1 **Plasticity of cold hardiness in the eastern spruce budworm, *Choristoneura fumiferana***

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11 **Abstract**

12 High latitude insect populations must cope with extreme conditions, particularly cold

13 temperatures. Insects use a variety of cold hardiness mechanisms to withstand this

14 temperature stress, and these can drive geographic distributions through overwintering

15 mortality. The degree of cold hardiness can be altered by two evolved responses:

16 phenotypic plasticity and local adaptation. Phenotypic plasticity can occur within or

17 between generations (transgenerational plasticity; TGP), and local adaptation can evolve

18 through directional selection in response to regional climatic differences. We used the

19 eastern spruce budworm, *Choristoneura fumiferana* (Lepidoptera: Tortricidae) as a model

20 to explore the role that variable winter temperatures play in inducing two aspects of

21 plasticity in cold hardiness: TGP and local adaptation in phenotypic plasticity. This species

22 is one of the most destructive boreal forest pests in North America, therefore accurately

23 predicting overwintering survival is essential for effective management. While we found no

24 evidence of TGP in cold hardiness, there was a long-term fitness cost to larvae that

25 experienced repeated cold exposures. We also found evidence of local adaptation in both

26 seasonal and short-term plasticity of cold hardiness. These findings provide evidence for the

27   importance of phenotypic plasticity and local adaptation when modelling species

28   distributions.

29   **Key words.** Winter ecology; phenotypic plasticity; transgenerational plasticity; local

30   adaptation; Lepidoptera; *Choristoneura fumiferana*

31

Introduction

Cold hardiness during overwintering is an important determinant of temperate insect survival. Body fluids of small ectotherms are especially at risk of freezing in winter environments due to their relatively low thermal inertia (Lee, 2010). Freezing is deadly for most species; ice crystal formation causes mechanical damage to cells and cellular dehydration results in an accumulation of solutes and altered biochemical gradients (Lee, 2010). Among cold hardy insects, cold hardiness strategies can be divided into freeze tolerance and freeze avoidance (Sinclair, 1999). While freeze tolerant insects can survive internal ice formation, freeze avoidant insects survive low temperatures by depressing their supercooling point (SCP; the temperature at which their internal fluid freeze. The SCP is depressed through a number of processes such as: the dehydration of body water content (Han and Bauce, 1998), expression of low-molecular weight cryoprotectants like glycerol (Churchill and Storey, 1989), and production of ice binding proteins (IBPs; Duman, 2015).

The ability to respond to cold stress can vary by region, and these differences can arise through a combination of basal genetic adaptations and phenotypic plasticity (Sinclair et al., 2012). Adaptive differences arise as environmental conditions select for regionally adapted genotypes, leading to localized genetic differentiation. Phenotypic plasticity – the capacity of a single genotype to produce multiple environmentally-induced phenotypes – is an induced response to thermal variability (Pigliucci, 2001). These basal and plastic responses to cold stress may coevolve (Calosi et al., 2008), or manifest as a trade-off where populations with high basal cold hardiness have low phenotypic plasticity in variable thermal conditions (Kellermann et al., 2018). Disentangling these processes requires exploring cold hardiness among populations within a common-garden framework or using reciprocal transplant experiments (Overgaard et al., 2010).

Plastic responses to temperature can occur over multiple timescales. In the short term, an individual can develop resistance to stressful cold temperatures by hardening, which occurs

59   rapidly after short exposures to sub-lethal conditions and is usually reversible (Kelty, 2007).

60   Rapid cold hardening (RCH) is a form of hardening that occurs within minutes to hours after

61   a cold exposure (Lee and Denlinger, 2010). Acclimation, on the other hand, is a more gradual

62   adjustment to cold temperatures that occurs over a period of days to months (Lee, 2010).

63   Both hardening and acclimation can lead to increased cold hardiness and may share

64   biochemical mechanisms such as cryoprotectants and membrane modifications to achieve

65   this phenotype, but there is evidence that they involve non-overlapping mechanisms

66   (Rajamohan and Sinclair, 2009). Furthermore, repeated cold exposure can cause significant

67   up-regulation of cryoprotectant concentrations beyond the effects of a single exposure

68   (reviewed in Marshall and Sinclair, 2012). Responses to cold can depend on developmental

69   stage and even age, with stage-specific tolerance shifting over time (Bowler & Terblanche

70   2008). For example, cold hardiness in *Drosophila melanogaster* varies across life stages

71   (Jensen et al., 2008), with eggs having greater cold tolerance than other life stages. Plastic,

72   stage-specific variation in temperature tolerance can contribute to mortality and population

73   dynamics, which may be critical in determining the geographic distribution of a species

74   (Bowler and Terblanche, 2008).

75   Plastic responses to cold stress can also extend beyond a single generation and impact

76   future offspring through transgenerational plasticity. Maternal effects are the most frequently

77   studied form of transgenerational plasticity (TGP; reviewed in Mousseau and Fox, 1998), but

78   can also include paternal, grandparental, and potentially earlier generational effects.

79   Transgenerational responses to temperature occur in many insect species (Woestmann and

80   Saastamoinen, 2016). For example, diapause in the offspring of the cabbage beetle,

81   *Colaphellus bowringi*, is modulated by the temperatures and photoperiods experienced by

82   their parents. Moreover, the expression of diapause itself in the parental generation predicts

83   the absence of diapause in the subsequent generation (He et al., 2018). Collectively, thermal

84   conditions experienced by a population of insects can alter their immediate phenotypic

85 response and have cascading impacts on the response and fitness of subsequent generations,

86 making it critical to examine the phenotypic responses to cold stress over a range of

87 timescales.

88 The ability to respond to temperature variability and the degree of that plastic response

89 can, in itself, show local adaptation (Calosi et al., 2008; Kawecki and Ebert, 2004), resulting

90 in variable expression among populations (Chown, 2001; Sinclair et al., 2012). For example,

91 *Daphnia magna,* a widely distributed zooplankton*,* exhibits both local adaptation and

92 adaptive plasticity in temperature tolerance (Yampolsky et al., 2013), with individuals from

93 warmer sites expressing higher heat tolerance than those from cooler locations. The

94 populations also showed variable degrees of acclimation to warmer temperatures that further

95 increased heat tolerance, suggesting adaptive plasticity. This form of plasticity may be

96 particularly important in species with large distributions, as climatic conditions can vary

97 substantially along latitudinal or altitudinal gradients, providing a mechanism for local

98 populations to respond to regional temperature conditions.

99 The eastern spruce budworm (*Choristoneura fumiferana* Clemens, 1865) is a destructive

100 forest pest found throughout the northern boreal forest (Fig. 1), whose distribution spans ~24

101 degrees of latitude and over 80 degrees of longitude. This distribution represents a large

102 winter temperature gradient, where populations in the southern range will experience a much

103 milder winter climate than those in the northern latitudes. Spruce budworm survives winter

104 as second instar larvae in silken overwintering structures (called hibernacula) and undergoes

105 a six to eight month dormancy (Han and Bauce, 1993; Marshall and Roe, n.d.; Régnière,

106 1990). Ice formation is lethal to *C. fumiferana*, so the SCP represents a physiological and

107 fitness limit for this freeze avoidant species.   Second instar larvae are the primary

108 overwintering stage and can depress their SCP to ~-35°C (Han and Bauce, 1995a) by

109 accumulating ~ 0.8 M glycerol (Han and Bauce, 1995b), expressing IBPs (Qin et al., 2007;

110 Tyshenko et al., 1997), and decreasing their internal water content (Bauce E and Han, 2001;

111   Han and Bauce, 1998; Marshall and Roe, n.d.). While capable of surviving very brief

112   exposures to temperatures near their SCP, exposure to -15 °C for more than ten days can

113   result in mortality (Han and Bauce, 1995a). There is also evidence that fluctuating

114   temperatures can further induce significant plasticity in cold tolerance on short time scales

115   (*i.e.* days, Marshall and Sinclair, 2015), even in seasonally-acclimated individuals. *C.*

116   *fumiferana* larvae overwintered outside have significantly lower SCPs than larvae

117   overwintering in laboratory conditions (Han and Bauce, 1993), suggesting that fluctuating

118   conditions may be a critical cue for inducing a greater response to cold stress than expressed

119   under constant temperatures.

120   The vast majority of cold tolerance work for *C. fumiferana* has been based on a

121   laboratory strain maintained at the Insect Production and Quarantine Laboratories (IPQL,

122   Natural Resources Canada, Great Lakes Forestry Centre, Sault Ste. Marie, Ontario, Canada).

123   The IPQL laboratory strain was established from multiple populations in Ontario and has

124   been in culture for over 60 years (Roe et al., 2017). Genetic drift and historical population

125   bottlenecks, as well as potential laboratory adaptation to constant temperatures, could limit

126   the general applicability this strain to characterize cold stress responses of wild populations.

127   The ability of *C. fumiferana* to withstand low temperature stress, particularly in northern

128   latitudes, is crucial to understanding their overwintering capacity, and by extension, their

129   geographic distribution (Gray, 2008; Régnière, 2009). Currently, winter conditions at the

130   northern edges of their range exceed these published limits, which suggests that cold

131   hardiness capacities in the eastern spruce budworm may be significantly under-reported and

132   vary throughout their range. Hence, we need to explore the intraspecific variation in basal

133   and plastic cold tolerance to determine the overwintering capacity of the spruce budworm.

134   Spruce budworm larvae do not feed after hatching, but prepare for diapause immediately

135   following eclosion. This means that the energy reserves needed to survive winter and initiate

136   spring development are entirely dependent on parental investment (Sanders, 1991).

