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Cold hardiness and deacclimation of overwintering *Papilio zelicaon* pupae

Caroline M. Williams
Western University

Nicolai Annegret
Western University

Brent J. Sinclair
Western University, bsincla7@uwo.ca

Laura V. Ferguson
Western University

Mark A. Bernards
Western University

See next page for additional authors

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Authors

Caroline M. Williams, Nicolai Annegret, Brent J. Sinclair, Laura V. Ferguson, Mark A. Bernards, and Jessica J. Hellmann

14 **Abstract**

15 Seasonally-acquired cold tolerance can be reversed by exposure to warm temperatures,
16 leaving temperate ectotherms vulnerable to cold snaps in spring. However, this process of
17 deacclimation, and its underlying mechanisms, has not been well-explored in insects.
18 Swallowtail butterflies are widely distributed globally but in some cases their range is limited by
19 low temperature and their cold tolerance seasonally acquired, implying that they may be
20 vulnerable to mortality resulting from deacclimation. We investigated cold tolerance and
21 hemolymph composition of Anise swallowtail (*Papilio zelicaon*) pupae during overwintering in
22 the laboratory, and after four days exposure to warm temperatures in spring. Overwintering
23 pupae had supercooling points around -20.5 °C and survived brief exposures to -30 °C,
24 suggesting partial freeze tolerance. Overwintering pupae had hemolymph osmolality of
25 approximately 920 mOsm, imparted by high concentrations of glycerol, K⁺ and Na⁺. After
26 exposure to spring warming, supercooling points increased to approximately -17 °C, and survival
27 of a 1h exposure to -20 °C decreased from 100 % to 0 %. This deacclimation was associated with
28 decreased hemolymph osmolality and reduced glycerol, trehalose, Na⁺ and Ca²⁺ concentrations.
29 We compared cold tolerance of pupae to weather conditions at and beyond the species' northern
30 range boundary. Minimum temperatures at the range boundary were close to the lower lethal
31 temperature of pupae, and temperatures north of the range were colder, suggesting that cold
32 hardiness may set northern range limits on the mainland. Minimum temperatures following
33 warm snaps were likely to cause mortality in at least one of the past three years. Cold snaps in
34 the spring are increasing in frequency as a result of global climate change, so are likely to be a
35 significant source of mortality for this species, and other temperate ectotherms.

- 36 **Keywords:** climate change, cold tolerance, cryoprotectant, deacclimation, insect, hemolymph,
- 37 Lepidoptera, winter

38 **Introduction**

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

55 The onset of low temperatures in winter can be unpredictable, so many temperate insects rely
56 on photoperiod cues to reliably initiate pre-winter cold hardening (Bradshaw and Holzapfel,
57 2010). However, thermal cues may still modulate the degree of cold hardiness acquired (Storey
58 and Storey, 1988). By contrast, the loss of cold hardiness and resumption of development at the
59 end of winter are often regulated solely by temperature cues (Košťál 2006), and warm snaps can

60 trigger the loss of cold hardiness (deacclimation). For example, Emerald Ash borer (*Agilus*
61 *planiplennis*; Coleoptera: Buprestidae) prepupae lose cold-hardiness in response to a mid-winter
62 warm snap (Sobek-Swant et al., 2012). Because the loss of cold tolerance is accompanied by
63 resumption of development, this deacclimation is irreversible (Sobek-Swant et al., 2012).
64 Deacclimation can thus leave insects vulnerable to cold snaps in the spring. Global climate
65 change is leading to earlier snow melt in the spring in many locations, which can expose
66 subnivean organisms to increased thermal variability in the spring (Brown and DeGaetano, 2011)
67 and induce deacclimation. This may underlie the increasing frequency of damaging spring frosts
68 over the past 100 years (Augsburger 2013). Thus, an essential part of predicting an organism's
69 response to changing winters is understanding the propensity for and consequences of
70 deacclimation.

