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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 by exposure to warm temperatures, leaving temperate ectotherms vulnerable to cold snaps in spring. However, this process of deacclimation, and its underlying mechanisms, has not been well-explored in insects. Swallowtail butterflies are widely distributed globally but in some cases their range is limited by low temperature and their cold tolerance seasonally acquired, implying that they may be vulnerable to 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 1h 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 were close to the lower lethal temperature of pupae, and temperatures north of the range were colder, suggesting that cold hardiness may set northern range limits on the mainland. 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.

Keywords: climate change, cold tolerance, cryoprotectant, deacclimation, insect, hemolymph, Lepidoptera, winter

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 (Sinclair et al., 2009; Zachariassen, 1985); 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 (Koštál 2006), and warm snaps can

trigger the loss of cold hardiness (deacclimation). For example, Emerald Ash borer (*Agrilus planiplennis*; 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., 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, and there are examples of both freeze-tolerant (e.g. *Papilio machaon*) and freeze-avoidant (e.g. *P. xuthus*) species (Kukal et al., 1991; Shimada, 1988). The four species examined to date (*P. machaon*, *P. xuthus*, *P. canadensis*, *P. glaucus*) use glycerol or trehalose as their primary cryoprotectants, and cold tolerance increases concurrently with the accumulation of these cryoprotectants during winter (Kukal et al., 1991; Shimada, 1988). 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 labelled 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 (Guppy and Shepard, 2012; Sims, 1980). *P. zelicaon* overwinter in a pupal diapause, and populations vary from univoltine (one generation per year) towards 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

105 for this species. British Columbia is experiencing warming that is faster than the global average,
106 and that warming is most pronounced in the north, and in the winter (Wang et al., 2006).

107 Winter warming is predicted to expand the range limits of animals and plants that are limited
108 by cold (Williams et al. 2014, in press). However, this effect may be mitigated or reversed if
109 winter warming causes energy drain, or if spring warming causes loss of winter cold acclimation,
110 leaving pupae vulnerable to spring cold snaps (Williams et al., in press). Previously observed
111 metabolic suppression means that *P. zelicaon* pupae are unlikely to be vulnerable to energy drain
112 induced by winter warming (Pelini et al., 2009). We therefore investigate here whether they are
113 at risk of increased mortality from cold snaps during spring, resulting from the loss of winter
114 acclimation.

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

125 **Methods**

Study species and rearing

Gravid 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 which were checked every second day for eggs. After collection, eggs were shipped to the University of Notre Dame. Larvae were reared in growth chambers (MTR-30; Conviron, Winnipeg, Manitoba, 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) in constant darkness. Pupae were transferred into 6-well tissue culture plates with a moist paper towel to maintain high humidity.

Cold tolerance experiment

During March and early April 2010, we estimated lower lethal temperatures of winter-acclimated pupae in response to one-hour or 12-hour cold exposures. To do this, pupae (n=5/ temperature) were held at -15, -20, -25, -30 and -35 °C for 1 h, or -20, -25 and -30 °C for 12 h. Individual pupae were placed in contact with 36-AWG type-T (copper-constantan) thermocouples into 12 mL plastic centrifuge tubes, which were inserted into an aluminum block cooled with methanol circulated from a refrigerated bath (Lauda Proline 3530, Würzberg,

149 Germany). The temperature was decreased from 8 °C at 0.1 °C·min^{-1} to the target temperature,
150 then maintained at the target temperature for either one or twelve hours. Temperatures from the
151 thermocouples were logged via a Picotech thermocouple interface using Picolog software for
152 Windows (v.5.2; Pico Technology, Cambridge, UK). The SCP was recorded as the lowest
153 temperature before the exotherm (release of heat) representing the release of the latent heat of
154 crystallization. Supercooling points from temperatures in which any pupae remained unfrozen
155 were discarded so as not to truncate the SCP distribution, yielding a total of 15 SCP
156 measurements. After cold exposure, pupae were placed into 200 mL plastic containers at 15 - 25
157 °C (night - day) on a 16:8 L:D cycle and monitored daily for emergence. Upon emergence from
158 the pupal case, their condition was recorded as alive (wings fully extended, no crumping or
159 deformity) or deformed (wings crumpled). Animals that failed to emerge were scored as dead.

160 *Deacclimation experiment*

161 In mid-April 2010, we split the remaining pupae (n=18) equally between winter-
162 acclimated (which remained in the same incubator) and spring-warmed treatments. Spring
163 warmed pupae (n=9) experienced four days at temperatures fluctuating between 15 - 25 °C
164 (night – day) on a 16:8 L:D cycle. We chose four days for the spring-warmed treatment, since
165 changes in the SCP and hemolymph constituents of deacclimating Emerald Ash Borer plateau by
166 that time, indicating that deacclimation is complete (Sobek-Swant et al., 2012). *Sitophilus*
167 *granarius* and *Cryptolestes ferrugineus* (Coleoptera) also lose their cold-acclimation within five
168 days of exposure to warm temperatures (Fields et al., 1998). After four days, we compared the
169 cold tolerance of pupae in the winter-acclimated and spring-warmed treatments (n=5 each) by
170 exposing them for one hour to the lowest temperature at which we saw 100% survival in winter-
171 acclimated pupae (-20 °C), and monitoring survival as described above. On the same day as cold

tolerance was assayed, we collected hemolymph samples from the remaining four pupae from each treatment (winter-acclimated and spring-warmed) for biochemical analyses.

