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Freeze tolerance of Cyphoderris monstrosa (Orthoptera: Prophalangopsidae)

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Abstract

The great grig, *Cyphoderris monstrosa* Uhler (Orthoptera: Prophalangopsidae), is a large (20-30 mm, >1 g), nocturnal ensiferan that inhabits montane coniferous forests in northwestern North America. *C. monstrosa* overwinters as a late-instar nymph, but its cold tolerance strategy has not previously been reported. We collected nymphs from near Kamloops, British Columbia, in late spring to determine their cold tolerance strategy. *C. monstrosa* nymphs were active at low temperatures until they froze at -4.6 ± 0.3 °C. The nymphs survived internal ice formation (i.e. are freeze tolerant), had a lethal temperature between -9 and -12 °C, and could survive for between five and ten days at -6 °C. Isolated *C. monstrosa* gut, Malpighian tubules and hind femur muscle tissues froze at temperatures similar to whole nymphs, and likely inoculate freezing *in vivo*. Hemolymph osmolality was 358 ± 51 mOsm, with trehalose and proline comprising approximately 10 % of that total. Glycerol was not detectable in hemolymph from field-fresh nymphs, but accumulated after freezing and thawing. The control of ice formation and presence of hemolymph cryoprotectants may contribute to *C. monstrosa* freeze tolerance and overwintering survival.

Introduction

The great grig, *Cyphoderris monstrosa* Uhler (Orthoptera: Prophalangopsidae), is a large (20 – 30 mm long, adults >1.5 g) ensiferan that inhabits montane coniferous forests of western North America (Morris and Gwynne 1978; Kumala *et al.* 2005). *C. monstrosa* is nocturnal, emerging from below-ground burrows and climbing conifers to feed on staminate cones (Caudell 1904; Morris and Gwynne 1978; Ladau 2003). Males sing after dusk via tegminal stridulation (Morris and Gwynne 1978) from late May or early June until late August (Mason 1996). *Cyphoderris* spp. are active at much lower temperatures than is typical for acoustic insects, singing at temperatures near 0 °C (Morris and Gwynne 1978; Dodson *et al.* 1983; Morris *et al.* 1989). *C. monstrosa* are thought to overwinter as late-instar nymphs in burrows below the leaf litter layer (Gwynne 1995), but nothing is known about their low temperature biology.

Insects employ two dominant strategies to survive subzero temperatures: freeze avoidant insects depress the temperature at which their fluids freeze, but die upon ice formation, while freeze tolerant insects can withstand internal ice formation. Although orthopteran eggs are freeze avoidant (e.g. Hao and Kang 2004), many nymphs and adults are freeze tolerant (e.g. Alexander 1967). The mechanisms underlying freeze tolerance are unclear, but many freeze-tolerant insects accumulate low molecular weight cryoprotectants, including the disaccharide trehalose and free amino acid proline, both detected in hemolymph of freeze-tolerant New Zealand

alpine weta, *Hemideina maori* Pictet & Saussure (Orthoptera: Anostosmatidae) (Neufeld and Leader 1998). Many freeze-tolerant insects accumulate glycerol (Lee 2010), but this cryoprotectant has not been detected in freeze-tolerant orthopterans (Ramløv *et al.* 1992; McKinnon 2015). Regulating the location and temperature of ice nucleation is thought to be essential for insect freeze tolerance (Zachariassen and Kristiansen 2000). These ice nucleators may be endogenous (e.g. proteins) or exogenous (e.g. ice nucleating-active bacteria or ice crystals), and can be located in the hemolymph (e.g. *H. maori*; Sinclair *et al.* 1999) and tissues (e.g. the Malpighian tubules and fat bodies of *E. solidaginis*; Mugnano *et al.* 1996).

Here, we characterize the cold tolerance strategy, the lower lethal limits, likely sites of ice nucleation, and common low molecular weight cryoprotectants of the overwintering stage of *C. monstrosa*.

Materials & Methods

We collected 40 nymphs by hand from tree trunks in pine forests near Kamloops, British Columbia (50.45°N, 120.07°W, c. 1000 m a.s.l) from 27 May – 2 June 2015. During this period, the air temperature ranged from 7.3 to 29.3 °C, with a daily mean of 17.8 °C (Environment Canada 2015). We placed nymphs in 100 ml perforated plastic containers, with apple pieces for food. We shipped the animals on ice to the University of Western Ontario, where we maintained them for 2-6 weeks at

4 °C until use in experiments. Nymphs fed in captivity, thus apple pieces were replaced weekly.