137 Therefore, conditions or stress experienced by the parental generation could affect the cold

138 tolerance of their offspring via TGP. For example, acclimating *Calliphora vicina* to lower

139 fall temperatures decreases the SCP of their offspring by 3 ℃ (Coleman et al., 2014). In *C.*

140 *fumiferana*, previous work has found transgenerational fitness effects on offspring fitness,

141 driven by parents' nutritional status as sixth-instars (Carisey and Bauce, 2002). It is,

142 however, unclear whether TGP influences the overwintering capacity of *C. fumiferana* or

143 contributes to its adaptive plasticity in cold tolerance.

144 The basic mechanisms of cold hardiness in *C. fumiferana* are well understood, however,

145 we know little about the role plasticity and local adaptation plays in the survival of this

146 widespread species. Using new laboratory strains of *C. fumiferana* (Perrault et al., 2021), we

147 tested two hypotheses: 1) that spruce budworm populations have local adaptation in the

148 plasticity of cold hardiness on either seasonal or short-term timescales, and 2) that this

149 plasticity induces TGP in cold hardiness. We found that while fluctuating temperatures

150 induced plasticity in cold hardiness, there was no evidence that this plasticity was

151 transgenerational. However, strains of *C. fumiferana* from higher latitudes exhibited greater

152 basal cold hardiness and significantly more plasticity than those from lower latitudes.

153 **Materials and methods**

154 *Experimental animals*

155 We performed all experiments on second-instar diapausing *Choristoneura fumiferana*

156 caterpillars obtained from IPQL. Transgenerational plasticity experiments were conducted

157 on the "IPQL" strain (Roe et al., 2017) established in 1961 (Glfc:IPQL:Cfum). Local

158 adaptation in plasticity experiments were conducted on colonies established from wild

159 populations as reported in Perrault et al. (2021; Fig. 1). Regional colonies used in this study

160 originated from Campbellton, New Brunswick (Glfc:IPQL:CfumBNB01, "New Brunswick"

161 47°59'13.1"N 66°40'37.8"W), Manic-Cinq, Quebec (Glfc:IPQL:CfumMQC01, "Quebec"

162   52°51'07.1"N 67°06'35.5"W), High Level, Alberta (Glfc:IPQL:CfumHAB, "Alberta"

163   58°50'71" N 117°14'03" W), Inuvik, Northwest Territories (Glfc:IPQL:CfumINT01,

164   "Inuvik" 68°21'56.2"N 133°42'04.9"W). The Alberta and Inuvik populations correspond to

165   the "Central" genomic cluster identified in Lumley et al. (2020), with the remaining

166   populations contained within the "Eastern" cluster. These regional strains were kept in

167   culture for one (late diapause measures) or two generations before use (all other measures).

168   We received dormant larvae in newly-spun hibernacula embedded in gauze from IPQL via

169   overnight shipping on ice packs to the University of British Columbia. Once received, larvae

170   were placed into an incubator (MIR-154, Sanyo, Bensenville, USA) held at 2 °C in constant

171   darkness.

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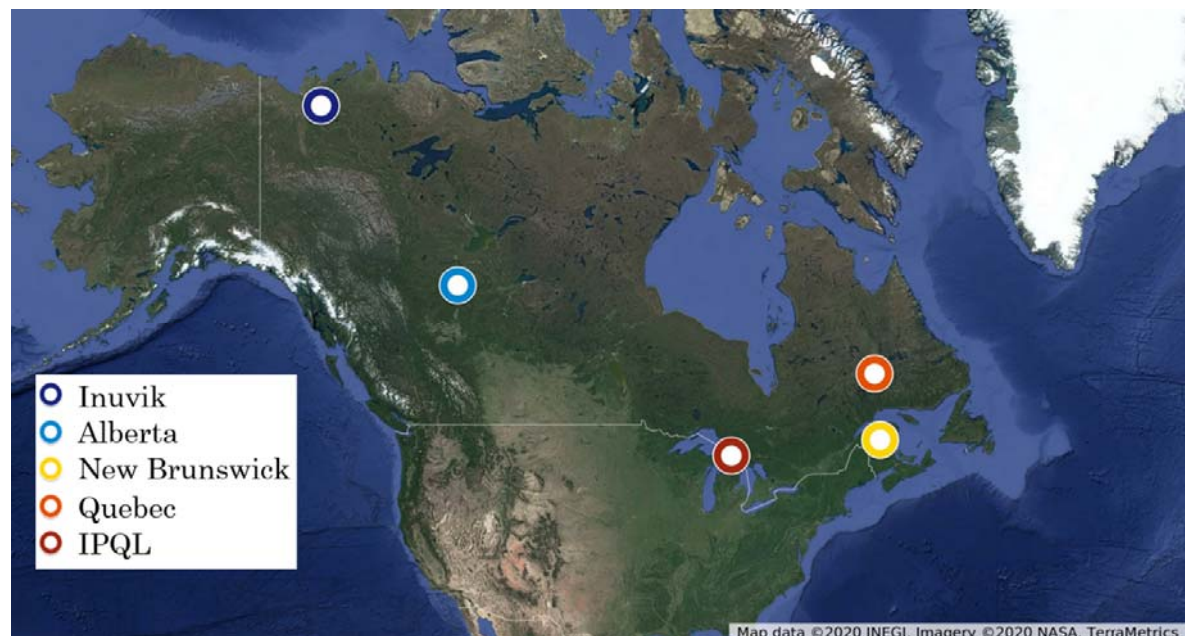
174   **Fig. 1.** Map of population sampling localities.

175   *Experimental design: local adaptation in phenotypic plasticity*

176   We assessed local adaptive plasticity in four colonies of eastern spruce budworm

177   recently derived from wild populations (Inuvik, Alberta, Quebec, New Brunswick). We

178   tested two aspects of locally adaptive plasticity in cold hardiness during diapause: 1) in

179   constant dormancy temperatures, and 2) following variable cold exposure regimes. The first

180   was tested by measuring larvae either six or twelve weeks into diapause (held at 2 °C under

181   constant darkness), constituting the "early" and "late" diapause groups, respectively. To test

182   the effect of variable temperatures, early diapause groups were further separated into "basal"

183   or "inducible". Individuals in the "inducible" treatment group were held at 2 °C for six

184   weeks, then exposed to five repeated cold events of -15 °C for 12 hours with a return to 2 °C

185   for 12 hours (ramp rate of 0.05 °C/minute). No caterpillars from the New Brunswick

186   population were exposed to the inducible treatment due to limited sample numbers.

187   *Experimental design: Transgenerational plasticity*

188   We explored whether parental cold stress would generate a transgenerational response in

189   *C. fumiferana* larval cold tolerance. First, we exposed the parental population to different

190   temperature regimes, then measured SCP, biochemical properties, and lower lethal

191   temperature (LLT). We exposed 980 diapausing IPQL caterpillars to four cold temperature

192   regimes: control, single 1, single 2, and repeated. "Control" insects were kept at 2°C for six

193   weeks. "Single 1" insects were kept at 2 °C for six weeks, then exposed to -10 °C for 12 hours

194   (including a ramp rate of 0.05 °C/minute) and measurements were made after 24 hours at 2

195   °C. "Single 2" insects were kept at 2 °C for six weeks, then exposed to -10 °C for 12 hours

196   (including a ramp rate of 0.05 °C/minute) and measurements were made after 152 hours (~ 6

197   days and 8 hours) at 2 °C to account for differences in the time since first cold exposure

198   between "single 1" and "repeated". "Repeated" insects were kept at 2 °C for six weeks, then

199   exposed to five repeated cold events of -10 °C for 12 hours with a return to 2 °C for 12 hours

200   (ramp rate of 0.05 °C/minute). We provide detailed descriptions of these cold exposure

201   techniques below (see *Cold exposures*). All temperature treatments were conducted in total

202   darkness. Following temperature exposures, we haphazardly selected 20 individual larvae for

203   SCP measurements, five sets of 10 larvae for biochemical assays, and five sets of 20 larvae

204   for LLT measurements. We also retained a subset of individuals (10 sets of 10 larvae) from

205   the parental treatment groups to generate F_1 progeny and repeated the cold tolerance

206   measurements on the F_1 progeny (i.e. SCP and biochemical properties).

207   *Cold exposures*

208   To conduct cold exposures, we placed microcentrifuge tubes containing larvae into a

209   milled aluminum block (Sinclair et al., 2015) connected to a programmable refrigerated

210   circulating bath (Lauda Proline RP 3530, Wurzburg, Germany) containing 50:50 ethylene

211   glycol:water. Ten 36 AWG Type T (copper-constantan) thermocouples (Omega Engineering

212   Inc., Laval, Canada) were placed in the block to monitor temperature. These thermocouples

213   were interfaced with PicoTech TC-08 thermocouple interfaces connected to a computer

214   running PicoLog software (Pico Technology, Cambridge, U.K.) taking temperature samples

215   in the block every 0.5 seconds. The bath was set to cycle between 2 and -15 °C (OR -10 °C

216   for TGP exposures) for 12 hours each including ramping rates between these temperatures of

217   0.05 °C/min for five full cycles. Larvae were returned to the incubator in diapausing

218   conditions for 24 hours to recover after the exposure.

219   *Measures of cold hardiness*

220   Supercooling point (SCP)

221   SCPs were measured as in (Strachan et al., 2011). Briefly, we removed larvae from their

222   hibernacula and stored them in 1.8 mL microcentrifuge tubes until SCP measurements. We

223   attached 20 individual caterpillars to 36 AWG Type T copper-constantan thermocouples

224   with a thin layer of vacuum grease. We threaded these thermocouples through the pierced top

225   of the 1.8 mL microcentrifuge tubes and held in place with adhesive putty.

