Freeze tolerance of *Cyphoderris monstrosa* (Orthoptera: Prophalangopsidae)

Jantina Toxopeus,[1] Jacqueline E. Lebenzon, Alexander H McKinnon, Brent J. Sinclair

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. *Cyphoderris monstrosa* overwinters as a late instar nymphs, but its cold tolerance strategy has not previously been reported. We collected nymphs from near Kamloops, British Columbia, Canada in late spring to determine their cold tolerance strategy. *Cyphoderris 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 °C and −12 °C, and could survive for between five and 10 days at −6 °C. Isolated *C. monstrosa* gut, Malpighian tubules, and metafemur 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 ~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). *Cyphoderris 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* Uhler species 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). *Cyphoderris monstrosa* are thought to overwinter as late instars 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 and 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 Orthoptera (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

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J. Toxopeus,[1] J.E. Lebenzon, A.H McKinnon, B.J. Sinclair, Department of Biology, University of Western Ontario, 1151 Richmond Street N, London, Ontario, N6A 5B7, Canada

[1]Corresponding author (e-mail: jtoxopeu@uwo.ca).
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(*e.g.*, *H. maori*; Sinclair *et al.* 1999) and tissues (*e.g.*, the Malpighian tubules and fat bodies of *Eurosta solidaginis*; Mugnano *et al.* 1996).

Here, we characterise 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 and methods

We collected 40 nymphs by hand from tree trunks in pine forests near Kamloops, British Columbia, Canada (50.45°N, 120.07°W, ~1000 m) from 27 May to 2 June 2015. During this period, the air temperature ranged from 7.3 °C 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 (London, Ontario, Canada), where we maintained them for two to six 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 (fresh mass range: 0.30–1.48 g) in 35 mL plastic vials in contact with a type T (copper-constantan) thermocouple and cooled them at 0.25 °C minute^{-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, United Kingdom). Our general approach to characterising cold tolerance is described by Sinclair *et al.* (2015). In all cases, we rewarmed the nymphs at 0.25 °C minute^{-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 hours after thawing. Because developmental stage of Orthoptera can modify parameters such as metabolite 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 hours 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 to both temperatures. We determined the lethal temperature by determining survival of nymphs exposed to temperatures between −9 °C and −16 °C for 1.5 hours. To determine lethal time, we monitored survival of nymphs kept frozen at −6 °C for time periods between 1.5 hours and 10 days, 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 metafemur 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 minute^{-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 analysis of variance

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(ANOVA) with planned contrasts in R version 3.0.3 (R Core Team 2013). Means are reported ± standard error (SEM).

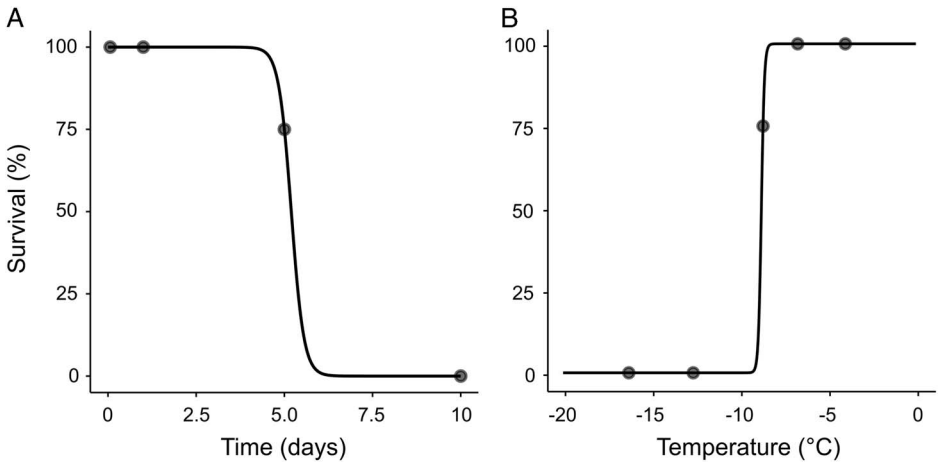
We also determined total hemolymph osmolality using a nanolitre osmometer (Otago Osmometers, Dunedin, New Zealand), as described previously (Crosthwaite *et al.* 2011). To quantify potential low molecular weight cryoprotectants in the hemolymph, we measured free proline (Carillo and Gibon 2011), glycerol (Crosthwaite *et al.* 2011), and trehalose (Tennessen *et al.* 2014) in 4 µL samples of hemolymph from three to eight nymphs (mass range: 0.90–1.48 g) using enzymatic spectrophotometric assays. Hemolymph was extracted from untreated nymphs, as well as nymphs that were frozen at −6 °C for one hour. Mean osmolality and cryoprotectant concentrations are reported ± SEM.

