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Cold hardiness and deacclimation of overwintering *Papilio zelicaon* pupae

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ABSTRACT

Seasonally-acquired cold tolerance can be reversed at warm temperatures, leaving temperate ectotherms vulnerable to cold snaps. However, deacclimation, and its underlying mechanisms, has not been well-explored in insects. Swallowtail butterflies are widely distributed but in some cases their range is limited by low temperature and their cold tolerance is seasonally acquired, implying that they experience mortality resulting from deacclimation. We investigated cold tolerance and hemolymph composition of Anise swallowtail (*Papilio zelicaon*) pupae during overwintering in the laboratory, and after four days exposure to warm temperatures in spring. Overwintering pupae had supercooling points around −20.5 °C and survived brief exposures to −30 °C, suggesting partial freeze tolerance. Overwintering pupae had hemolymph osmolality of approximately 920 mOsm, imparted by high concentrations of glycerol, K^+ and Na^+. After exposure to spring warming, supercooling points increased to approximately −17 °C, and survival of a 1 h exposure to −20 °C decreased from 100% to 0%. This deacclimation was associated with decreased hemolymph osmolality and reduced glycerol, trehalose, Na^+ and Ca^{2+} concentrations. We compared cold tolerance of pupae to weather conditions at and beyond the species' northern range boundary. Minimum temperatures at the range boundary approached the lower lethal temperature of pupae, and were colder north of the range, suggesting that cold hardiness may set northern range limits. Minimum temperatures following warm snaps were likely to cause mortality in at least one of the past three years. Cold snaps in the spring are increasing in frequency as a result of global climate change, so are likely to be a significant source of mortality for this species, and other temperate ectotherms.

1. Introduction

The body temperatures of small ectotherms generally reflect those of the environment, particularly in immobile and/or quiescent overwintering stages (Harrison et al., 2012). At sub-freezing temperatures, insects risk ice formation in their body fluids. They mitigate this risk using cold tolerance strategies that are generally divided into freeze tolerance (those that withstand internal ice formation) and freeze avoidance (those that maintain the body fluids in a liquid state at low subzero temperatures) (Lee, 2010). Freeze-avoidance and -tolerance are both typically associated with a suite of biochemical adaptations, including carbohydrate and polyol cryoprotectants, and antifreeze proteins (Lee, 2010). The strategies differ in the control of ice nucleation (Zachariassen, 1985; Sinclair et al., 2009); freeze-tolerant insects generally have high supercooling points (SCP, the temperature at which ice formation begins), while freeze avoiders have depressed SCPs. For example, the SCP of freeze-tolerant larvae of *Pyrrharctia isabella* (Lepidoptera: Arctiidae) ranges from −3 to −12 °C (Marshall and Sinclair, 2011), while the SCP of overwintering freeze-avoidant *Phyllocnistis populiella* adults (Lepidoptera: Gracillariidae) averages −32 °C (Wagner et al., 2012). Few temperate insects maintain extensive cold tolerance year-round, but instead increase cold tolerance in preparation for winter (Leather et al., 1995; Lee, 2010).

The onset of low temperatures in winter can be unpredictable, so many temperate insects rely on photoperiod cues to reliably initiate pre-winter cold hardening (Bradshaw and Holzapfel, 2010). However, thermal cues may still modulate the degree of cold hardiness acquired (Storey and Storey, 1988). By contrast, the loss of cold hardiness and resumption of development at the end of winter are often regulated solely by temperature cues (Košťál, 2006), and warm snaps can trigger the loss of cold hardiness (deacclimation). For example, Emerald Ash borer (*Agrilus planipennis*; Coleoptera: Buprestidae) prepupae lose cold-hardiness in response to a mid-winter warm snap (Sobek-Swant et al., 2012). Because the loss of cold tolerance is accompanied by resumption of development, this deacclimation is irreversible (Sobek-Swant et al.,

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2012). Deacclimation can thus leave insects vulnerable to cold snaps in the spring. Global climate change is leading to earlier snow melt in the spring in many locations, which can expose subnivean organisms to increased thermal variability in the spring (Brown and DeGaetano, 2011) and induce deacclimation. This may underlie the increasing frequency of damaging spring frosts over the past 100 years (Augspurger, 2013). Thus, an essential part of predicting an organism's response to changing winters is understanding the propensity for and consequences of deacclimation.

Swallowtail butterflies (Lepidoptera: Papilionidae) have a broad global distribution, and in North America occupy climates ranging from tropical to sub-arctic (Lederhouse et al., 1995). There is a steep decline in species diversity with latitude: only 2 of ~500 papilionid species occur north of 60° latitude (Lederhouse et al., 1995). Cold hardiness limits the northern distribution of some species (Kukal et al., 1991). Swallowtails are thought to be susceptible to climate change; moving northward in warm years only to be knocked back in cold ones (Scriber and Gage, 1995). Globally, all swallowtails that occupy environments with a cold winter overwinter as pupae. There are examples of both freeze-tolerant (e.g. *Papilio machaon*) and freeze-avoidant (e.g. *Papilio xuthus*) species (Shimada, 1988; Kukal et al., 1991). The four species examined to date (*P. machaon*, *P. xuthus*, *Papilio canadensis*, *Papilio glaucus*) use glycerol or trehalose as their primary cryoprotectants, and cold tolerance increases concurrently with the accumulation of these cryoprotectants during winter (Shimada, 1988; Kukal et al., 1991). This seasonal acclimation is more pronounced in more cold-hardy species (such as *P. canadensis*), and is modified by the severity of the cold experienced. For example, cold-hardy *P. canadensis* pupae decrease their supercooling points when overwintering in Alaska compared to Michigan, but cold-susceptible *P. glaucus* pupae do not, and correspondingly suffer higher mortality in Alaska than do *P. canadensis* (Kukal et al., 1991). These interspecific differences in cold tolerance or acclimation ability may stem from differences in carbohydrate metabolism: *P. canadensis* synthesizes cryoprotectants from isotopically labeled glucose, while *P. glaucus* does not (Kukal et al., 1991). Since their distributions are set by cold, and their cold tolerance is seasonally acquired through plastic changes to physiology, swallowtail butterflies are an ideal system in which to study deacclimation and its impacts on survival.