226   We floated each tube on a 60:40 methanol: water solution in a programmable

227   refrigerated circulating bath (Lauda ECO RE 1050, Wurzburg, Germany). The refrigerated

228   circulating bath was cooled from 2 to -38 °C at a rate of 0.09 °C/minute. SCP was recorded as

229   the temperature immediately prior to the onset of the exotherm (Lee, 2010; Sinclair et al.,

230   2015). Late diapausing Inuvik caterpillars could not be reliably frozen using the

231   abovementioned method. In this case, SCPs were estimated by placing computer-interfaced

232   thermocouples attached to individual caterpillars in microcentrifuge tubes in Styrofoam

233   freezer boxes and then into a -80 °C freezer. The cooling rate for this exposure can only be

234   estimated in this case as 9 °C/minute.

235   *Biochemical assays for energetics and metabolites*

236   We removed larvae from their hibernacula and stored them as in SCP treatment. We then

237   homogenized groups of 10 caterpillars with approximately 90 0.5 mm Zirconium oxide

238   beads (Next Advance Inc., Averill Park, USA) in a Bullet Blender (Storm 24, Next Advance

239   Inc., Averill Park, USA) for two minutes at the highest speed. After initial blending, we

240   added 50 µL 0.05% Tween 20 and repeated blending. An additional 250 µL 0.05% Tween 20

241   was added and the sample was mixed using a vortexer (Vortex-Genie 2, Scientific Industries

242   Inc., Bohemia, USA). Following mixing, we centrifuged each sample (Allegra 64R,

243   Beckman Coulter Canada Inc., Mississauga, Canada) for 10 min at $15,000 \times g$. We removed

244   two aliquots of the supernatant and stored the samples at -80 °C for later assays.

245   Glycerol, glucose, glycogen and protein content was measured using spectrophotometric

246   assays following (Marshall and Sinclair, 2015) using glycerol, glucose and Type II glycogen

247   from oyster and bovine serum albumin as standards, respectively. Briefly, glycerol was

248   measured using a Free Glycerol kit (MAK117, Sigma-Aldrich Canada Co., Oakville,

249   Canada). Glucose was measured using a hexokinase-based Glucose assay kit (GAHK20,

250   Sigma-Aldrich Canada Co.). Glycogen content was measured using the same kit following

251   an 8 h amyloglucosidase (A9228, Sigma-Aldrich Canada Co.) digestion in a dark drawer at

252   room temperature. Soluble protein was measured using a Bicinchronicinc acid kit (BCA1,

253   Sigma-Aldrich Canada Co.). Absorbance of each reaction was measured in a

254   spectrophotometer (Spectra Max M2, Molecular Devices, San Jose, USA) and calculated

255   concentrations are reported in µmol/individual.

256   *Additional measures for TGP experiment: Lower lethal temperature (LLT) and rearing F_1*

257   *progeny*

258   To estimate LLT, five groups of 20 caterpillars within their hibernacula from each

259   treatment group were exposed to either -15, -20, -25, -30, or -35 °C, as described in (Sinclair

260   et al., 2015). Groups of larvae were exposed to their corresponding temperature treatment for

261   4 hours, by putting the pierced 1.8 mL microcentrifuge tubes into a milled aluminum block as

262   described for the cold exposure treatments. After exposure, they were transferred to Petri

263   dishes. Mortality was assessed under the microscope (MEB126, Leica, Wetzlar, Germany)

264   by checking if caterpillars were dehydrated, out of their hibernaculum or immobile, at five

265   different time points throughout development (one week after exposure, at the end of

266   diapause (20 weeks from the onset of diapause), at thinning (10 days after ending diapause,

267   between instar 3 and 4) and at pupation, following the rearing protocols explained below.

268   Dead caterpillars were removed at each time point. The temperature at which 50% of the

269   group survived (LT_{50}) was calculated at each time point by assessing mortality to determine

270   both change in LT_{50} after different cold exposures (treatments) and change in LT_{50} over

271   development (time).

272   After the cold exposures, the mating group was returned to diapausing conditions (2 °C

273   in constant darkness) for a total of 20 diapausing weeks (including the initial six weeks

274   pretreatment and treatment time). Following this, they were put into feeding/developing

275   conditions (24 °C, with a 16:8 light: dark cycle) as detailed in (Marshall and Sinclair, 2015).

276   Briefly, groups of 10 caterpillars were put onto diet cups with artificial diet (McMorran,

277   1965)   purchased   through   Insect   Production   and   Quarantine   Services

278   (https://www.nrcan.gc.ca/science-data/research-centres-labs/forestry-research-centres/great-

279   lakes-forestry-centre/insect-production-services/13467). Insects were placed on fresh diet

280   once a week to avoid the accumulation of frass, microbes and mold. Ten days later, one

281   caterpillar was put into each cup (thinning). At pupation, we sterilized pupae with a 10%

282   bleach solution under a fume hood, rinsed with deionized water and left to dry on paper

283   towels. They were then sexed under a microscope and the first 40 individuals were weighed

284   on a microbalance (CP 124 S, Sartorius, Göttingen, Germany). Males and females were

285   moved to separate ventilated plastic containers to emerge. These emergence chambers were

286   checked daily for emerged adult moths. The first 20 male and female moths (total of 40

287   moths) that emerged were put into 20 L clear plastic bags with three sets of five 5×5 cm

288   strips of waxed paper stapled together. A 50:50 female and male ratio was maintained in each

289   bag. Each treatment's mating chamber was sprayed with deionized water before and after

290   take-down. The three mating chambers were kept in environmental chambers ($23 \pm 3°C$, $55 \pm$

291   10% RH, 16L:8D) in the Faculty of Forestry at the University of British Columbia. Mating

292   chambers were set-up for a week, after which moths died and egg masses were collected.

293   Emergence pans for egg masses were set-up using $30 \times 25 \times 5$ cm baking trays lined with

294   gauze and sealed with Parafilm and electrical tape to avoid larval escape. Once second-instar

295   caterpillars began spinning their hibernacula, the emergence pans were opened to count the

296   number of larvae that had emerged and spun their hibernacula. The gauze was then removed,

297   wrapped it with Parafilm, and put it in an incubator held at 2 °C in constant darkness.

298   After six weeks into diapause, all measures of cold hardiness were repeated with F_1

299   second-instar larvae.

300   *Statistics*

301   All statistical tests and data plots were conducted in RStudio (version 1.1.463, 2018). To

302   test for transgenerational plasticity, a Type II ANOVA model was fitted for each cold

303   hardiness measure using the aov function in the car package (Fox and Weisberg, 2019) with

304   generation and treatment as predictors. To test for local adaptation in cold hardiness, the

305   same model was fitted with population, diapause stage and treatment as predictors. For

306   biochemical assays, we used protein concentration as a covariate in the model. LLTs were

307   calculated at each assessed time point using a generalized linear model with binomial error

308   distribution and the dose.p function with p set to 0.5 from the MASS package (Venables and

309   Ripley, 2003). Alpha was set to 0.05 for all tests. p-values less than 0.01 are reported as such.

310   All significant interactions were further investigated using TukeyHSD posthoc tests.

311   **Results**

312   *Local adaptation in phenotypic plasticity of cold hardiness*

313   Spruce budworm populations showed significant differences in cold hardiness,

314   particularly as larvae progressed in diapause. Late diapause individuals from the Inuvik

315   population had a significantly lower SCP compared to other populations and times tested

316   (population: $F_{(2,119)}$=4.87, p<0.001; time: $F_{(1,119)}$=16.06, p<0.001; population $\times$ time:

317    $F_{(2,119)}$=5.43, p<0.01; Fig. 2A). Population had a significant effect on basal glycerol content

318   (population: $F_{(2,29)}$=13.85, p<0.001; Fig. 2B). In this case, larvae from Inuvik significantly

319   increased their glycerol by the late diapause time point, whereas larvae from New Brunswick

320   significantly decreased glycerol content during the same timeframe. Glycogen

321   concentrations were significantly affected by time (time: $F_{(1,29)}$=11.27, p<0.01; Fig. 2C).

322   Population and time in diapause also had a significant effect on basal total carbohydrate

323   concentrations (population: $F_{(2, 29)}$=3.196, p=0.020; time: $F_{(1, 29)}$=15.96, p<0.001), with New

324   Brunswick larvae in early diapause having lower total carbohydrate content than the early

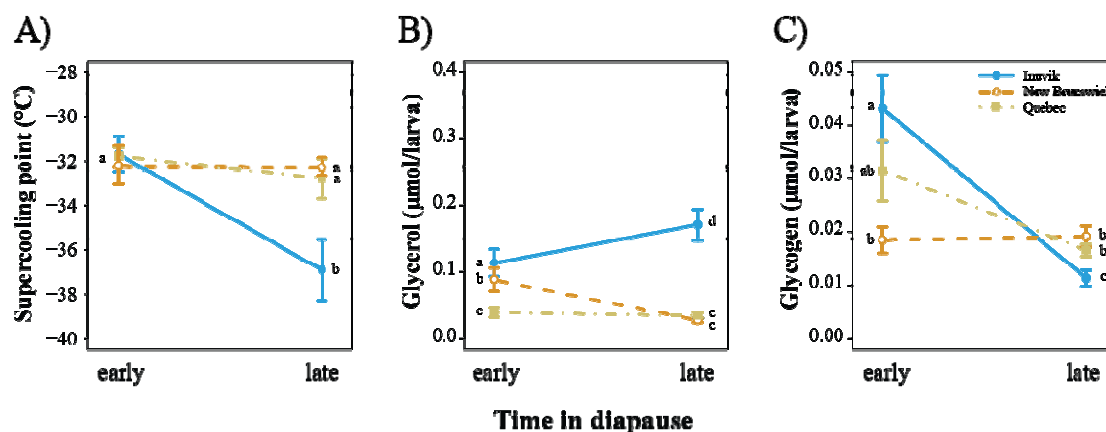325   diapause larvae from Inuvik and Quebec (Fig. A2).