Hemolymph composition analyses

All biochemical analyses were performed on the same (n=4) individuals from each treatment (winter-acclimated and spring-warmed). Pupae were punctured at the first abdominal sternite with an insect pin and, without delay, placed cremaster-down in a 1.5 mL Eppendorf tube. Pupae were spun briefly (5 s, 8 rpm) in a microcentrifuge to expel hemolymph (50-100 µL), which was pipetted into 0.6 mL Eppendorf tubes, snap-frozen in liquid nitrogen and stored at -80 °C until biochemical analysis. Aliquots were taken from these tubes for each analysis.

Hemolymph osmolality and thermal hysteresis were measured using the method of Crosthwaite at al. (2011) on a Clifton Nanolitre Osmometer (Clifton Technical Physics, Hartford, NY, USA). Thermal hysteresis was defined as the difference between the freezing point and the melting point, and is an indication of antifreeze activity. The melting point was used to determine osmolality (1 mole = -1.86 °C). Spicular and angular crystal morphology during regrowth was noted as an indicator of hemolymph antifreeze activity (Scotter et al. 2006).

Sugars and polyols were measured using gas chromatography with a flame ionization detector (GC-FID) or a spectrophotometric assay for free glycerol as described in Crosthwaite et al. (2011), with modifications to allow quantification of trehalose, the primary insect hemolymph sugar. Briefly, sugars and polyols were extracted from 20 µL hemolymph samples with 1 mL methanol:chloroform:water mixture (2:1:2) (Nicolai et al., 2011), with xylitol (in methanol) added as internal standard used to correct for derivatization efficiency (1 $mg.mL^{-1}$ final concentration in the extract). A 350 µL aliquot from the upper aqueous phase was dried in a

194 vacuum concentrator (Genevac, Suffolk, UK) and re-dissolved in 20 µL deionized water, then
195 incubated with trehalase (20 µL of 10% [v/v]) from porcine kidney (Sigma Aldrich product
196 number T8778, in 135 mM Citric Acid buffer, pH 5.7 [Sigma product number C7129]) at 37°C
197 for 30 min (Flatt et al., 2008), to produce glucose. Carbohydrates and polyols were derivatized
198 using the alditol acetate method (Blakeney et al., 1983) and analyzed using GC-FID as modified
199 in Crosthwaite et al. (2011).

200 Hemolymph glycerol was quantified spectrophotometrically from a further 350 µL
201 aliquot from the upper aqueous phase (modified from Crosthwaite et al., 2011). The aliquot was
202 dried in a vacuum concentrator as described above and reconstituted in 0.05 % Tween 20 in
203 distilled water to achieve original hemolymph concentration and then diluted them to the
204 appropriate concentration based on pilot studies (deacclimated samples, 1:20; winter-acclimated
205 samples, 1:500). Standards were generated by diluting 99 % glycerol in 0.05 % Tween 20 to
206 yield concentrations ranging from 0.005 mg/mL to 0.04 mg/mL. Samples and standards were
207 added in triplicate to a 96-well microtitre plate (10 µL of sample or standard per well) and 100
208 µL of free glycerol reagent (Sigma-Aldrich® St. Louis, Missouri, USA) was added to each well.
209 Plates were incubated at room temperature for 5 min and absorbance was read at 540 nm in a
210 SpectraMax® M2e (Molecular Devices, Sunnyvale, California, USA) spectrophotometer, and
211 quantified against glycerol standard curves.

212 We measured ion concentrations in the hemolymph using atomic absorption spectroscopy
213 (iCE 3300; Thermo Scientific, Waltham MA, USA) as described by MacMillan and Sinclair
214 (2011). We digested 10 µL hemolymph with 100 µL nitric acid for 24 h at room temperature.
215 We determined Na^+ and K^+ concentrations in a 1% solution and Mg^{2+} and Ca^{2+} in a 0.2%

solution of the digested hemolymph diluted with double-distilled water. Standard curves of ions (0.2 to 2 ppm) were generated from diluted standards containing the same amount of nitric acid.

Weather data

To test for cold-induced range limitation, we obtained daily weather data from three years (2011, 2012 and 2013), including daily minima and maxima, from Environment Canada's Daily Data reports (climate.weather.gc.ca). We chose stations at 450 - 650 m elevation at three equally-spaced points along the northern range boundary from the western to the eastern edge of their distribution in British Columbia and Alberta, that were within 50 km of a northernmost *P. zelicaon* locality (Guppy and Shepard, 2012, Fig. 2). The range on the mainland, and thus the weather sites we used, extends 5-10° latitude further north of the Vancouver Island sites where our animals were collected. Thus, we are making an assumption that cold hardiness is relatively invariant across the northern portion of the range (see discussion). For each of these three weather stations, we chose a matched "outside range" station 300-400 km north at approximately the same longitude, also within the same elevational range where possible (Table 1). This provided three comparisons of conditions inside versus outside the range, at controlled elevation, replicated over three years at each longitude.

