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The relative importance of number, duration, and intensity of cold stress events in determining survival and energetics of an overwintering insect

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Citation of this paper:
Marshall, Katie E. and Sinclair, Brent J, "The relative importance of number, duration, and intensity of cold stress events in determining survival and energetics of an overwintering insect" (2014). *Biology Publications*. 74.
https://ir.lib.uwo.ca/biologypub/74

Functional Ecology
British Ecological Society

The relative importance of number, duration, and intensity of cold stress events in determining survival and energetics of an overwintering insect

For Peer Review

Journal:	*Functional Ecology*
Manuscript ID:	FE-2014-00297.R1
Manuscript Type:	Standard Paper
Date Submitted by the Author:	n/a
Complete List of Authors:	Marshall, Katie; University of British Columbia, Zoology Sinclair, Brent; University of Western Ontario, Biology
Key-words:	abiotic stress, chilling injury, eastern spruce budworm, fluctuating thermal regimes, freeze avoidance, repeated cold exposure

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1 # The relative importance of number, duration, and intensity of
2 cold stress events in determining survival and energetics of an
3 overwintering insect

4

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13

14 **Running Headline:** Impacts of repeated stress events

15

"This is the pre-peer reviewed version of the following article: [Marshall, K. E. and Sinclair, B. J. (2015), The relative importance of number, duration and intensity of cold stress events in determining survival and energetics of an overwintering insect. Funct Ecol, 29: 357–366. doi:10.1111/1365-2435.12328], which has been published in final form at [http://onlinelibrary.wiley.com/doi/10.1111/1365-2435.12328/full]. This article may be used for non-commercial purposed in accordance with Wiley Terms and Conditions for Self-Archiving."

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16 ## Summary

17 1. The relationship between abiotic stress and fitness in an individual is usually
18 described by the intensity and duration of stress. Yet in natural systems, variability in
19 abiotic stress is common. Since individuals have physiological and fitness responses to
20 single bouts of stress, frequency of stress may also determine the lifetime success of an
21 organism. However the majority of laboratory studies have focused only on the effects of
22 single stress events.
23 2. In this study we investigated the relative importance of stress parameters
24 including duration, intensity, and number of cold events on the short-term physiology and
25 long-term fitness in the freeze avoiding eastern spruce budworm *Choristoneura*
26 *fumiferana* (Lepidoptera: Tortricidae, Clemens).
27 3. We exposed overwintering 2nd instar larvae of *C. fumiferana* to -5 °C, -10 ºC, -15
28 °C, or -20 ºC, for either a single exposure of 120 hours or repeated 12 h exposures (three,
29 six, or 10 exposures). Changes in short-term physiology were quantified by
30 cryoprotectant content, energetic stores, and supercooling point. Long-term fitness
31 effects were measured by rearing individuals after overwintering and recording
32 successful eclosion as adults, development time from 2nd instar to adult, and adult size.
33 4. We found that long-term survival of *C. fumiferana* was most strongly affected by
34 the number of low temperature stress events rather than intensity or duration, despite
35 increased investment into cryoprotection at the expense of glycogen reserves. Sub-lethal
36 measures such as adult size were unaffected by low temperature stress.
37 5. Here we show that frequency of stress is an important, yet frequently neglected,
38 parameter in the study of the effects of abiotic stress. The responses we documented here

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39 suggest that frequency of stress may be an additional important parameter for modelling

40 the effects of abiotic stress on populations.

41 **Key-words:** abiotic stress, chilling injury, eastern spruce budworm, fluctuating thermal

42 regimes, freeze avoidance, repeated cold exposure

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43 **Introduction**

44 Abiotic stress drives population processes and species' geographic range boundaries
45 (Helmuth, Kingsolver & Carrington 2005; Bozinovic, Calosi & Spicer 2011). The
46 impact of abiotic stress on fitness is often explored in the laboratory, where variation in
47 the stressor is usually parameterized by intensity and duration, such that more intense
48 exposure and/or longer exposure result in decreased fitness (Nedvěd, Lavy & Verhoef
49 1998; Chown & Terblanche 2007). This approach adequately represents the impact of
50 rare extreme events that occur once, such as a single extreme temperature event (Gaines
51 & Denny 1993). However, abiotic conditions fluctuate in natural systems such that an
52 individual organism will likely experience a series of stress events interspersed with less-
53 stressful episodes over the course of its lifetime. For example, temperature fluctuates on
54 multiple interacting temporal scales from the daily through to decadal cycles (reviewed
55 by Stenseth *et al.* 2002). These stressful events vary in duration and intensity, and may
56 also be clustered; for example low temperature stress is more common in winter (Chown
57 & Terblanche 2007; Marshall & Sinclair 2012). Repeated stress events allow organisms
58 to respond physiologically during the benign period between successive exposures, which
59 can modify the fitness consequences of the stress in a manner that will depend on the
60 duration and intensity of that prior exposure, as well as the time between exposures
61 during which the animal can respond (Feder & Hofmann 1999; Dowd & Somero 2013).

62 Stressful events may thus occur in multiple dimensions encompassing the number of
63 events and the return time of those events, as well as intensity and duration of the stress.
64 This multivariate variation in exposure to even a single abiotic stressor makes

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65 experiments and extrapolation through modelling intimidating and unwieldy (see
66 discussion by Gaston *et al.* 2009) – an experiment with three durations, three intensities
67 and three different numbers of events will have 27 treatment groups. However, some of
68 these dimensions of variation in stress events may be predictive of fitness effects than
69 others. This simplification of the key components of repeated stress could therefore be
70 useful both in experimental design and in interpreting estimates of stresses experienced in
71 the field.

72 Winter conditions can drive ecological and evolutionary processes, particularly in
73 ectotherms (Williams, Sinclair & Henry), setting northern range limits (e.g. Stahl, Moore
74 & McKendry 2006; Gray 2008; Calosi *et al.* 2010) and affecting population processes
75 (e.g. Breed, Stichter & Crone 2012). Winter conditions are changing rapidly in temperate
76 regions, shifting cold stress regimes from their historical frequencies (Williams *et al.*
77 2014) ; nevertheless, organismal responses to repeated stress events are poorly
78 characterized, and models of insect populations frequently assume that responses to low
79 temperature stress remain static, despite previous cold stress events (*e.g.* Régnière 1990;
80 Knight 2007; Gray 2008; Régnière *et al.* 2012). Low temperature exposure can induce
81 chilling injury in insects, which leads to mortality unrelated to freezing as the duration
82 and intensity of cold exposure increases (Chen & Walker 1994; Nedvěd *et al.* 1998).
83 Low temperature stress is therefore ecologically relevant, and provides a tractable system
84 for understanding the impacts of repeated stress.

