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Cold tolerance of the montane Sierra leaf beetle, *Chrysomela aeneicollis*

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ABSTRACT

Small ectothermic animals living at high altitude in temperate latitudes are vulnerable to lethal cold throughout the year. Here we investigated the cold tolerance of the leaf beetle *Chrysomela aeneicollis* living at high elevation in California's Sierra Nevada mountains. These insects spend over half their life cycle overwintering, and may therefore be vulnerable to winter cold, and prior studies have demonstrated that survival is reduced by exposure to summertime cold. We identify overwintering microhabitat of this insect, describe cold tolerance strategies in all life stages, and use microclimate data to determine the importance of snow cover and microhabitat buffering for overwinter survival. Cold tolerance varies among life history stages and is typically correlated with microhabitat temperature: cold hardiness is lowest in chill-susceptible larvae, and highest in freeze-tolerant adults. Hemolymph osmolality is higher in quiescent (overwintering) than summer adults, primarily, but not exclusively, due to elevated hemolymph glycerol. In nature, adult beetles overwinter primarily in leaf litter and suffer high mortality if early, unseasonable cold prevents them from entering this refuge. These data suggest that cold tolerance is tightly linked to life stage. Thus, population persistence of montane insects may become problematic as climate becomes more unpredictable and climate change uncouples the phenology of cold tolerance and development from the timing of extreme cold events.

1. Introduction

Montane habitats impose a range of environmental stresses on the organisms that inhabit them, including high ultraviolet radiation and insolation that cause high daytime body temperatures in exposed habitats, and low atmospheric pressure that cause desiccation stress and hypoxia (Sømme, 1989). In these high elevation habitats, low environmental temperatures occur year-round and winter temperatures may be especially cold, even at low latitude (Sømme, 1995). Environmental cold may have significant impacts on small ectotherms such as insects, affecting activity and growth, and causing mortality both through the action of cold on cells and molecules, and because of the likelihood of internal ice formation (Harrison et al., 2012). Prolonged exposure of montane insects to sub-zero temperatures may lead to overwintering mortality and sub-lethal reduction of fitness characters, like growth and reproductive output (Sinclair et al., 2003). Unfortunately, the physiology of cold tolerance in montane insects has been incompletely explored (e.g. Kohshima, 1984; Sinclair and Chown, 2005; Sømme, 1989; Vrba et al., 2012; Wharton, 2011; Zettel and Zettel, 1994), especially in the mountains of the Americas (but see Edwards, 1986; Ring, 1982; Sømme et al., 1996).

Low temperatures experienced by montane insects may be particularly influenced by the duration and depth of snow cover. Paradoxically, extreme low temperatures and freeze–thaw cycles are more common in montane habitats during climatically-warm winters, which often lack significant snowfall relative to cooler, wetter winters, during which accumulated snow cover provides significant insulation to overwintering insects (e.g. Sinclair, 2001b). Interactions between snow cover and extreme low temperature can be especially important in spring and autumn, when early accumulation or late melt of snow cover can extend the period during which environmental temperatures are buffered. In contrast, a reduced snow pack can expose overwintering organisms to extreme low temperatures in the spring and autumn, at times and/or life stages when the insect's cold tolerance may be inadequate for survival through the episode (Williams et al., 2015). Predicted winter climate change, with modified temperature and

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precipitation regimes, may therefore have a substantial impact on montane insects by modifying both the occurrence and intensity of cold exposure (Gu et al., 2008; Williams et al., 2015). Thus, understanding mechanisms of cold tolerance, and how it varies through the life history of an animal, is essential for understanding the potential impacts of climate change on organisms in montane environments.

Cold-hardy insects primarily use one of two strategies to survive low temperatures; they either withstand the formation of internal ice (freeze tolerance), or depress their supercooling point (SCP, the temperature at which they freeze) to remain unfrozen at low temperatures (freeze avoidance). Many insects, however, are killed by cold at temperatures where they remain unfrozen, and these are termed chill-susceptible (Lee, 2010). The biochemistry associated with insect cold tolerance is fairly well-described. Briefly, low molecular weight cryoprotectants (for example, polyols such as glycerol or sugars such as trehalose) can protect cells and membranes against the osmotic dehydration associated with freeze tolerance or colligatively depress the SCP in freeze avoidant insects. Improvements in cold tolerance have been associated with even small (10–100 mM) increases in the concentration of cryoprotectants (Lee et al., 1987), although the mechanisms of this protection are less well-understood (see MacMillan et al., 2015, for a possible relationship with water balance in the cold). Thermal hysteresis agents (including antifreeze proteins and glycolipids) inhibit recrystallization in freeze-tolerant species, or retard nucleation in freeze-avoidant insects (Walters et al., 2011; Zachariassen and Kristiansen, 2000). Finally, control of ice nucleation is a key difference between strategies, with freeze tolerant species generally initiating ice formation at consistently high (above −10 °C) sub-zero temperatures (Sinclair et al., 2009).

