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Winter 2019

Laboratory acclimation to autumn-like conditions induces freeze tolerance in the spring field cricket Gryllus veletis (Orthoptera: Gryllidae)

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Citation of this paper:
Toxopeus, Jantina; Mckinnon, Alexander H.; Štětina, Tomáš; Turnbull, Kurtis F.; and Sinclair, Brent J, "Laboratory acclimation to autumn-like conditions induces freeze tolerance in the spring field cricket Gryllus veletis (Orthoptera: Gryllidae)" (2019). *Biology Publications*. 113.
https://ir.lib.uwo.ca/biologypub/113

1 **Laboratory acclimation to autumn-like conditions induces freeze tolerance in the spring**
2 **field cricket *Gryllus veletis* (Orthoptera: Gryllidae)**

3

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20

21 **Abstract**

22 Many temperate insects encounter temperatures low enough to freeze their body fluids.
23 Remarkably, some insects are freeze-tolerant, surviving this internal ice formation. However, the
24 mechanisms underlying freeze tolerance are not well-understood, in part due to a lack of
25 tractable model organisms. We describe a novel laboratory model to study insect freeze
26 tolerance, the spring field cricket *Gryllus veletis* (Orthopera: Gryllidae). Following acclimation
27 to six weeks of decreasing temperature and photoperiod, *G. veletis* become freeze-tolerant,
28 similar to those exposed to natural autumn conditions in London, Ontario, Canada. Acclimated
29 crickets suppress their metabolic rate by c. 33%, and survive freezing for up to one week at -8°C,
30 and to temperatures as low as -12°C. Freeze-tolerant *G. veletis* protect fat body cells from freeze
31 injury *in vivo*, and fat body tissue from freeze-tolerant cricket survives brief freeze treatments
32 when frozen *ex vivo*. Freeze-tolerant crickets freeze at c. -6°C, which may be initiated by
33 accumulation of ice-nucleating agents in hemolymph or gut tissue. We hypothesize that control
34 of ice formation facilitates freeze tolerance, but initiating ice formation at high subzero
35 temperatures does not confer freeze tolerance on freeze-intolerant nymphs. Acclimation
36 increases hemolymph osmolality from c. 400 to c. 650 mOsm, which may facilitate freeze
37 tolerance by reducing ice content. Hemolymph ion concentrations do not change with
38 acclimation, and we therefore predict that freeze-tolerant *G. veletis* elevate hemolymph
39 osmolality by accumulating other molecules. *Gryllus veletis* is easily reared and manipulated in a
40 controlled laboratory environment, and is therefore a suitable candidate for further investigating
41 the mechanisms underlying freeze tolerance.

42 **Key Words:** freeze tolerance; cold tolerance; acclimation; ice nucleation; metabolic rate; insect
43

44 **1 Introduction**

45 Ectotherms in temperate and polar regions can be exposed to temperatures low enough to freeze
46 their body fluids. Freezing is lethal in most cases (Sinclair et al., 2015), and many insects have
47 evolved strategies to avoid freezing (Lee, 2010). Strikingly, some insects are freeze-tolerant,
48 surviving internal ice formation (Lee, 2010), a strategy that has evolved multiple times in a
49 diversity of insects (Dennis et al., 2015; Sinclair and Chown, 2010; Walters et al., 2009). Some
50 of these freeze-tolerant insects survive in the frozen state for extended periods [e.g. the
51 cockroach *Cryptocercus punctulatus* survives at least 205 days at -10°C (Hamilton et al., 1985)]
52 or to extremely low temperatures [e.g. larvae of the drosphilid fly *Chymomyza costata* can
53 survive immersion in liquid nitrogen, -196°C (Koštál et al., 2011)]. Research on most freeze-
54 tolerant species (e.g. *Eurosta solidaginis* and *Belgica antarctica*) has been restricted to field-
55 collected individuals (Toxopeus and Sinclair, 2018). Studies in lab-reared *C. costata* have
56 improved our understanding of freeze tolerance through manipulative experiments (e.g. Koštál et
57 al., 2011), but one lab model is unlikely sufficient for uncovering the potentially diverse
58 mechanisms underlying insect freeze tolerance. Thus despite the prevalence of insect freeze
59 tolerance, a lack of tractable laboratory model systems has limited investigations of the
60 physiological mechanisms underlying this cold tolerance strategy (Storey and Storey, 1988;
61 Toxopeus and Sinclair, 2018).

62 Freezing is usually harmful to biological systems (Muldrew et al., 2004; Toxopeus and Sinclair,
63 2018). Ice crystals can physically damage tissues, and intracellular ice formation (IIF) is often
64 lethal (Lee, 2010; Zachariassen, 1985). Extracellular ice formation causes osmotic dehydration
65 of cells, imposing challenges on structural integrity, macromolecule conformation, and function
66 of diverse macromolecular structures [nucleoprotein complexes, enzymatic complexes,
67 cytoskeleton, phospholipid bilayers, etc. (Lee, 2010; Toxopeus and Sinclair, 2018)]. In addition,
68 ice crystals can grow over time *via* recrystallization (Ramløv et al., 1996), and metabolic waste
69 products (e.g. lactate) can accumulate in the frozen state (Storey and Storey, 1985). Many insects
70 suppress their metabolism over winter (diapause; Hahn and Denlinger, 2011) which may
71 mitigate some of these metabolic impacts. For example, diapausing *E. solidaginis* suppress their
72 metabolic rate by c. 67% (Irwin et al., 2001). Freeze-tolerant insects will die if cooled to their
73 lower lethal temperature (LLT) or held in the frozen state beyond a lethal time (Lt), suggesting

74 that low temperatures and long freezing periods exacerbate these challenges (Toxopeus and
75 Sinclair, 2018).

76 To minimize damage from ice, it is hypothesized that freeze-tolerant insects control the
77 distribution and quality (size and shape) of ice crystals. Ice-nucleating agents (INAs) can control
78 where and at what temperature ice begins to form (Zachariassen and Kristiansen, 2000), and
79 many freeze-tolerant insects elevate the temperature at which ice formation begins [supercooling
80 point; SCP (Toxopeus and Sinclair, 2018)]. Aquaporins (AQPs) facilitate transmembrane water
81 transport and may therefore facilitate effective osmotic dehydration of cells, preventing IIF (Lee,
82 2010; Toxopeus and Sinclair, 2018). Other ice-binding molecules, such as antifreeze proteins
83 (AFPs), control the size and shape of ice crystals, and are hypothesized to prevent ice crystal
84 growth (recrystallization) over time (Duman, 2015). Manipulating the conditions of ice
85 formation (e.g. temperature and cooling rate) can have a strong impact on whether insects
86 survive freezing. For example, larvae of *C. costata* are only freeze tolerant if ice formation is
87 nucleated at a high subzero temperature (c. -1°C) by contact with external ice, and cooling rate is
88 slow (Koštál et al., 2011). Many freeze-tolerant insects elevate their hemolymph osmolality by
89 accumulating low molecular weight cryoprotectants (e.g. glycerol), which reduces the amount of
90 ice that forms (Layne and Blakely, 2002; Rozsypal et al., 2018; Toxopeus and Sinclair, 2018).