85 The impact of repeated cold exposures on insects has been investigated in a range of
86 systems; however these have usually manipulated only the number of exposures often
87 lack adequate controls, confounding repeated cold with other factors, such as cumulative

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88 cold exposure and age of the experimental animals (reviewed by Marshall & Sinclair
89 2012). Broadly, repeated cold exposure usually increases investment in polyol
90 cryoprotectants such as glycerol (Churchill & Storey 1989; Marshall & Sinclair 2011)
91 and the rewarming phase allows repair of cold-induced damage between low temperature
92 exposures; this effect of rewarming is particularly important in the less-ecologically-
93 relevant fluctuating thermal regimes, where prolonged cold exposures are interspersed
94 with brief periods at benign temperatures (Koštál *et al.* 2007; Lalouette *et al.* 2011). The
95 deleterious consequences of repeated cold stress are often subtle, sub-lethal, effects such
96 as reduced reproductive output and growth rate (Bale, Worland & Block 2001; Sinclair &
97 Chown 2005; Marshall & Sinclair 2010). Few studies examine the impacts of additional
98 dimensions such as period between exposures (but see Bale, Worland & Block 2001),
99 and none have encompassed intensity, period between exposures, and number of
100 exposures within the same experimental design.

101 Here, we investigate the relative importance of intensity, duration, number of, and period
102 between, cold stress events using overwintering 2nd-instar larvae of the freeze-avoiding
103 eastern spruce budworm *Choristoneura fumiferana* as a model system. Our immediate
104 measures included cryoprotectant content and carbohydrate energy reserves, and our
105 long-term measures included development time, survival to maturity, and adult body
106 mass and size, all of which reflect long term performance, and are reasonable proxies of
107 fitness. We found that the number of cold exposures was the most important factor
108 determining performance, and this response to repeated cold was mediated by increased
109 investment in cryoprotection at the expense of energetic reserves. Thus, we suggest that

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110 the most important aspect to consider when examining responses to stress may be the
111 number of exposures, rather than the more commonly-explored intensity and duration.

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112 ## Materials and methods

113 *Study system*

114 The eastern spruce budworm (*Choristoneura fumiferana*, Lepidoptera: Tortricidae) is a
115 natural pest found throughout the North American boreal forest, from Alaska to the island
116 of Newfoundland (Gray 2008; Régnière, St-Amant & Duval 2012b). *Choristoneura*
117 *fumiferana* feeds on several boreal tree species, with balsam fir (*Abies balsamea*) as its
118 preferred host (reviewed by Régnière, St-Amant & Duval 2012). Models of the effects of
119 climate change on future population distribution and outbreak cycles of the eastern spruce
120 budworm have explicitly included the overwintering stage (Gray 2008; Régnière *et al.*
121 2012b). These models have indicated that spring and summer population dynamics may
122 be strongly mediated by fitness in the overwintering stage.

123 The eastern spruce budworm is a freeze avoiding species which survives the winter by
124 depressing its supercooling point (SCP, the temperature at which supercooling of body
125 fluids stops and the insect freezes, Lee 2010). This is accomplished by accumulating *ca.*
126 0.8 M glycerol synthesized from glycogen reserves (Han & Bauce 1995a; b; Lee 2010),
127 expressing a suite of antifreeze proteins (Tyshenko *et al.* 1997; Qin *et al.* 2007), and
128 reducing body water to *ca.* 35% of fresh mass (Bauce & Han 2001). The 2nd instar larvae
129 of *C. fumiferana* overwinter in thermally-exposed microenvironments that receive
130 repeated exposures to -20 ºC through the winter (Han & Bauce 1998). Overwintering
131 larvae are highly cold tolerant with SCPs as low as -35 ºC, although exposures to -15 ºC
132 for longer than ten days can induce mortality, and cold hardiness is reduced in early and
133 late stages of diapause (Han & Bauce 1995a). By the end of winter, glycogen reserves

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134 are nearly depleted while lipid reserves remain intact, suggesting that overwinter
135 metabolism in *C. fumiferana* larvae is fueled by carbohydrate stores rather than lipids
136 (Han & Bauce 1993). Glycogen stores therefore represent both fuel and potential
137 cryoprotectant.

138 Animal source and rearing

139 We ordered pre-diapause 2nd instar *C. fumiferana* larvae (diapausing strain) from Insect
140 Production Services at the Great Lakes Forestry Centre (Sault St. Marie, Ontario,
141 Canada) in October 2010. Eggs are oviposited on gauze at this facility, then 1st instars
142 emerge, molt into 2nd instars without feeding, and spin hibernacula on the gauze, in which
143 they diapause. Larvae in hibernacula were shipped on ice to the University of Western
144 Ontario (London, Ontario, Canada). Upon receipt, we placed larvae in an incubator at
145 2/0 °C 12/12 h, in constant darkness. After allowing a month of acclimation to these
146 conditions, we extracted larvae from their hibernacula on a Petri dish filled with ice. We
147 then randomly placed larvae into 0.2 mL microcentrifuge tubes with perforated lids in
148 groups of 20 (for metabolite assays) or 24 (for supercooling point assays). We kept
149 additional larvae (in groups of 50) for adult fitness assays in their hibernacula on the
150 gauze they were shipped in, placed into 50 mL plastic bottles. All larvae were then
151 allowed to acclimate to 2/0 °C 12/12 h, in constant darkness, for an additional month
152 before our low temperature exposures.

153 Experimental design

154

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155 Low temperature exposures were conducted between the final week of December 2010
156 and late March 2011. Four different temperature treatments (-5, -10, -15, and -20 °C)
157 were used, which constituted the "intensity" treatment (Fig. 1). Larvae received either a
158 single 120 h exposure ("prolonged" exposure) or "repeated" 12 h exposures, which
159 constituted the difference in "frequency" (Fig.1). The larvae that received a single 120 h
160 exposure were exposed in either January or March ("time of year"). Larvae that received
161 repeated 12 h exposures received them daily, every five days, or every 10 days ("period"
162 of exposure return time), and received 3, 6, or 10 of these exposures ("number" of
163 exposures, Fig. 1). All repeated cold treatments began at the beginning of the
164 experimental period (late December 2010). Because the 10 exposure × 10 d interval
165 experiment extended over a long duration, we also sampled control individuals at the
166 beginning, middle, and end of the experimental period (late December, mid-February,
167 and late March, Fig. 1). Immediate impacts (glycerol, protein, and glycogen content, as
168 well as supercooling point) were measured 24 hours after the last cold exposure, while
169 sex was determined and long-term impacts (survival, wing length, mass, development
170 time) measured after placing larvae on food in the spring (Fig. S1).

