *E. pronoe*. CrPC again showed a different pattern, with the lowest value recorded for the lowland *E. medusa*, followed by the submountain *E. aethiops*, and then by *E. pronoe* and *E. cassioides*.

The average values of SCP and CrPC were negatively correlated across species and treatments (N = 12), revealing that high CrPC is associated with a low temperature at which the body fluids freeze, but this was not statistically significant (Pearson's $r = –0.428$, $P = 0.165$).

The factorial ANOVA of SCP for the four species exposed to the two winter treatments (Table 1) pointed to a marginally significant increase in SCP in the subalpine *E. pronoe* and alpine *E. cassioides* subject to the WinterCold treatment (cf. Fig. 1). For CrPC, the low CrPC recorded in submountain *E. aethiops*, and the already high autumn CrPC recorded in subalpine *E. pronoe*, substantially increased in winter (Fig. 1). The comparison for the three species subjected to all three treatments (Table 1), revealed the following changes among treatments: SCPs, which did not differ in the AutumnWarm treatment, diverged in the AutumnCold treatment (it increased in *E. medusa*, remained unchanged in *E. aethiops*, and decreased in subalpine *E. pronoe*) and further diverged in the WinterCold treatment (increased in *E. pronoe*). CrPC of *E. aethiops* and *E. pronoe*, but not *E. medusa*, significantly increased from the AutumnCold to WinterCold treatment.

Table 1. Results of two-way ANOVA tests relating the super cooling points (SCP; sample size per species and month: N = 16) and total concentration of cryoprotectants (CrPC; N = 10) to species (4 spp.: *Erebia medusa*, *E. aethiops*, *E. pronoe*, *E. cassioides*; 3 spp.: *E. medusa*, *E. aethiops*, *E. pronoe*) and treatment (AW – Autumn-Warm, AC – AutumnCold, WC – WinterCold).

	Supercooling point (SCP)			Cryoprotectant concentration (CrPC)		
	df	*F*	P	df	*F*	P
4 species						
Intercept	1	1139.9	<0.001	1	1650.0	<0.001
Species	3	19.3	<0.001	3	123.3	<0.001
Treatment (AC, WC)	1	4.9	0.03	1	38.2	<0.001
Species*Treatment	3	2.4	0.07	3	9.7	<0.001
Error	108			72		
3 species						
Intercept	1	1029.8	<0.001	1	983.4	<0.001
Species	2	4.2	0.02	2	208.9	<0.001
Treatment (AW, AC, WC)	2	2.9	0.06	2	36.7	<0.001
Species*Treatment	4	3.6	0.008	4	13.4	<0.001
Error	125			81		
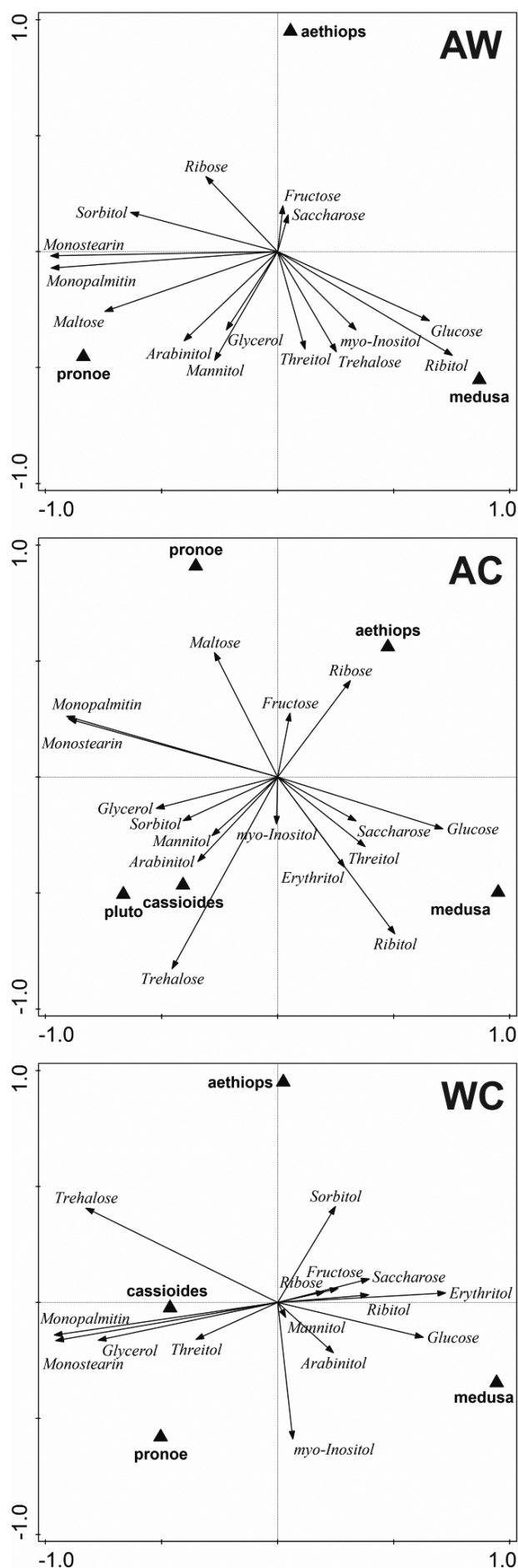
Cryoprotectant profiles