91 Here we develop the spring field cricket, *Gryllus veletis* (Alexander & Bigelow) (Orthoptera:
92 Gryllidae), as a laboratory model system to study freeze tolerance. *Gryllus veletis* overwinters as
93 a late-instar nymph in north eastern North America (Alexander and Bigelow, 1960). We
94 demonstrate that the overwintering nymphs of *G. veletis* are freeze tolerant, characterize the
95 physiological changes associated with laboratory-induced freeze tolerance, and test how control
96 of ice nucleation contributes to survival of internal ice formation at the whole animal and cellular
97 level.

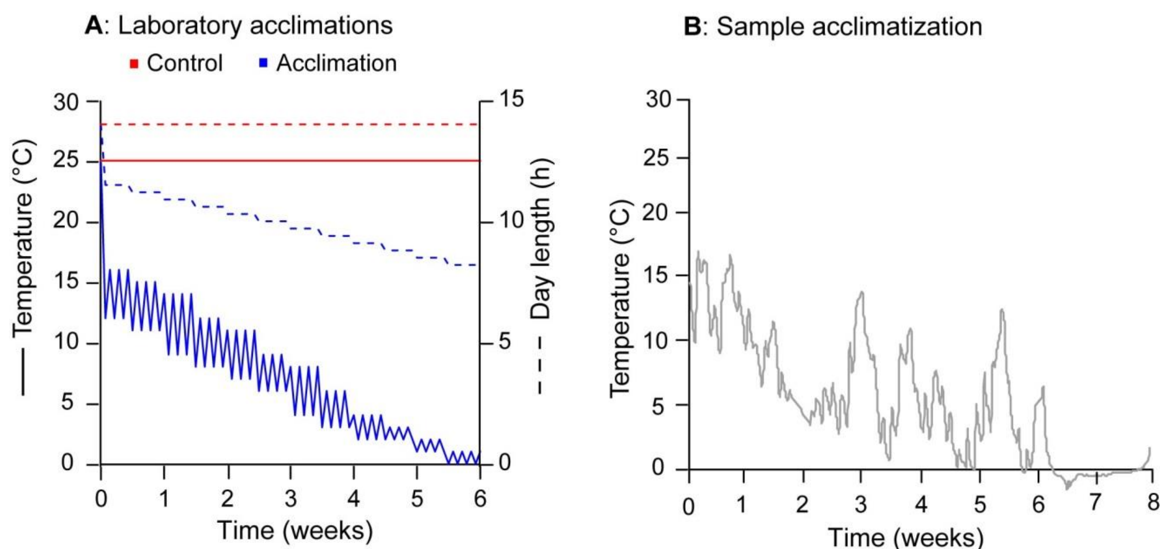
98 2 Materials and Methods

99 *2.1 Rearing and acclimation conditions*

100 Our laboratory colony of *G. veletis* originated from individuals collected in 2010 from the
101 University of Lethbridge campus, Alberta, Canada and was reared under constant summer-like

102 conditions (25ºC, 14:10 L:D photoperiod, 70% RH), as described previously (Coello Alvarado et
103 al., 2015). We haphazardly assigned fifth-instar male *G. veletis* approximately eight weeks post-
104 hatch to remain in rearing (control) conditions, or to undergo acclimatization or acclimation
105 conditions, and tested if these conditions induced freeze tolerance. For subsequent experiments,
106 we used the six-week acclimation in Fig. 1A.

107 For outdoor acclimatizations, we placed crickets in enclosed mesh cages (60 cm × 60 cm × 75
108 cm) in a shaded suburban garden (42°59'N, 81°17'W, 251 m elevation) in London, Ontario,
109 Canada, along with soil, rabbit food pellets, chicory (*Cichorium endivia*) leaves, black locust
110 (*Robinia pseudoacacia*) leaves, and grass from a cultivated lawn. The acclimitizations lasted for
111 six to eight weeks, and included 'Outdoor 2013-a' (10 Oct – 3 Dec 2013), 'Outdoor 2013-b' (10
112 Oct 2013 – 9 Jan 2014), and 'Outdoor 2014' (1 Oct – 5 Dec 2014). We used a HOBO Pro v2
113 U23-003 data logger (Onset Computer Corporation, Bourne, MA, USA) to record surface soil
114 temperature every 30 min, and obtained day length data for London from the National Research
115 Council of Canada website (http://www.nrc-cnrc.gc.ca/eng/services/sunrise/). The temperature
116 data for 'Outdoor 2013-a' and '-b' are presented in Fig. 1B.



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118 **Figure 1. Acclimation and acclimatization regimes that induce freeze tolerance in *Gryllus*
119 *veletis.* (A)** Temperature (solid line) and photoperiod (dashed line) of laboratory control (red)
120 and acclimation (blue) conditions. **(B)** Air temperature in London, Canada from 10 Oct 2013 – 9
121 Jan 2014 ('Outdoor-2013b'). The change in photoperiod over that time period was from
122 11.5:12.5 to 7.9:16.1 L:D.
123

124 For laboratory acclimations, we isolated crickets into individual 180 ml plastic cups (Polar
125 Plastics, Summit Food Distributors, London, ON, Canada) with mesh covering and shelters made
126 from egg cartons, and transferred them to a Sanyo MIR 154 incubator (Sanyo Scientific,
127 Bensenville, IL, USA) or kept them in the rearing incubator. We provided rabbit food and water
128 *ad libitum*. In the control conditions (Fig. 1A), we tested cricket freeze tolerance within 2 h of
129 isolation ('zero weeks control'), and after three weeks ('three weeks control') and six weeks ('six
130 weeks control'). Acclimation (Fig. 1A) was designed to mimic autumn conditions in London,
131 Ontario, Canada: temperature fluctuated daily (12 h at daily high and low temperatures, based on
132 the recorded upper- and lower-quartile values obtained from the 'Outdoor-2013b' soil surface
133 temperatures; Fig. 1B), and decreased over six weeks from 16/12°C to 1/0°C (high/low).
134 Photoperiod decreased by 36 minutes per week (from 11.5:12.5 to 7.9:16.1 L:D). We tested
135 cricket freeze tolerance after three weeks ('three weeks acclimation') and six weeks ('six weeks
136 acclimation') of acclimation. In addition, we tested whether crickets were freeze tolerant after a
137 six week acclimation that included only the decreases in temperature ('six weeks acclimation
138 temperature only;' constant photoperiod 14:10 L:D) or photoperiod ('six weeks acclimation
139 photoperiod only;' constant temperature 25°C) used in the six week acclimation. To determine if
140 freeze tolerance could be induced by short acclimations, we also exposed crickets to one week of
141 low temperatures ('one week cold shock;' constant temperature -2°C, constant photoperiod 11:13
142 L:D), and a rapid cold exposure ('rapid cold hardening;' 15 min at -2°C, 40 min at 22°C).