171 *Low temperature exposures*

172 We conducted all low temperature exposures by placing larvae (still in 0.2 mL Eppendorf
173 tubes or on gauze) in 20 mL plastic tubes in wells drilled in an insulated aluminum block
174 chilled by a Proline 3530C programmable refrigerated circulator (Lauda, Wurzburg,
175 Germany) containing 50:50 methanol:water. We monitored the temperature at 0.5 s
176 intervals inside randomly-chosen tubes using 36 AWG Type-T thermocouples (Omega,
177 Laval, Quebec, Canada) that were interfaced via TC-08 thermocouple interfaces (Pico

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178 Technology, Cambridge, UK) to a computer running PicoLog Software (Picotech). All
179 low temperature exposures began at 8 pm EST, started at 0 °C, and cooling rate was 0.1
180 °C/min to -5, -10, -15, or -20 °C (Fig. 1). Larvae received low temperature exposures for
181 either 12 h or 120 h, and then were rewarmed to 0 °C at 0.1 °C/min. After the conclusion
182 of low temperature treatment, we placed all individuals back (still in their 0.2 mL
183 microcentrifuge tubes or on gauze) in the maintenance incubator for recovery at 12/12 h
184 2/0 °C.

185 We allowed larvae a 24 h recovery at 12/12 h 2/0 °C following exposure (Fig. S1), and
186 transferred five sets of 20 individuals to 1.7 mL microcentrifuge tubes, snap-froze them
187 in liquid nitrogen vapour, and stored them at -80 °C for subsequent assays. At this time,
188 we also measured the supercooling point (SCP, temperature at which ice formation is
189 initiated) of 24 individuals (after Strachan *et al.* 2011, see Supplementary Methods for
190 additional detail). We returned individuals still on gauze to the incubator at 2/0 °C 12/12
191 h in constant darkness for later life history assays. To measure the long-term effects of
192 prolonged cold exposure on glycerol and glycogen stores, we also allowed an additional
193 group of individuals that received 120 h of low temperature exposure in January to
194 recover in the same incubator until they were sampled in late March (Fig. S1).

195 Glycerol and glycogen assays

196 Larvae were homogenized in groups of 20 in a Bullet Blender (Next Advance, Averill
197 Park, NY, USA) in 50 µL of 0.05% Tween 20 with eight 1 mm glass beads as in Marshall
198 and Sinclair (2010). We then added 450 µL of 0.05% Tween 20, before centrifugation
199 (15 min at 15,000 × *g*). We separated the supernatant (~350 µL) into three equal aliquots

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200 and stored them at -80 °C for use in subsequent assays. We measured protein, glucose,
201 glycogen (from undiluted homogenate), and glycerol (diluted 1:9 in 0.05% Tween 20)
202 content spectrophotometrically as in Gefen *et al.*(2006) using bovine serum albumin,
203 glucose, Type II glycogen from oyster, and glycerol standards respectively. Briefly,
204 soluble protein content was measured using a Bicinchoninic Acid Kit (BCA1, Sigma-
205 Aldrich, Oakville, ON, Canada). Glucose content was measured using a hexokinase-
206 based glucose assay kit (GAHK20, Sigma-Aldrich), while glycogen content was
207 measured using the same kit following overnight amyloglucosidase (A9228, Sigma-
208 Aldrich) digestion at room temperature. Glycogen is expressed in glucose units
209 throughout. Glycerol content was measured using Free Glycerol Reagent (F6425, Sigma-
210 Aldrich).

211 Life history measures

212 After the conclusion of the low temperature treatments, we kept all remaining larvae (45-
213 60 per group) in constant darkness at 12:12 h 2/0 °C for an additional month. We then
214 transferred the larvae (on gauze, 50/cup) to 30 mL plastic cups containing 22 mL
215 McMorran diet (Insect Production Services) and placed them in an incubator in constant
216 light at 23 °C. After two weeks, we randomly selected 24 larvae from each group and
217 placed them onto new McMorran diet in groups of six ('thinning'); additional larvae
218 were counted and discarded at this point, and survival at this point constituted 'survival to
219 thinning' (Fig. S1). We checked cups daily for pupae, which were immediately removed
220 and placed individually into empty 22 mL plastic cups. Pupae were monitored daily and
221 the eclosion date noted (leading to our measure of 'survival from thinning to eclosion').

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222 We recorded sex, mass (Mettler-Toledo MX5, Columbus, Ohio, USA, d = 0.1 µg), and
223 wing length (digital calipers ± 0.5 mm, Mastercraft, Toronto, Ontario, Canada) of adults.

224 *Statistical analyses*

225 In all statistical comparisons, we first fitted a Type II ANOVA model (implemented
226 using the Anova function from the car package in R v.2.15.0, Fox & Weisberg 2011; R
227 Core Team 2013) with all possible terms and interactions. Then, using the step algorithm
228 implemented in R (Venables & Ripley 2002), we simplified to the model with the lowest
229 Akaike's Information Criterion (AIC) by sequentially removing the highest-order
230 interaction terms regardless of p-value. Since the step algorithm will halt when removing
231 a term causes an increase in AIC, even if that increase is non-significant (*i.e.* ΔAIC <2,
232 Crawley 2005), we compared AIC values between the best-fit model from the step
233 function with the next-simplest model (*i.e.* best fit model from the step function with the
234 highest-order interaction term removed) using the extractAIC function in R. If the
235 increase in AIC was <2, we restarted the step algorithm with the next simplest model
236 (Crawley 2005).

237 In all analyses, we first compared all larvae that had experienced repeated exposures
238 using a three-way ANOVA with period (length of time between exposures), intensity
239 (temperature of cold exposure), and frequency (number of low temperature exposures) as
240 fixed factors. We also used a three-way ANOVA to compare larvae that experienced a
241 single prolonged exposure using a one-way ANOVA to determine the effects of intensity
242 and time of year, and we compared control larvae among sampling points using one-way
243 ANOVA to determine the effects of time of year. If the period between exposures was

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244 not a significant predictor, we pooled all the larvae that received ten × 12 h exposures
245 regardless of period within each temperature. Similarly, if time of year was not a
246 significant predictor in larvae that had experienced prolonged exposures or control
247 conditions, these individuals were also pooled within temperatures. We then compared
248 larvae that had received cold exposures using a two-way ANOVA with temperature and
249 experimental group as predictors (where experimental group could include separate
250 groups for repeatedly-exposed larvae with different periods of exposure and larvae that
251 had experienced prolonged exposure at different times of year).

252 This model-reduction approach was repeated for all analyses, with the following
253 exceptions. To compare survival to eclosion and sex ratio, we used generalized linear
254 models with a binomial distribution rather than ANOVA, and glycerol and glycogen
255 analyses were conducted on μmol/sample values, with protein mass as a covariate. Means
256 ± SE are reported throughout and alpha was set to 0.05 in all tests.