71 Swallowtail butterflies (Lepidoptera: Papilionidae) have a broad global distribution, and in
72 North America occupy climates ranging from tropical to sub-arctic (Lederhouse et al., 1995).
73 There is a steep decline in species diversity with latitude: only 2 of ~500 papilionid species occur
74 north of 60° latitude (Lederhouse et al., 1995). Cold hardiness limits the northern distribution of
75 some species (Kukal et al., 1991). Swallowtails are thought to be susceptible to climate change;
76 moving northward in warm years only to be knocked back in cold ones (Scriber and Gage,
77 1995). Globally, all swallowtails that occupy environments with a cold winter overwinter as
78 pupae, and there are examples of both freeze-tolerant (e.g. *Papilio machaon*) and freeze-avoidant
79 (e.g. *P. xuthus*) species (Kukal et al., 1991; Shimada, 1988). The four species examined to date
80 (*P. machaon*, *P. xuthus*, *P. canadensis*, *P. glaucus*) use glycerol or trehalose as their primary
81 cryoprotectants, and cold tolerance increases concurrently with the accumulation of these
82 cryoprotectants during winter (Kukal et al., 1991; Shimada, 1988). This seasonal acclimation is

83 more pronounced in more cold-hardy species (such as *P. canadensis*), and is modified by the
84 severity of the cold experienced. For example, cold-hardy *P. canadensis* pupae decrease their
85 supercooling points when overwintering in Alaska compared to Michigan, but cold-susceptible
86 *P. glaucus* pupae do not, and correspondingly suffer higher mortality in Alaska than do *P.*
87 *canadensis* (Kukal et al., 1991). These interspecific differences in cold tolerance or acclimation
88 ability may stem from differences in carbohydrate metabolism: *P. canadensis* synthesizes
89 cryoprotectants from isotopically labelled glucose, while *P. glaucus* does not (Kukal et al.,
90 1991). Since their distributions are set by cold, and their cold tolerance is seasonally acquired
91 through plastic changes to physiology, swallowtail butterflies are an ideal system in which to
92 study deacclimation and its impacts on survival.

93 *Papilio zelicaon* belongs to the *Papilio (sensu stricto)* lineage, which dispersed to North
94 America across Beringia before and during the Pleistocene (Zakharov et al., 2004). Beringia
95 comprised Alaska, the Yukon Territory, and the now-submerged Bering Land Bridge, and as
96 such was home to a cold and harsh climate (Elias 2000). This biogeographic history makes this
97 group ideal for investigations of cold hardiness. *P. zelicaon* inhabits fields, oak savannahs, and
98 roadsides throughout western North America (extending to North Dakota, 100°W) up to 60°N
99 and feed on plants in the Apiaceae (Guppy and Shepard, 2012; Sims, 1980). *P. zelicaon*
100 overwinter in a pupal diapause, and populations vary from univoltine (one generation per year)
101 towards the northern range edge, to multivoltine (several generations per year) further south
102 (Sims, 1980; Thorne et al., 2006). *P. zelicaon* is the most abundant swallowtail butterfly in
103 British Columbia (BC); its range extends north into boreal climates on the mainland (Peel et al.,
104 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**

126 *Study species and rearing*

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

142 *Cold tolerance experiment*

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

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 crumpling 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

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

174 *Hemolymph composition analyses*

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

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

187 Sugars and polyols were measured using gas chromatography with a flame ionization
188 detector (GC-FID) or a spectrophotometric assay for free glycerol as described in Crosthwaite et
189 al. (2011), with modifications to allow quantification of trehalose, the primary insect hemolymph
190 sugar. Briefly, sugars and polyols were extracted from $20\text{ }\mu\text{L}$ hemolymph samples with 1 mL
191 methanol:chloroform:water mixture (2:1:2) (Nicolai et al., 2011), with xylitol (in methanol)
192 added as internal standard used to correct for derivatization efficiency ($1\text{ mg}\cdot\text{mL}^{-1}$ final
193 concentration in the extract). A $350\text{ }\mu\text{L}$ 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%

216 solution of the digested hemolymph diluted with double-distilled water. Standard curves of ions
217 (0.2 to 2 ppm) were generated from diluted standards containing the same amount of nitric acid.

218 *Weather data*

219 To test for cold-induced range limitation, we obtained daily weather data from three years
220 (2011, 2012 and 2013), including daily minima and maxima, from Environment Canada's Daily
221 Data reports (climate.weather.gc.ca). We chose stations at 450 - 650 m elevation at three equally-
222 spaced points along the northern range boundary from the western to the eastern edge of their
223 distribution in British Columbia and Alberta, that were within 50 km of a northernmost *P.*
224 *zelicaon* locality (Guppy and Shepard, 2012, Fig. 2). The range on the mainland, and thus the
225 weather sites we used, extends 5-10° latitude further north of the Vancouver Island sites where
226 our animals were collected. Thus, we are making an assumption that cold hardiness is relatively
227 invariant across the northern portion of the range (see discussion). For each of these three
228 weather stations, we chose a matched "outside range" station 300-400 km north at approximately
229 the same longitude, also within the same elevational range where possible (Table 1). This
230 provided three comparisons of conditions inside versus outside the range, at controlled elevation,
231 replicated over three years at each longitude.