We next assessed the frequency of lethal cold snaps following deacclimation. To estimate the timing of the first warm spell that might initiate development and deacclimation, we parsed the weather data (for each year at each site) for the first occurrence of a four day stretch of daily maxima above the threshold for development in closely-related swallowtail species (11 °C) (Scriber and Lederhouse, 1983). Four days was chosen to match the time used in our deacclimation experiments. We recorded the date at which the warm spell started, the minimum temperature between January 1 and that date for that year, and the minimum temperature in the

16 days following that date – this period was chosen because it reflects the average time for adult emergence in the laboratory under the deacclimation conditions. This yields a conservative estimate of the minimum temperature, since development is likely slower under spring conditions in the field, which we expect to be cooler than the 25 °C in the laboratory.

Statistical analyses

We compared concentration of carbohydrates and ions in the hemolymph as well as hemolymph osmolality and thermal hysteresis between acclimated and deacclimated pupae using two-tailed t-tests in Microsoft Excel 2010 (Microsoft, Redmond, Washington, USA). We compared survival of acclimated and deacclimated pupae using a chi square test in R 3.0.2 (R Core Team 2013). Data are presented as mean ± standard error (SEM). We tested the hypothesis that minimum low temperatures both before and after a spring warm period were more severe outside the range using paired t-tests in R v3.0.2.

Results

Cold tolerance strategy

No mortality was observed in winter-acclimated pupae after 1 h at -15 or -20 °C, despite some individuals freezing at -20 °C. Sixty percent of individuals survived 1 h at -25 and -30 °C, during which all pupae froze, but no pupae survived a 1h exposure at -35 °C (Fig. 3A). Half the pupae survived a 12 h exposure at -20 °C, including one individual that froze during the ramping period and thus likely reached equilibrium ice formation during the 12 h exposure. No healthy adults emerged after 12 h at either -25 or -30 °C (most died, and one emerged deformed; Fig. 3B). The average time to adult emergence after transfer into warm conditions was 15.9 ± 0.8 days (range 11-26 days) and did not noticeably differ among treatments.

Deacclimation experiment

After four days of simulated spring warming, no pupae survived 1 h at -20 °C, compared to 100% for winter-acclimated pupae tested at the same time. Concurrently, the SCP increased slightly but significantly from -20.5 for winter-acclimated pupae to -17.4 °C for spring-warmed (henceforth referred to as deacclimated) pupae (Table 2).

Hemolymph osmolality of winter-acclimated pupae ranged from 690 - 1193 mOsm, and decreased by 45 % in deacclimated pupae (Table 2). Preliminary analyses without the trehalase digestion did not detect any free glucose in either winter-acclimated or deacclimated pupae. The predominant sugar/ sugar alcohol in the hemolymph of winter-acclimated pupae was glycerol, with smaller amounts of trehalose (Table 2). After four days of simulated warming, glycerol – and to a lesser extent trehalose - concentrations decreased significantly relative to winter-acclimated pupae (93 and 54% decreases for glycerol and trehalose, respectively), resulting in glycerol and trehalose concentrations being approximately equal in deacclimated pupae (Table 2). Hemolymph of winter-acclimated pupae contained approximately equal concentrations of Na^+ and K^+, with lower concentrations of Mg^{2+} and Ca^{2+} (Table 2). Hemolymph Na^+ and Ca^{2+} concentrations decreased significantly following deacclimation, while $[K^+]$ and $[Mg^{2+}]$ did not change. The 58 % decrease in $[Na^+]$ decreased the Na^+:K^+ ratio from 0.96 (winter-acclimated) to 0.38 (deacclimated). Thermal hysteresis in the hemolymph was present, but low even in winter-acclimated pupae, and decreased slightly but significantly upon deacclimation (Table 2).

Weather data

Mean minimum daily temperatures during the winter were significantly colder outside the range compared to the northern range edge (mean -31.5 ± 1.6 °C compared to -37.3 ± 1.5 °C; t_8=3.09, p=0.007, Table 3). A four-day warm spell (that we presumed would cause

deacclimation, see methods) occurred earlier at the northern range edge compared to outside the range on 95 % of occasions (Table 3). The mean daily minimum temperature following a warm spell was -7.0 ± 1.7 °C (ranging from -0.2 to -18.3 °C), and did not differ between inside and outside of the range (t_8=1.09, p=0.153).

Discussion

Papilio zelicaon pupae are tolerant of transient low temperature exposure, and appear to survive at least some internal ice formation. This cold hardiness stems from the accumulation of glycerol and cations that contribute to the low supercooling point; glycerol likely also stabilizes biological macromolecules (Zachariassen 1985). A four-day warm spell caused a sharp decrease in hemolymph sugars, sugar alcohols, cations, and thus total osmolality, and a concordant drop in cold hardiness. Weather data show that temperatures following a warm spell drop as low as -18.6 °C, close to the temperature at which we saw 0 % survival in the laboratory, suggesting that deacclimation could lead to cold-induced mortality in the wild, particularly outside the range.