257 Results

258 Survival

259 A total of 1505 larvae (out of 2340 initially counted and placed on McMorran diet)
260 emerged from their hibernacula, and were viable two weeks following placement on diet.
261 Intensity, duration, or period between exposures did not significantly affect survival to
262 thinning of larvae that received repeated 12 h exposures (Table 1). The interaction
263 between exposure temperature and time of year exposed (either January or March)
264 significantly affected survival in larvae that received 120 h cold exposure (Table 1). This
265 effect was driven by extremely low survival (one larva out of 48 initially placed on

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266 McMorran diet) of larvae that received a 120 h exposure to -20 ºC in March (Fig. 2A).
267 When survival was compared among larvae in all experimental groups (repeated 12 h
268 exposures *vs*. one 120 h exposure), the only significant deviation from the overall high
269 survival (64.3%) was in those larvae that received prolonged exposure to the most
270 extreme temperature, -20 ºC, in March (Fig. 2A, Table 1). Similarly, intensity, duration,
271 and period between cold exposures did not reduce survival from thinning to eclosion in
272 larvae that received repeated low temperature exposures (Table 1). Larvae given a single
273 120 h cold exposure in March had significantly lower survival than larvae exposed to 120
274 h of cold in January (Fig. 2B, Table 1). Frequency of cold exposure significantly
275 impacted larval survival from thinning to eclosion (Fig. 2B, Table 1). Larvae that
276 received repeated exposures had significantly lower survival (difference in coefficients, p
277 $= 0.027$) than those that received 120 h in January, but no difference compared with
278 larvae that received 120 h cold exposures in March ($p = 0.068$). A total of 716 adult *C.*
279 *fumiferana* (378 female, 338 male) eclosed successfully from all experimental groups,
280 with overall survival from thinning to eclosion of 65%.

281 *Glycerol and glycogen content*

282 The interactions between exposure temperature, period between exposures, and number
283 of exposures significantly affected glycogen content in larvae that received repeated 12 h
284 cold exposures (Table S7). Increased number of exposures significantly decreased
285 glycogen content, and this was exacerbated when there were longer periods between cold
286 exposures (Figs 3A-B). Lower exposure temperature and longer period between
287 exposures also reduced glycogen content (Figs 3A-C). Similarly, exposure temperature
288 and time of year sampled (regardless of whether the larvae were exposed in January or

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289 March) had a significant effect on glycogen content (Fig. 3D, Table S8). Lower exposure
290 temperature, and sampling later in the experimental period reduced glycogen content in
291 larvae (Fig. 3D). Glycogen content significantly decreased in control larvae through the
292 experimental period (from 0.52 µmol/20 larvae to 0.07 µmol/20 larvae over the three
293 month period, Fig. 3E, Table S9). When all experimental groups were compared, there
294 were significant effects of both exposure temperature and exposure type on glycogen
295 content (Table 2) whereby lower temperature exposure generally resulted in lower
296 glycogen content (Fig. 3F). Larvae that received a prolonged exposure in January had
297 significantly higher glycogen content than any other group, while larvae that received
298 repeated 12 h exposures either daily or every five days had significantly decreased
299 glycogen content relative to those that received prolonged exposure in January (Fig. 3F).

300 Glycerol content was dependent on almost every aspect of low temperature exposure.
301 Increased number of exposures at shorter periods and lower temperatures significantly
302 increased glycerol concentration in larvae that received repeated 12 h cold exposures
303 (Figs 4A-C, Table S7). Glycerol concentration was dependent on time of year only in
304 larvae given a single 120 h cold exposure (Fig. 4D, Table S8). Glycerol concentrations
305 of control larvae peaked in February (Fig. 4E, Table S9). When all exposure groups were
306 compared, larvae that received repeated daily exposures in January had the highest
307 glycerol concentration, while larvae that received repeated exposures every five or ten
308 days or a prolonged exposure in January had the next highest concentrations. Larvae that
309 received prolonged exposures and were sampled in March had the lowest glycerol
310 concentrations (Fig. 4F, Table 2).

311 Discussion

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312 Predicting species' range shifts in response to climate change requires the incorporation
313 of relevant physiological data into models (Kearney & Porter 2009; Buckley *et al.* 2010).
314 Here we show that – within the range of survivable temperatures – the number of low
315 temperature stress events is a consistent determinant of the overwintering survival of an
316 important natural pest species (summarized in Table 3). This result points to a cost of
317 repeated low temperature events that accumulates with each exposure. Including the
318 number of stress events may therefore improve models of organisms' responses to abiotic
319 stress that currently only account for intensity and duration of stress.

320 Second instar *C. fumiferana* larvae that received a single cold exposure in early winter
321 mobilized glycerol from glycogen stores, and experienced little subsequent mortality. By
322 contrast, a prolonged cold exposure in March reduced glycogen stores, reducing glycerol
323 mobilization, which resulted in mortality later in development. By contrast, repeated
324 cold exposure increased glycerol mobilization, although survival was still reduced. Sub-
325 lethal measures of stress, such as mass and development time, were not affected by
326 repeated cold exposure, whereas survival to eclosion was strongly affected by the nature
327 of larval low temperature exposure. Thus, increased frequency of exposure, rather than
328 intensity, duration, or period between stresses drives the relationship between cold
329 exposure and survival in *C. fumiferana.* Importantly, this effect of stress in early
330 development affects lifetime performance of *C. fumiferana* and cannot be compensated
331 for during larval feeding and development.

332 *Energy stores and cryoprotection*

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333 The seasonal production of glycerol from glycogen stores is well-characterized in *C.*
334 *fumiferana* (Han & Bauce 1995b), but here we show that glycerol content increases
335 following low temperature exposure, likely due to increased mobilization from glycogen,
336 and in common with many other insect species (reviewed by Marshall & Sinclair 2012).
337 A prolonged cold exposure in January reduced glycerol content of *C. fumiferana* larvae in
338 March; we therefore suggest that the interaction between period and number of exposures
339 is due to glycerol catabolism during winter. We cannot determine whether this additional
340 glycerol is directly metabolized or recycled into other energy pools, nor can we
341 differentiate between the energetic costs of repair of chilling injury or preparation for
342 subsequent low temperature events in this study.

343 Individuals exposed to repeated (rather than single) stress increased their investment in
344 stress resistance (cryoprotectants) at the expense of energy reserves. This suggests that
345 studies that focus on a single stress event likely do not encompass the full stress response,
346 reducing the generalizability to field conditions. For example, the freeze tolerant midge
347 *Belgica antarctica* upregulates *hsp70* only in response to repeated freeze-thaw events, not
348 single events (Teets *et al.* 2011). Moreover, energetic trade-offs induced by repeated
349 stress events could reduce investment in reproductive output (Petavy *et al.* 2001;
350 Marshall & Sinclair 2010); these effects could strongly impact models of population
351 growth and expansion.