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

239 16 days following that date – this period was chosen because it reflects the average time for adult
240 emergence in the laboratory under the deacclimation conditions. This yields a conservative
241 estimate of the minimum temperature, since development is likely slower under spring
242 conditions in the field, which we expect to be cooler than the 25 °C in the laboratory.

243 *Statistical analyses*

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

251 **Results**

252 *Cold tolerance strategy*

253 No mortality was observed in winter-acclimated pupae after 1 h at -15 or -20 °C, despite
254 some individuals freezing at -20 °C. Sixty percent of individuals survived 1 h at -25 and -30 °C,
255 during which all pupae froze, but no pupae survived a 1h exposure at -35 °C (Fig. 3A). Half the
256 pupae survived a 12 h exposure at -20 °C, including one individual that froze during the ramping
257 period and thus likely reached equilibrium ice formation during the 12 h exposure. No healthy
258 adults emerged after 12 h at either -25 or -30 °C (most died, and one emerged deformed; Fig.
259 3B). The average time to adult emergence after transfer into warm conditions was 15.9 ± 0.8
260 days (range 11-26 days) and did not noticeably differ among treatments.

261 *Deacclimation experiment*

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

266 Hemolymph osmolality of winter-acclimated pupae ranged from 690 - 1193 mOsm, and
267 decreased by 45 % in deacclimated pupae (Table 2). Preliminary analyses without the trehalase
268 digestion did not detect any free glucose in either winter-acclimated or deacclimated pupae. The
269 predominant sugar/ sugar alcohol in the hemolymph of winter-acclimated pupae was glycerol,
270 with smaller amounts of trehalose (Table 2). After four days of simulated warming, glycerol –
271 and to a lesser extent trehalose - concentrations decreased significantly relative to winter-
272 acclimated pupae (93 and 54% decreases for glycerol and trehalose, respectively), resulting in
273 glycerol and trehalose concentrations being approximately equal in deacclimated pupae (Table
274 2). Hemolymph of winter-acclimated pupae contained approximately equal concentrations of
275 Na⁺ and K⁺, with lower concentrations of Mg²⁺ and Ca²⁺ (Table 2). Hemolymph Na⁺ and Ca²⁺
276 concentrations decreased significantly following deacclimation, while [K⁺] and [Mg²⁺] did not
277 change. The 58 % decrease in [Na⁺] decreased the Na⁺:K⁺ ratio from 0.96 (winter-acclimated) to
278 0.38 (deacclimated). Thermal hysteresis in the hemolymph was present, but low even in winter-
279 acclimated pupae, and decreased slightly but significantly upon deacclimation (Table 2).

280 *Weather data*

281 Mean minimum daily temperatures during the winter were significantly colder outside
282 the range compared to the northern range edge (mean -31.5 ± 1.6 °C compared to -37.3 ± 1.5 °C;
283 $t_8=3.09$, $p=0.007$, Table 3). A four-day warm spell (that we presumed would cause

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

288 **Discussion**

289 *Papilio zelicaon* pupae are tolerant of transient low temperature exposure, and appear to
290 survive at least some internal ice formation. This cold hardiness stems from the accumulation of
291 glycerol and cations that contribute to the low supercooling point; glycerol likely also stabilizes
292 biological macromolecules (Zachariassen 1985). A four-day warm spell caused a sharp decrease
293 in hemolymph sugars, sugar alcohols, cations, and thus total osmolality, and a concordant drop in
294 cold hardiness. Weather data show that temperatures following a warm spell drop as low as -18.6
295 °C, close to the temperature at which we saw 0 % survival in the laboratory, suggesting that
296 deacclimation could lead to cold-induced mortality in the wild, particularly outside the range.