We detected 16 compounds with a putative cryoprotectant function (Table 2). Three of these (myo-inositol, erythritol and ribose) never reached a concentration of > 0.1 μg/mg fresh mass. The three compounds with highest average concentrations, across all treatments, were: trehalose, glucose and saccharose in *E. medusa*; and trehalose, monostearin and monopalmitin in the remaining four species.

Correlating concentrations of individual polyols with SCPs (N = 12 in all the following cases) revealed three significant, counterintuitively positive, correlations: with erythritol (r = 0.660, P = 0.02), glucose (r = 0.658, P = 0.02) and saccharose (r = 0.750, P = 0.005). For the three most abundant compounds, we detected negative but statistically not significant correlations (monopalmitin: r = –0.416, P = 0.18; monostearin: r = –0.445, P = 0.15; trehalose: r = –0.315, P = 0.32).

All three RDA analyses comparing cryoprotectant profiles among species within individual treatments revealed significant patterns. In all three treatments, the first ordination axes differentiated low altitude species (with *E. medusa* always the most extreme) from alpine/subnivean species (Fig. 2). The second ordination axes pointed to a difference between *E. aethiops* and the remaining species in AutumnWarm and WinterCold treatments, and between *E. aethiops* and *E. pronoe* relative to the remaining three species in the AutumnCold treatment.

The AutumnWarm treatment (3 species) revealed that lowland *E. medusa* contained high concentrations of glucose, but also trehalose and ribitol. The concentrations of fructose, ribose and saccharose were relatively high in *E. aethiops*. Arabinitol, maltose, monopalmitin, monostearin and sorbitol were characteristic of the subalpine *E. pronoe* (Fig. 2). In the AutumnCold treatment (5 species), the lowland *E. medusa* again had high concentrations of glucose, which was now accompanied by erythritol, ribitol, saccharose and threitol. *E. aethiops* had high concentrations of fructose and ribose, while *E. pronoe*, the alpine grasslands dweller, contained relatively high concentrations of maltose, plus monopalmitin and monostearin. The two species presumably adapted to the harshest conditions, *E. pluto* and *E. cassioides*, had the highest concentrations of compounds believed to be responsible for cold hardiness: glycerol and trehalose, plus other common cryoprotectants: arabinitol, mannitol and sorbitol (Fig. 2). The situation was similar in the WinterCold treatment (Fig. 2), except for the high concentration of glycerol (still accompanied by monopalmitin, monostearin, trehalose and threitol) in *E. pronoe* and *E. cassioides*. Sorbitol and trehalose were high

Fig. 2. RDA ordination biplots, analyses of the concentrations of individual cryprotectants, with respect to species. Pseudo-F and P values obtained using Monte-Carlo tests (999 permutations). Plot AW – AutumnWarm treatment: Canonical axes eigenvalues: 0.712, 0.043; first axis pseudo-F = 64.2, P< 0.001; all axes pseudo-F = 40.0, P < 0.001. AC – AutumnCold treatment: Eigenvalues 0.542, 0.270, 0.024, 0.003; first axis pseudo-F = 53.1, P < 0.001; all axes pseudo-F = 58.5, P < 0.001. WC – WinterCold treatment: Eigenvalues 0.802, 0.061, 0.013; first axis pseudo-F = 146.0, P < 0.001; all axes pseudo-F = 85.2, P < 0.001.