352 *Causes of mortality*

353 There was little evidence that the mortality we observed in response to repeated cold
354 exposure was driven by freezing injury or direct chilling injury. The supercooling points

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355 we measured (no individual SCP above -21.8 ºC) were all lower than the lowest exposure
356 temperature (-20 ºC), and previous work shows that *C. fumiferana* can remain
357 supercooled for ten days at -23 ºC (Han & Bauce 1995a). We did observe substantial
358 pre-thinning mortality after five days of exposure to -20 ºC in March, likely due to direct
359 chilling injury, perhaps because depleted glycogen reserves reduced glycerol availability,
360 and therefore cold tolerance (Han & Bauce 1995a; b). This hypothesis is supported by
361 elevated glycerol content in larvae exposed to low temperature early in winter (Figs 4A-
362 D), and by the observations of reduced glycerol mobilization late in diapause (as in Han
363 & Bauce 1995b). By contrast, larvae exposed to less stressful conditions (milder
364 temperatures or shorter durations) in March had similar survival to control larvae.

365 Survival to eclosion – a measure of mortality late in development – was higher in larvae
366 that received repeated exposures; this may be due to either accrued indirect chilling
367 injury or energetic depletion due to investment in glycerol. Low temperature reduces
368 metabolic rate and thus energetic demands during diapause; however, production of
369 cryoprotectants in response to repeated low temperature stress may offset these savings
370 (Hahn & Denlinger 2011); in particular glycerol mobilization from glycogen requires
371 ATP (Storey & Storey 1983), and therefore may lead to a trade-off between glycerol and
372 glycogen contents. Decreased energy reserves could be important in nature: energy drain
373 due to warm temperatures may cause spring mortality of *C. fumiferana* (Han & Bauce
374 1998). Although *C. fumiferana* larvae feed after winter, it is possible that repeated cold
375 exposure reduces feeding performance through tissue damage, as observed in larvae of
376 *Pringleophaga marioni* (Sinclair & Chown 2005).

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377 The delayed mortality we observed in control larvae could result from energy drain due
378 to the increased temperature (and therefore metabolic rate) relative to groups that
379 received cold exposure (Han & Bauce 1998; Irwin & Lee 2003). In this case, we would
380 expect control larvae to have lower glycogen content than cold-exposed larvae at the end
381 of winter, which does appear to be the case (Fig. 3). Alternatively, insufficient chilling
382 exposure may have prevented appropriate diapause termination (Hodek & Hodková
383 1988). Clearly, however, survival was not determined by duration or intensity of stress
384 exposure, which is how abiotic stress is usually modelled (*e.g.* Turnock, Lamb &
385 Bodnaryk 1983; Régnière *et al.* 2012a). Instead, the number and timing of stress
386 exposures predicted mortality, suggesting that intensity, duration, and time between stress
387 exposures do not by themselves capture responses to abiotic stress.

388 *Implications for* C. fumiferana *population modelling*

389 Individual larvae overwintering in the field are exposed to repeated low temperature
390 stress consistent with our treatments (Han & Bauce 1998). Existing correlative and
391 mechanistic models of *C. fumiferana* populations (Gray 2008; Régnière *et al.* 2012a)
392 utilize intensity and duration of cold stress as predictive variables; however, we show that
393 repeated cold exposure modifies the response of this species to cold stress, reducing the
394 predictive power of duration and intensity of low temperature exposure for overwinter
395 success of this species. Future models could incorporate the number and duration of cold
396 exposures by utilizing fine-temporal-scale temperature records. The northern range edge
397 of *C. fumiferana* is correlated with the minimum temperature (Gray 2008). However, we
398 found that number of exposures, rather than minimum temperature, determines cold
399 survival. It is possible that minimum temperature is correlated with the number of low

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400 temperature exposures in nature, leading to the relationship between minimum
401 temperature and range edge; however, the nature of repeated cold exposure has not been
402 explored in the context of species distributions.

403 *Conclusions*

404 This study is the first examination of the relative importance of intensity and duration of
405 stress compared to number of abiotic stress events. We show that the energetic costs of
406 physiological processes following a stress event accumulate with each stress event and
407 lead to long-term fitness declines. Of the factors relating to abiotic stress—number,
408 intensity, and period between exposures—it appears that the number of exposures has the
409 strongest impact on long-term fitness. This suggests that understanding the energetic
410 costs and physiological impacts of repeated stress events may improve prediction of the
411 effects of climate change on animal population densities and distributions.

412 Acknowledgements

413 We thank Áron Roxin, Tilly MacRae, and Laura Chee for technical assistance, Heath
414 MacMillan, Daniel Doucet, and Rick Lee for helpful discussion, and two anonymous
415 referees for constructive comments that improved the manuscript. This research was
416 supported by the Natural Science and Engineering Research Council of Canada via a
417 Discovery grant to BJS and a postgraduate scholarship to KEM, and by a grant from the
418 Canadian Foundation for Innovation to BJS.

419 Data Accessibility

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420 All data will be made publically accessible on Dryad following acceptance of the
421 manuscript.

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561 **Table 1**. The effects of low temperature exposure during the 2nd instar on long-term survival and sex ratio of *Choristoneura*
562 *fumiferana.* Values are ANOVA statistics comparing survival and sex ratio among moths as a result of exposure temperature
563 frequency (ten 12 h cold exposures or one 120 h cold exposure) as 2nd instar larvae. Retained terms with significant p-values
564 ($p < 0.05$) are in bold typeface. In the case of survival and sex ratio, a generalized linear model was fitted with a binomial
565 distribution. If there were no significant effects (other than intercept), this is indicated by "Y = null model". If there were no
566 significant effects (other than intercept), this is indicated by "null model" and there are no statistical tests associated with it.

Groups tested	Y	Initial model	Minimal adequate model	Terms from minimal adequate model	F	Df	P
Repeated: pooled **Prolonged**: time of year exposed	Survival to thinning =	exposure temperature × exposure type	exposure temperature × exposure type	Temperature Exposure type **Temperature × Type**	0.81 2.48 **6.16**	3, 39 2, 37 **6, 31**	0.486 0.084 **<0.001**
Repeated: pooled **Prolonged**: time of year exposed	Survival from thinning to eclosion =	exposure temperature × exposure type	exposure type	**Exposure type**	**4.66**	**2, 40**	**0.009**
Repeated: pooled **Prolonged**: pooled	Sex ratio =	exposure temperature × exposure type	null model	**-**	**-**	**-**	**-**

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569 **Table 2.** The effects of low temperature exposure on glycerol and glycogen content in 2nd instar *Choristoneura fumiferana*.
570 Values are ANOVA statistics comparing glycerol and glycogen content among *Choristoneura fumiferana* 2nd instar larvae as a
571 result of cold exposure type (ten 12 h exposures or one 120 h exposure) as 2nd instar larvae. Retained terms with significant p-
572 values ($p < 0.05$) are in bold typeface.