Cold tolerance strategy and physiological mechanisms

Some pupae survived internal ice formation (indicative of freeze tolerance), while others were killed by, or before, ice formation (indicative of freeze avoidance or chill susceptibility) (Lee, 2010). In addition, the SCPs were low (around -20 °C in winter-acclimated individuals) which would normally be associated with freeze avoidance (Lee, 2010). Freeze tolerance by species with low SCPs has been reported previously (Ring, 1982), and the SCP of -20 places this species in that category (Sinclair, 1999), along with other species that show only partial tolerance of internal ice formation (Ring, 1982). The SCP of *P. zelicaon* is not low enough for a freeze

avoidant strategy to allow survival in nature (at least after laboratory rearing), so it seems that this partial freeze tolerance forms a part of their strategy to survive low winter temperatures.

The primary hemolymph cryoprotectant in *P. zelicaon* is glycerol, in contrast to overwintering *P. glaucus* and *P. canadensis*, which contain trehalose but no measureable glycerol (Kukal et al., 1991), but consistent with *P. machaon* and *P. xuthus* which have 160-220 mM hemolymph glycerol (Shimada 1988). This conforms to the phylogeny, which places *P. zelicaon*, *P. xuthus* and *P. machaon* in the *P. machaon* species group in the *Papilio* (*sensu stricto*) lineage, which diverged from the clade containing *P. glaucus* and *P. canadensis* over 55 million years ago (Zakharov et al., 2004). Thus, pupal cold hardiness may have evolved convergently in these clades using different mechanisms. Glycerol has a well-documented role as a cryoprotectant (Lee, 2010), but the concentrations we describe are relatively low compared to some insect species. For example, overwintering prepupae of the Emerald Ash Borer accumulate 2-4 M glycerol (Crosthwaite et al., 2011), while freeze-tolerant *Pyrrharctia isabella* caterpillars (Arctiidae) accumulate 200-300 mM (Layne and Blakeley, 2002; Marshall and Sinclair, 2012b). Trehalose is generally the dominant blood sugar in most Lepidoptera, making up 90% of hemolymph sugars, and our acclimated and deacclimated values are all within the normal range reported for other pupal Lepidoptera (Wyatt and Kalf, 1957).

Plasticity of cold tolerance

Four days of spring warming significantly reduced the cold tolerance of *P. zelicaon* pupae (i.e. caused deacclimation). Loss of cold tolerance in response to short exposures to warm temperatures (as opposed to longer-term exposures in the context of seasonal transitions) has been observed in several insect species (e.g. Fields et al., 1998, Sobek-Swant et al. 2012). Here, we show that this deacclimation was accompanied by an increase in SCP and a decrease in total

328 osmolality due in part to declines in the concentrations of glycerol, trehalose, Na^+ and Ca^{2+} in the
329 hemolymph. Similar mechanisms seem to underpin deacclimation in emerald ash borer, which
330 also experiences an increase in SCP and decrease in total osmolality and glycerol concentration
331 (although trehalose and cations were not measured; Sobek-Swant et al., 2012). A decline in
332 hemolymph cryoprotectants has been documented during transition from winter to the growing
333 season (e.g. Li et al. 2001, Vanin et al. 2008, Crosthwaite et al. 2011). In emerald ash borer,
334 deacclimation is irreversible, since it is associated with the resumption of development (Sobek-
335 Swant et al., 2012), which may also be the case in *P. zelicaon*.

336 Although cryoprotectants and osmolytes play important roles in depressing the SCP and
337 stabilizing proteins and macromolecules when there is a risk of cold damage, it is energetically
338 costly to maintain high levels of these molecules in the hemolymph. We found good support for
339 our hypothesis that the costs of that high osmolality would lead to a rapid decline upon
340 rewarming. Declines in sugars and polyols likely represent the recycling of energetically dense
341 molecules to fuel development, once they are no longer required for cryoprotection (Storey
342 1997). In the present study, hemolymph glycerol concentration showed the most pronounced
343 decline with deacclimation, suggesting that it may have a causal relationship with cold hardiness
344 as is seen with other polyols at similar concentrations in *Pyrrhocoris apterus* (Hemiptera:
345 Heteroptera) (Koštál et al., 2001). We also documented a decline in trehalose concentration. In
346 the fall webworm (Lepidoptera: Arctiidae), a decrease in trehalose is paralleled by an increase in
347 glycogen, suggesting that free sugars are sequestered into energy reserves after winter (Li et al.
348 2001). This may also be the case in *P. zelicaon*. Contrary to our findings, trehalose does not
349 decrease after 10 days acclimation to 25 °C in *P. glaucus* and *P. canadensis*; perhaps indicating

that the primary function of accumulated trehalose in those species is energetic rather than cryoprotective (Kukal et al. 1991).