Results and 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 °C to −6.8 °C). All *C. monstrosa* nymphs survived exposure to −4 °C ($n = 4$, unfrozen) and −6 °C ($n = 4$, frozen), thus we conclude that they are freeze-tolerant. Most (75%) *C. monstrosa* survived being frozen at −6 °C for 5 days (Fig. 1A), demonstrating survival of equilibrium ice formation (which can take several hours in large Orthoptera; Ramløv and Westh 1993). However, they did not survive acute (1.5 hour) exposures at or below −12 °C (Fig. 1B). This pattern is similar to other freeze-tolerant ensiferans, such as *H. maori* (Ramløv *et al.* 1992), that freeze at moderate subzero temperatures, but have a relatively high lower lethal temperature (Sinclair *et al.* 2003).

The mean fresh mass of *C. monstrosa* nymphs was 0.95 ± 0.08 g (range: 0.30–1.52 g), and SCP was independent of fresh mass (linear regression, $F_{1,21} = 0.207$, $P = 0.65$), suggesting that ice formation is initiated by ice nucleating agents (Sinclair *et al.* 2009). The relationship between dry mass and SCP could be examined to verify this trend (*e.g.*, Ditrich and Koštál 2011). *Cyphoderris monstrosa* hemolymph froze at −8.5 °C, 8 °C higher than the ascorbic acid anticoagulant (Fig. 2), indicating the presence of a hemolymph ice nucleator (Sømme 1986; Sinclair *et al.* 1999), although the low SCP of hemolymph suggests that it is not the source of the high SCP we observe in the whole animal. Fat body did not substantially increase the SCP of ascorbic acid, but gut tissues, metafemur muscle and Malpighian tubules in ascorbic acid froze at temperatures similar to whole-body SCP (Fig. 2). Thus, it appears that although there is a nucleating agent in the hemolymph, ice formation is initiated by one or more of these tissues, similar to the

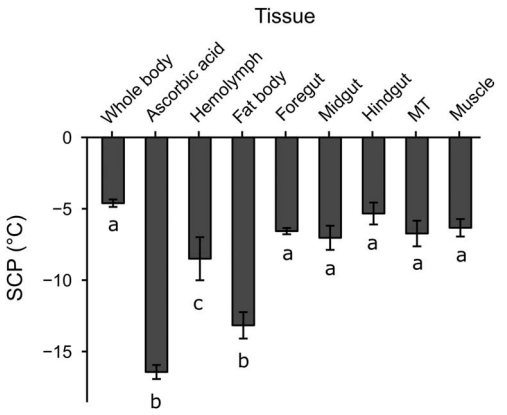
Fig. 1. Survival of *Cyphoderris monstrosa* nymphs 48 hours after being frozen for different periods of time at −6 °C (A) or at different temperatures for 1.5 hours (B). $n = 4$ for each temperature and time point. Survival curves were calculated using a generalised linear model.