326

Fig. 2. Testing for local adaptation in seasonal plasticity without pretreatment. A) Supercooling point (SCP; °C), B) glycerol per larva (μmol) and C) glycogen per larva (μmol) of second-instar *Choristoneura fumiferana* across different populations measured at either early (6 weeks) or late (12 weeks) into diapause. Glycogen is measured in glucose units. Points with the same letter are not statistically significantly different in an ANCOVA with protein mass as a covariate.

We exposed larvae to repeated cold events to examine the differences between basal cold hardiness and an inducible plastic response. We found a significant interaction between population and repeated cold exposures on SCP (population: $F_{(3,169)}=10.32$, $p<0.001$; treatment: $F_{(1,169)}=15.80$, $p<0.001$; population × treatment: $F_{(3,169)}=3.654$, $p=0.014$; Fig. 3A). A significant effect of population and treatment was found on glycerol concentrations (population: $F_{(3,39)}=36.47$, $p<0.001$; treatment: $F_{(1,39)}=31.31$, $p<0.001$; population × treatment: $F_{(3,39)}=7.24$, $p<0.001$; Fig. 3B). Alberta and Inuvik decreased their SCP after exposure, whereas Quebec and the IPQL remained the same. For glycogen; there was no effect of population or treatment (population: $F_{(3,39)}=0.123$, $p=0.12$; treatment: $F_{(1,39)}=0.534$, $p=0.47$; Fig. 3C). The same was true for total carbohydrate, there was no significant effect of population or treatment (population: $F_{(3,39)}=2.765$, $p=0.058$; treatment: $F_{(1,39)}=0.88$, $p=0.35$; Fig. A3).
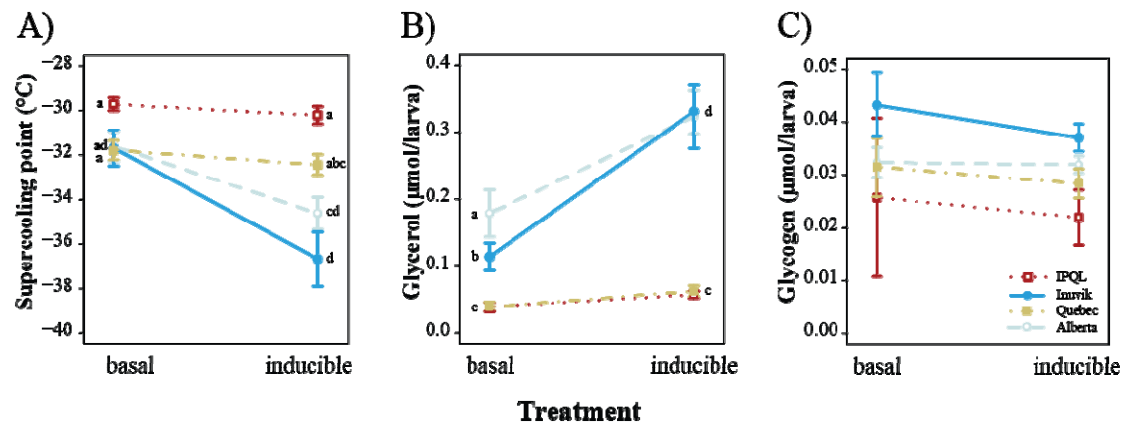
Fig. 3. Testing for local adaptation in short-term response to temperature variation. A) Supercooling point (SCP; °C), B) glycerol per larva (µmol) and C) glycogen per larva (µmol) of second-instar *Choristoneura fumiferana* before ("basal") and after ("induced") five exposures to -15 °C. Different letters indicate statistically significant comparisons (p≤α). All exposures were conducted on early diapause larvae. Glycogen is measured in glucose units.   Points with the same letter are not statistically significantly different in an ANCOVA with protein mass as a covariate.

Transgenerational plasticity of cold hardiness

In the IPQL strain, repeated cold exposures did not significantly change SCP of either generation, although SCP was higher in the F_1 generation (generation: $F_{(1,139)}$=63.77, p<0.01; Fig. 4).
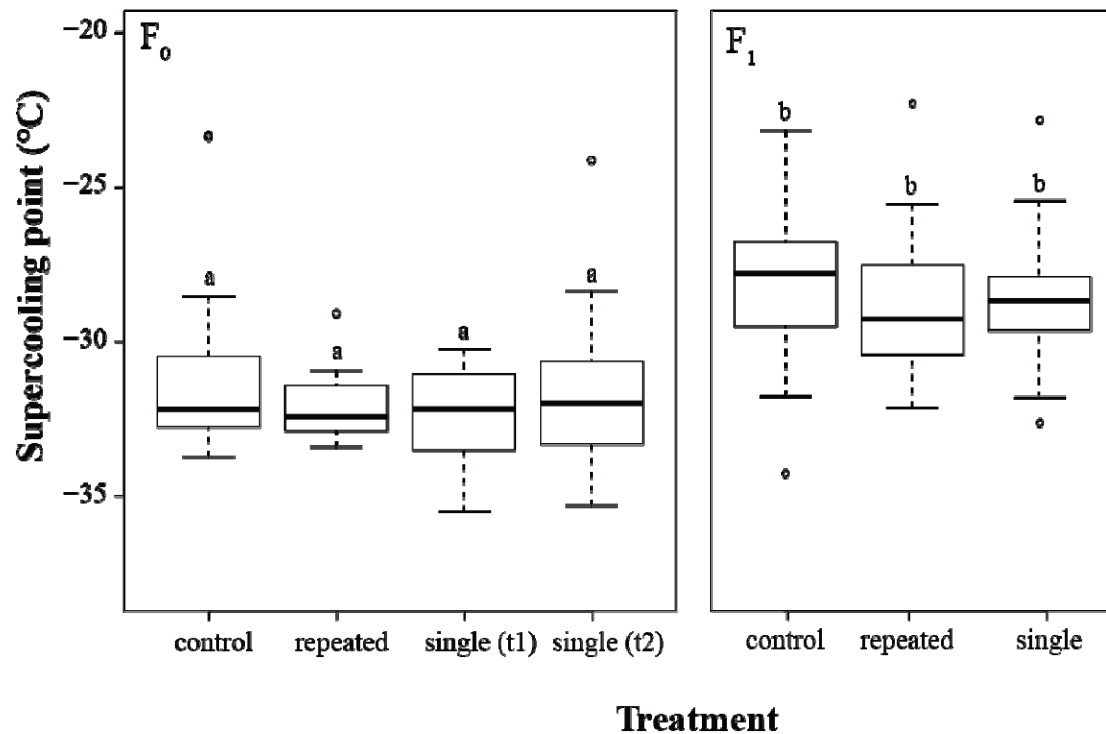
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Fig. 4. Supercooling points (SCP) (°C) as a result of cold exposures and generation in second instar IPQL *Choristoneura fumiferana* larvae. Bold line inside box shows median, lower and upper box boundaries show 25th and 75th percentile, respectively, lower and upper error lines show 10th and 90th percentile, respectively. Treatment groups are Single T1 (larvae exposed to a single cold exposure of -10 °C for 12 hrs and assessed 24 hours after), Single T2 (larvae exposed to a single cold exposure of -10 °C for 12 hrs and assessed at the same time as the repeated group) and Repeated (larvae exposed to five cold exposures of -10 °C for 12 hrs and assessed 24 hours after). Different letters indicate statistically significantly different comparisons ($p \leq \alpha$).

We calculated LT_{50} for each treatment group at four time points following exposure to five temperatures. We assessed mortality one week after exposure, at the end of diapause (20 weeks from the onset of diapause), thinning (between instar 3 and 4) and following pupation (Fig.s A4-7). The effect of cold exposure on LT_{50} depended on the time of assessment and treatment (time point: $F_{(3,15)}=58.63$, $p<0.001$; treatment: $F_{(3,15)}=4.18$, $p=0.047$). Individuals exposed to a single cold event (but sampled at the same time as the repeated group, Single 2) had a higher LT_{50} at every time-point assessed, except at the end of diapause, compared to other treatments (Fig.. 5). Additionally, LT_{50} increased with time as mortality effects accrued

381   through development. Larvae that experienced repeated cold exposures had the lowest LT_{50}

382   at every time point.