297 *Cold tolerance strategy and physiological mechanisms*

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

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

307 The primary hemolymph cryoprotectant in *P. zelicaon* is glycerol, in contrast to
308 overwintering *P. glaucus* and *P. canadensis*, which contain trehalose but no measureable
309 glycerol (Kukal et al., 1991), but consistent with *P. machaon* and *P. xuthus* which have 160-220
310 mM hemolymph glycerol (Shimada 1988). This conforms to the phylogeny, which places *P.*
311 *zelicaon*, *P. xuthus* and *P. machaon* in the *P. machaon* species group in the *Papilio* (*sensu*
312 *stricto*) lineage, which diverged from the clade containing *P. glaucus* and *P. canadensis* over 55
313 million years ago (Zakharov et al., 2004). Thus, pupal cold hardiness may have evolved
314 convergently in these clades using different mechanisms. Glycerol has a well-documented role as
315 a cryoprotectant (Lee, 2010), but the concentrations we describe are relatively low compared to
316 some insect species. For example, overwintering prepupae of the Emerald Ash Borer accumulate
317 2-4 M glycerol (Crosthwaite et al., 2011), while freeze-tolerant *Pyrrharctia isabella* caterpillars
318 (Arctiidae) accumulate 200-300 mM (Layne and Blakeley, 2002; Marshall and Sinclair, 2012b).
319 Trehalose is generally the dominant blood sugar in most Lepidoptera, making up 90% of
320 hemolymph sugars, and our acclimated and deacclimated values are all within the normal range
321 reported for other pupal Lepidoptera (Wyatt and Kalf, 1957).

322 *Plasticity of cold tolerance*

323 Four days of spring warming significantly reduced the cold tolerance of *P. zelicaon*
324 pupae (i.e. caused deacclimation). Loss of cold tolerance in response to short exposures to warm
325 temperatures (as opposed to longer-term exposures in the context of seasonal transitions) has
326 been observed in several insect species (e.g. Fields et al., 1998, Sobek-Swant et al. 2012). Here,
327 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 Ca²⁺ 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 *Pyrhocoris apterus* (Hemiptera:
345 Heteroptera) (Košťál 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

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

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

372 *Implications for geographic range limits*

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

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

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

402 Third, the position of the northern range boundary may be overestimated due to sampling
403 of migrants: these butterflies are strong fliers and can travel up to 20 km (Peterson and Denno,
404 1998), and range boundary information comes from reported sightings of adults rather than
405 overwintering stages (Guppy and Shepard, 2012).

406 Fourth, due to logistical constraints, we sourced individuals from Vancouver Island,
407 which are genetically-distinct from individuals on the mainland, south of Vancouver Island
408 (Zakharov and Hellmann, 2008). We do not know whether northern range edge populations on
409 the mainland are genetically distinct from Vancouver Island, but it remains possible that
410 populations may be locally adapted in their cold-hardiness. Temperatures on the mainland are
411 generally more severe than those on Vancouver Island, which may make our estimates of cold
412 tolerance conservative (i.e. mainland populations would be expected to be more cold-hardy).
413 However, snow cover is much deeper and more persistent on the mainland compared to
414 Vancouver Island meaning that exposure to the elements may be higher on Vancouver Island,
415 resulting in conditions being more similar than would be expected based on weather data.
416 Deacclimation processes may also differ between populations, but since deacclimation results
417 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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590 **Figures**

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

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

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

600 **Tables**

601 Table 1– Details of weather stations used to compare weather parameters inside and outside the
602 range limit of *Papilio zelicaon*. Station IDs and location information from Environment Canada
603 (climate.weather.gc.ca). Station number corresponds to numbers on Fig. 1, and position is
604 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

605

606 Table 2 – Biochemical and physiological measurements of winter-acclimated and deacclimated
 607 *Papilio zelicaon* pupae (mean \pm 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 \pm 2.0 (n=15)	-17.4 \pm 3.6 (n=5)	t ₃₃ =2.42, p=0.026
Survival (1h @ -20 °C)	100 % (n=5)	0 % (n=5)*	χ^2 =6.4, p=0.011
Osmolality (mOsm)	924 \pm 105	411 \pm 19*	t ₆ =4.8, p=0.003
Thermal hysteresis (°C)	0.08 \pm 0.002	0.05 \pm 0.007*	t ₆ =3.5, p=0.012
Glycerol (mM)	119.5 \pm 21.7	8.1 \pm 3.6*	t ₆ =5.0, p=0.002
Trehalose (mM)	18.9 \pm 2.1	8.7 \pm 0.7*	t ₆ =4.6, p=0.004
[Na ⁺] (mM)	76 \pm 6	32 \pm 13*	t ₆ =2.97, p=0.025
[K ⁺] (mM)	79 \pm 4	84 \pm 14	t ₆ =0.39, p=0.713
[Mg ²⁺] (mM)	18 \pm 2	12 \pm 2	t ₆ =1.67, p=0.145
[Ca ²⁺] (mM)	29 \pm 5	17 \pm 0.1*	t ₆ =2.5, p=0.046