143 *2.2 Determining freeze tolerance*

144 To freeze crickets, we put them individually into 1.7 ml microcentrifuge tubes, which we placed
145 in an aluminium block cooled by 50% methanol circulated from a programmable refrigerated
146 bath (Lauda Proline 3530, Würzburg, Germany). We equilibrated the crickets at 6°C for 10 min,
147 followed by cooling to the target temperature at 0.25°C min^{-1}. Crickets were held at the target
148 temperature for 1.5 h or more, followed by rewarming to 6°C at 0.25°C min^{-1}. To detect the SCP,
149 each cricket was in contact with a 36-AWG type-T copper-constantan thermocouple (Omega,
150 Laval, QC, Canada). Temperature was recorded at 0.5 s intervals by Picolog v5.24.1 software
151 (Pico Technology, Cambridge, UK) *via* a Pico Technology TC-08 interface. The SCP was
152 defined as the lowest temperature before the exotherm caused by the latent heat of crystallisation
153 (Sinclair et al., 2015). After thawing, crickets were transferred to individual mesh-covered 180

154 ml transparent cups containing rabbit food, water, and shelters made from egg cartons for
155 recovery at 15°C. We assessed survival as the ability of crickets to move in response to gentle
156 prodding within 48 h of recovery. Crickets were classified as freeze-tolerant if more than 75% of
157 individuals in a treatment group (control, acclimated, acclimatized) survived a freeze treatment
158 of 1.5 h at -8°C, and were otherwise classified as freeze-intolerant.

159 We estimated the acute LLT of 'six-weeks acclimated' (hereafter referred to as freeze-tolerant)
160 *G. veletis* by determining survival after freezing them to target temperatures between -6°C and -
161 15°C for 1.5 h. We estimated the Lt of freeze-tolerant *G. veletis* by determining survival after
162 freezing them to -8°C for between 1.5 h and 7 d. The range of temperatures/times encompassed 0
163 to 100% mortality. We conducted all statistical analyses in R version 3.4.1 (R Core Team, 2017).
164 We calculated the LLT_{80} (temperature at which 80% of crickets will die after a 1.5 h exposure)
165 and Lt_{80} (lethal time at which 80% die when kept frozen at -8°C) using a generalized linear
166 model with a binomial distribution, and we tested the fit with Wald's χ^2 using the package
167 MASS in R (Venables and Ripley, 2002).

168 *2.3* In vivo *and* ex vivo *cellular freeze tolerance*

169 To test whether freeze-tolerant and control (hereafter referred to as freeze-intolerant) crickets
170 protected cells during freezing (*in vivo*), we conducted cell viability assays on fat body tissue
171 dissected from crickets before or after they underwent a freeze treatment. These freeze
172 treatments included -8°C for 1.5 h (freeze-tolerant and freeze-intolerant crickets), as well as -
173 12°C for 1.5 h (LLT) and -8°C for 7 d (Lt) treatments (freeze-tolerant crickets only). We placed
174 fat body tissue in a 0.6 ml microcentrifuge tube containing 10 µl *G. veletis* Ringer's solution
175 (160 mM NaCl, 11 mM KCl, 8.4 mM $CaCl_2$, 5.9 mM $MgCl_2$, 5 mM HEPES, pH 7.6) for
176 subsequent cell viability assays.

177 To test whether cells from freeze-tolerant and freeze-intolerant crickets survived freezing *ex*
178 *vivo*, we dissected fat body tissue from *G. veletis*, and performed cell viability assays on fat body
179 tissue after a freeze treatment. These freeze treatments included -8°C for 10 min (freeze-tolerant
180 and freeze-intolerant fat body), the cellular LLT (-16°C for 10 min) and cellular Lt (-8°C for 24
181 h), as determined in Fig. S1. To freeze fat body tissue *ex vivo*, we transferred the tissue into 0.6
182 ml tubes containing 10 µl Grace's Insect Medium and 2 µl 25 mg/ml silver iodide (AgI) slurry in

183 water (Sigma Aldrich, Mississauga, ON, Canada). Silver iodide is a potent INA, which we added
184 to tubes to ensure the medium froze. Within 15 min of dissection, we placed these tubes in an
185 aluminium block as described above. Thermocouples were attached to the outside of the tube,
186 and temperature was recorded to detect the SCP when the medium froze. Samples were
187 equilibrated for 1 min at 6°C, cooled at 0.25°C min^{-1} to the target temperature, held at the target
188 temperature for 10 min or more, and rewarmed at 0.25°C min^{-1} to 6°C. After thawing, we
189 transferred fat body samples to tubes containing 10 µl *G. veletis* Ringer's solution, and
190 determined cell viability by live-dead staining.

191 *2.4 Cell viability assays*

192 We added 10 µl staining solution [33 µg/ml DAPI and 33 µg/ml mM propidium iodide in
193 phosphate buffered saline (PBS)] (ThermoFisher Scientific, Missisauga, ON, Canada) to fat body
194 samples in *G. veletis* Ringer's solution. We incubated the samples at room temperature (c. 22°C)
195 for 5 min, washed fat body in PBS, and imaged samples with the Axio Imager Z1 upright
196 compound fluorescence microscope (Carl Zeiss Canada, North York, ON, Canada), using
197 excitation/emission wavelengths of 538/461 nm for DAPI, and 488/585 nm for propidium iodide.
198 We imaged a single field of view of each fat body sample under 50× magnification,
199 encompassing an area of c. 8.5 mm^2 (~300 cells; example micrographs in Fig. S2). We measured
200 cell survival by estimating the proportion of tissue area stained by DAPI only (live cells) with
201 ImageJ (Abramoff et al., 2004; Marshall and Sinclair, 2011). We calculated the average
202 proportion of live cells from fat body in each treatment, and compared the effect of acclimation
203 and time/temperature spent frozen on cell survival using generalized linear models with a
204 binomial distribution.

205 *2.5 Biochemical composition of hemolymph and tissues*

206 We compared biochemical parameters of freeze-tolerant and freeze-intolerant *G. veletis* to
207 identify potential mechanisms of freeze tolerance. We measured osmolality and thermal
208 hysteresis (TH) of hemolymph from freeze-tolerant and freeze-intolerant *G. veletis* using a
209 nanolitre osmometer (Otago Osmometers, Dunedin, New Zealand) as described previously
210 (Crosthwaite et al., 2011). Hemolymph was extracted from crickets, diluted 1:3 in an
211 anticoagulant (3% ascorbic acid), overlaid with type B immersion oil, flash frozen in liquid

212 nitrogen and stored at -80°C until analysis. We determined osmolality from the melting point
213 (T_m) of hemolymph ice crystals (accounting for dilution in ascorbic acid), and TH from the
214 difference between melting and freezing point (Crosthwaite et al., 2011). We determined
215 differences in osmolality between freeze-tolerant and freeze-intolerant cricket hemolymph using
216 a one-tailed Welch's t-test.

217 We measured concentrations of sodium (Na^+) and potassium (K^+) in hemolymph from freeze-
218 tolerant and freeze-intolerant crickets using an atomic absorption spectrometer (iCE 3000,
219 Thermo Scientific, Waltham, USA) as previously described (MacMillan and Sinclair, 2011). We
220 incubated 4 µl hemolymph samples in 20 µl 3% nitric acid, at room temperature for 24 h,
221 centrifuged the samples (600 × g for 1 minute), and diluted 20 µl of supernatant with 10 ml
222 distilled deionized water (ddH_2O). We determined [Na^+] and [K^+] in each sample by comparing
223 absorbance to Na^+ and K^+ standards diluted in 3% nitric acid. We tested for differences in ion
224 concentrations between freeze-tolerant and freeze-intolerant crickets ($N = 8$ per treatment) with
225 two-tailed Welch's t-tests. We estimated Cl^- concentrations as the sum of Na^+ and K^+
226 concentrations, assuming a relationship similar to that observed in hemolymph of another freeze-
227 tolerant orthopteran (Ramløv et al., 1992).