Table 2. Putative cryoprotectants: mean concentrations (and their standard deviations) expressed in µg/mg fresh body mass of all the five species tested and three treatments (AW – AutumnWarm, AC – AutumnCold, WC – WinterCold). Only compounds reaching a higher concentration than 0.1 µg/mg fresh body mass are listed.

	E. medusa			E. aethiops			E. pronoe			E. cassioides		E. pluto
	AW	AC	WC	AW	AC	WC	AW	AC	WC	AC	WC	AC
Trehalose	3.30±4.66	4.07±2.26	1.28±1.35	0.37±0.43	0.88±1.03	11.90±3.12	0.90±0.44	0.95±0.28	8.68±0.88	11.28±2.40	11.83±3.64	13.19±1.90
Monostearin	0.37±0.16	0.51±0.14	0.43±0.19	2.87±0.59	3.43±1.23	3.75±1.60	12.31±3.82	13.81±2.33	14.55±3.14	9.33±4.15	12.09±3.72	12.60±4.63
Monopalmitin	0.20±0.08	0.26±0.06	0.23±0.10	1.63±0.31	2.05±0.79	2.36±1.00	7.16±2.08	8.13±1.64	8.88±1.68	5.34±2.64	6.70±2.18	7.74±2.69
Glycerol	0.42±0.41	0.27±0.05	0.35±0.09	0.30±0.05	0.40±0.15	0.57±0.22	0.49±0.10	0.68±0.10	0.86±0.05	0.51±0.08	0.63±0.06	2.40±2.75
Sucrose	1.26±0.77	1.58±0.66	1.25±0.77	1.36±0.56	1.25±0.67	1.19±0.49	1.12±0.21	0.69±0.27	0.96±0.27	0.45±0.17	0.34±0.24	1.44±0.20
Glucose	1.37±0.76	1.29±0.65	2.23±1.31	0.47±0.19	0.60±0.19	1.06±0.44	0.38±0.26	0.42±0.03	0.99±0.34	0.43±0.09	0.61±0.24	0.40±0.20
Fructose	0.28±0.72	0.04±0.02	0.10±0.09	0.30±0.21	0.35±0.40	0.10±0.05	0.17±0.06	0.08±0.03	0.09±0.03	0.02±0.02	0.02±0.02	0.17±0.06
Arabinitol	0.03±0.02	0.04±0.01	0.11±0.04	0.02±0.02	0.03±0.03	0.09±0.07	0.05±0.01	0.02±0.02	0.11±0.03	0.04±0.02	0.05±0.02	0.15±0.12
Mannitol	0.02±0.01	0.02±0.01	0.04±0.01	0.01±0.01	0.02±0.03	0.04±0.02	0.03±0.00	0.01±0.02	0.05±0.01	0.01±0.02	0.02±0.02	0.07±0.07
Threitol	0.14±0.11	0.14±0.03	0.10±0.04	0.08±0.01	0.11±0.03	0.10±0.03	0.12±0.02	0.07±0.03	0.13±0.02	0.12±0.04	0.13±0.05	0.09±0.07
Ribitol	0.13±0.05	0.11±0.03	0.10±0.05	0.03±0.01	0.04±0.01	0.07±0.03	0.02±0.00	0.01±0.01	0.05±0.01	0.06±0.02	0.08±0.02	0.05±0.01
Sorbitol	0	0	0.02±0.02	0.02±0.02	0.03±0.03	0.04±0.04	0.03±0.01	0.01±0.02	0.01±0.02	0	0	0.10±0.03
Maltose	0.01±0.02	0.02±0.07	0	0.03±0.06	0.02±0.03	0	0.14±0.05	0.13±0.02	0	0	0	0.04±0.05

in *E. aethiops*, while the lowland *E. medusa* contained high concentrations of arabinitol, erythritol, glucose, ribitol and sucrose.

Comparing the effects of treatments for each species separately pointed to differences in the polyol profiles recorded in the three treatments for *E. medusa*, *E. aethiops* and *E. pronoe*, but not in the two cold treatments for *E. cassioides*. The distinctions between WinterCold and the two autumn treatments were always greater (i.e., fitted by the first canonical axes) than those separating the two autumn treatments (Fig. 3).