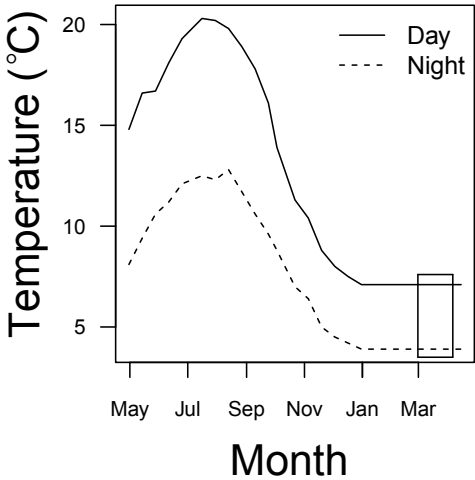
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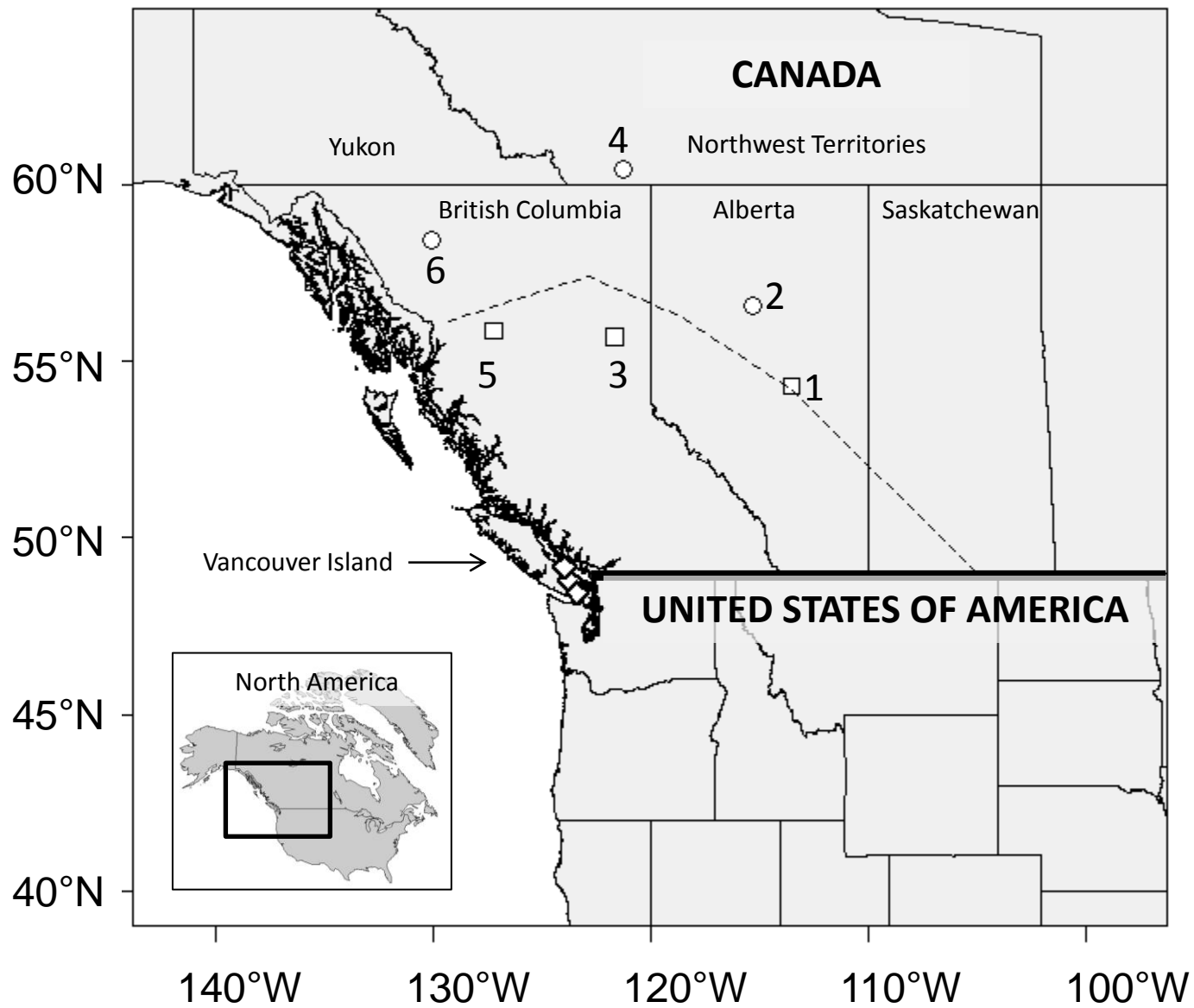
611