228 To measure ice nucleator activity, we determined the SCP of hemolymph and tissues (gut,
229 Malpighian tubules, fat body) extracted from live *G. veletis* at room temperature following
230 previously described methods (Toxopeus et al., 2016). We blotted tissues with tissue paper to
231 remove hemolymph prior to transferring each tissue to a 0.2 ml microcentrifuge tube. We added
232 20 µl of anticoagulant (3% ascorbic acid) to each sample, and included a control of *G. veletis*
233 Ringer's solution similarly diluted with 3% ascorbic acid. We cooled the samples at 0.25°C min^{-1}
234 to -35°C, and measured the SCP of each sample with a thermocouple attached to the outside of
235 the tube. We compared the SCP of each sample to that of the Ringer's solution in 3% ascorbic
236 acid using a one-way ANOVA with planned contrasts. We compared SCPs of freeze-tolerant and
237 freeze-intolerant crickets using a one-tailed Welch's t-test with a Bonferroni correction.

238 *2.6 Ice nucleation manipulations*

239 To determine if manipulating the temperature and site of ice nucleation could confer freeze
240 tolerance, we induced freezing of freeze-intolerant crickets with AgI, both externally ('external

241 AgI'), and in the gut ('gut AgI') and the hemolymph ('hemolymph Ag'I). To promote external
242 ice formation, the crickets were briefly submerged in a 25 mg/ml AgI in water slurry prior to
243 freezing (SCP = -5.0 ± 0.4°C). To initiate ice formation in the gut, we dusted their diet (rabbit
244 food) with AgI for four weeks prior to freezing crickets (SCP = -5.8 ± 0.2°C). To manipulate
245 hemolymph ice nucleation, we injected 4 µl of a 25 mg/ml AgI slurry under the cricket pronotum
246 using a 5 µl gastight Hamilton syringe with a 25 gauge needle (Hamilton Company, Reno, NV,
247 USA) (SCP = -3.6 ± 0.4°C). Crickets were placed in 180 ml transparent plastic cups to recover at
248 room temperature for 40 min prior to a freeze treatment of -8°C for 1.5 h.

249 *2.7 Measuring metabolic rate and water loss rate*

250 We measured CO_2 and H_2O emission by freeze-tolerant and freeze-intolerant *G. veletis* nymphs
251 using Sable Systems flow-through respirometry (Sable Systems International, Las Vegas, NV,
252 USA) as described previously, with air scrubbed of CO_2 and H_2O and a flow rate of 80 ml min^{-1}
253 (Lake et al., 2013). Crickets were starved for 24 h (Sinclair et al., 2011) at 25°C (control) or 0°C
254 (acclimated) prior to respirometry measurements for 40 min each at 5°C and 15°C, with a 0.25°C
255 min^{-1} ramp rate between temperatures (example respirometry traces in Fig. S3). The order of
256 temperatures was randomized for each individual, and activity was recorded throughout data
257 collection to ensure the calculations were based on resting animals. Both CO_2 and H_2O
258 production were corrected to 5 min baseline measurements. We calculated the rate of CO_2
259 production ($\dot{V}CO_2$) at both temperatures, and calculated the Q_{10} (the slope of log-transformed
260 mass-specific $\dot{V}CO_2$ as a function of temperature) as described previously (Lake et al. 2013). We
261 determined the water loss rate (WLR), and cuticular and respiratory water loss using the method
262 of Gibbs and Johnson (2004) as described previously (Williams et al. 2010). Briefly, we
263 regressed WLR against $\dot{V}CO_2$ and used the intercept (where $\dot{V}CO_2 = 0$, presumably because the
264 spiracles are closed) to estimate the cuticular water loss rate. Respiratory water loss rate was then
265 calculated as the difference between total and cuticular water loss rates. We compared the effect
266 of acclimation on $\dot{V}CO_2$ and WLR at both temperatures using ANCOVAs with mass as a
267 covariate. We compared Q_{10} of freeze-tolerant and freeze-intolerant crickets using a two-tailed
268 Welch's t-test.

269 **3 Results**

270 *3.1 Conditions that induce freeze tolerance*

271 Fifth-instar (juvenile) male *G. veletis* acclimated to decreasing, fluctuating, temperature and
272 photoperiod for six weeks were freeze-tolerant: 92 ± 6% survived being frozen for 1.5 h at -8°C
273 (Table 1). None of their counterparts maintained under control (rearing) conditions over the same
274 period survived freezing, and were therefore freeze-intolerant (Table 1). Lab-reared crickets
275 acclimatized outside in autumn in London, Ontario, Canada for eight weeks were also freeze-
276 tolerant (Table 1). We could not induce freeze tolerance in crickets with short acclimation
277 treatments (less than six weeks), nor with six-week acclimations in which only temperature or
278 photoperiod decreased (Table 1).

279 Acclimated *G. veletis* were moderately freeze-tolerant (*sensu* Sinclair, 1999): they died if frozen
280 for 1.5 h below -12°C or for 7 d at -8°C, which set the bounds for the LLT (Fig. 2A) and Lt (Fig.
281 2B), respectively. When we examined *in vivo* tissue damage, fat body cell survival in *G. veletis*
282 was high prior to freezing, and in freeze-tolerant crickets frozen for 1.5 h at -8°C (Fig. 3A).
283 Conversely, freeze-intolerant crickets frozen for 1.5 h at -8°C and freeze-tolerant crickets frozen
284 to their LLT or for their Lt had low fat body cell survival (Fig. 3A). Fat body cells from freeze-
285 tolerant crickets frozen *ex vivo* in Grace's Insect Medium for 10 min at -8°C survived better than
286 those frozen to the cellular LLT or Lt, or those from freeze-intolerant crickets (Fig. 3B).
287

288 **Table 1. Freeze tolerance of fifth-instar *Gryllus veletis* following acclimation,**
289 **acclimatization, or laboratory manipulations.** Treatments are described in the methods
290 section. Crickets were defined as freeze tolerant if > 75% survived being frozen at -8°C for 1.5 h.
291 *N*, sample size; AgI, silver iodide
292

Treatment	*N*	*N* frozen	*N* survived	Freeze tolerant
Laboratory acclimations				
Zero weeks control[a]	24	24	0	No
Three weeks control[a]	24	22	0	No
Six weeks control[a]	48	43	0	No
Three weeks acclimation[b]	24	24	3	No
Six weeks acclimation[b]	24	24	22	**Yes**
Six weeks acclimation temperature only[c]	8	8	0	No
Six weeks acclimation photoperiod only[d]	8	8	0	No
One week cold shock	8	8	0	No
Rapid cold hardening	8	8	0	No
Field acclimatizations				
Outdoor 2013-a[e]	6	6	6	**Yes**
Outdoor 2013-b[e]	8	8	8	**Yes**
Outdoor 2014	5	5	5	**Yes**
Laboratory manipulations				
External AgI (inoculation)	8	8	0	No
Gut AgI (diet)	24	24	0	No
Hemolymph AgI (injection)	8	8	0	No

293
294 [a]Fig. 1A, red temperature and photoperiod; [b]Fig. 1A, blue temperature and photoperiod;
295 [c]Fig. 1A, blue temperature, red photoperiod; [d]Fig. 1A, red temperature, blue photoperiod; [e]Fig. 1B.

