Contrasting Supercooling Ability in Lowland and Mountain European *Colias* Butterflies[1]

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Abstract We report different values of supercooling points (SCP) of overwintering larvae of 4 European species belonging to the globally-distributed butterfly genus *Colias*. The selected species represent diverse habitat and altitude preferences, from dry and warm steppe grasslands to alpine meadows and peat bogs. The Mediterranean migrant *C. crocea* Geoffroy did not survive the acclimation temperature of 5°C. All 4 remaining species were freeze-susceptible. *Colias palaeno* (L.), a peat bogs species, showed a high degree of cold hardiness (mean SCP: –24.8°C). It was followed by the steppe grassland specialist *C. alfacariensis* Ribbe (–18.6°C). Alpine *C. phicomone* (Esper) (–13.8°C) and lowland generalist *C. hyale* (L.) (–14.5°C) exhibited similar high values. We argue that besides ambient temperature, the specific microclimate at overwintering sites, and continentality influencing snow cover influence the diversity of cold hardiness in *Colias* butterflies.

Key Words butterfly ecology, diapause, frost survival, mountain habitats, grasslands, Palaerctic region, Pieridae

Temperature is one of the most important environmental factors influencing insect survival. This is particularly prominent in cold mountain or polar regions, where insect activity is restricted to only short periods of the year and complex strategies of winter period survival have evolved (Roff 1980, Boggs and Inouye 2012). The bulk of Lepidoptera cold hardiness studies target pest species (e.g., Andreadis et al. 2008, Hou et al. 2009), with only few studies focusing on species native to arctic or alpine (Dillon et al. 2006, Vrba et al. 2012) environments. These species, however, may be particularly sensitive to temperature-mediated habitat loss caused by recent climatic warming (Boggs and Murphy 1997, Dirnböck et al. 2011, Konvička et al. 2003). The ecophysiological adaptations and constraints restricting cold-adapted specialists to cold environments remain largely unknown.

Each of the 2 insect cold hardiness strategies (freeze tolerance and freeze avoidance) seems to be advantageous in certain climatic contexts (Turnock and Fields 2005). The role of microclimate at overwintering sites is also important, mainly the buffering role of snow cover or moss hummocks (Marshall and Sinclair 2011b, Vrba et al. 2012). Winter survival, thus, depends on combinations of physiological mechanisms and microhabitat use (Layne et al. 1999, Wagner et al. 2012). Because freezing

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is lethal for freeze avoidant species, their SCP is slightly lower than, or equal to, their lower lethal temperature, and SCP gives a rough estimation of their cold hardiness.

The species-rich globally-distributed butterfly genus *Colias* (F.) (Lepidoptera: Pieridae) is remarkable for its diversification in a wide range of nonforested habitats, from tropical savannas and temperate grasslands to arctic and alpine tundras (Verhulst 2000 - 2001). Out of about 80 species described to date, 9 species occur in Central Europe, but even here, they display remarkable diversity of altitude ranges (lowland to alpine), habitats (wetlands to dry steppes), and life histories (sedentary, partial and obligatory migrants) (Tolman and Lewington 2008). All species overwinter as larvae, which facilitates comparison of larval cold hardiness.

We report here how overwintering larvae of 5 ecologically divergent European *Colias* species differ in their cold hardiness, mainly supercooling points, and related cold-survival strategies. We initially hypothesised that species inhabiting cooler mountain environments should exhibit lower SCP values than lowland species. Alternatively (*cf.* Vrba et al. 2012), species occurring in regions and habitats with reliable insulation by snow cover may abandon the investment to antifreeze adaptations, which may result in higher SCP values than in species overwintering in habitats with little snow.

Materials and Methods

The 5 studied species form an altitudinal cline from alpine cold habitats to warm lowlands (Verhulst 2000 - 2001, Tolman and Lewington 2008).

Colias phicomone (Esper) is an alpine species, restricted to Europe and distributed in the Cantabrian Mts., Pyrenees, Alps and Eastern Carpathians. Its altitudinal range is 900 - 2500 m, its habitats include alpine grasslands and pastures, its larvae feed on various Fabaceae (e.g., *Astragalus*, *Medicago*, *Trifolium*). The development is univoltine, with one generation per year.

Colias palaeno (L.) is a widely-distributed species of the Eurasian Boreal zone, with more southerly outposts in mountainous regions between France and Japan. Its habitats include open taiga woodlands, peat-bogs and alpine heathlands, provided that its single host plant, *Vaccinium uliginosum* L., is present. The altitude range in Central Europe is 400 - 2200 m. A single generation occurs per year.

Colias hyale (L.) is distributed from Central Europe to NE China. In Central Europe, it occurs from sea level up to approx.. 1800 m on a wide-range of nonforested habitats, preferring farmlands with Fabaceae fodder crops used as larval host plants (e.g., *Medicago sativa*, *Trifolium* spp.). The species is polyvoltine, regularly forming three generations per year in lowland Central Europe, and behaves as a highly mobile partial migrant in northern part of its range.