Hemolymph composition of deacclimated pupae is in line with previous reports for Lepidoptera. The total osmolality of the hemolymph of deacclimated pupae is within the range reported for other Lepidoptera (258-629 mOsm) (Sutcliffe, 1963). The dominant ions in the hemolymph are Na^+ and K^+, with smaller contributions from Ca^{2+} and Mg^{2+}. The components that we measured account for ~36% of the measured total osmolality, thus there are other major contributors to total osmotic pressure that we did not quantify. These likely include amino acids, organic and inorganic anions (Pastor et al., 1997), which contribute up to half of hemolymph osmolality in other Lepidoptera (Sutcliffe, 1963). The Na^+:K^+ ratio in this study was 0.4 for deacclimated pupae, in line with values for other Lepidoptera (Sutcliffe, 1963), but increased to nearly 1 for winter-acclimated pupae due to an increase in [Na^+]. This is the opposite to the low hemolymph [Na^+] associated with increased cold hardiness among *Drosophila* species (MacMillan, 2013), where low [Na^+] is hypothesized to counter migration of water from the hemocoel to the gut during cold exposure (MacMillan and Sinclair, 2011). It is likely that any role of Na^+ in cold tolerance of *P. zelicaon* differs from that in *Drosophila*, in keeping with the atypical hemolymph chemistry of Lepidoptera, particularly with respect to low [Na^+] and Na^+:K^+ ratios, (Sutcliffe, 1963). However, prior to the present study the role of ion balance during cold exposure in Lepidoptera has not been well-explored, although hemolymph [Na^+] does not change after freezing in larvae of *Pyrrharctia isabella* (Arctiidae), and remains low during the winter (Boardman et al., 2011). This emphasizes the importance of taking a broad phylogenetic approach when studying the evolution of cold tolerance mechanisms.

Implications for geographic range limits

Average minimum temperatures dropped below the temperature that caused 100 % mortality in winter-acclimated individuals (-35 °C) on average once out of the past three years inside the northern range boundary, but that number increased to two out of three years 300-400 km north of the current *P. zelicaon* range boundary. This suggests that low winter temperatures may limit the northern range boundary of this species. The presence of potentially suitable host plants north of the current range edge (Natural Resources Conservation Service, USDA, plants.usda.gov), suggests that the range boundary is not set by biotic interactions. This conclusion of cold-limitation depends on the validity of our estimates of 1) cold exposure, 2) cold hardiness, 3) range boundary and 4) representativeness of our study organisms to the species as a whole. We will address each of these points in turn.

First, cold exposure will be determined by overwintering microhabitat, which can be buffered by snow cover (Williams et al., in press). Thus, pupae overwintering beneath the snow pack will experience milder temperatures than the air temperatures we used, at least for part of the winter. Natural pupation sites of *P. zelicaon* have not been studied, and related species use a range of pupation sites ranging from beneath the leaf litter to tree trunks high above the snowpack (West and Hazel, 1979). Thus, the degree to which overwintering individuals experience microclimate temperatures comparable to our weather data records is unknown, and we may have overestimated the occurrence of potentially-lethal temperature exposures. However, the pupae tie themselves to twigs during pupation, suggesting that they may be exposed to the elements during overwintering (JJH and CMW, personal communication). Additionally, low temperatures and snow cover do not always coincide (Williams et al., in press), further suggesting that at least some exposure to low air temperature likely occurs.

Second, cold hardiness can be enhanced by physiological plasticity in response to temperatures experienced during the winter (cues not present in the laboratory experiment). For example, many insect species are capable of rapid cold-hardening, wherein a prior (mild) cold exposure increases tolerance of subsequent cold snaps (Lee et al., 1987), and repeated exposure to cold generally improves cold tolerance (Marshall and Sinclair, 2012a). Thus, it is possible that the persistence of northern populations could be enhanced by physiological plasticity, resulting in low temperature tolerance not being challenged even outside the range boundary.

Third, the position of the northern range boundary may be overestimated due to sampling of migrants: these butterflies are strong fliers and can travel up to 20 km (Peterson and Denno, 1998), and range boundary information comes from reported sightings of adults rather than overwintering stages (Guppy and Shepard, 2012).

Fourth, due to logistical constraints, we sourced individuals from Vancouver Island, which are genetically-distinct from individuals on the mainland, south of Vancouver Island (Zakharov and Hellmann, 2008). We do not know whether northern range edge populations on the mainland are genetically distinct from Vancouver Island, but it remains possible that populations may be locally adapted in their cold-hardiness. Temperatures on the mainland are generally more severe than those on Vancouver Island, which may make our estimates of cold tolerance conservative (i.e. mainland populations would be expected to be more cold-hardy). However, snow cover is much deeper and more persistent on the mainland compared to Vancouver Island meaning that exposure to the elements may be higher on Vancouver Island, resulting in conditions being more similar than would be expected based on weather data. Deacclimation processes may also differ between populations, but since deacclimation results from resumption of development, and northern range edge populations are under seasonal time

418 constraints, we expect that northern range edge populations would also show a robust
419 deacclimation response.

420 Thus, although our estimates suggest that populations could survive at the northern-most
421 range limit but not beyond, increased certainty would require better estimates of microclimate
422 conditions (based on knowledge of natural pupation sites), knowledge of the degree of
423 physiological plasticity in cold hardiness in natural conditions, more nuanced information on the
424 range boundary of stable overwintering populations, and information on intra-specific variation
425 in cold hardiness among populations. Nevertheless, despite all these potential limitations of the
426 available data, given that the match between measured lower lethal temperatures and
427 temperatures near the best-estimated northern range boundary is close, we believe that there is
428 good support for the hypothesis of cold limitation for this species.