612 Table 3 - Daily minimum temperatures during 2011-2013 at three points longitudinally spanning
613 the northern range edge of *Papilio zelicaon* (East, Mid, West, and [Vancouver] Island). Stations
614 were chosen that were close to the northern range edge (inside range), with a paired station 300-
615 400km to the north at the same longitude (outside range) (Table 2). Date of warm spell is the
616 first day of a four-day stretch of maximum temperatures above 11 °C. Minimum temperatures
617 are in °C and are absolute minima between Jan 1st and date of warm spell (before) or in the 16
618 days following the last day of the warm spell (after).

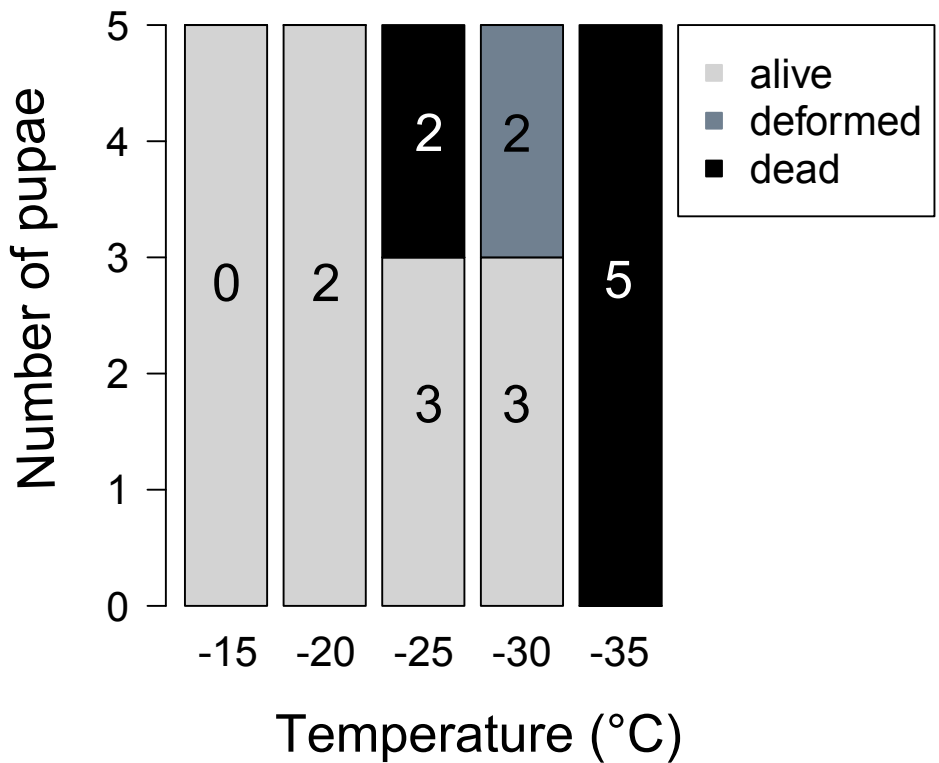
Position in range	Minimum temp. before (°C)			Date of warm spell			Minimum temp. after (°C)		
	2011	2012	2013	2011	2012	2013	2011	2012	2013
<i>East</i>									
inside	-30.0	-31.0	-31.7	11-Apr	19-Apr	25-Apr	-0.2	-0.7	-10.4
outside	-39.0	-32.4	-37.5	25-Apr	1-Mar	24-Apr	-3.6	-15.1	-10.6
<i>Mid</i>									
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
<i>West</i>									
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

619





A - One hour exposure



B - 12 hour exposure