For low temperature exposures, we placed nymphs (wet mass range: 0.3-1.48 g) in 35 ml plastic vials in contact with a type T (copper-constantan) thermocouple and cooled them at 0.25 °C min^{-1} to the target temperature in an aluminum block through which 50% methanol was circulated from a programmable refrigerated circulator (Proline RP 55, Lauda, Wurzburg, Germany). We monitored the temperature from the thermocouple using PicoLog software via a Picotech TC-08 thermocouple interface (Pico Technology, Cambridge, UK). Our general approach to characterizing cold tolerance is described by Sinclair *et al.* (2015). In all cases, we rewarmed the nymphs at 0.25 °C min^{-1} to 4 °C, weighed them (fresh mass ± 0.01 g), and transferred them to individual 100 ml containers with apple pieces at 15 °C for recovery. Nymphs were considered 'alive' if they could stand and move in a coordinated fashion 48 h after thawing. Because developmental stage of orthopterans can modify parameters such as metabolic composition (e.g. Anand and Lorenz 2008), we restricted subsequent experiments to larger nymphs (> 0.9 g).

To determine the temperature at which ice formation begins (supercooling point, SCP), we cooled nymphs in 35 ml plastic vials as described above, and recorded the lowest temperature before the exotherm due to ice formation (Sinclair *et al.* 2015). The survival of these nymphs was monitored (details below). To determine

the critical thermal minimum (CT_{min}), or the temperature at which the nymphs entered chill coma, we cooled six nymphs from 25 °C to the SCP as described previously (MacMillan and Sinclair 2011). Nymphs were monitored continuously, and the CT_{min} was the temperature at which nymphs could no longer exhibit coordinated movement in response to probing. We determined cold tolerance strategy by monitoring survival of nymphs held for 1.5 h at -4 °C (unfrozen) or -6 °C (frozen), with freezing confirmed by detection of the SCP exotherm of each nymph. We considered them freeze tolerant if they survived both temperatures, freeze avoidant if they survived at -4 °C but not -6 °C, or chill-susceptible if they were killed by exposure both temperatures. We determined the lethal temperature by determining survival of nymphs exposed to temperatures between -9 °C and -16 °C for 1.5 h. To determine lethal time, we monitored survival of nymphs kept frozen at -6 °C for time periods between 1.5 h and 10 d, and subsequently thawed. Each nymph was exposed to only one cold treatment.

To identify likely sites of ice nucleation, we compared the SCP of hemolymph and several excised tissues (foregut, midgut, hindgut, Malpighian tubules, fat bodies, and hind femur muscle) to whole body SCP. We extracted 4 µl of hemolymph from each of three nymphs (mass 1.16, 1.25, and 1.48 g) using a 20 µl pipette, and diluted it with 12 µl 3 % ascorbic acid to prevent coagulation (McKinnon 2015). We dissected tissues from the same three nymphs, and placed them in 20 µl 3 % ascorbic

acid. We cooled hemolymph, tissue samples, and 20 µl 3 % ascorbic acid in 1.7 ml microcentrifuge tubes at 0.25 °C min^{-1} from 4 °C to -30 °C, with thermocouples attached to the external surface of tubes to detect temperature. We compared the mean SCP of hemolymph (in 3 % ascorbic acid) to 3 % ascorbic acid alone, as well as the mean SCP of hemolymph and each tissue to whole-body SCP using a one-way ANOVA with planned contrasts in R version 3.0.3 (R Core Team 2013). Means are reported ± s.e.m.

We also determined total hemolymph osmolality using a nanolitre osmometer (Otago Osmometers, Dunedin, New Zealand), as described previously (Crosthwaite *et al.* 2011). To quantify potential low molecular weight cryoprotectants in the hemolymph, we measured free proline (Carillo and Gibon 2011), glycerol (Crosthwaite *et al.* 2011) and trehalose (Tennessen *et al.* 2014) in 4 µl samples of hemolymph from three to eight nymphs (mass range: 0.9-1.48 g) using enzymatic spectrophotometric assays. Hemolymph was extracted from untreated nymphs, as well as nymphs that were frozen at -6 °C for 1 h. Mean osmolality and cryoprotectant concentrations are reported ± s.e.m.