In lowland *E. medusa* (Fig. 3A), a decrease in temperature (AutumnCold) was associated with an increase in the concentration of monopalmitin, monostearin, saccharose, threitol and trehalose. With continuing winter, there was not only a remarkable increase in erythritol, but also an increase in arabinitol, glucose, mannitol and sorbitol. In *E. aethiops* (Fig. 3B), maltose prevailed in the AutumnWarm, followed by fructose in the AutumnCold treatment. Then, in the WinterCold treatment, there was big increases in multiple cryoprotective compounds, mainly arabinitol, erythritol, glycerol, ribitol and trehalose, which were similar to the increase in total CrPC concentration (see Table 1, Fig. 1). Similar changes occurred in *E. pronoe* (Fig. 3). Here, the main AutumnWarm compound was maltose, accompanied by fructose and sorbitol. The increase in CrPC in WinterCold was due to arabinitol, glucose, glycerol, mannitol, ribitol and trehalose. Finally, the analysis for *E. cassioides* indicated a non-significant increase in all cryoprotectants between AutumnCold and WinterCold (Fig. 3D).

DISCUSSION

The findings regarding the mean super cooling point did not support our initial expectation, derived from Vrba et al. (2012) that species experiencing less predictable snow cover (i.e., the lowland *E. medusa* and submountain *E. aethiops*) should be more cold hardy than the alpine and subnivean species (*E. pronoe*, *E. cassioides* and *E. pluto*). The only (weak) support for such a pattern was the lower SCP recorded for *E. cassioides*, a species associated with stony substrates, than in the alpine grasslands dwelling *E.*

pronoe (cf. Kleckova et al., 2014). Our second prediction that the highest levels of cold hardiness would be recorded during winter was not the case for the two alpine species, *E. cassioides* and *E. pronoe*. Their high autumn super cooling ability could be due to a less predictable snow cover in autumn than later in winter. In the lowland *E. medusa* and submountain *E. aethiops*, no changes in cold hardiness were recorded between the AutumnCold and WinterCold treatments. Our third prediction, i.e. that a higher cryoprotectant content would be associated with a lower SCP, was also only partly supported; the two values were correlated negatively, but not statistically significantly. Arguably, the patterns in cold hardiness were complicated by the two different cold hardiness strategies of the species studied and hence differences in polyol profiles among individual species. Individual cryoprotectants also tend to replace their precursors during the season, complicating their long term comparison.

Variation in cold hardiness and cryoprotectant content

Consistently with Vrba et al. (2012) we recorded the freeze tolerant cold hardiness strategy in lowland *E. medusa* and freeze-avoidant strategies in the three high-altitude species (*E. pronoe*, *E. cassioides*, *E. pluto*). A mixed situation was recorded in submountain *E. aethiops*, in which some of the larvae (those with high SCP) survived ice formation in their body fluids.

Freeze-avoidance seems to be an evolutionarily older and more common strategy (Vernon & Vannier, 2002). The apparently less common freeze tolerance occurs either in habitats with prolonged periods of extremely low temperatures (Turnock & Fields, 2005) or in unpredictable climates with repeated freeze-thaw events (Sinclair et al., 2003). We speculated earlier (Vrba et al., 2012) that lowland *E. medusa* is more likely to experience freezing temperatures without snow cover and freeze-thaw cycles than the alpine species. The results recorded here document that freeze tolerance in *E. medusa* occurs independently of pre-winter acclimation conditions.