429 Deacclimation has important implications for population persistence in a variable world.
430 We found that "warm snaps", defined as four or more days above the threshold for development,
431 occurred between the end of March and the end of April at the northern range edge, and in some
432 years were followed by low temperatures approaching the temperature that caused 100 %
433 mortality after a 1 h exposure in the laboratory for deacclimated pupae. It thus seems likely that
434 cold snaps following deacclimation could be an important selective pressure on natural
435 populations near the northern range limit, at least in the recent past. Frost events after the start of
436 the growing season are increasing in frequency as a consequence of climate change in the eastern
437 United States (Augspurger, 2013); thus, mortality resulting from deacclimation is likely to
438 become more important in the future. Cold snaps following deacclimation were no more severe
439 outside the current putative range limit, although the "warm snaps" occurred later in the year at
440 higher latitudes. Given the potential importance of deacclimation in determining overwinter

441 survival and thus fitness, further research is needed to elucidate the performance consequences
442 of, and conditions that elicit, deacclimation.

443

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Figures

Figure 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).

Figure 2 – Map showing collection locations of *Papilio zelicaon* (grey circles), the northern range limit (dotted line, from Guppy and Shepard, 2012), and locations of weather stations (squares = inside range, triangles = outside range; Table 2) in Northwestern North America.

Figure 3 - Survival to adulthood of *Papilio zelicaon* exposed to A) one hour or B) 12 hours of cold as winter-acclimated pupae. Number of pupae that froze is on each bar.

Tables

Table 1– Details of weather stations used to compare weather parameters inside and outside the range limit of *Papilio zelicaon*. Station IDs and location information from Environment Canada (climate.weather.gc.ca). Station number corresponds to numbers on Fig. 1, and position is relative to northern range edge (Figure 1).

Station number	Site	Station ID	Latitude	Longitude	Position	Elevation (m)
1	Tawatinaw	3066367	54.30 N	113.52 W	inside-east	611
2	Red Earth	3075488	56.55 N	115.28 W	outside-east	546
3	Chetwynd A	1187335	55.69 N	121.63 W	inside-mid	610
4	Trout Lake	220CQHR	60.44 N	121.24 W	outside-mid	498
5	Smithers A	1077500	55.82 N	127.18 W	inside-west	522
6	Dease Lake	119BLM0	58.43 N	130.03 W	outside-west	802

606 Table 2 – Biochemical and physiological measurements of winter-acclimated and deacclimated
607 *Papilio zelicaon* pupae (mean ± SEM). Asterisks indicate variables that are significantly lower in
608 deacclimated pupae ($\alpha<0.05$). All biochemical assays (osmolality and hemolymph composition)
609 were performed on the same four individuals per acclimation treatment, thus, unless noted, n=4.

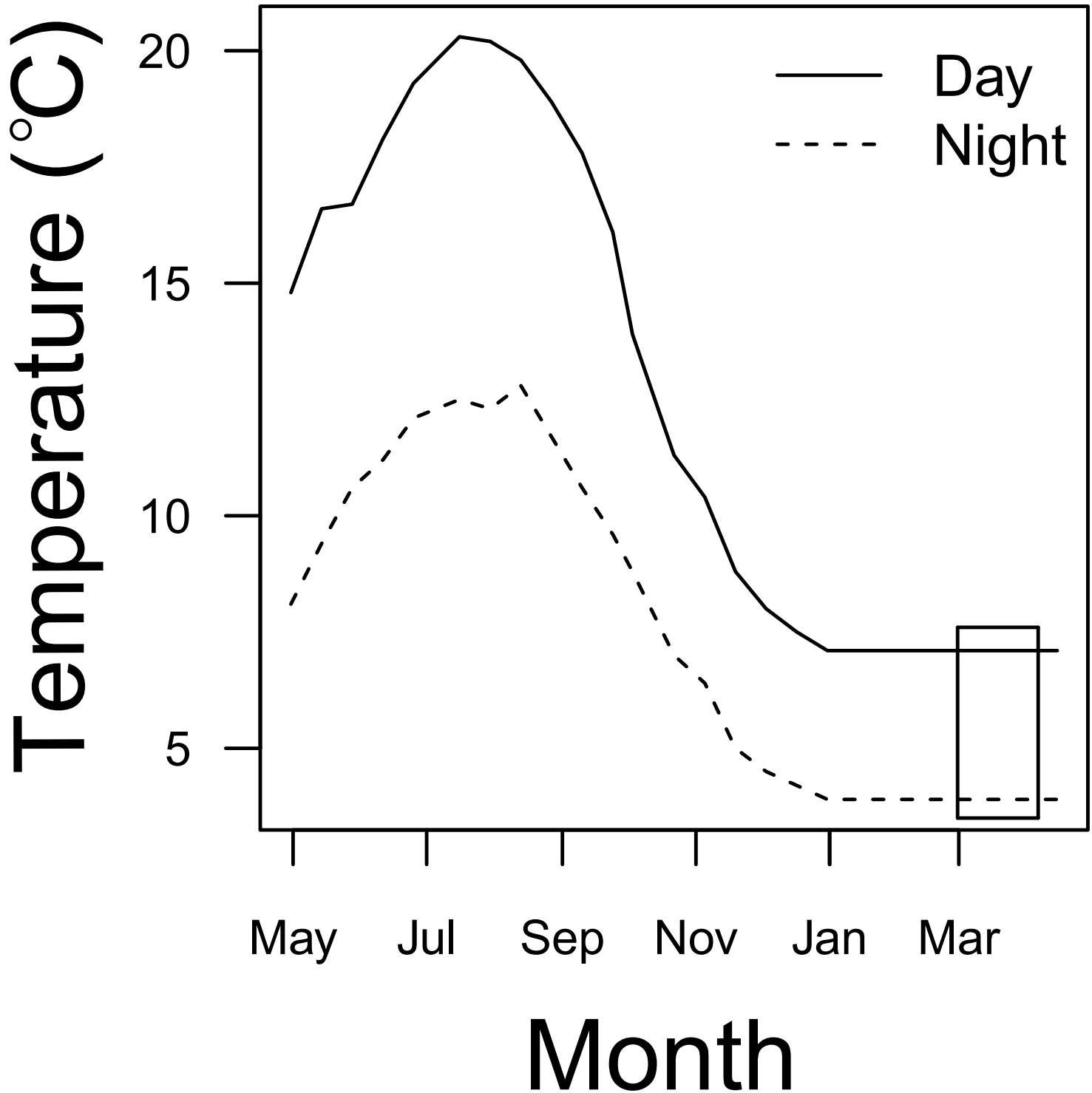
	Acclimated	**Deacclimated**	**Stats**
SCP (°C)	-20.5 ± 2.0 (n=15)	-17.4 ± 3.6 (n=5)	t_{33}=2.42, p=0.026
Survival (1h @ -20 °C)	100 % (n=5)	0 % (n=5)*	χ2=6.4, p=0.011
Osmolality (mOsm)	924 ± 105	411 ± 19*	t_6=4.8, p=0.003
Thermal hysteresis (°C)	0.08 ± 0.002	0.05 ± 0.007*	t_6=3.5, p=0.012
Glycerol (mM)	119.5 ± 21.7	8.1 ± 3.6*	t_6=5.0, p=0.002
Trehalose (mM)	18.9 ± 2.1	8.7 ± 0.7*	t_6=4.6, p=0.004
[Na^+] (mM)	76 ± 6	32 ± 13*	t_6=2.97, p=0.025
[K^+](mM)	79 ± 4	84 ± 14	t_6=0.39, p=0.713
[Mg^{2+}] (mM)	18 ± 2	12 ± 2	t_6=1.67, p=0.145
[Ca^{2+}] (mM)	29 ± 5	17 ± 0.1*	t_6=2.5, p=0.046