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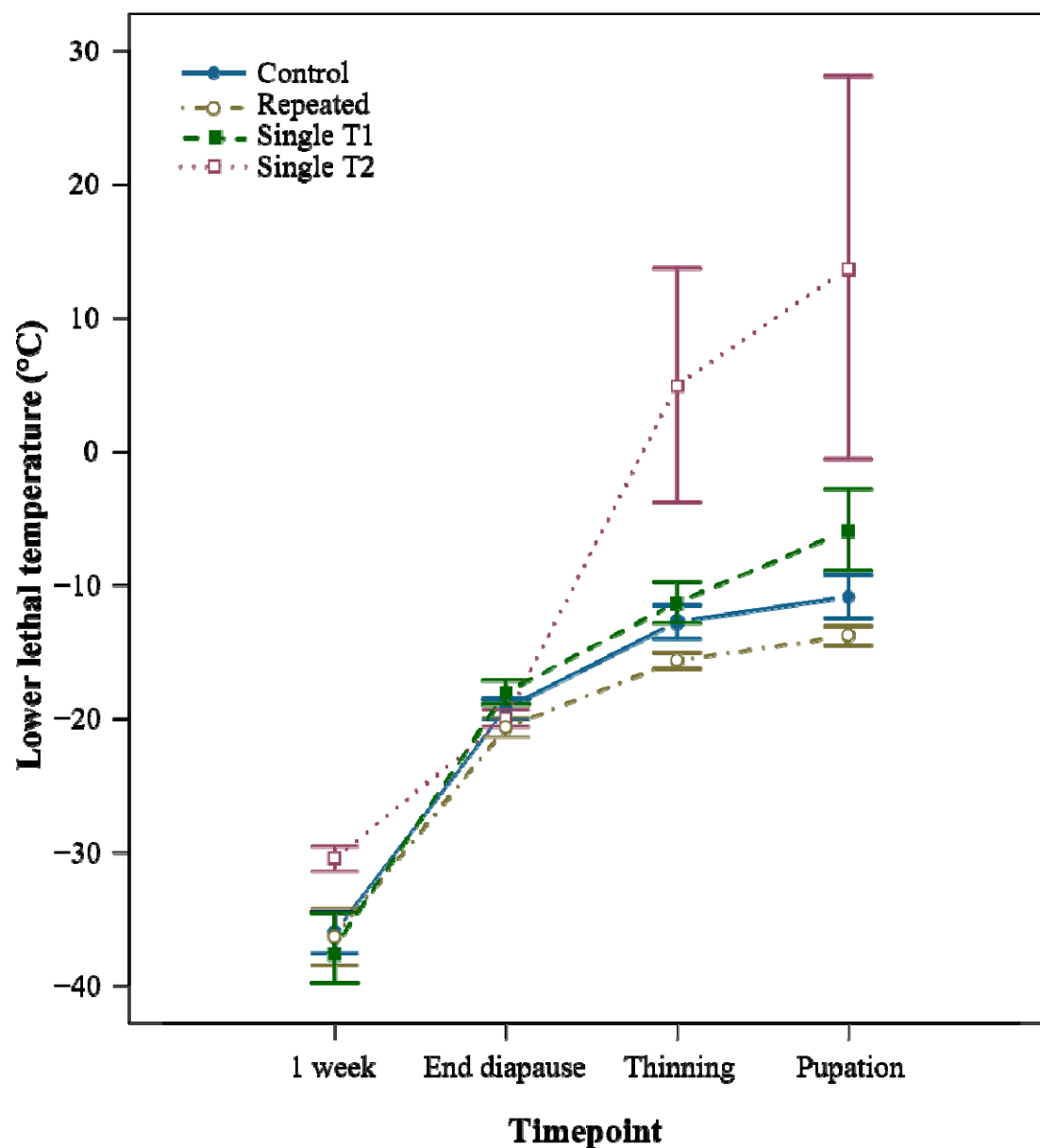
Fig. 5. Lower lethal temperature (LLT), displayed as LT_{50}, of F_0 second-instar IPQL (*Choristoneura fumiferana*) larvae after different cold exposures shown in color. Error bars show standard error of the LT_{50} estimate. Treatment groups are Single 1 (larvae exposed to a single cold exposure of -10 °C for 12 hrs and assessed 24 hours after), Single 2 (larvae exposed to a single cold exposure of -10 °C for 12 hrs and assessed at the same time as the repeated group) and Repeated (larvae exposed to five cold exposures of -10 °C for 12 hrs and assessed 24 hours after).

392

393   Cold exposure and generation had a significant effect on energy reserves and metabolites

394   measured in our biochemical assays. Cold exposure caused no significant differences in

395   glycerol content (treatment: $F_{(3,34)}=1.039$, p=0.39). This can be attributed to the large

396   variance (sd=0.08) compared to the other treatment groups in F_0 (sd<0.01; Fig. 6A).

397   Glycogen content significantly differed among treatments (treatment: $F_{(3,34)}=7.60$, p<0.001),

398   with control and single 1 being significantly lower than single 2 and repeatedly cold exposed

399   larvae (Fig. 6B). There was no significant interaction between treatment and protein mass

400   (treatment × total protein: $F_{(3,34)}=2.03$ p=0.13). Glycogen concentrations differed with

401   protein mass (total protein: $F_{(1,34)}=43.81$, p<0.001). Total carbohydrate significantly

402   differed among treatments (treatment: $F_{(3,34)}=5.42$, p<0.01). Post-hoc tests indicated larvae in

403   treatments single 2 and repeated had significantly higher total carbohydrate content than

404   larvae in the control and single 1 treatments (Fig. A1).
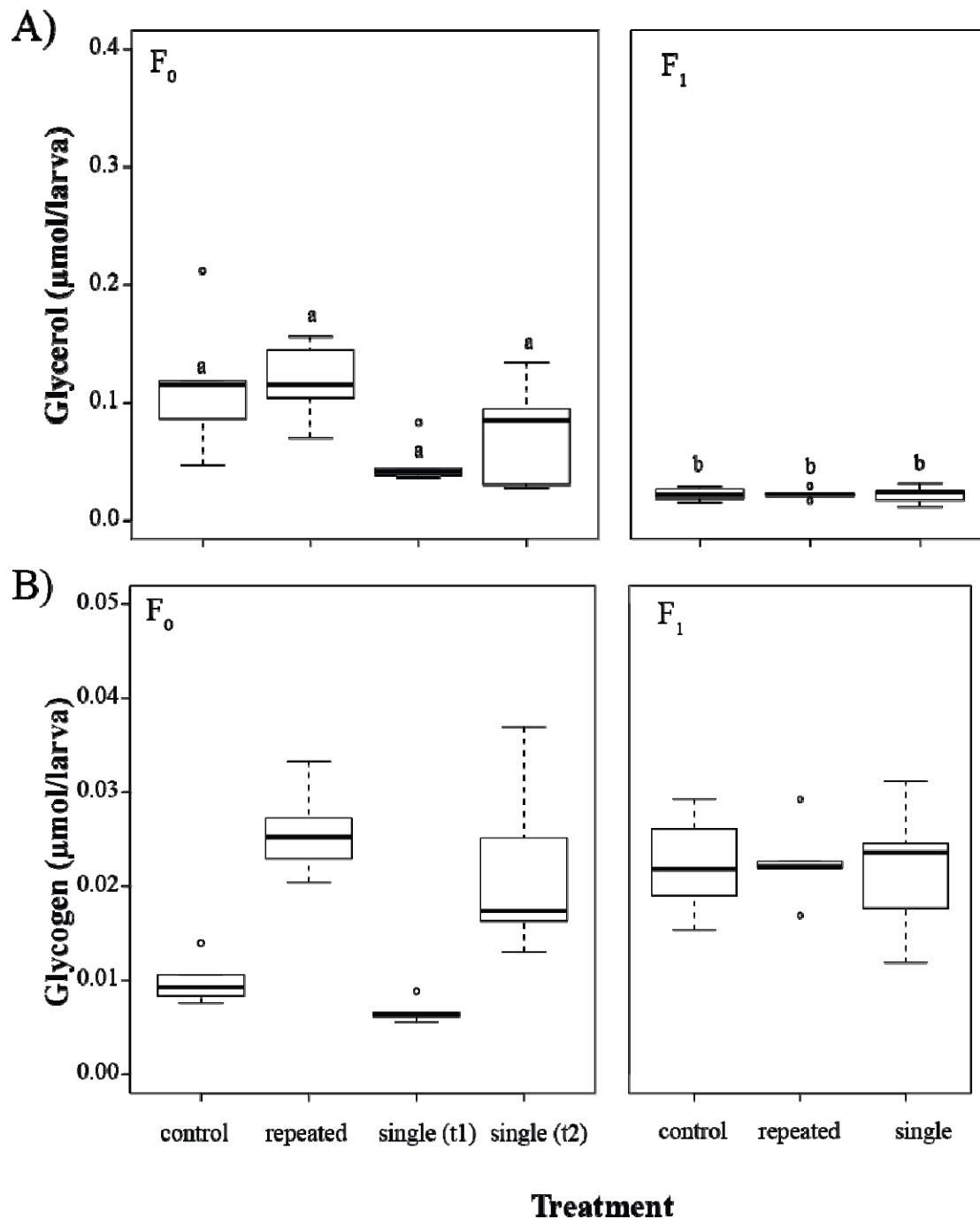
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Fig. 6. A) Glycerol per larva (μmol) and B) Glycogen per larva (μmol) across the different treatments and two generations in second-instar IPQL *Choristoneura fumiferana* larvae. Refer to Fig. 2 caption for explanation of boxplot display and treatment groups. Letters indicate statistically significant comparisons ($p \leq \alpha$). Glycogen is measured in glucose units. Points with the same letter are not statistically significantly different in an ANCOVA with protein mass as a covariate.

Discussion

415   Geographic differences in cold tolerance can arise from genetic variation, phenotypic

416   plasticity, or their interaction (reviewed in Chevin et al., 2010). Here, we explored the

417   relationship between local adaptation and plasticity in overwintering capacity using *C.*

418   *fumiferana*, an ecologically and economically important forest insect pest. We found

419   regional differences in both seasonal and short-term plasticity of cold hardiness in *C.*

420   *fumiferana*, suggesting local adaptation to winter climates. Regional differences in

421   short-term plasticity were greater than differences in basal cold hardiness in early diapause

422   larvae, emphasizing the importance of inducible responses in high latitude populations. We

423   found no evidence for TGP despite a significant reduction in F_0 fitness following repeated

424   cold exposures. Our results highlight the importance of regional differences in cold tolerance,

425   particularly the capacity for northern populations to respond to variable winter conditions.