Exposure type	Y	Initial model	Minimal adequate model	Terms from minimal adequate model	F	df	P
Repeated: All periods **Prolonged:** Time of year sampled and exposed	Glycogen =	protein mass + exposure temperature × exposure type	protein mass + exposure temperature × exposure type	**Protein mass**	**76.55**	**1, 109**	**<0.001**
				Temperature	**17.94**	**1, 109**	**<0.001**
				Exposure type	**75.56**	**4, 109**	**<0.001**
				Temperature × type	**2.95**	**4, 109**	**0.024**
Repeated: All periods **Prolonged:** Time of year sampled and exposed	Glycerol =	protein mass + exposure temperature × exposure type	protein mass + exposure temperature × exposure type	Protein mass	0.80	1, 95	0.374
				Temperature	2.33	3, 95	0.093
				Exposure type	**31.98**	**5, 95**	**<0.001**
				Temperature × type	**2.22**	**15, 95**	**0.010**

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575 **Table 3.** Summary of the significant main effects of cold exposure on eastern spruce budworm larvae (see Figs. 1 and S1 for
576 experimental details). Each number represents a regression coefficient between the main effect and the measured trait,
577 normalized to the largest absolute regression coefficient for each measure. "n.s." indicates coefficients that are not statistically
578 different from zero. Time of year was only tested in individuals that received prolonged exposures, while period and duration
579 were only tested in individuals that received repeated exposures. Number of exposures was compared between individuals that
580 received prolonged exposures and repeated exposures, while intensity was tested in all larvae that were cold-exposed. See
581 Tables 1, 2, and S1 – S10 for full models and Figs. 2-4 and S3 – S6 for data.

Timescale	Trait	Number	Period	Intensity	Duration	Time of year
Long term	Female development time	+0.29	-0.26	n.s.	n.s.	+1.00
	Female mass	n.s.	n.s.	n.s.	n.s.	n.s.
	Sex ratio of survivors	n.s.	n.s.	n.s.	n.s.	n.s.
	Survival to thinning	+1.00	n.s.	n.s.	n.s.	n.s.
	Survival to eclosion	-0.16	n.s.	n.s.	n.s.	+1.00
Short term	Glucose content	n.s.	n.s.	n.s.	n.s.	n.s.
	Glycerol content	+1.00	-0.20	-0.02	+0.04	-0.56
	Glycogen content	+0.53	n.s.	+0.03	-0.17	-1.00
	Supercooling point	-1.00	-0.09	n.s.	+0.03	n.s.

582 **Figure Legends:**

583 **Fig. 1.** Experimental design for studying the effects of frequency, intensity, duration, and period
584 of low temperature exposure on *Choristoneura fumiferana*. Terms in boldface indicate predictive
585 terms used in statistical models.

586 **Fig. 2.** The effects of cold exposure on long-term survival of *Choristoneura fumiferana.* All
587 significant effects are indicated by the bold terms in each figure, and accompanying statistics are
588 presented in Tables S5-9, and Table 1. "Intensity" = temperature of cold exposure, "Type" =
589 either "Repeated" 12 h exposures, or a single "Prolonged" 120 h cold exposure (see Figs. 1 and
590 S1 for details of experimental design). Solid and dotted horizontal grey lines indicate mean ±
591 standard error of the proportion survival of controls, respectively. A) Survival from placement
592 on McMorran diet to thinning after two weeks at 23 ºC. N = 48 – 185. B) Survival from thinning
593 until eclosion as adults. N = 24 – 72 (N = 1 for larvae exposed to -20 °C for 120 h in March).

594 **Fig. 3.** The effects of cold exposure on mean (± SE) glycogen content of 2nd instar
595 *Choristoneura fumiferana* larvae. Significant effects are indicated by the bold terms in each
596 figure (see Tables 2 and S7-9 for statistics). "Period" = days between cold exposures,
597 "Intensity" = temperature of cold exposure, "Number" = number of cold exposures, "Time of
598 Year" = time of year exposed or sampled. "Exposure Type" = either "Repeated" exposures, or a
599 single "Prolonged" cold exposure (see Figs. 1 and S1 for details of experimental design). Solid
600 and dotted horizontal grey lines represent mean ± SE of control larvae in January (upper),
601 February (middle) and March (lower). A) Larvae that received 10 twelve hour exposures. B-C)

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602 Larvae that received repeated 12 h cold exposures. D) Larvae that received a single 120 h cold
603 exposure. E) Control larvae. F) All exposure types together.

604 **Fig. 4.** The effects of cold exposure on mean (±SE) glycerol content of 2nd instar *Choristoneura*
605 *fumiferana* larvae. ignificant effects are indicated by the bold terms in each figure, see Tables 2
606 and S7-9 for statistics. “Period” = days between cold, “Intensity” = temperature of cold
607 exposure, “Number” = number of cold exposures, “Time of Year” = time of year exposed or
608 sampled. “Exposure Type” = either “Repeated” exposures, or a single “Prolonged” cold exposure
609 (see Figs. 1 and S1 for details of experimental design). Solid and dotted horizontal lines
610 represent mean and standard error of all control larvae. A) Larvae that received 10 twelve hour
611 exposures. B-C) Larvae that received repeated 12 h cold exposures. D) Larvae that received a
612 single 120 h cold exposure. E) Control larvae. F) All exposure types together.