A fifth of the individuals of the submountain *E. aethiops* assayed for SCP survived the freezing of their body fluids,
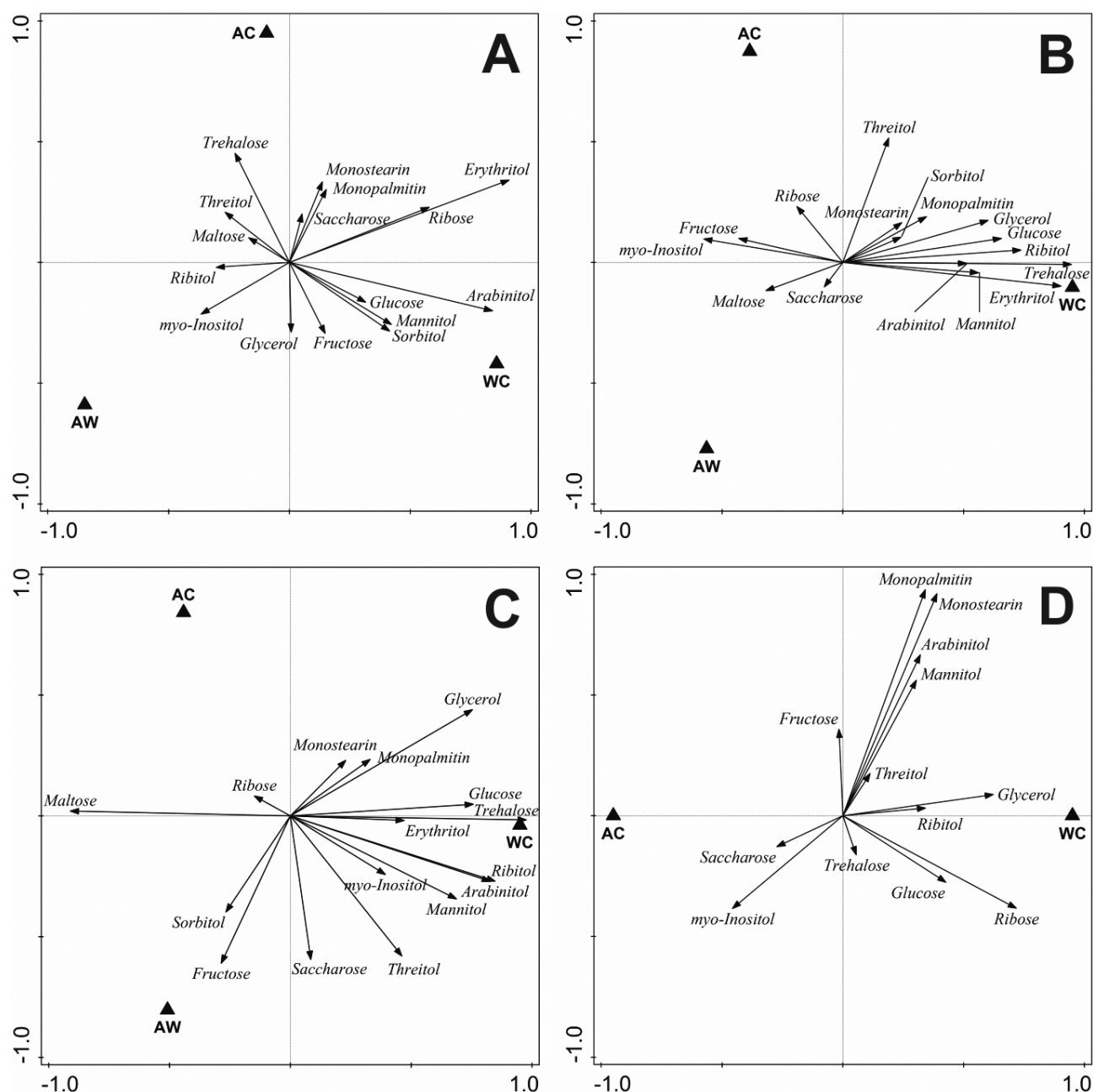
Fig. 3. RDA ordination biplots, analyses of the concentrations of individual cryoprotectants, with respect to different treatments (AW – AutumnWarm, AC – AutumnCold, WC – WinterCold). Monte-Carlo tests results: A – *Erebia medusa* (eigenvalues 0.223, 0.085; first axis pseudo-F = 7.5, P < 0.001; all axes pseudo-F = 5.8, P < 0.001). B – *Erebia aethiops* (eigenvalues 0.753, 0.004; first axis pseudo-F = 82.1, P < 0.001; all axes pseudo-F = 41.9, P < 0.001). C – *Erebia pronoe* (eigenvalues 0.785, 0.022; first axis pseudo-F = 98.6, P < 0.001; all axes pseudo-F = 56.6, P < 0.001). D – *E. cassioides* (eigenvalue 0.113; first axis pseudo-F = 2.3 NS).