Results & Discussion

Cyphoderris monstrosa nymphs remained active as they were cooled, until they froze at a mean SCP of -4.6 ± 0.3 °C (range: -2.4 to -6.8 °C). All *C. monstrosa* nymphs survived exposure to -4 °C (*N*=4, unfrozen) and -6 °C (*N*=4, frozen), thus we

126 conclude that they are freeze-tolerant. Most (75%) *C. monstrosa* survived being
127 frozen at -6 °C for 5 days (Fig. 1a), demonstrating survival of equilibrium ice
128 formation (which can take several hours in large Orthoptera; Ramløv and Westh
129 1993). However, they did not survive acute (1.5 h) exposures at or below -12 °C (Fig.
130 1b). This pattern is similar to other freeze-tolerant ensiferans, such as *H. maori*
131 (Ramløv *et al.* 1992), that freeze at moderate subzero temperatures, but have a
132 relatively high lower lethal temperature (Sinclair *et al.* 2003).
133
134 The mean fresh mass of *C. monstrosa* nymphs was 0.95 ± 0.08 g (range: 0.30
135 to 1.52 g), and SCP was independent of fresh mass (linear regression, $F_{1,21} = 0.207$, p
136 = 0.65), suggesting that ice formation is initiated by ice nucleating agents (Sinclair *et*
137 *al.* 2009). The relationship between dry mass and SCP could be examined to verify
138 this trend (e.g. Ditrich and Koštál 2011). *C. monstrosa* hemolymph froze at -8.5 °C, 8
139 °C higher than the ascorbic acid anticoagulant (Fig. 2), indicating the presence of a
140 hemolymph ice nucleator (cf. Sømme 1986; Sinclair *et al.* 1999), although the low
141 SCP of hemolymph suggests that it is not the source of the high SCP we observe in
142 the whole animal. Fat body did not substantially increase the SCP of ascorbic acid,
143 but gut tissues, hind femur muscle and Malpighian tubules in ascorbic acid froze at
144 temperatures similar to whole-body SCP (Fig. 2). Thus, it appears that although there
145 is a nucleating agent in the hemolymph, ice formation is initiated by one or more of
146 these tissues, similar to the ice-nucleating Malpighian tubules and fat bodies of *E.*
147 *solidaginis* (Mugnano *et al.* 1996).

148

149 The hemolymph osmolality of *C. monstrosa* nymphs was 358 ± 51 mOsm
150 (*N*=4). This is lower than that of other freeze tolerant ensiferans, *H. maori* (700
151 mOsm; Ramløv 1999) and *Gryllus veletis* Alexander & Bigelow (Orthoptera:
152 Gryllidae) (615 mOsm; McKinnon 2015). *C. monstrosa* hemolymph contained 17.4 ±
153 3.2 mM trehalose (*N*=4) and 12.7 ± 2.6 mM proline (*N*=8), accounting for
154 approximately 10% of total hemolymph osmolality. The concentrations of these
155 cryoprotectants are lower than in *H. maori* (Ramløv *et al.* 1992; Neufeld and Leader
156 1998) and *G. veletis* (McKinnon 2015). Like *G. veletis* and *H. maori*, we detected no
157 hemolymph glycerol in field-fresh nymphs (*N*=3). However, hemolymph sampled 2
158 to 4 weeks after the nymphs had been frozen at -6 °C contained 14.6 ± 5.7 mM
159 glycerol (*N*=3). No such changes in hemolymph concentrations of trehalose or proline
160 were observed after freezing. The increase in glycerol suggests that *C. monstrosa*
161 cryoprotectant composition is plastic, and that they may also be able to enhance
162 freeze tolerance in response to short cold exposures, such as frosts in the fall or late
163 spring (cf. Marshall and Sinclair 2015). Thus, although the hemolymph osmolality we
164 measured in *C. monstrosa* was not high in our spring-collected specimens, there is
165 potential for an increase in hemolymph osmolality prior to or during the winter
166 months, which may support lower lethal temperatures and tolerance to longer
167 durations frozen than we observed in this study.

168

169 To our knowledge, this is the first report of freeze tolerance in
170 Prophalangopsidae. The minimum air temperature in Kamloops during the 2014-2015
171 winter was -19.6 °C (Environment Canada 2015), well below the lethal temperature
172 of *C. monstrosa* nymphs. However, their overwintering habitat is likely buffered by
173 snow cover (Petty *et al.* 2015), such that burrow temperatures likely do not approach
174 these low air temperatures. Future investigations could determine whether *C.*
175 *monstrosa* exhibits seasonal plasticity in freeze tolerance, and which mechanisms
176 (e.g. cryoprotectant accumulation) drive this plasticity.
177

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286 **Figure Legends**

287

288 **Figure 1.** Survival of *C. monstrosa* nymphs 48 h after being frozen for different

289 periods of time at -6 °C (A) or at different temperatures for 1.5 h (B). *N=4* for each

290 temperature and time point. Survival curves were calculated using a generalized

291 linear model.