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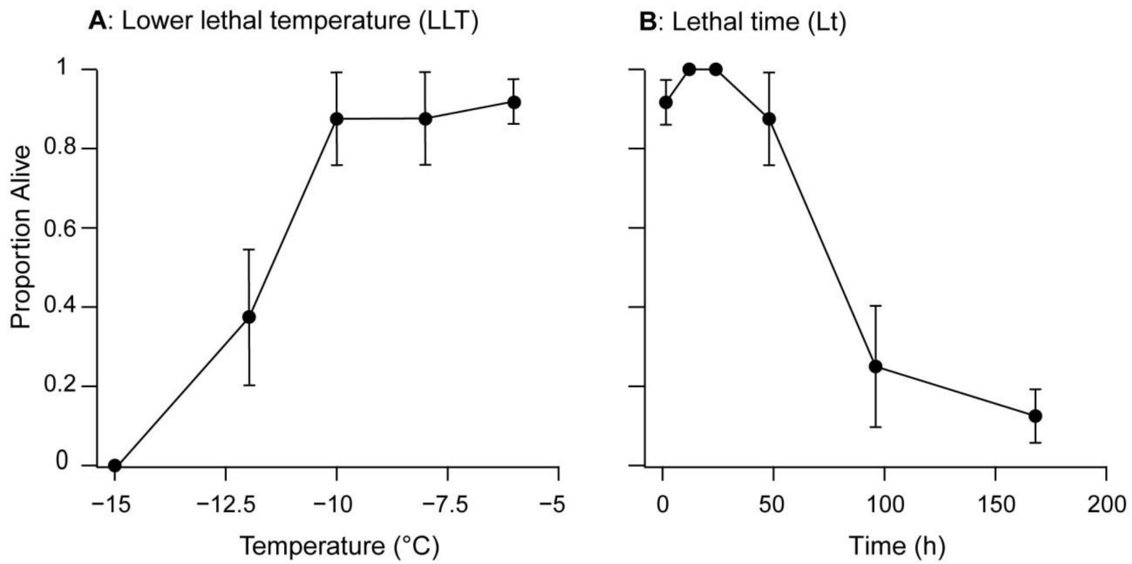
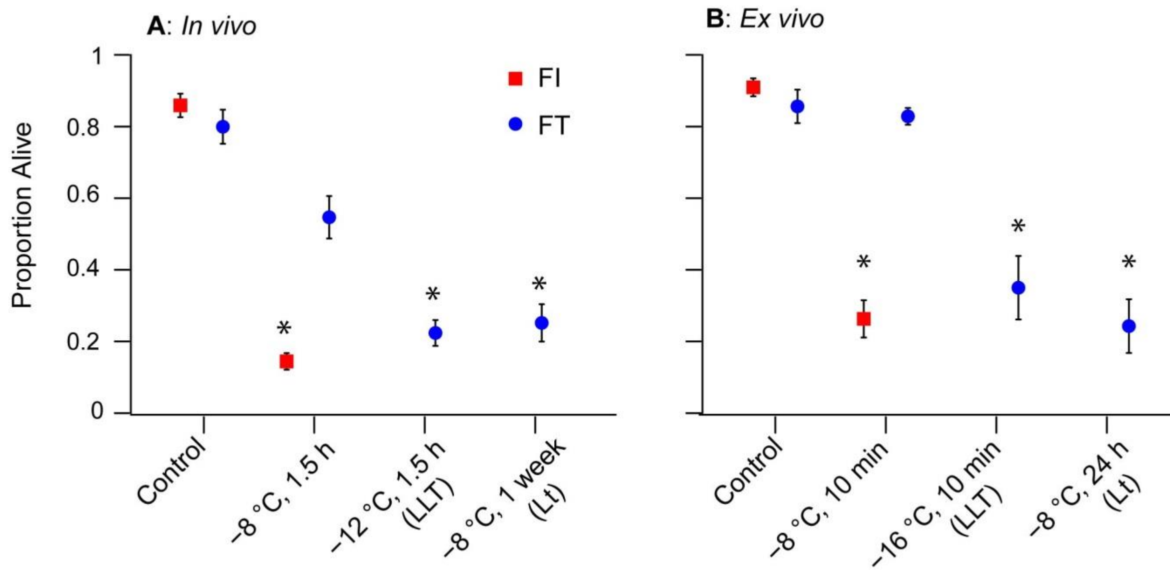


298 **Figure 2. Lethal limits of freeze-tolerant (FT) *Gryllus veletis*. (A)** Proportion of FT crickets
299 that survived following 1.5 h exposures to temperatures ranging from -6°C to -15°C. **(B)**
300 Proportion of FT crickets that survived following exposure to -8°C for times ranging from 1.5 h
301 to 7 d. Each point represents the proportion of 24 crickets ± s.e. Low temperatures reduced
302 survival (Wald $\chi^2 = 2.763$, $P = 0.006$; $LLT_{80} = -13.8$°C). Prolonged exposure to -8°C reduced
303 survival (Wald $\chi^2 = 3.956$, $P < 0.001$; with $Lt_{80} = 123$ h).
304



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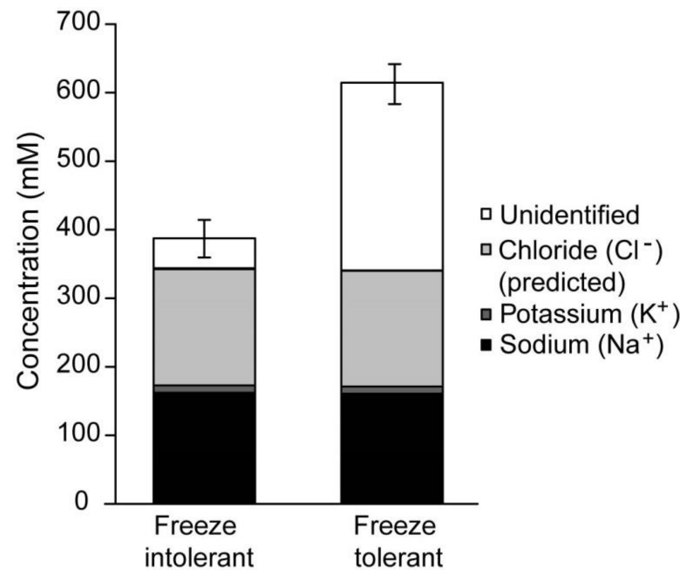
306 **Figure 3. Fat body cell (FBC) survival following freezing (A) *in vivo* and (B) *ex vivo*. (A)** The
307 proportion of live FBCs dissected from freeze-tolerant (FT) and freeze-intolerant (FI) crickets
308 that were never frozen (control), or frozen for 1.5 h at -8°C, the LLT, or the Lt. **(B)** The
309 proportion of live FBCs from FI and FT crickets after *ex vivo* freezing in Grace's Insect Medium
310 to -8°C for 10 min, or the cellular LLT or Lt. Control samples were dissected from crickets, and
311 never frozen. The mean proportion of live FBCs was lower in all treatments relative to controls
312 ($P < 0.05$, denoted by asterisks), except FBCs from FT crickets frozen *in vivo* for 1.5 h at -8°C
313 ($\chi^2 = 1.77$, $P = 0.077$) and FT FBCs frozen *ex vivo* for 10 min at -8°C ($\chi^2 = 0.266$, $P = 0.790$).
314 Each point represents the mean ± s.e.m. proportion of live FBCs from 18 (*in vivo*) or 24 (*ex vivo*)
315 crickets. Example micrographs (from which survival was quantified) are in Fig. S2.