which indicates the existence of a mixed strategy. So far, evidence for alternating strategies within one species, or co-existence of strategies within a single population, are extremely scarce. A lepidopteran example of a mixed strategy might be *Papilio zelicaon* Lucas, 1858 (Lepidoptera: Papilionidae), in which winter-acclimated pupae partly survive and are partly killed by internal ice formation (Williams et al., 2014). Two beetle species, *Dendroides canadensis* Latreille, 1810 (Pyrochroidae) (Horwath & Duman, 1984) and *Cucujus clavipes* Fabricius, 1781 (Cucujidae) (Kukal & Duman, 1989) may adapt their cold hardiness strategies along a latitudinal gradient, or accord-

ing to acclimation conditions (Sformo et al., 2010). In the case of *E. aethiops*, this species' range includes warm non-alpine habitats, such as piedmont forest steppes and lowland wooded savannas (Franco et al., 2006; Slamova et al., 2011), as well as mountain forests openings and grasslands (Sonderegger, 2005). In diverse areas such as the Alps, such contrasting habitats may well occur within an individual female's dispersal range (cf. Slamova et al., 2013) and the species hence could have evolved either mechanisms to adapt strategies according to external conditions or polymorphism within populations. The freeze tolerant strategy is less energy demanding and does not require

emptying the gut, which combines the possibility of food intake and ability to survive unpredictable cold snaps at the same time. On the contrary, the predictable, cold subnivean conditions in montane habitats may favour an alternative freeze-avoiding strategy via investment in super cooling (cf. Sinclair et al., 2003). More detailed, family controlled studies of this phenomenon are needed.

The recorded SCP values were nearly identical for the three *Erebia* species subjected to the treatment mimicking pre-freezing in early autumn (AutumnWarm), but diverged in the treatment mimicking onset of autumn freezing (AutumnCold). Moreover, for two species earlier assayed (Vrba et al., 2012), *E. cassioides* and *E. medusa*, our present AutumnCold values differed dramatically from the previously reported values, being much lower for the alpine *E. cassioides* (–19.0°C vs. –8.4 ± 2.8°C in the earlier study) and much higher for the lowland *E. medusa* (–17.0 ± 2.3°C vs. –11.5°C in present study).

This difference is clearly due to the acclimation conditions and nicely mirrors the contrasting cold hardiness strategies. In Vrba et al. (2012) the larvae were simply acclimated to constant 5°C. Assaying the cold hardiness of animals acclimated to a constant 5°C is frequently used in cold hardiness studies, as it facilitates rapid inter-species comparisons (Denlinger et al., 1992). In natural settings, however, external conditions change during winter, causing the changes in SCP values recorded here and elsewhere (Vrba et al., 2017).

The AutumnCold treatment temperatures, to which the alpine and freeze-avoidant *E. cassioides* larvae were exposed, likely crossed a threshold for a decrease in SCP (Vesala et al., 2012). The larvae dramatically increased their cryoprotectant concentration and decreased their SCP. Due to limited material, we were not able to assay the SCP of *E. cassioides* in the AutumnWarm treatment, which might be particularly informative.

In *E. medusa*, it is likely that the exposure to freezing temperatures initiated mechanisms that allow controlled freezing at relatively high subzero temperatures (Turnock & Fields, 2005). Although repeated freezing and thawing increases risks of tissue damage (cf. Marshall & Sinclair, 2011), freezing at higher temperatures decreases energy costs due to repeated synthesis of cryoprotectants (Voituron et al., 2002). Increase in SCP occurred also in the subalpine *E. pronoe*, in this case between AutumnCold and WinterCold treatments, which differed in the length of acclimation.

All these differences in SCP recorded under slightly different conditions imply that standardized cold hardiness measuring procedures can convey some information about the physiological capacities of the species studied, but are of limited value for understanding an insects' ability to survive extremely cold conditions.

Cryoprotectant concentrations mirrored SCP only to a limited extent. Alpine species always had a higher CrPC than lowland species. The two high altitude species, *E. pronoe* and *E. cassioides*, had high CrPCs already during the warm autumn treatment. In this respect, they resembled the boreomontane *Colias palaeno* (Linnaeus, 1761) (Lepidoptera: Pieridae), a species that has a high CrPC in autumn, but can decrease its cryoprotectant content in mild winters (Vrba et al., 2017). In contrast, the submountain *E. aethiops* doubled its cryoprotectant content between AutumnCold and WinterCold treatments, probably in association with the plastic reactions to external cues in this mixed-strategy species. Correlating individual cryprotectant content with SCPs provided some indication of why total cryoprotectant content did not mirror cold hardiness. Compounds such as glucose or sucrose, used as precursors in the synthesis of other polyols (Kostal et al., 2004), reached high concentrations under conditions resulting in a high SCP (i.e., in AutumWarm treatment). Also, the presence of two alternative cold hardiness strategies in the species studied precluded a straightforward association between total cryoprotectant content and supercooling point, because freeze tolerant and freeze avoidant species use cryoprotectants in different ways (Turnock & Fields, 2005). On the other hand, we showed earlier that individual populations of the boreomontane *C. palaeno* may dramatically differ in cryoprotectant profiles but not in their cold hardiness (Vrba et al., 2014a). The role of specific cryoprotectants on the level of cold hardiness may differ across species and climatic conditions, as can be demonstrated, e.g., by the different role of glycerol content on cold hardiness in several species of Lepidoptera (Andreadis et al., 2008; Hou et al., 2009; Trudeau et al., 2010). In addition, the synthesis of cryoprotectants is only a part of a more complex mechanism of increasing cold hardiness. Other important components are the synthesis of antifreeze proteins and active removal of ice-nucleating agents from the body (Somme, 1982; Duman, 2001). Clearly, surveying changes in cryoprotectant profiles may also provide more precise insights into the overwintering of alpine Lepidoptera.