Colias alfacariensis Ribbe, 1905

This steppe grasslands specialist is distributed from southwestern Europe to Central Asia. In Central Europe, it displays a distinct association with dry and warm seminatural grasslands in lower-elevated regions. Its larval hosts include *Coronilla varia* and *Hippocrepis comosa*. Polyvoltine species with three generations per year in Central Europe.

Colias crocea (Fourcroy, 1785)

An obligatory migrant, permanently surviving in the Mediterranean region and regularly advancing toward Central and Northern Europe with progression of season. It can reach Central Europe already in early summer, but the highest numbers are encountered from late August to October, corresponding to the third or fourth annual generation. Often encountered on Fabaceae field crops.

Wild-captured fertilized females of each studied species were left to oviposit in an outdoor rearing facility in lowland seminatural conditions in Ceske Budejovice, Czech Republic (49°00'N, 14°25'E, 400 m a.s.l.). *Colias crocea, C. hyale* and *C. alfacariensis* were captured as the autumn generation in lowlands of the Czech Republic. The two univoltine species, *C. palaeno* and *C. phicomone*, originated from mountain peat-bogs in Sumava Mts., SW Czech Republic (49°00'N, 13°34'E, altitude 400 m) and Otztaler Alps, Austria (46°58'N, 11°05'E, altitude 2000 m), respectively.

The rearing facility, situated in a half-shaded garden corner, consisted of wired cages (50 × 50 × 100 cm) covered by nylon mesh. Each cage contained 2 flower pots with the respective host plants. We released up to 5 females into each cage, fed them by 5% sugar solution to maximize their life span and hence the number of eggs laid. After the larvae hatched, we left them to develop without disturbance, except for watering the host plants.

During November, we transferred the dormant larvae from outdoor cages to a constant laboratory temperature of 5°C for standardized acclimation. They remained stored at this temperature until early January.

Sixteen larvae per species were used to estimate the supercooling point (SCP), using a thermocouple device (Brunnhofer et al. 1991). The cooling rate was controlled at 1 degree per minute. After the exotherm appeared, the larva was kept in the cooling device until its body temperature decreased again to the value of the crystallization temperature. The chamber was then removed from the cooling device, the body ice left to melt and the body temperature was left to increase to 5°C. The larva was then removed from the chamber and kept in a Petri dish at 5°C for 24 h. Subsequently, it was transferred into the laboratory, and survival was checked as presence of spontaneous movement or reaction to mechanical stimuli.

Results

Of the 46 *C. crocea* larvae tested, none survived the acclimation temperature (5°C). They were gradually dying in the cold room, and no surviving larvae were found after 2 weeks.

The remaining 4 species exhibited the freeze-avoidant strategy, not surviving freezing of their body fluids. The mean SCP differed among species ($F = 11.73$, $df = 3$, $P < 0.0001$). The strongest degree of cold hardiness, with the extremely low value of SCP = –24.8 ± 3.9°C, was found in the mountain butterfly *C. palaeno*, whose SCP was significantly lower than in all 3 remaining species (Tukey HSD: $P < 0.05$). The steppe grasslands specialist *C. alfacariensis* displayed the second lowest value (–18.6 ± 8.1°C); it differed from *C. palaeno*, but not from the remaining 2 species (Tukey HSD: $P < 0.05$). Still higher and similar values were found in the alpine species *C. phicomone* (–13.8 ± 3.5°C) and the common farmland species *C. hyale* (–14.5 ± 4.0°C) (Fig. 1).

Discussion

Identification of supercooling point and cold hardiness strategy represents just the necessary first step toward understanding of how insects survive cold parts of the year. The differences among 5 *Colias* butterflies inhabiting Central Europe, however, nicely reflect the diversity of conditions within their distribution ranges.

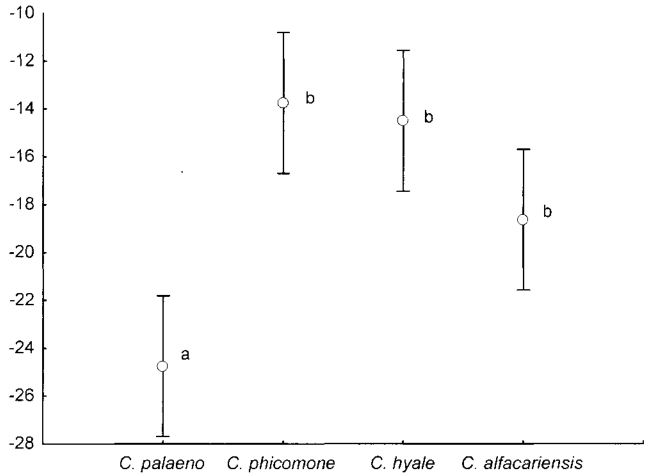

Fig. 1. ANOVA comparison of supercooling points among four European species of *Colias* butterflies. Means and SD of values measured in sixteen diapausing 3rd instar larvae per species.