316

317

318 *3.2 Physiological correlates of freeze tolerance*

319 Freeze-tolerant *G. veletis* elevated hemolymph osmolality by c. 250 mOsm relative to freeze-
320 intolerant crickets (Fig. 4). Sodium, potassium, and chloride ions accounted for c. 88% of freeze-
321 intolerant *G. veletis* hemolymph osmolality. Ion concentrations did not differ between freeze-
322 tolerant and freeze-intolerant crickets, and the solutes accounting for c. 300 mOsm of freeze-
323 tolerant cricket hemolymph remained unidentified (Fig. 4).



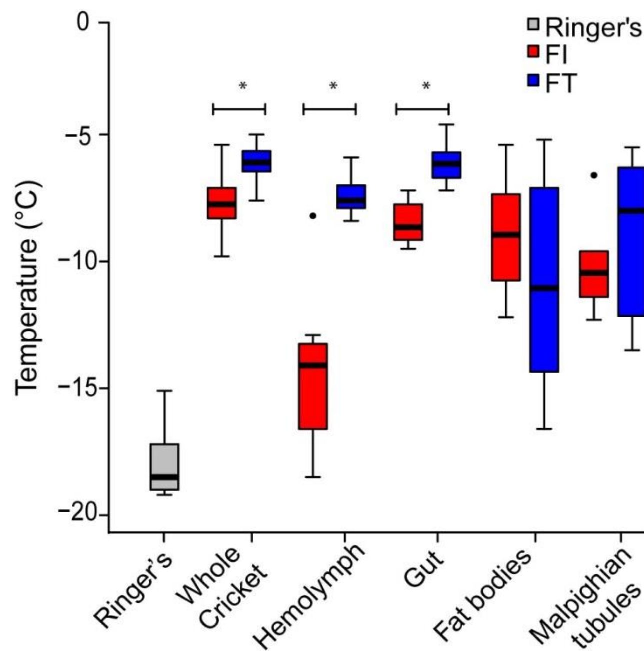
324

325 **Figure 4. Hemolymph composition of freeze-tolerant (FT) and freeze-intolerant (FI)**
326 ***Gryllus veletis*.** Mean concentrations of ions (Na^+ and K^+) were determined *via* atomic
327 absorption spectrometry, and are represented as a fraction of the total hemolymph osmolality in
328 FT and FI *G. veletis* ($N = 8$). Cl^- concentrations were estimated to be equal to the sum of Na^+ and
329 K^+ concentrations (Ramløv et al., 1992). Errors bars represent the s.e.m. of osmolality.
330 Osmolality was higher in FT than FI crickets ($t_{14} = 5.64$, $P < 0.001$). Mean Na^+ and K^+
331 concentrations were similar between treatment groups (Na^+: $t_{14} = 0.10$, $P = 0.54$; K^+: $t_{14} = 0.12$, P
332 $= 0.55$).

333

334

335 Although high osmolality colligatively depresses the freezing point of fluids, freeze-tolerant *G.*
336 *veletis* had a higher mean SCP (-6.1°C) than freeze-intolerant crickets (-7.7°C; Fig. 5). Nanolitre
337 osmometry of *G. veletis* hemolymph did not reveal either thermal hysteresis (TH) or
338 spicular/angular ice crystal growth characteristic of ice-binding protein activity (Table S1),
339 suggesting a lack of hemolymph proteins that regulate ice growth. When frozen *ex vivo*, the SCP
340 of the hemolymph and gut of freeze-tolerant crickets was significantly higher than those of
341 freeze-intolerant individuals (Fig. 5). Supercooling points of fat body and Malpighian tubules did
342 not differ between freeze-tolerant and freeze-intolerant crickets (Fig. 5). Although adding AgI to
343 the cuticle, hemolymph or gut of freeze-intolerant crickets increased the SCP to c. -4°C, none of
344 these crickets survived a freeze treatment of 1.5 h at -8°C (Table 1).



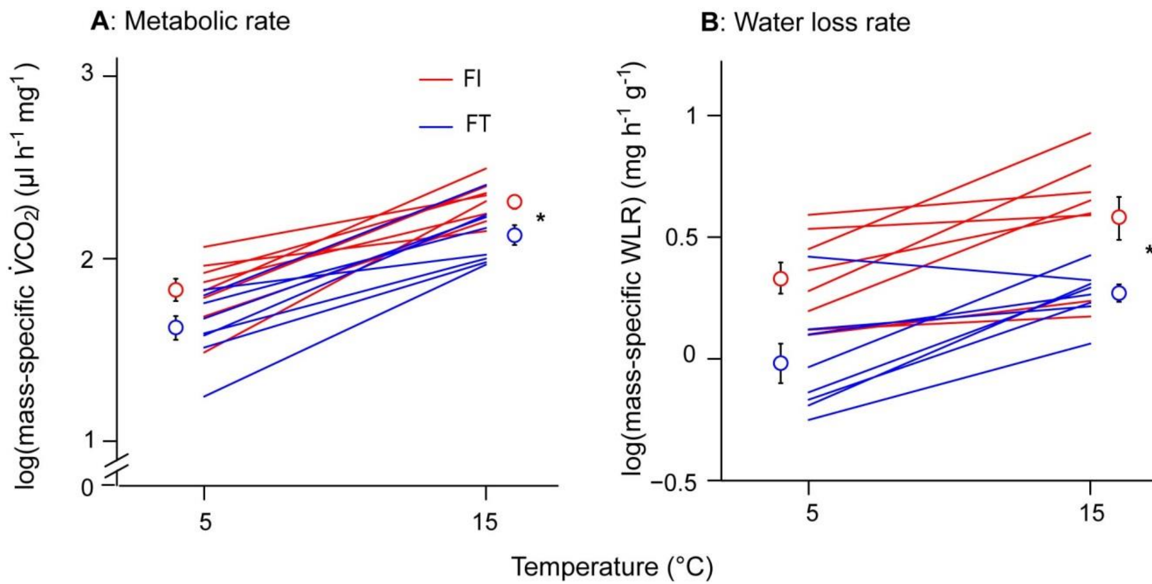
345

346 **Figure 5. Supercooling points (SCPs) of *Gryllus veletis* and its tissues**. The SCP was
347 determined for freeze-tolerant (FT; $N = 24$) and freeze-intolerant (FI; $N = 64$) crickets, as well as
348 samples of hemolymph and tissues (gut, fat bodies and Malpighian tubules) in anticoagulant ($N =$
349 8 per hemolymph or tissue type). The mean SCPs of hemolymph and all tissues differed from *G.*
350 *veletis* Ringer's solution ($F_{9,70} = 27.18$, $P < 0.001$; all contrasts $P < 0.05$). The top and bottom of
351 each box represents the upper and lower quartile, respectively; the horizontal line represents the
352 median; the vertical lines extend to the minimum and maximum values within 1.5 times the
353 inter-quartile range; and black dots indicate outliers. Mean SCPs that are significantly different
354 between FT and FI crickets are denoted with an asterisk ($t_{56}= 8.36$, $P < 0.001$).