292

293 **Figure 2**. Mean ± s.e.m. SCP of whole *C. monstrosa* nymphs, 20 µl 3% ascorbic

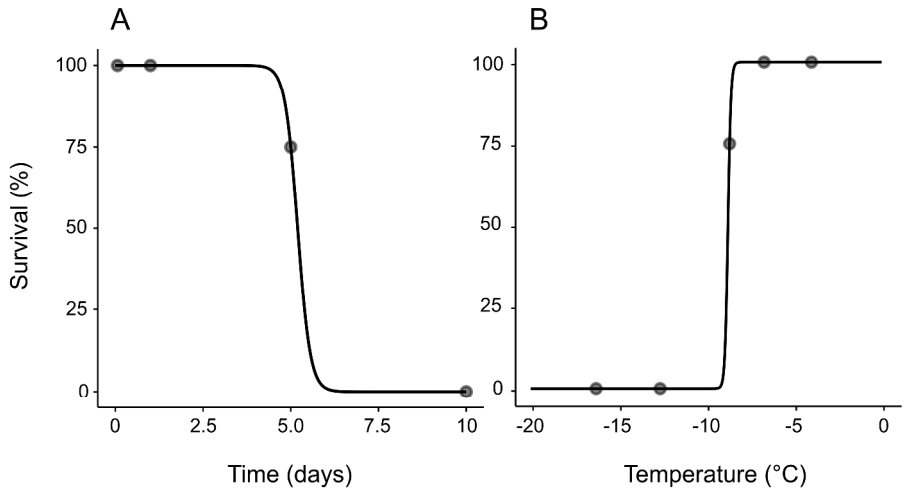
294 acid, hemolymph diluted 1:3 with 3% ascorbic acid, and tissues (c. 10 mg) in 20 µl

295 3% ascorbic acid. *N=23* for whole body SCP, *N=3* for all other samples. Different

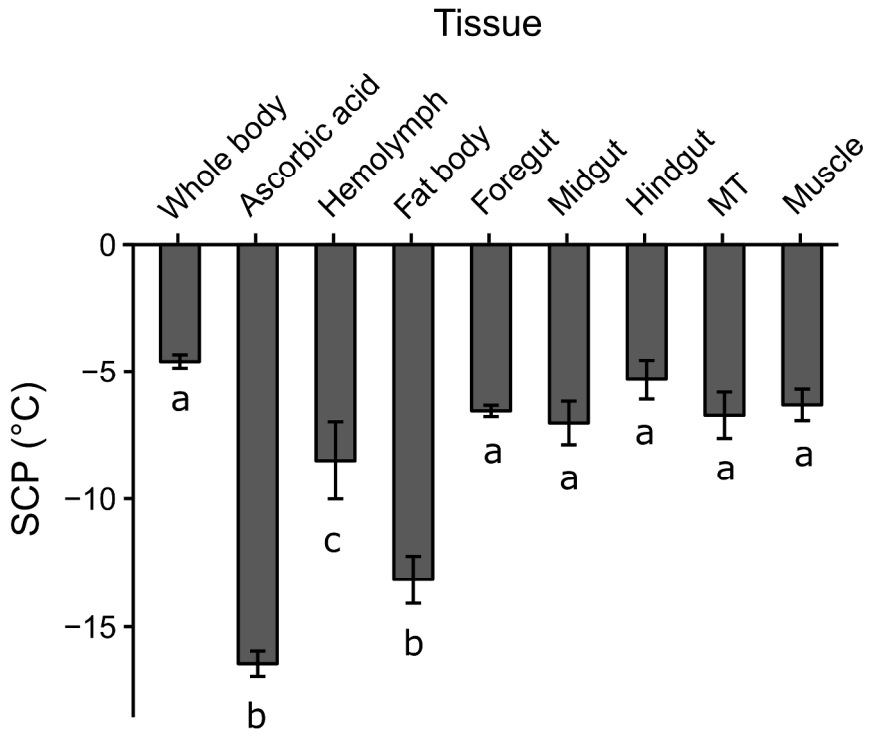
296 letters indicate significant differences (α=0.05) in SCP (ANOVA with planned

297 contrasts: $F_{8,21} = 5.671$, $p < 0.001$).

298



. Survival of *C. monstrosa* nymphs 48 h after being frozen for different periods of time at -6 °C (A) or at different temperatures for 1.5 h (B). N=4 for each temperature and time point. Survival curves were calculated using a generalized linear model.
1979x1013mm (96 x 96 DPI)



Mean ± s.e.m. SCP of whole *C. monstrosa* nymphs, 20 µl 3% ascorbic acid, hemolymph diluted 1:3 with 3% ascorbic acid, and tissues (c. 10 mg) in 20 µl 3% ascorbic acid. N=23 for whole body SCP, N=3 for all other samples. Different letters indicate significant differences (α=0.05) in SCP (ANOVA with planned contrasts: $F_{8,21} = 5.671$, $p < 0.001$).
941x757mm (96 x 96 DPI)