Cryoprotectant profiles

In all three treatments, the analyses of differences in cryoprotectant profiles among species revealed a lowland–alpine gradient, partly supporting our prediction that species inhabiting similar conditions should employ similar cryprotective compounds. The lowland and freeze-tolerant *E. medusa* differed from the high altitude and freeze-avoiding *E. cassioides*, *E. pluto* and *E. pronoe*.

All the species studied had high contents of trehalose, a compound commonly found in insects, while the glycerol content, frequently cited as major cryoprotectant in Lepidoptera (Cha & Lee, 2016), including the boreomontane butterfly *Colias palaeno* (Vrba et al., 2017), was surprisingly low. The four high altitude species contained high volumes of monopalmitin and monostrearin, two compounds rarely associated with cryoprotective functions (Vrba et al., 2017), while the lowland *E. medusa* contained metabolically active precursors (glucose, sucrose) and rarer compounds, such as ribitol, erythritol and threitol. A different situation occurred in *E. aethiops*, which contained precursors or constitutive cryoprotectants different from both *E. medusa* and the alpine species, such as a relatively high concentration of fructose in both autumn treatments.

Autumn and winter samples were differentiated along the main ordination axis, whereas the vertical axis, indicating secondary gradients in cryoprotectant variation, corresponded to differences within autumn. For the three species with significant results, the patterns corroborated the differences between lowland and mountain/alpine species outlined above. Thus, *E. medusa* synthesized trehalose already in autumn, similar to species in which cold hardiness is achieved before the onset of freezing [e.g. *C. palaeno* in Vrba et al. (2017)]. In contrast, *E. aethiops* and *E. pronoe* increased their cryoprotectants only after exposure to freezing.

The contents of cryoprotectants in overwintering *Erebia* larvae thus vary considerably both among species and during the overwintering period. Putatively, this diversity seems to be linked both with the diversity of cold hardiness strategies (tolerant, mixed, avoidant), which in turn probably allows *Erebia* butterflies to exist in a high diversity of climatic conditions, from lowlands to subnivean zones and from wetlands to semiarid conditions. Notably, Shimada (1988) and Williams et al. (2014) also demonstrate that a high diversity of cryoprotective strategies is linked with diversity of cryoprotectant profiles in another widely distributed butterfly genus, *Papilio* Linnaeus, 1758.

CONCLUSION

Our study of five ecologically different species of *Erebia* revealed a high level of diversity in the physiology of cold hardiness within this butterfly genus, including different profiles of cryoprotectants, which may or may not provide similar levels of cold hardiness in the different species. It is tempting to speculate that switching among biosynthetic pathways, leading to synthesis of species-specific cryoprotectant mixtures, allowed *Erebia* to adapt to a diversity of extreme conditions. Such an inference, however, requires a better understanding of inter-specific cryoprotectants and diversity of cold hardiness in other insect taxa. Such knowledge is currently fragmentary, and the modest sampling of taxa used in this study is still much wider than that used in studies on other lepidopteran genera [cf. Pullin & Bale (1989a): two Nymphalidae species; Kukal et al. (1991): four species of *Papilio*; Vrba et al. (2017): two species of *Colias*]. It is now necessary to undertake a wider and more systematic sampling of taxa in order to distinguish whether the diversity of strategies and cryoprotectant profiles found in *Erebia* is particular to this genus, or perhaps Satyrinae butterflies in general, or a general characteristic of groups adapted to climatically extreme environments.

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