355

356 Acclimated crickets decreased their metabolic rate by c. 33%, as estimated from CO_2 emission
357 using flow-through respirometry (Fig. 6A, Table 2). Acclimation did not change thermal
358 sensitivity of metabolic rate, expressed as Q_{10} (Fig. 6A, Table 2). Freeze-tolerant *G. veletis* had
359 c. 50% lower water loss rates than freeze-intolerant crickets, most of which (>80%) was
360 accounted for by reduced cuticular water loss (Fig. 6B, Table 2).

361



362

363 **Figure 6. (A) CO_2 emission ($\dot{V}CO_2$) and (B) water loss rate (WLR) of freeze-intolerant (FI)**
364 **and freeze-tolerant (FT) *Gryllus veletis* at two temperatures.** Each line represents a single
365 cricket ($N = 8$ per treatment), and open circles represent the mean ± s.e.m. for that group of
366 crickets at 5°C or 15°C. Small error bars are obscured by symbols. Asterisks indicate a
367 difference between FT and FI crickets at both temperatures ($\dot{V}CO_2$, ANCOVA: mass: $F_{1,31}$ =
368 12.01, $P = 0.002$; acclimation: $F_{1,31} = 9.67$, $P = 0.004$; temperature: $F_{1,31} = 63.00$, $P < 0.001$;
369 acclimation × temperature: $F_{1,31} = 1.00$, $P = 0.327$; WLR, ANCOVA: mass: $F_{1,31} = 8.35$, P =
370 0.007; acclimation: $F_{1,31} = 16.45$, $P < 0.001$; temperature: $F_{1,31} = 8.54$, $P < 0.001$; acclimation ×
371 temperature: $F_{1,31} = 1.39$, $P = 0.248$). Example respirometry traces are in Fig. S3.

372

373

374 **Table 2. Gas exchange and water loss parameters in freeze-tolerant and freeze-intolerant**
375 **fifth instar juvenile *Gryllus veletis*.** Mean ± s.e.m. at each temperature is presented. CWL,
376 cuticular water loss; *N*, sample size; Q_{10}, measure of thermal sensitivity; RWL, respiratory water
377 loss; $\dot{V}CO_2$, rate of CO_2 emission; WLR, water loss rate.
378

Parameter	Freeze-intolerant		Freeze-tolerant	
	5°C	15°C	5°C	15°C
N	8		8	
Fresh mass (mg)	173.9 ± 19.5		178.7 ± 11.7	
Q_{10}	2.27 ± 0.29		1.77 ± 0.38	
VCO_2 (µl h^{-1})^{a,b}	12.8 ± 2.4	35.5 ± 3.6	7.9 ± 1.0	25.6 ± 4.2
WLR (mg h^{-1}) ^{a,b}	0.411 ± 0.100	0.739 ± 0.144	0.194 ±0.043	0.334 ±0.09
RWL (mg h^{-1})^{a,b}	0.030 ± 0.022	0.114 ±0.031	0.013 ±0.010	0.055 ±0.019
CWL (mg h^{-1})^{a,b}	0.382 ±0.079	0.625 ±0.128	0.192 ±0.038	0.278 ± 0.035
RWL as a percentage total water loss (%)^{c}	4.0 ± 2.0	16.6 ± 2.8	3.9 ±2.2	17.4 ± 6.3

379
380 [a]Significant difference ($P < 0.05$) in parameter between freeze-intolerant and freeze-tolerant crickets.
381 [b]Significant difference ($P < 0.05$) in parameter between crickets at 5°C and 15°C.
382 [c]Significant interaction ($P < 0.05$) of acclimation and test temperature on parameter.

383

384 **4 Discussion**

385 *4.1 Acclimation induces freeze tolerance in* G. veletis *nymphs*

386 We induced freeze tolerance in a laboratory colony of *G. veletis* by mimicking temperature and
387 photoperiod conditions that juvenile nymphs experience as winter approaches. Changes in
388 temperature or photoperiod alone did not induce freeze tolerance, indicating that the
389 physiological changes required for freeze tolerance are induced by both cues in concert.
390 Although seasonal photoperiod patterns are consistent from year to year, warm fall weather (e.g.
391 due to climate change; Gallinat et al., 2015) may prevent crickets from becoming freeze tolerant,
392 potentially increasing overwintering mortality. Crickets did not become freeze-tolerant until after
393 at least six weeks of acclimation, suggesting that acquiring freeze tolerance requires
394 physiological changes that take many weeks to complete.

395 These lethal limits of freeze-tolerant *G. veletis* are similar to those of other orthopterans that are
396 freeze-tolerant as juveniles (Toxopeus et al., 2016) or adults (Ramløv et al., 1992; Sinclair et al.,
397 1999). Winter air temperatures are likely to approach or exceed these lethal limits across much of
398 the *G. veletis* geographical range (north eastern North America). However, *G. veletis* can likely
399 still survive in these regions by overwintering in thermally-buffered microhabitats, such as
400 beneath snow or leaf litter (Sinclair, 2015). Alternatively, *G. veletis* may further decrease their
401 LLT or increase their Lt upon exposure to lower temperatures during winter, either via seasonal
402 plasticity (cf. the alpine cockroach *Celatoblatta quinquemaculata*; Sinclair, 1997), or through
403 other hardening responses, as shown by *B. antarctica* (Teets et al., 2008).

404 Freeze-tolerant insects presumably protect their cells and tissues from freeze injury, but fail to do
405 so at the lethal limits (LLT, Lt; Toxopeus and Sinclair, 2018). We observed that high fat body
406 cell damage *in vivo* was associated with whole animal mortality of freeze-intolerant crickets and
407 freeze-tolerant crickets frozen to their lethal limits. Therefore, whole animal freeze tolerance
408 appears to be correlated with cellular freeze tolerance. These results are similar to seasonal
409 acquisition of freeze tolerance by *E. solidaginis*, which is associated with improved tissue freeze
410 tolerance *ex vivo* (Yi and Lee, 2003). In addition, when we froze fat body cells *ex vivo*, cell
411 viability paralleled the trends we observed *in vivo* (high mortality of freeze-intolerant cells, and
412 freeze-tolerant cells frozen to their lethal limits). Thus, these *ex vivo* experiments can inform our
413 understanding of *in vivo* freeze tolerance, and vice versa.