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Fig. 2. Mean ± standard error (SEM) supercooling point (SCP) of whole *C. monstrosa* nymphs, 20 µL 3% ascorbic acid, hemolymph diluted 1:3 with 3% ascorbic acid, and tissues (~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 ($\alpha = 0.05$) in SCP (ANOVA with planned contrasts: $F_{8,21} = 5.671$, $P < 0.001$). MT, Malpighian tubules.



ice-nucleating Malpighian tubules and fat bodies of *E. solidaginis* (Mugnano *et al.* 1996).

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

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

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References

Alexander, G. 1967. Cold hardiness in overwintering juvenile grasshoppers. Entomological News, **78**: 147–154.

Anand, A.N. and Lorenz, M.W. 2008. Age-dependent changes of fat body stores and the regulation of fat body lipid synthesis and mobilisation by adipokinetic hormone in the last larval instar of the cricket, *Gryllus bimaculatus*. Journal of Insect Physiology, **54**: 1404–1412.

Carillo, P. and Gibon, Y. 2011. Protocol: extraction and determination of proline [online]. Available from: http://prometheuswiki.publish.csiro.au/tiki-index.php?page=Extraction%20and%20determination%20of%20proline&refresh=1 [accessed 20 December 2015].

Caudell, A.N. 1904. The genus *Cyphoderris*. Journal of the New York Entomological Society, **12**: 47–53.

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https://doi.org/10.4039/tce.2016.21 Published online by Cambridge University Press

Crosthwaite, J.C., Sobek, S., Lyons, D.B., Bernards, M.A., and Sinclair, B.J. 2011. The overwintering physiology of the emerald ash borer, *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae). Journal of Insect Physiology, **57**: 166–173.

Ditrich, T. and Koštál, V. 2011. Comparative analysis of overwintering physiology in nine species of semi-aquatic bugs (Heteroptera: Gerromorpha). Physiological Entomology, **36**: 261–270.

Dodson, G.N., Morris, G.K., and Gwynne, D.T. 1983. Mating behavior of the primitive orthopteran genus *Cyphoderris* (Haglidae). *In* Orthopteran mating systems: sexual competition in a diverse group of insects. *Edited by* D.T. Gwynne and G.K. Morris. Westview Press, Boulder, Colorado, United States of America. Pp. 305–318.

Environment Canada. 2015. Historical climate data, Kamloops, British Columbia [online]. Available from http://climate.weather.gc.ca/index_e.html [accessed 4 February 2016].

Gwynne, D.T. 1995. Phylogeny of the Ensifera (Orthoptera): a hypothesis supporting multiple origins of acoustical signalling, complex spermatophores and maternal care in crickets, katydids, and weta. Journal of Orthoptera Research, **4**: 203–218.

Hao, S.-G. and Kang, L. 2004. Supercooling capacity and cold hardiness of the eggs of the grasshopper *Chorthippus fallax* (Orthoptera: Acrididae). European Journal of Entomology, **101**: 231–236.

Kumala, M., McLennan, D.A., Brooks, D.R., and Mason, A.C. 2005. Phylogenetic relationships within hump-winged grigs, *Cyphoderris* (Insecta, Orthoptera, Tettigonioidea, Haglidae). Canadian Journal of Zoology, **83**: 1003–1011.

Ladau, J. 2003. Territoriality and singing-site preferences in the cricket, *Cyphoderris monstrosa* (Orthoptera: Haglidae) in western North America. Entomological News, **114**: 197–204.

Lee, R.E. 2010. A primer on insect cold-tolerance. *In* Low temperature biology of insects. *Edited by* D.L. Denlinger and R.E. Lee. Cambridge University Press, New York, New York, United States of America. Pp. 3–34.

MacMillan, H.A. and Sinclair, B.J. 2011. The role of gut in insect chilling injury: cold-induced disruption of osmoregulation in the field fall cricket, *Gryllus pennsylvanicus*. Journal of Experimental Biology, **214**: 726–734.