426   We suggest that future studies consider regional differences in spruce budworm populations

427   and include seasonal and short-term plasticity when quantifying overwintering capacity.

428   Seasonal plasticity in insect cold tolerance is well described (Bale, 2002). Generally, our

429   populations reduced their SCPs and shifted metabolite concentration as diapause progressed.

430   Similar results were previously found in the IPQL laboratory strain, with cold tolerance and

431   metabolites changing over the dormancy period (Marshall and Sinclair, 2015). However, our

432   results indicated that these patterns of seasonal plasticity varied among populations. While

433   our populations did not differ in their basal SCP in early diapause (Fig. 2A, 3A), late diapause

434   larvae from high latitudes had significantly lower SCPs compared to lower latitude

435   populations (Fig. 2A). These larvae also had significantly higher glycerol content compared

436   to lower latitude populations and their increased glycerol content corresponded to reductions

437   in SCP. Our results suggest that *C. fumiferana* has significant local adaptation to seasonal

438   winter conditions.

439   High latitude populations experience significantly longer and colder winters than

440   populations at lower latitudes (Marshall et al., 2020). Recent studies have shown that local

441   adaptation in the degree of seasonal cold tolerance occurs across a range of native and

442   invasive insects (Batz et al., 2020; Marshall et al., 2020; Williams et al., 2015). As expected,

443   spruce budworm populations from high latitudes (e.g. Inuvik, Northwest Territories and

444   High Level, Alberta) had greater overwintering capacity than eastern populations at lower

445   latitudes. The basal SCP for late diapause Inuvik insects is lower than results published for *C.*

446   *fumiferana* larvae from the IPQL lab colony under identical experimental and rearing

447   conditions (Han and Bauce, 1995a; Marshall and Sinclair, 2015). In fact, we actually

448   encountered methodological challenges measuring SCPs for these northern individuals. We

449   were unable to freeze late-diapause Inuvik larvae using a refrigerated circulating bath, a

450   method successful for other populations in our study. Therefore, we had to use a -80 °C

451   freezer to reach temperatures low enough to initiate freezing in these larvae. An important

452   difference in these two methods is that the cooling rate in the freezer was faster (9 °C/min)

453   than the programmable bath (0.09 °C/min). There is evidence that cooling rates can influence

454   absolute thermal limits (Terblanche et al., 2007), so the faster cooling rate of the freezer

455   could have artificially depressed the SCP of the Inuvik insects. However, given that we

456   switched to this method because the Inuvik population would not freeze under conditions

457   used for other populations, and they consistently had lower SCP measurements than southern

458   populations in other experiments (i.e. following repeated cold exposures, Fig. 6A), we are

459   confident that the lower SCP in late diapause larvae is accurate.

460   Short-term plasticity allows organisms to respond rapidly to variable environmental

461   conditions through inducible responses. We verified that fluctuating temperatures induced

462   greater cold tolerance in *C. fumiferana* than constant temperatures alone (Marshall and

463   Sinclair, 2015), and then demonstrated that high latitude populations expressed a

464   significantly greater magnitude of plasticity than lower latitude populations (Fig 3).

465   Short-term plasticity allows insects to respond to variable temperatures in a short temporal

466   timeframe (reviewed in Marshall and Sinclair, 2012). The ability to rapidly respond to

467 temperature shifts would be advantageous in northern climates where winter conditions are

468 significantly more variable than southern latitudes (Marshall et al., 2020). Both seasonal and

469 short-term plasticity combine to provide high latitude populations greater overwintering

470 capacity than their southern counterparts.

471   Theoretical models often pit local adaptation against phenotypic plasticity (e.g. Sultan

472 and Spencer, 2002), however these results indicate that phenotypic plasticity itself may be

473 under local adaptation, particularly in insect overwintering. Spruce budworm populations

474 in our study represented two distinct genetic backgrounds: Central and Eastern subclusters

475 (Lumley et al., 2020). Our high latitude populations belong to the Central cluster, while New

476 Brunswick and Quebec belong within the Eastern cluster. In fact, the IPQL population was

477 founded from populations in eastern Canada, giving it the same genetic background as our

478 other eastern populations. Even though the IPQL strain has been maintained in colony since

479 the 1960s (Roe et al., 2017) and does not experience cold temperature stress, it was

480 functionally equivalent to Eastern populations. Genomic differences between these central

481 and eastern *C. fumiferana* populations are defined by a block of single nucleotide

482 polymorphisms located on a single linkage group (LG4, Lumley et al., 2020). The authors

483 noted that one of these variant loci was associated with glycerol-3-phosphate dehydrogenase

484 (GPDH). GPDH is known to be involved in the glycerol synthesis pathway in other insects

485 (Park and Kim, 2013), which is a key cryoprotectant in *C. fumiferana* (Han and Bauce,

486 1995b; Marshall and Sinclair, 2015) and insects in general (Lee, 2010). Larvae from the

487 Central cluster produced elevated glycerol concentrations, so this genomic variation could be

488 linked to the functional differences we observed. Further exploration of these genomic

489 variants in the context of glycerol production will be critical to resolving the underlying

490 mechanisms driving local adaptation in *C. fumiferana*.

491   Transgenerational plasticity (TGP) allows insects to survive in variable climates by

492 inducing increased tolerance to temperature stress in future generations. First, however, we

493   needed to demonstrate that repeated cold exposures triggered a cold stress response in the F_0

494   generation. While we found that SCP did not change among the F_0 treatment groups, we

495   noted a decrease in LT_{50} in caterpillars that received repeated cold exposures. As such, we

496   took this as a response to temperature stress and the insects altered their cold hardiness.

497   Additionally, we found that treatment groups differed in mortality over time, suggesting that

498   cold temperatures caused injury that manifested at later developmental stages. Although we

499   did not observe a significant increase in glycerol content as a result of cold exposure, it is

500   possible that unmeasured cold hardiness mechanisms (e.g. heat shock proteins or antifreeze

501   proteins) could be responsible for this increased cold hardiness. Our results align with

502   previous work (Han and Bauce, 1995a) and although tolerance to acute cold temperatures is

503   very high as measured by SCP, the resultant chilling injury resulting from temperatures

504   above the SCP is still significant. These results suggest that we induced a significant cold

505   stress response in our F_0 generation and established the selective environment for a

506   transgenerational response.

507   Despite inducing a cold stress response in our F_0 generation, we did not detect evidence

508   of TGP in cold tolerance in our F_1 generation. This lack of TGP after repeated cold exposures

509   could be due to post-diapause feeding which masked the effect of stress-induced TGP

510   (Tauber and Tauber, 1986). We did, however, find that F_0 parents who experienced

511   repeated cold exposures experienced a substantial decrease in fecundity (>50%), similar to

512   results observed in the vinegar fly *Drosophila melanogaster* (Marshall and Sinclair, 2010)

513   even when food was not limiting. Variable, stressful environments can impact many fitness

514   traits, including growth, body size, energy stores, and development, ultimately leading to

515   reduced fecundity (Buckley et al., 2021). High latitude or high elevation populations are

516   exposed to highly variable temperature environments, which can lead to a combination of

517   short-term mortality and long-term reproductive effects that impact overall population

518   demographics (Buckley et al., 2021). Mechanistically, this lowered reproductive output

519   could be due to an inability to replenish depleted energetic reserves or long-term damage. We

520   did not detect differences in pupal weights, which suggests that individuals were likely able

521   to replenish themselves during the post-stress feeding stage, and instead it is more likely that

522   chronic damage was incurred that was not reflected in overall weight. Larval exposure to

523   heat stress in *D. melanogaster* can cause developmental defects in eclosing adults which is

524   ameliorated in strains with higher HSP70 copy number (and therefore expression),

525   suggesting that unrepaired protein damage may persist through metamorphosis (Roberts and

526   Feder, 1999). It is, therefore, not inconceivable that the same is true for cold stress in

527   overwintering larvae (reviewed in King and MacRae, 2015), and would be worthwhile

528   exploring in greater detail.

529   TGP can only be adaptive when environmental conditions are predictable between

530   parent and offspring lifecycles. Larval *C. fumiferana* are short-distance dispersers; they are

531   capable of dispersing between a few trees by "ballooning" on silken threads or walking to

532   different parts of the tree crown (Johns and Eveleigh, 2013; Nealis, 2016). By comparison,

533   adult moths disperse much larger distances, sometimes hundreds of kilometers (Sturtevant et

534   al., 2013). Therefore, the probability of shared environmental conditions between

535   generations is low, and TGP of cold hardiness in second-instars is not favored by selection. It

536   is also possible that TGP occurs in other winter-related traits rather than in absolute cold

537   hardiness or supplied energetic reserves. For example, Harvey (1961) found that some

538   fourth-instar *C. fumiferana* undergo a second diapause. The expression of a second diapause

539   may be a plastic response to environmental variability. If so, TGP could be observed in

540   diapause expression among larval instars since it is likely that environmental predictability

541   between instars is higher than between generations. The lack of TGP in our measures of cold

542   hardiness could also be due to using a lab-selected strain. The IPQL strain has been in culture

543   since 1961 and has not been augmented with wild material for the past 20 years (Roe et al.,

544   2017). Therefore, the relaxed selection of cold hardiness under lab conditions could have

545   resulted in a very different response compared to wild populations. Wild populations may

546   differ in this respect, and would be an important area for further study.