414 *4.2 Freeze-tolerant* G. veletis *likely control ice formation*

415 To identify parameters that support whole animal and cellular freeze tolerance in *G. veletis*, we
416 compared factors that could impact ice formation and quality in freeze-tolerant and freeze-
417 intolerant crickets. The hemolymph osmolality elevation in freeze-tolerant *G. veletis* may
418 colligatively reduce ice content. For example, a c. 300 mM increase in hemolymph metabolite
419 concentration decreases maximum ice content in *C. costata* from 76% to 68% (Rozsypal et al.,
420 2018). We therefore hypothesize that high hemolymph osmolality in *G. veletis* reduces the
421 dehydration stress associated with high ice contents (Toxopeus and Sinclair, 2018), thereby
422 reducing freeze injury. Hemolymph ion concentrations did not differ between freeze-tolerant and
423 freeze-intolerant *G. veletis*. Low molecular weight cryoprotectants are responsible for seasonal

424 changes in hemolymph composition in other freeze tolerant insects (Storey and Storey, 1988).
425 Toxopeus (2018) found that freeze-tolerant *G. veletis* accumulate *myo*-inositol, proline and
426 trehalose, and these species likely contribute to their elevated hemolymph osmolality.

427 Freeze tolerance is often accompanied by elevated SCP, which may facilitate freeze tolerance by
428 slowing ice formation and minimizing IIF (Zachariassen and Kristiansen, 2000). While the mean
429 SCP of freeze-tolerant and freeze-intolerant crickets differed by a relatively small amount (c.
430 1.5°C), small differences in SCP can considerably impact the location of ice formation. For
431 example ice propagates into cells of the alpine cockroach *C. quinquemaculata* tissues frozen *ex*
432 *vivo* at -4°C, but ice remains extracellular if freezing begins at -2°C (Worland et al., 2004).
433 Elevated SCPs of hemolymph and gut tissue suggests that freeze-tolerant *G. veletis* elevate their
434 SCP by accumulating extracellular INAs. We tested the hypothesis that controlling the
435 temperature and location of ice formation was sufficient to confer freeze tolerance on control
436 crickets. However, elevating the SCP with AgI to c. -4°C in gut, hemolymph, or on the cuticle
437 did not induce freeze tolerance, indicating that controlling the temperature and location of ice
438 formation is not sufficient for surviving internal ice. These results are similar to observations of
439 *Eleodes blanchardi* beetles, which lose their freeze tolerance if deacclimated despite retaining a
440 high (c. -6°C) SCP (Zachariassen et al., 1979). However, initiating ice formation at a very high
441 subzero temperature (c. -0.5°C due to inoculation with external ice) is necessary for *C. costata*
442 freeze tolerance (Koštál et al. 2011), and we therefore suggest that more subtle manipulations of
443 ice formation and milder freeze treatments may yet reveal a role for this mechanism in *G. veletis*.

444 In addition to controlling the initiation of ice formation, freeze-tolerant insects may need to
445 restrict the growth and recrystallization of ice once it has formed, for example by accumulating
446 TH factors with recrystallization inhibition activity (Knight and Duman, 1986; Toxopeus and
447 Sinclair, 2018). *Gryllus veletis* hemolymph did not exhibit TH or evidence of ice-binding
448 proteins that modify crystal shape. However, TH factors can be intracellular or bound to epithelia
449 in other freeze-tolerant arthropods (Duman, 2015; Tursman and Duman, 1995; Wharton et al.,
450 2009). In addition, recrystallization inhibitors do not always exhibit TH activity (Wharton et al.,
451 2005; Toxopeus and Sinclair, 2018).

452 *4.3 Acclimation reduces metabolic rate and water loss*

453 Many overwintering insects suppress their metabolic rate (e.g. in diapause; Hahn and Denlinger,
454 2011), and the reduced CO_2 production in freeze-tolerant *G. veletis* conforms to this pattern,
455 similar to freeze-tolerant *E. solidaginis* (Irwin et al., 2001). Decreased CO_2 production may
456 partially reflect a change of fuel to solely lipids (Sinclair et al., 2011), although the crickets were
457 all fasted for 24 h prior to measurement to control for this. Reduced metabolic rate likely
458 conserves energy reserves during winter (Sinclair, 2015), and we hypothesize that this facilitates
459 successful post-thaw recovery. In addition to suppressing metabolic rate, freeze-tolerant *G.*
460 *veletis* decreased cuticular water loss. We hypothesize that *G. veletis* modifies its cuticle
461 structure or hydrocarbons during acclimation to reduce water loss, which may mitigate
462 dehydration stress during winter when animals are not able to replenish their body water by
463 drinking (Bazinet et al., 2010; Stinziano et al., 2015).

464 *4.4 Conclusion*

465 Here we present a new model for studying insect freeze tolerance. *Gryllus veletis* is easily reared,
466 facultatively acquires freeze tolerance with acclimation, and is amenable to laboratory
467 manipulations and tissue-specific work. We identify physiological changes associated with
468 acclimation that may control ice formation, and reduce challenges associated with energy drain
469 and dehydration challenges. Few freeze-tolerant insects are amenable to laboratory rearing and
470 manipulation (Toxopeus and Sinclair, 2018), and we therefore expect that further work with the
471 *G. veletis* model will be crucial for uncovering the mechanisms underlying insect freeze
472 tolerance.

473 **Abbreviations**

474 AFP – antifreeze protein
475 AQP – aquaporin
476 DAPI – 4',6-diamidino-2-phenylindole
477 ddH_2O – distilled deionized water
478 IIF – intracellular ice formation
479 INA – ice-nucleating agent
480 L:D – light: dark
481 LLT – lower lethal temperature

482 Lt – lethal time

483 PBS – phosphate buffered saline

484 Q_{10} – rate of change as a function of temperature

485 RH – relative humidity

486 SCP – supercooling point

487 TH – thermal hysteresis

488 T_m – melting point

489 $\dot{V}CO_2$ – rate of CO_2 production

490 WLR – water loss rate

491 **Acknowledgements**

492 Thank you to Laura V. Ferguson, whose initial observations on overwintering *G. veletis*
493 motivated this study of freeze tolerance. We are grateful to many lab assistants, for their help
494 with cricket maintenance, especially Haonan (Johnny) Jiang, Michelle Lim, Nicole Martin-
495 Kenny and Aldina Sertovic. We thank James F. Staples, Vladimír Koštál, and two anonymous
496 reviewers for their constructive comments on earlier versions of this manuscript.

497 **Declarations of interest**

498 None. The authors declare that they have no completing interests.

499 **Funding**

500 This work was supported by a Discovery Grant from the Natural Sciences and Engineering
501 Research Council of Canada (NSERC) to BJS, by NSERC Canada Graduate Scholarships and
502 Ontario Graduate Scholarships (OGS) to JT and KFT, and by an OGS to AHM. These funding
503 bodies played no role in study design; collection, analysis and interpretation of data; writing the
504 manuscript; or in the decision to submit the article for publication.

505 **Data availability**

506 The datasets supporting this article are available in supplementary Dataset S1.

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