Most species of temperate insects have a specific life history stage in which they overwinter. Not surprisingly, insects are usually most cold tolerant during these overwintering stages, developing cold tolerance during the autumn (or during diapause development) and losing it upon resumption of activity in the spring (Lee, 2010). Not all overwintering insects must develop extreme cold tolerance. For example, larvae of acorn weevils have limited cold tolerance, but overwinter successfully in Southern Canada by relying on the buffering effect of being buried in the soil (Udaka and Sinclair, 2014). This is in contrast to species that are exposed to near-ambient temperatures in unprotected, above-snow, wintering microsites, like goldenrod gall flies, which have evolved robust strategies of freeze tolerance (Irwin and Lee, 2003). The high degree of plasticity of cold tolerance among different life history stages and among species suggests energetic and evolutionary trade-offs in cold tolerance strategy and capacity (see also Sinclair, 1999; Sinclair and Chown, 2010; Voituron et al., 2002; Zachariassen, 1985). Thus, understanding how cold tolerance changes among life stages and seasons is critical to understanding the constraints on cold tolerance. Unfortunately, there are surprisingly few studies of cold tolerance in montane insects that investigate cold tolerance of all life history stages, even those that provide year-round comparisons (e.g. Ramløv, 1999; Ramløv et al., 1992; Sinclair, 1997).

In this study, we investigate cold tolerance in the leaf beetle *Chrysomela aeneicollis*, an ideal species in which to investigate cold tolerance of montane species. This beetle lives on willows along streams, lakes, and bogs throughout Western North America (Brown, 1956; Dellicour et al., 2014); at the southern edge of their range in the southern Sierra Nevada Mountains of Eastern California at high elevation (2800–3200 m). Sierra willow beetles overwinter as adults, emerging from winter diapause in mid-May and completing a single cycle of reproduction and larval development during the summer. Adults mate and females lay eggs in June, eggs hatch and larvae mature through three instars in July and August, pupating in August and September. Newly-eclosed adults feed in August and September, before entering a winter quiescence (or possibly diapause) in early October (Smiley and Rank, 1986). Beetles living in high elevation habitats in the Sierra Nevada are challenged by exposure to potentially-lethal cold temperatures throughout the year. We have observed: (1) cold mortality in adults emerging from diapause after a single night time cold exposure to temperatures between −8 and −10 °C in June (Bruce, 2005; Dahlhoff and Rank, 2007); (2) cold-induced mortality in first- and second-instar larvae during exposure to temperatures between −4 and −6 °C in July (McMillan et al., 2005; Smiley and Rank, 1986); and (3) high mortality of pupae due to a single early frost in October (present study). Furthermore, long-term field observations of Sierra willow beetle populations show that abundance declines precipitously after extremely cold winters. For example, there was virtually no snowpack at most mid- and low-elevation sites in 2007, and beetle populations declined by 80% in the subsequent year, especially at mid- and low elevation localities (Smiley, Dahlhoff and Rank, unpublished observations). Thus, it appears that cold tolerance may be important year-round in *C. aeneicollis*.

2. Materials and methods

2.1. Study animals

We hand-collected *C. aeneicollis* adults and larvae from willow (primarily the Sierra willow *Salix orestera*) during the summer at sites around 3200 m elevation from three study drainages in the Eastern Sierra Nevada mountains of California, which have been well-described (e.g. Dahlhoff et al., 2008; Dahlhoff and Rank, 2000; Rank, 1992a): Big Pine Creek (BPC), Bishop Creek (BC) and Rock Creek (RC). We did not examine the well-described polymorphism at the glycolytic enzyme phosphoglucose isomerase (PGI) described in earlier studies (Dahlhoff and Rank, 2007; Rank, 1992a; Wheat and Hill, 2014), and preliminary experiments did not indicate significant differences in supercooling points of larvae or adults among drainages, so individuals from different drainages were pooled for all experiments. Eggs and first instar larvae were collected as full clutches by plucking the whole leaf on which the clutch was laid; no more than two individuals per clutch were used in any experiment. All other life stages were collected as individuals from willow leaves and returned in an insulated backpack within 12 h to the Owens Valley Laboratory of University of California's White Mountain Research Center (Bishop, CA), where all experiments were conducted. Beetles were maintained in groups of approximately 100 individuals in 5 l plastic containers at 20 °C, 12 h D: 4 °C, 12 h N (20 min ramp-up and -down time) inside an incubator (Percival, Perry, IA, USA). Beetles were fed on a diet of fresh *S. orestera* collected from 3000 m in Bishop Creek every 2–3 days. After laboratory manipulation, individuals used for later biochemical analysis were placed in 0.6 mL microcentrifuge tubes and frozen at −80 °C.

Summer adults, eggs and first instar larvae were collected and frozen for future analyses between July and August 2011. Second instar larvae, third instar larvae, pupae and newly-eclosed adults were collected and frozen between July and August 2012. Quiescent beetles were obtained either as field-collected pupae or newly-eclosed adult beetles and shipped to University of Western Ontario. Adult beetles were reared in 40 × 30 × 40 cm clear plastic cages with a mesh top for ventilation and were provided with *Salix amygdaloides* for food. *S. amygdaloides* is similar to *S. orestera* in that it has a high salicylate content that is preferred by *C. aeneicollis* (Rank, 1992b). In all cases, willow was changed 2–3 times per week, misted with water daily, and water provided