547   **Conclusions**

548   The eastern spruce budworm has a broad geographic range in North America, so

549   populations are exposed to very different temperature regimes across this range. It was

550   hypothesized that populations would exhibit local adaptation in seasonal as well as

551   short-term plasticity. Two populations tested, Alberta and Inuvik, showed a similar degree of

552   short-term plasticity, which we attribute to the geographic connectivity between the

553   populations. Generally, there were no differences in basal cold hardiness among the

554   populations we tested, however seasonal and repeated cold exposures revealed evidence for

555   local adaptation of plasticity in the populations. These results have direct implications for the

556   predicted population growth, range shifts, and current species distribution modelling for the

557   species. The IPQL lab strain, which forms the basis of our current understanding of cold

558   hardiness in the species, shows comparatively low plasticity. Therefore, shifting thermal

559   regimes in local environments due to climate change, leading to increased temperature

560   extremes that select for plasticity, will result in highly variable regional responses among

561   spruce budworm populations.

562   Winter and higher latitudes have experienced the most change in temperature under new

563   climate conditions (Marshall et al., 2020; Zhang et al., 2019), exposing populations to novel

564   thermal environments across their ranges. Understanding the regional responses of

565   populations across a species' range is critical for predicting future changes in population

566   growth and species distribution throughout the boreal forest. Absence of evidence for cold

567   hardiness TGP in the IPQL strain suggests that TGP does not need to be incorporated into

568   population growth or species range models in *C. fumiferana*. However, it does provide

569   further evidence that repeated cold exposure may drive population growth effects and that

570   fitness trade-offs in overwintering insects exist. Further work should focus on untangling the

571   potential mechanisms of these trade-offs, and future modelling should include fitness effects

572   of repeated cold exposures.

573   Local adaptation and phenotypic plasticity can and should be included in species

574   distribution models for *C. fumiferana*. Bush et al. (2016) highlights the use of hybrid species

575   distribution models, which uses trait mean, variability, heritability and the plasticity of a trait

576   to determine range shifts, therefore incorporating both plasticity and evolution. Diamond

577   (2018) tested this *AdaptR* model and found that range loss for 17 species of *Drosophila*

578   decreased by 33% in 2105 when incorporating these additional traits. For *C. fumiferana*,

579   accurately modelling species distributions with these spatio-temporal adaptive models could

580   prove useful for species management in future climates.

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586

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591   experiments, S. B.; analyses, S.B. and K.E.M.; manuscript preparation, S.B., K.E.M.

592   **Data availability statement** Data and code is available on the Open Science Framework.

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594   **References**

595 Bale, J.S., 2002. Insects and low temperatures: from molecular biology to distributions and
596   abundance. Philos. Trans. R. Soc. Lond. B. Biol. Sci. 357, 849–862.
597   https://doi.org/10.1098/rstb.2002.1074

598 Batz, Z.A., Clemento, A.J., Fritzenwanker, J., Ring, T.J., Garza, J.C., Armbruster, P.A.,
599   2020. Rapid adaptive evolution of the diapause program during range expansion of an
600   invasive   mosquito.   Evolution   (N.   Y).   74,   1451–1465.
601   https://doi.org/10.1111/evo.14029

602 Bauce E, Han, E., 2001. Desiccation resistance in pre-diapause, diapause and post-diapause
603   larvae of *Choristoneura fumiferana* (Lepidoptera: Tortricidae). Bull. Entomol. Res. 91,
604   321–326. https://doi.org/10.1079/BER2001119

605 Bowler, K., Terblanche, J.S., 2008. Insect thermal tolerance: What is the role of ontogeny,
606   ageing   and   senescence?   Biol.   Rev.   83,   339–355.
607   https://doi.org/10.1111/j.1469-185X.2008.00046.x

608 Buckley, L.B., Schoville, S.D., Williams, C.M., 2021. Shifts in the relative fitness
609   contributions of fecundity and survival in variable and changing environments. J. Exp.
610   Biol. 224, jeb228031. https://doi.org/10.1242/jeb.228031

611 Bush, A., Mokany, K., Catullo, R., Hoffmann, A., Kellermann, V., Sgrò, C., McEvey, S.,
612   Ferrier, S., 2016. Incorporating evolutionary adaptation in species distribution
613   modelling reduces projected vulnerability to climate change. Ecol. Lett. 19, 1468–1478.
614   https://doi.org/10.1111/ele.12696

615 Calosi, P., Bilton, D.T., Spicer, J.I., 2008. Thermal tolerance, acclimatory capacity and
616   vulnerability   to   global   climate   change.   Biol.   Lett.   4,   99–102.
617   https://doi.org/10.1098/rsbl.2007.0408

618 Carisey, N., Bauce, E., 2002. Does nutrition-related stress carry over to spruce budworm,
619   *Choristoneura fumiferana* (Lepidoptera: Tortricidae) progeny? Bull. Entomol. Res. 92,
620   101–108. https://doi.org/10.1079/BER2001148

621 Chevin, L.M., Lande, R., Mace, G.M., 2010. Adaptation, plasticity, and extinction in a
622   changing environment: Towards a predictive theory. PLoS Biol. 8, e1000357.
623   https://doi.org/10.1371/journal.pbio.1000357

624 Chown, S.L., 2001. Physiological variation in insects: hierarchical levels and implications. J.
625   Insect Physiol. 47, 649–660. https://doi.org/10.1016/S0022-1910(00)00163-3

626 Churchill, T.A., Storey, K.B., 1989. Metabolic correlates to glycerol biosynthesis in a
627   freeze-avoiding insect, *Epiblema scudderiana*. J. Comp. Physiol. B 159, 461–472.
628   https://doi.org/10.1007/BF00692418

629 Coleman, P.C., Bale, J.S., Hayward, S.A.L., 2014. Cross-generation plasticity in cold
630   hardiness is associated with diapause, but not the non-diapause developmental pathway,
631   in   the   blow   fly   *Calliphora   vicina*.   J.   Exp.   Biol.   217,   1454–1461.
632   https://doi.org/10.1242/jeb.098053

633 Diamond, S.E., 2018. Contemporary climate-driven range shifts: Putting evolution back on

634   the table. Funct. Ecol. 32, 1652–1665. https://doi.org/10.1111/1365-2435.13095

635 Duman, J.G., 2015. Animal ice-binding (antifreeze) proteins and glycolipids: an overview
636   with emphasis on physiological function. J. Exp. Biol. 218, 1846–55.
637   https://doi.org/10.1242/jeb.116905

638 Fox, J., Weisberg, S., 2019. An R Companion to Applied Regression:, Third. ed, Robust
639   Regression in R. SAGE Publications, Thousand Oaks, California.

640 Gray, D.R., 2008. The relationship between climate and outbreak characteristics of the
641   spruce budworm in eastern Canada. Clim. Change 87, 361–383.
642   https://doi.org/10.1007/s10584-007-9317-5

643 Han, E.-N., Bauce, E., 1993. Physiological changes and cold hardiness of spruce budworm
644   larvae, *Choristoneura fumiferana* (Clem.), during pre-diapause and diapause
645   development under laboratory conditions. Can. Entomol. 125, 1043–1053.
646   https://doi.org/doi:10.4039/Ent1251077-6

647 Han, E.N., Bauce, E., 1995a. Non-Freeze Survival of Spruce Budworm Larvae,
648   *Choristoneura fumiferana*, at Subzero Temperatures during Diapause. Entomol. Exp.
649   Appl. 75, 67–74.

650 Han, E.N., Bauce, E., 1995b. Glycerol synthesis by diapausing larvae in response to the
651   timing of low temperature exposure, and implications for overwintering survival of the
652   spruce budworm, *Choristoneura fumiferana*. J. Insect Physiol. 41, 981–985.
653   https://doi.org/10.1016/0022-1910(95)00049-Z

654 Han, E.R.N., Bauce, E., 1998. Timing of diapause initiation, metabolic changes and
655   overwintering survival of the spruce budworm, *Choristoneura fumiferana*. Ecol.
656   Entomol. 23, 160–167. https://doi.org/10.1046/j.1365-2311.1998.00111.x

657 Harvey, G.T., 1961. Second Diapause in Spruce Budworm from Eastern Canada. Can.
658   Entomol. 93, 594–602. https://doi.org/10.4039/Ent93594-7

659 He, H.M., Xiao, H.J., Xue, F.S., 2018. Parental effect of diapause in relation to photoperiod
660   and temperature in the cabbage beetle, *Colaphellus bowringi* (Coleoptera:
661   Chrysomelidae). Bull. Entomol. Res. 108, 773–780.
662   https://doi.org/10.1017/S0007485318000019

663 Johns, R.C., Eveleigh, E.S., 2013. Ontogeny and Stand Condition Influence the Dispersal
664   Behavior of a Defoliating Specialist Caterpillar. Environ. Entomol. 42, 1329–1337.
665   https://doi.org/10.1603/EN13083

666 Kawecki, T.J., Ebert, D., 2004. Conceptual issues in local adaptation. Ecol. Lett. 7,
667   1225–1241. https://doi.org/10.1111/j.1461-0248.2004.00684.x

668 Kellermann, V., Hoffmann, A.A., Overgaard, J., Loeschcke, V., Sgrò, C.M., 2018. Plasticity
669   for desiccation tolerance across *Drosophila* species is affected by phylogeny and
670   climate in complex ways. Proc. R. Soc. B Biol. Sci. 285.
671   https://doi.org/10.1098/rspb.2018.0048

672 Kelty, J., 2007. Rapid cold-hardening of *Drosophila melanogaster* in a field setting. Physiol.

673   Entomol. 32, 343–350. https://doi.org/10.1111/j.1365-3032.2007.00584.x

674 King, A.M., MacRae, T.H., 2015. Insect Heat Shock Proteins During Stress and Diapause.
675   Annu. Rev. Entomol. 60, 59–75. https://doi.org/10.1146/annurev-ento-011613-162107

676 Lee, R.E., Denlinger, D.L., 2010. Rapid cold-hardening: Ecological significance and
677   underpinning mechanisms. Low Temp. Biol. Insects 35–58.