Papilio zelicaon belongs to the Papilio (*sensu stricto*) lineage, which dispersed to North America across Beringia before and during the Pleistocene (Zakharov et al., 2004). Beringia comprised Alaska, the Yukon Territory, and the now-submerged Bering Land Bridge, and, as such, was home to a cold and harsh climate (Elias, 2000). This biogeographic history makes this group ideal for investigations of cold hardiness. *P. zelicaon* inhabits fields, oak savannahs, and roadsides throughout western North America (extending to North Dakota, 100°W) up to 60°N and feed on plants in the Apiaceae (Sims, 1980; Guppy and Shepard, 2012). *P. zelicaon* overwinter in a pupal diapause, and populations vary from univoltine (one generation per year) toward the northern range edge, to multivoltine (several generations per year) further south (Sims, 1980; Thorne et al., 2006). *P. zelicaon* is the most abundant swallowtail butterfly in British Columbia (BC); its range extends north into boreal climates on the mainland (Peel et al., 2007; Guppy and Shepard, 2012), but the factors that limit the northern distribution are unknown for this species. British Columbia is experiencing warming that is faster than the global average, and that warming is most pronounced in the north, and in the winter (Wang et al., 2006).

Winter warming is predicted to expand the ranges of animals and plants that are limited by cold (Williams et al., in press). However, this effect may be mitigated or reversed if winter warming causes energy drain, or if spring warming causes loss of winter cold acclimation, leaving pupae vulnerable to spring cold snaps (Williams et al., in press). Previously observed metabolic suppression means that *P. zelicaon* pupae are unlikely to be vulnerable to energy drain induced by winter warming (Pelini et al., 2009). We therefore investigate here whether they are at risk of increased mortality from cold snaps during spring, resulting from the loss of winter acclimation.

Here we report the cold tolerance strategy and hemolymph composition of *P. zelicaon*, as well as plasticity in cold tolerance and hemolymph composition in the face of a short warming period, similar to that which might be experienced during a late winter or early spring warm spell. We hypothesize that cryoprotectants are essential for cold tolerance, but that there are costs to maintaining high cryoprotectant concentration such that cryoprotectant concentrations will be reduced quickly at the end of winter. We predict, therefore, that exposure to warm spring temperatures will lead to deacclimation (loss of cold tolerance), which will be accompanied by a decrease in cryoprotectants. We then combine our physiological measurements with recent weather data to test the hypothesis that spring deacclimation could lead to mortality of this species in the wild.

2. Materials and methods

2.1. Study species and rearing

Gravid *P. zelicaon* females were wild-collected from multiple sites on Vancouver Island, B.C. at a latitudes between 48 and 50°N (see Pelini et al., 2009 for details), between April and June 2009, then maintained in greenhouses at the University of Western Ontario in individual cages and fed a 10% solution of honey water twice daily. The adults had constant access to potted parsley (*Petroselinum crispum*) plants that were checked every second day for eggs. After collection, eggs were shipped to the University of Notre Dame, IN, USA. Larvae were reared in growth chambers (MTR-30; Conviron, Winnipeg, MB, Canada) on potted parsley under temperatures approximating Vancouver Island conditions based on long-term climate data (1997–2006) from Victoria International Airport (The Weather Underground, Inc.) on a 12:12 L:D cycle (Pelini et al., 2009). Temperatures in the growth chambers cycled between average maximum and minimum temperatures, and were adjusted every two weeks to reflect seasonal changes (Fig. 1). In late August 2009, pupae and remaining larvae were returned to the University of Western Ontario where they were maintained under the same conditions in incubators (MIR-153, Sanyo Scientific, San Diego, CA, USA) in constant darkness. Pupae were transferred into 6-well tissue culture plates with a moist paper towel to maintain high humidity.

2.2. Cold tolerance experiment

During March and early April 2010, we estimated lower lethal temperatures of winter-acclimated pupae in response to 1-hour or 12-hour

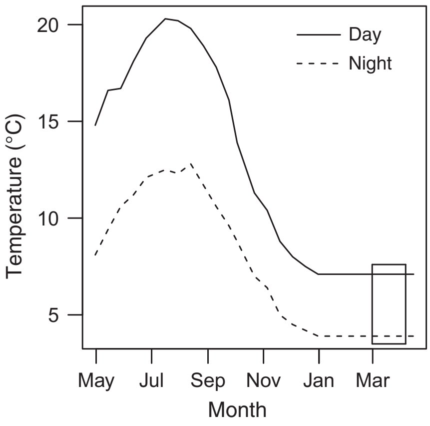

Fig. 1. Incubator temperature regimes for *Papilio zelicaon* pupae, based on historical mean biweekly highs and low from a weather station near collection locales. Temperatures cycled daily between daytime highs (solid line) and nighttime lows (dotted line). Experiments were performed during March and April (indicated with a box).