Colias palaeno, the most cold-hardy species, inhabits a huge northern geographic range, within which it occupies a variety of habitats including humid bogs of European low mountains, heathlands above the timberline, boreal forest openings, and treeless tundra (Verhulst 2000 - 2001). Whereas, snow cover insulates the diapausing larvae from deep frosts in oceanic parts of the range, the frosts become deeper and snow cover less reliable in more continental regions of Eurasia. We show elsewhere (Vrba et al., unpubl data) that *C. palaeno*'s lowest lethal temperature (–26°C) is very close to the SCP in this species and that, at this temperature, 50% of individuals survive for almost 10 days. *Colias palaeno* larvae, thus, appear adapted for surviving extremely cold temperatures, even without insulation by snow, which makes it one of the most cold-hardy butterfly species assessed so far.

The distribution of *C. alfacariensis*, with the second-lowest SCP value, includes steppic grasslands of continental Eurasia. In these continental areas, its larvae likely encounter deep continental frosts with little snow cover (*cf.* Li and Zachariassen 2006), requiring good cold hardiness adaptations. The SCP value found for this species, however, is within the range commonly reported for temperate Lepidoptera (e.g., Dennis 1993).

A counterintuitive situation applies to *C. phicomone*, a species of alpine grasslands with low ambient winter temperatures. We have shown elsewhere, using the predominately mountain butterfly genus *Erebia* Dalman, that species of the high alpine habitats can display higher SCP (*E. tyndarus* Esper: –8.4 ± 2.8°C) than related species of the mountain belt (*E. sudetica* Staudinger: –15.1 ± 4.4°C) or lowland species (*E. medusa* Denis & Schiffermüller: –17.0 ± 2.3°C) (Vrba et al. 2012). The most obvious explanation is again insulation by snow cover (Marshall and Sinclair 2011a,b), which is durable and hence reliable at high altitudes in European mountians, probably allowing *C. phicomone* and other high alpine butterflies to abandon their freeze protection, in contrast to congeneric butterflies of continental steppes. A corresponding difference was found in 2 syntopic ladybird beetles; whereas *Ceratomegilla undecimnotata* (Schneider) overwintering exposed to air had a mean SCP of −19°C, *Coccinella septempunctata* (L.) overwintering at ground level insulated in plant material had a SCP of −15°C (Nedvěd 1993).

Due to unpredictable snow cover, *C. hyale* frequently encounters snowless episodes in lowlands of temperate Europe. Its SCP value, however, was similar to that of alpine *C. phicomone*, seemingly contradicting the snow cover explanation for the alpine species. On the other hand, *C. hyale* is a highly mobile species, considered as "partial migrant" by some authors and not able to overwinter in more northerly countries (Asher et al. 2001). Its high SCP value provides indirect evidence that its occurrence in more northerly locations depends on immigration from regions with milder winters.

The Mediterranean migrant *C. crocea* did not survive the acclimation temperature of 5°C. At such a temperature, the majority of lepidopteran larvae would stop feeding, entering a quiescence, if not already being in diapause. Ability to increase cold hardiness during cold acclimation is generally dependent on previously entering the diapause state (Košťál et al. 2011). This result confirms that no proper diapause exists in *C. crocea* (*cf.* Tolman and Lewington 2008), but also suggests that *C. crocea* winter larvae cannot survive in those parts of the Mediterranean basin where temperatures drop below 5°C for continuous periods of several weeks, precluding them to heat up and feed for at least parts of days. This effectively restricts *C. crocea* winter distribution to such thermally favorable parts of the Meditarranean as islands and coastal lowlands.

Given the diveristy of *Colias* SCP values, it is tempting to speculate on phylogenetic apsects of the pattern. Unfortunately, no comprehensive *Colias* phylogeny has been published, and the existing studies focus on either North American (Pollock et al. 1998, Wheat and Watt 2008, Schoville et al. 2011) or European (Brunton 1998) representatives, with minimum overlaps in taxa sampling. The phylogeny by Brunton (1998) suggests that northern and presumably cold-hardy species (represented by *C. palaeno* in this study) should be ancestral, with subsequent radiation into a clade containing temperate species with moderate SCP values (including *C. hyale*, *C. alfacariensis* and *C. phicomone*), and a clade containing Mediterranean and/or migrant species (including *C. crocea*). From this hypothesis, efficient cold hardiness in ancestral *Colias* spp., weakened as their descendants dispersed toward warmer areas, is expectable. This is contradicted by Pollock et al. (1998) or Wheat and Watt (2008), who proposed the Palaearctic migrants *C.crocea* and *C. erate* (Esper) as ancestral. Until a more comprehensive phylogeny encompassing both Eurasian and American representatives is available, speculations onto the evolution of cold hardiness variation within *Colias* remain premature.

In conclusion, the values of the supercooling point in the freeze avoiding larvae of European *Colias* butterflies do not follow a simple temperature-related gradient, and both very low and moderate SCP values exist in both mountain and lowland species. Besides ambient temperature, individual species thermal niches are influenced by the reliability of snow cover (continentality gradient), and the migration ability allowing some species to regularly recolonise thermally suboptimal habitats.

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