678 Lee, Richard E., 2010. A primer on insect cold tolerance, in: Denlinger, D.L., Lee, R.E.
679   (Eds.), Low Temperature Biology of Insects. Cambridge University Press, Cambridge,
680   UK, pp. 3–34.

681 Lumley, L.M., Pouliot, E., Laroche, J., Boyle, B., Brunet, B.M.T., Levesque, R.C., Sperling,
682   F.A.H., Cusson, M., 2020. Continent☐wide population genomic structure and
683   phylogeography of North America's most destructive conifer defoliator, the spruce
684   budworm (*Choristoneura fumiferana*). Ecol. Evol. ece3.5950.
685   https://doi.org/10.1002/ece3.5950

686 Marshall, K., Roe, A.D., n.d. Surviving in a frozen forest: the physiology of eastern spruce
687   budworm overwintering. Physiology.

688 Marshall, K.E., Gotthard, K., Williams, C.M., 2020. Evolutionary impacts of winter climate
689   change on insects. Curr. Opin. Insect Sci. 41, 54–62.
690   https://doi.org/10.1016/j.cois.2020.06.003

691 Marshall, K.E., Sinclair, B.J., 2015. The relative importance of number, duration and
692   intensity of cold stress events in determining survival and energetics of an
693   overwintering insect. Funct. Ecol. 29, 357–366.
694   https://doi.org/10.1111/1365-2435.12328

695 Marshall, K.E., Sinclair, B.J., 2012. The impacts of repeated cold exposure on insects. J. Exp.
696   Biol. 215, 1607–1613. https://doi.org/10.1242/jeb.059956

697 Marshall, K.E., Sinclair, B.J., 2010. Repeated stress exposure results in a
698   survival-reproduction trade-off in *Drosophila melanogaster*. Proc. R. Soc. B Biol. Sci.
699   277. https://doi.org/10.1098/rspb.2009.1807

700 McMorran, A., 1965. A Synthetic Diet for the Spruce Budworm, *Choristoneura fumiferana*.
701   Can. Entomol. 97, 58–62. https://doi.org/10.4039/Ent9758-1

702 Mousseau, T.A., Fox, C.W., 1998. The adaptive significance of maternal effects. Trends
703   Ecol. Evol. https://doi.org/10.1016/S0169-5347(98)01472-4

704 Nealis, V.G., 2016. Comparative ecology of conifer-feeding spruce budworms (Lepidoptera:
705   Tortricidae). Can. Entomol. 148, S33–S57. https://doi.org/10.4039/tce.2015.15

706 Overgaard, J., Sørensen, J.G., Loeschcke, V., 2010. Genetic variability and evolution of
707   cold-tolerance, in: Low Temperature Biology of Insects. Cambridge University Press,
708   pp. 276–296. https://doi.org/10.1017/CBO9780511675997.012

709 Park, Y., Kim, Y., 2013. RNA interference of glycerol biosynthesis suppresses rapid cold
710   hardening of the beet armyworm, *Spodoptera exigua*. J. Exp. Biol. 216, 4196–4203.

711   https://doi.org/10.1242/jeb.092031

712   Perrault, K., Wardlaw, A. A., Candau, J.-N., Irwin, C. L., Demidovich, M., MacQuarrie,
713   C.J.K., Roe, A. D., 2021. From branch to bench: establishing wild spruce budworm
714   populations into laboratory colonies for the exploration of local adaptation and
715   plasticity. Can. Entomol. [online early]. https://doi.org/10.4039/tce.2021.1

716   Pigliucci, M., 2001. Phenotypic Plasticity: Beyond Nature and Nurture. JHU Press,
717   Baltimore.

718   Qin, W., Doucet, D., Tyshenko, M.G., Walker, V.K., 2007. Transcription of antifreeze
719   protein genes in *Choristoneura fumiferana*. Insect Mol. Biol. 16, 423–434.
720   https://doi.org/10.1111/j.1365-2583.2007.00743.x

721   Rajamohan, A., Sinclair, B.J., 2009. Hardening trumps acclimation in improving cold
722   tolerance of *Drosophila melanogaster* larvae. Physiol. Entomol. 34, 217–223.
723   https://doi.org/10.1111/j.1365-3032.2009.00677.x

724   Régnière, J., 2009. Predicting insect continental distributions from species physiology.
725   Unasylva 60, 37–42.

726   Régnière, J., 1990. Diapause termination and changes in thermal responses during
727   postdiapause development in larvae of the spruce budworm, *Choristoneura fumiferana*.
728   J. Insect Physiol. 36, 727–735. https://doi.org/10.1016/0022-1910(90)90046-I

729   Roberts, S.P., Feder, M.E., 1999. Natural hyperthermia and expression of the heat shock
730   protein Hsp70 affect developmental abnormalities in *Drosophila melanogaster*.
731   Oecologia 121, 323–329. https://doi.org/10.1007/s004420050935

732   Roe, A.D., Demidovich, M., Dedes, J., 2017. Origins and History of Laboratory Insect
733   Stocks in a Multispecies Insect Production Facility, With the Proposal of Standardized
734   Nomenclature and Designation of Formal Standard Names. J. Insect Sci. 18.
735   https://doi.org/10.1093/JISESA/IEY037

736   Sanders, C.J., 1991. Biology of North American spruce budworm., in: van der Geest, L.P.S.,
737   Evenhuis, H.H. (Eds.), Tortricid Pests, Their Biology, Natural Enemies and Control.
738   Elsevier Science, Amsterdam, The Netherlands, pp. 579–620.

739   Sinclair, B.J., 1999. Insect cold tolerance: How many kinds of frozen? Eur. J. Entomol. 96,
740   157–164.

741   Sinclair, B.J., Coello Alvarado, L.E., Ferguson, L. V., 2015. An invitation to measure insect
742   cold tolerance: Methods, approaches, and workflow. J. Therm. Biol. 53, 180–197.
743   https://doi.org/10.1016/j.jtherbio.2015.11.003

744   Sinclair, B.J., Williams, C.M., Terblanche, J.S., 2012. Variation in Thermal Performance
745   among Insect Populations. Physiol. Biochem. Zool. 85, 594–606.
746   https://doi.org/10.1086/665388

747   Strachan, L.A., Tarnowski-Garner, H.E., Marshall, K.E., Sinclair, B.J., 2011. The evolution
748   of cold tolerance in *Drosophila* larvae. Physiol. Biochem. Zool. 84, 43–53.
749   https://doi.org/10.1086/657147

750   Sturtevant, B.R., Achtemeier, G.L., Charney, J.J., Anderson, D.P., Cooke, B.J., Townsend,
751   P.A., 2013. Author ' s personal copy Agricultural and Forest Meteorology
752   Long-distance dispersal of spruce budworm (*Choristoneura fumiferana* Clemens) in
753   Minnesota (USA) and Ontario (Canada) via the atmospheric pathway. Agric. For.
754   Meteorol. 168, 186–200.

755   Sultan, S.E., Spencer, H.G., 2002. Metapopulation structure favors plasticity over local
756   adaptation. Am. Nat. 160, 271–283. https://doi.org/10.1086/341015

757   Tauber, C.A., Tauber, M.J., 1986. Ecophysiological responses in life-history evolution:
758   evidence for their importance in a geographically widespread insect species complex.
759   Can. J. Zool. 64, 875–884. https://doi.org/10.1139/z86-131

760   Terblanche, J.S., Deere, J. a, Clusella-Trullas, S., Janion, C., Chown, S.L., 2007. Critical
761   thermal limits depend on methodological context. Proc. Biol. Sci. 274, 2935–2942.
762   https://doi.org/10.1098/rspb.2007.0985

763   Tyshenko, M.G., Doucet, D., Davies, P.L., Walker, V.K., 1997. The antifreeze potential of
764   the spruce budworm thermal hysteresis protein. Nat. Biotechnol. 15, 887–890.
765   https://doi.org/10.1038/nbt0997-887

766   Venables, W.N., Ripley, B.D., 2003. Modern Applied Statistics With S, 4th ed,
767   Technometrics. Springer, New York. https://doi.org/10.1198/tech.2003.s33

768   Williams, C.M., Henry, H.A.L., Sinclair, B.J., 2015. Cold truths: How winter drives
769   responses of terrestrial organisms to climate change. Biol. Rev. 90, 214–235.
770   https://doi.org/10.1111/brv.12105

771   Woestmann, L., Saastamoinen, M., 2016. The importance of trans-generational effects in
772   Lepidoptera. Curr. Zool. 62, 489–499. https://doi.org/10.1093/cz/zow029

773   Yampolsky, L.Y., Schaer, T.M.M., Ebert, D., 2013. Adaptive phenotypic plasticity and local
774   adaptation for temperature tolerance in freshwater zooplankton. Proc. R. Soc. B Biol.
775   Sci. 281. https://doi.org/10.1098/rspb.2013.2744

776   Zhang, X., Flato, G., Kirchmeier-Young, M., Vincent, L.A., Wan, H., Wang, X., Rong, R.,
777   Fyfe, J.C., L, G., Kharin, V. V., 2019. Changes in Temperature and Precipitation Across
778   Canada. Canada's Chang. Clim. Rep. 112–193.

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- Inuvik
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