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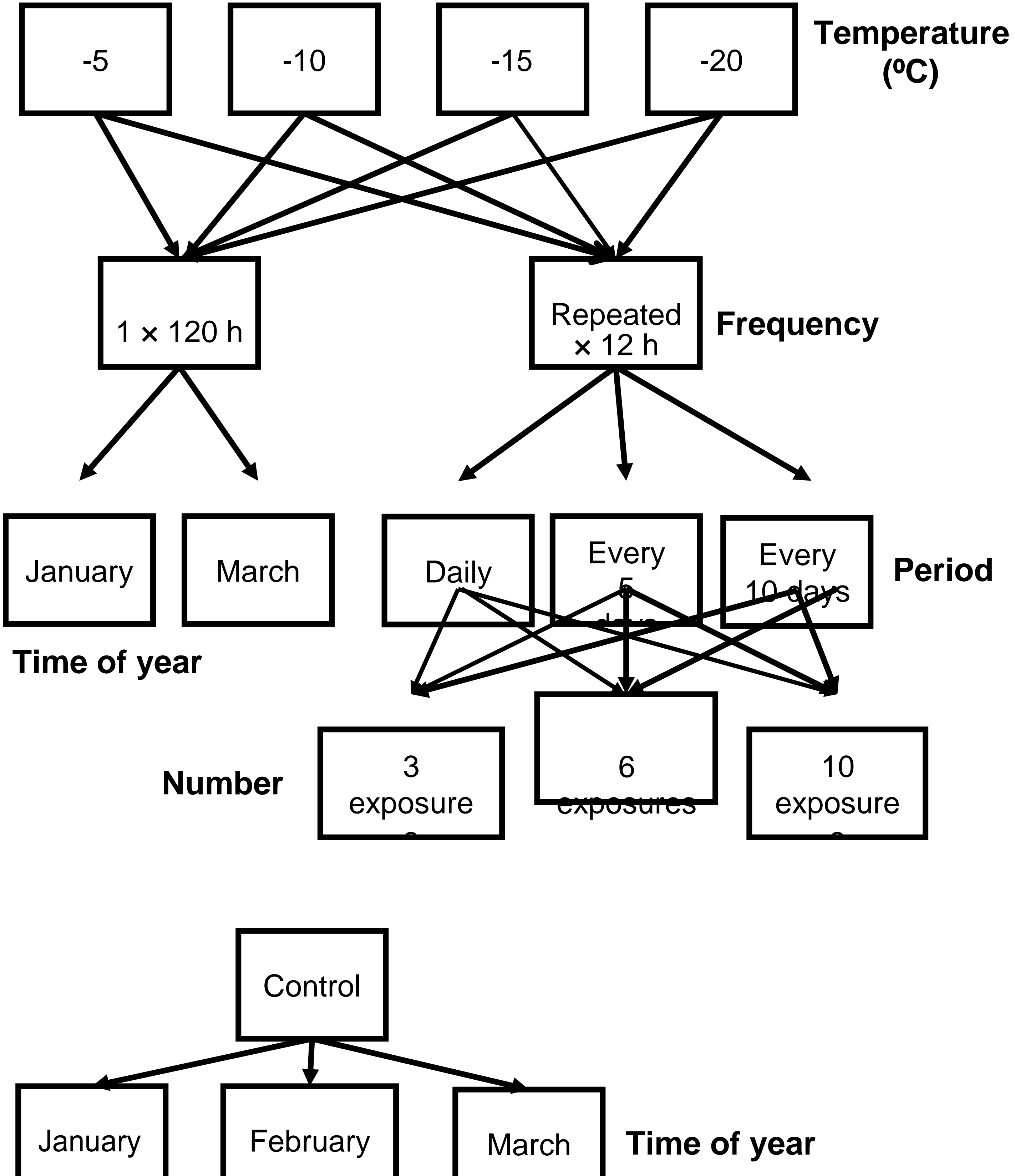
-5
-10
-15
-20
Temperature (⁰C)
1 × 120 h
Repeated × 12 h
Frequency
January
March
Daily
Every 5
Every 10 days
Period
Time of year
Number
3 exposure
6 exposures
10 exposure
Control
January
February
March
Time of year

A

Proportion surviving to thinning

1.0
0.8
0.6
0.4
0.2
0.0

Repeated
Prolonged January
Prolonged March

B

Proportion surviving to eclosion

1.0
0.8
0.6
0.4
0.2

Repeated
Prolonged January
Prolonged March

20−15−10−5

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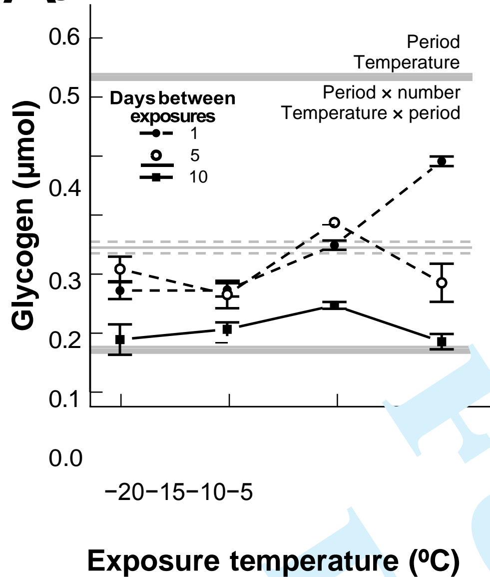
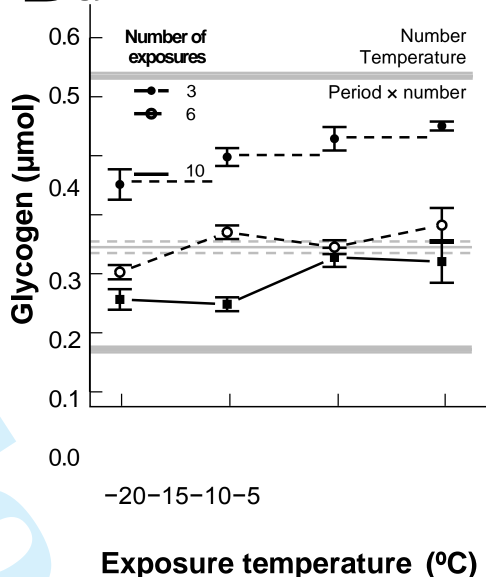
Temperature exposed (ºC)