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. Functional Ecology, **29**: 357–366.

Mason, A.C. 1996. Territoriality and the function of song in the primitive acoustic insect *Cyphoderris monstrosa* (Orthoptera: Haglidae). Animal Behaviour, **51**: 211–214.

McKinnon, A.H. 2015. Freeze tolerance in the spring field cricket, *Gryllus veletis*. M.Sc. thesis. University of Western Ontario, London, Ontario, Canada.

Morris, G.K. and Gwynne, D.T. 1978. Geographical distribution and biological observations of *Cyphoderris* (Orthoptera: Haglidae) with a description of a new species. Psyche, **85**: 147–167.

Morris, G.K., Gwynne, D.T., Klimas, D.E., and Sakaluk, S.K. 1989. Virgin male mating advantage in a primitive acoustic insect (Orthoptera: Haglidae). Journal of Insect Behavior, **2**: 173–185.

Mugnano, J.A., Lee, R.E., and Taylor, R.T. 1996. Fat body cells and calcium phosphate spherules induce ice nucleation in the freeze-tolerant larve of the gall fly *Eurosta solidaginis* (Diptera, Tephritidae). Journal of Experimental Biology, **199**: 465–471.

Neufeld, D.S. and Leader, L.P. 1998. Freezing survival by isolated Malpighian tubules of the New Zealand alpine weta *Hemideina maori*. Journal of Experimental Biology, **201**: 227–236.

Petty, S.K., Zuckerberg, B., and Pauli, J.N. 2015. Winter conditions and land cover structure the subnivium, a seasonal refuge beneath the snow. PLoS One, **10**: e0127613.

R Core Team. 2013. R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. Available from http://www.R-project.org [accessed 21 March 2016].

Ramløv, H. 1999. Microclimate and variations in haemolymph composition in the freezing-tolerant New Zealand alpine weta *Hemideina maori* Hutton (Orthoptera: Stenopelmatidae). Journal of Comparative Physiology B, **169**: 224–235.

Ramløv, H., Bedford, J., and Leader, J. 1992. Freezing tolerance of the New Zealand alpine weta, *Hemideina maori* Hutton [Orthoptera; Stenopelmatidae]. Journal of Thermal Biology, **17**: 51–54.

Ramløv, H. and Westh, P. 1993. Ice formation in the freeze tolerant alpine weta *Hemideina maori* Hutton (Orthoptera, Stenopelmatidae). Cryo-Letters, **14**: 169–176.

Sinclair, B.J., Addo-Beddiako, A., and Chown, S.L. 2003. Climatic variability and the evolution of insect freeze tolerance. Biological Reviews, **78**: 181–195.

Sinclair, B.J., Alvarado, L.E.C., and Ferguson, L.V. 2015. An invitation to measure insect cold tolerance: methods, approaches, and workflow. Journal of Thermal Biology, **53**: 180–197.

Sinclair, B.J., Gibbs, A.G., Lee, W.-K., Rajamohan, A., Roberts, S.P., and Socha, J.J. 2009. Synchrotron x-ray visualisation of ice formation in insects during lethal and non-lethal freezing. PLoS One, **4**: e8259.

Sinclair, B.J., Worland, M.R., and Wharton, D.A. 1999. Ice nucleation and freezing tolerance in New Zealand alpine and lowland weta, *Hemideina* spp. (Orthoptera; Stenopelmatidae). Physiological Entomology, **24**: 56–63.

Sømme, L. 1986. Tolerance to low temperatures and desiccation in insects from Andean paramos. Arctic and Alpine Research, **18**: 253–259.

Tennessen, J.M., Barry, W.E., Cox, J., and Thummel, C.S. 2014. Methods for studying metabolism in *Drosophila*. Methods, **68**: 105–115.

Zachariassen, K.E. and Kristiansen, E. 2000. Ice nucleation and antinucleation in nature. Cryobiology, **41**: 257–279.

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