610

611

Table 3 - Daily minimum temperatures during 2011-2013 at three points longitudinally spanning the northern range edge of *Papilio zelicaon* (East, Mid, West, and [Vancouver] Island). Stations were chosen that were close to the northern range edge (inside range), with a paired station 300-400km to the north at the same longitude (outside range) (Table 2). Date of warm spell is the first day of a four-day stretch of maximum temperatures above 11 °C. Minimum temperatures are in °C and are absolute minima between Jan 1st and date of warm spell (before) or in the 16 days following the last day of the warm spell (after).

Position in range	Minimum temp. before (°C)			Date of warm spell			Minimum temp. after (°C)		
	2011	2012	2013	2011	2012	2013	2011	2012	2013
East									
inside	-30.0	-31.0	-31.7	11-Apr	19-Apr	25-Apr	-0.2	-0.7	-10.4
outside	-39.0	-32.4	-37.5	25-Apr	1-Mar	24-Apr	-3.6	-15.1	-10.6
Mid									
inside	-37.4	-35.2	-31.6	22-Apr	7-Apr	25-Mar	-4.9	-6.8	-14.8
outside	-42.7	-30.2	-35.1	1-May	7-Apr	28-Apr	-4.1	-18.3	-8.3
West									
inside	-27.0	-37.1	-22.8	27-Mar	7-Apr	26-Mar	-5.7	-4.3	-4.4
outside	-40.7	-43.4	-34.3	5-May	8-Apr	3-May	-3.8	-6.2	-3.9