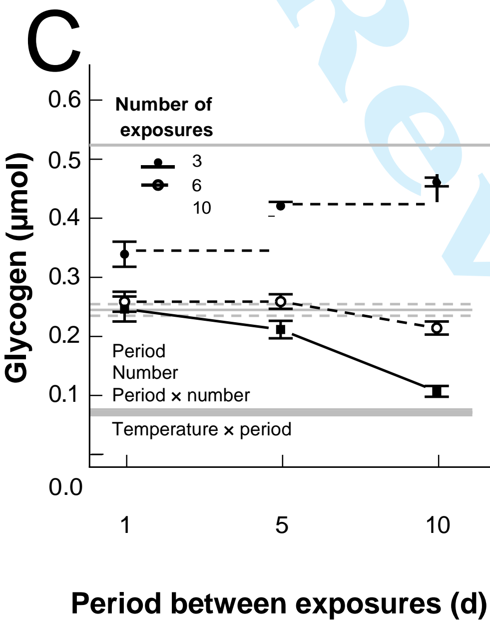
0.0

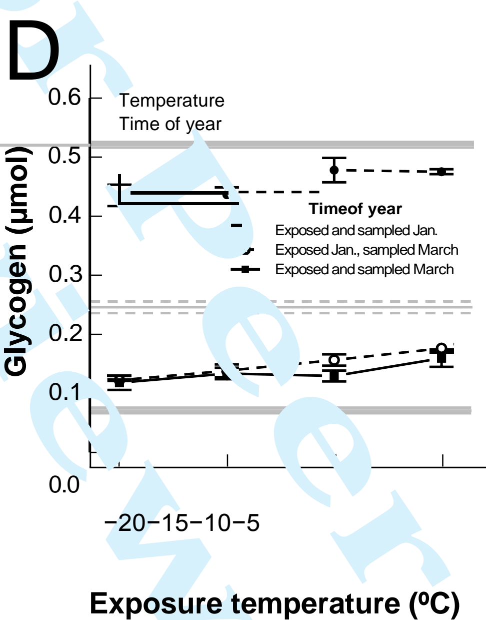
−20−15−10−5

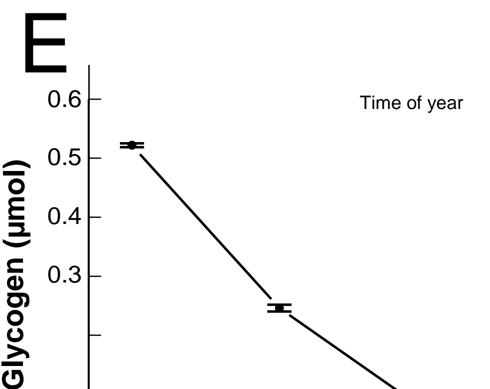
Temperature exposed (ºC)

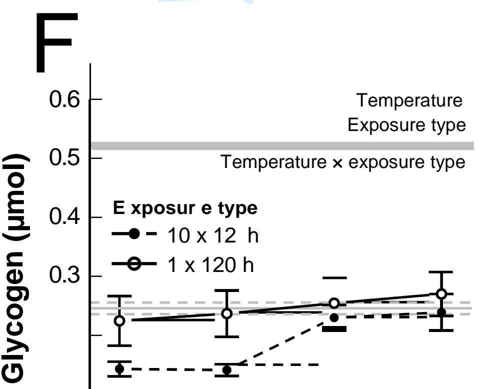












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0.2

0.1

0.0

January February March

Time of year sampled

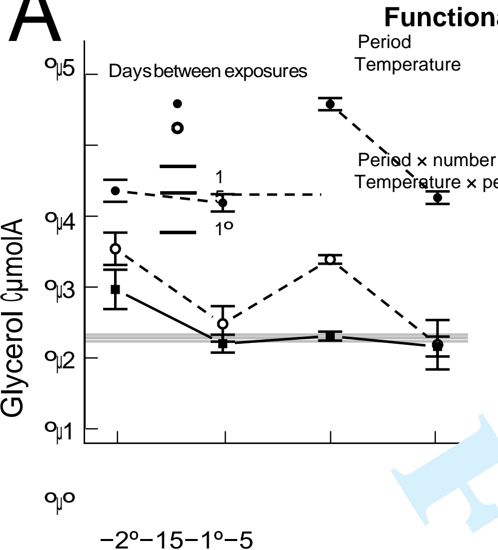
0.2

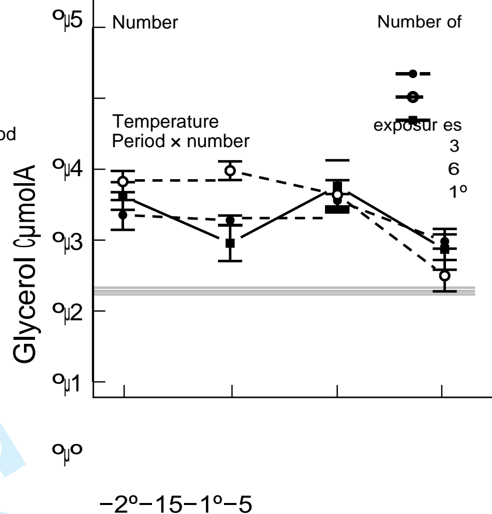
0.1

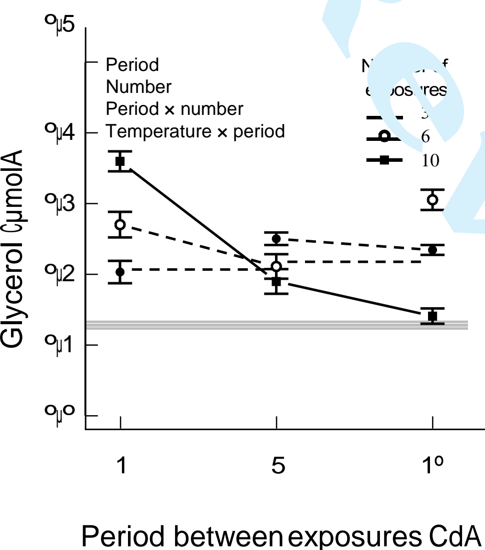
0.0

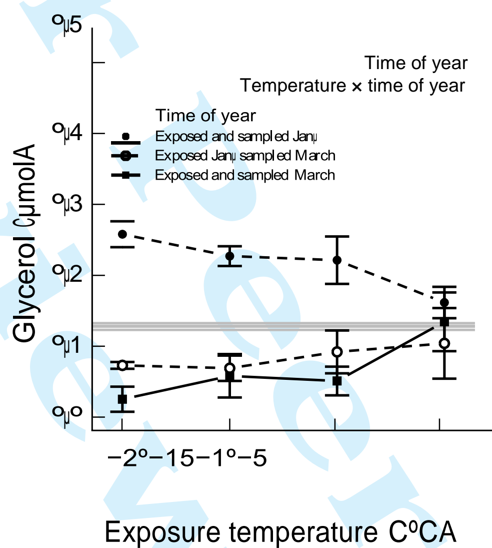
−20−15−10−5

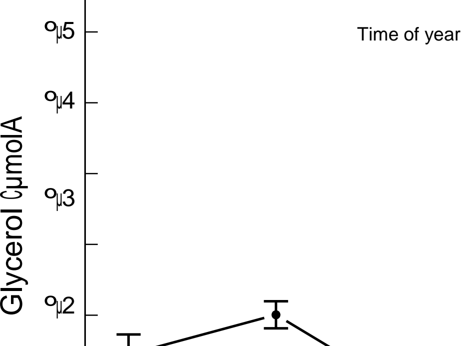
Exposure temperature (ºC)

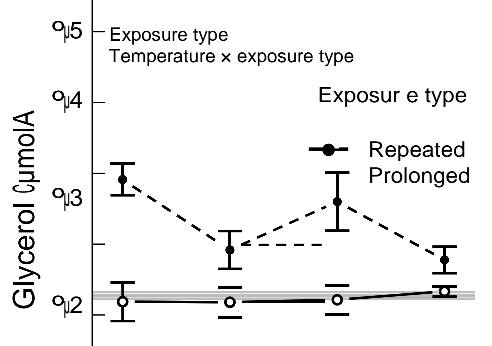












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oµ1

oµo

January February March

Time of year sampled

oµ1

oµo

−2°−15−1°−5

Exposure temperature CºCA