Temperature (°C)
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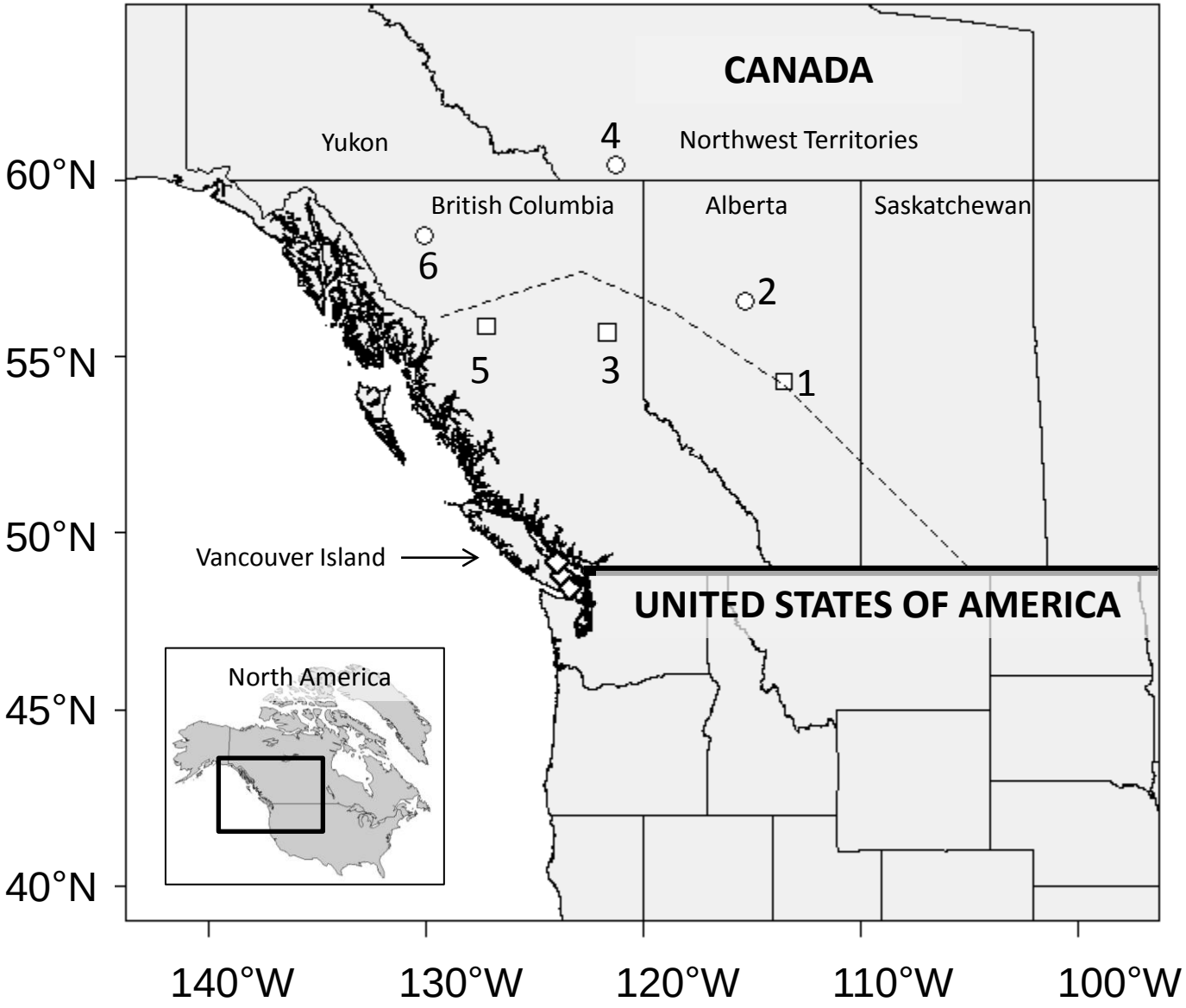
CANADA
Yukon
Northwest Territories
British Columbia
Alberta
Saskatchewan
UNITED STATES OF AMERICA
Vancouver Island
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60°N
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50°N
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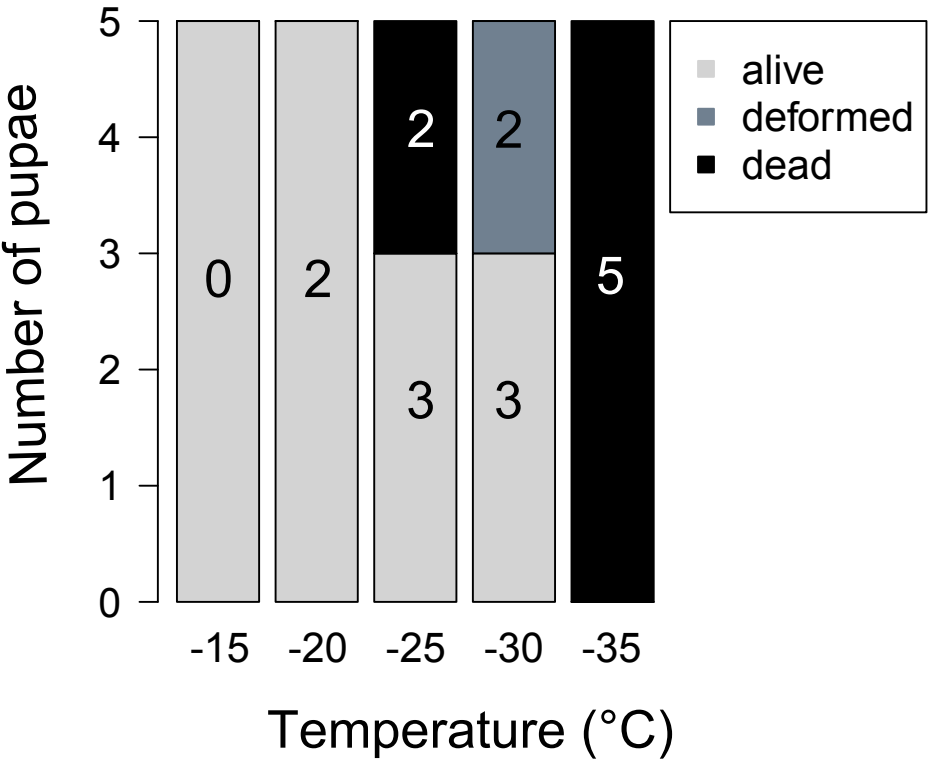
40°N
140°W
130°W
120°W
110°W
100°W

A - One hour exposure



B - 12 hour exposure