Supplementary material for

Evidence for non-colligative function of small cryoprotectants in a freeze-tolerant insect

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SI Methods

To measure cryoprotectant concentrations in haemolymph samples from *Gryllus veletis*, we used the following spectrophotometric assays:

Glucose – We determined haemolymph glucose concentrations using the Glucose Assay Reagent (Sigma Aldrich, Mississauga, ON, Canada), according to manufacturer's instructions. Briefly, we diluted haemolymph samples by 1:50 to 1:1000 in Tris buffer (TB; 5 mM Tris, 137 mM NaCl, 2.7 mM KCl), vortexed to mix, incubated at 70 °C for 10 min, and centrifuged at 20,000 × g for 3 min. We added 10 µl of supernatant to 90 µl of Glucose Assay Reagent (Sigma Aldrich), incubated the reaction in flat-bottomed 96-well-plates for 20 min at room temperature (c. 22 °C), and measured absorbance in a microplate spectrophotometer (SpectraMax 340PC, Molecular Devices, Sunnydale, CA, USA) at 340 nm. We determined glucose concentration in each sample by comparison to a standard curve range of 0.16 to 0.01 mg/ml glucose diluted in TB.

Glycerol – We determined haemolymph glycerol concentrations using Free Glycerol Reagent (Sigma Aldrich), according to manufacturer's instructions [1]. Briefly, we diluted haemolymph samples by 1:50 to 1:1000 in 0.05 % Tween-20, and vortexed to mix. We added 30 µl of sample to 100 µl of Free Glycerol Reagent (Sigma Aldrich), incubated the reaction in flat-bottomed 96-well-plates for 15 min at room temperature, and measured absorbance at 540 nm. We determined glycerol concentration in each sample by comparison to a standard curve range of 0.5 mM to 0.031 mM glycerol diluted in 0.05 % Tween-20.

myo-Inositol – We determined haemolymph *myo*-inositol concentrations using the *myo*-Inositol Assay Kit (Megazyme, Bray, Ireland), scaled down from manufacturer's instructions. Briefly, we diluted haemolymph samples by 1:50 to 1:1000 in distilled deinoised H_2O (ddH_2O), vortexed to mix, incubated at 70 °C for 10 min, and centrifuged at 20,000 × g for 3 min. We added 15 µl of solution A (hexokinase solution) to 20 µl of sample, and incubated the reaction at room temperature for 15 min. We then added 100 µl of solution B (inositol dehydrogenase/diaphorase solution), and incubated the reaction at room temperature for 10 min. We transferred 100 µl of this mixture into flat-bottomed 96-well-plates, and measured absorbance at 492 nm. We

determined *myo*-inositol concentration in each sample by comparison to a standard curve range of 0.25 to 0.016 mg/ml *myo*-inositol diluted in ddH_2O.

Polyethylene glycol – We measured haemolymph polyethylene glycol (PEG) concentrations using a previously described assay [2]. Briefly, we diluted haemolymph samples 1:25 in ddH_2O, and vortexed to mix. We added 500 µl of chloroform and 500 µl of ammonium ferrothiocyanate reagent (0.1 M $FeCl_3$, 0.4 M NH_4SCN) to 50 µl of sample in 1.7 ml microcentrifuge tubes. We mixed the sample vigorously on a benchtop shaker (700 rpm) for 30 min at room temperature, and centrifuged at 600 × g for 2 min. We transferred 100 µl of the lower chloroform layer into flat-bottomed 96-well-plates, and measured absorbance at 510 nm. We determined PEG concentration in each sample by comparison to a standard curve range of 1.25 to 0.078 mM PEG-8000 (Sigma Aldrich) diluted in ddH_2O.

Proline – We measured haemolymph proline concentration using a previously described assay [3]. Briefly, we diluted haemolymph samples by 1:50 to 1:1000 in 40 % ethanol, and vortexed to mix. Samples were incubated at 4 °C overnight (18 to 24 h), and then centrifuged at 14,000 × g for 5 min. We added 40 µl of sample to 100 µl of ninhydrin solution [1 % (w/v) ninhydrin in 60 % acetic acid (v/v) and 20 % ethanol (v/v)], and incubated the reaction at 95 °C for 20 min. We centrifuged samples at 600 × g for 1 min, transferred 100 µl of supernatant into flat-bottomed 96-well-plates, and measured absorbance at 520 nm. We determined proline concentration in each sample by comparison to a standard curve range of 0.5 mM to 0.016 mM proline diluted in 40 % ethanol.

Trehalose – We measured haemolymph trehalose concentrations using a previously described assay [4]. Briefly, we diluted haemolymph samples by 1:50 to 1:1000 in Tris buffer (TB), vortexed to mix, incubated at 70 °C for 10 min, and centrifuged at 20,000 × g for 3 min. For each sample, we added 30 µl of trehalase stock [TS; 0.3 % (v/v) porcine trehalase enzyme (Sigma Alrich) in TB] to a 30 µl aliquot of supernatant to lyse trehalose into glucose monomers. We added 30 µl of TB to a second aliquot of supernatant. Samples were incubated at 37 °C overnight (18 to 24 h), centrifuged at 20,000 × g for 3 min, and processed using the Glucose Assay Reagent, as described above. We determined baseline glucose haemolymph concentration by comparing samples incubated in TB to a standard curve range of 0.16 to 0.1 mg/ml glucose

diluted in TB. We calculated trehalose haemolymph concentration by comparing samples incubated in 1:1 TB:TS to a standard curve range of 0.16 to 0.1 mg/ml trehalose diluted in 1:1 TB:TS, and subtracting the signal from baseline glucose haemolymph.

SI References

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2. Nag A, Mitra G, Ghosh PC 1996 A colorimetric assay for estimation of polyethylene glycol and polyethylene glycolated protein using ammonium ferrothiocyanate. *Analytical Biochemistry*. **237**, 224-231.
3. Carillo P, Gibon Y. Protocol: extraction and determination of proline https://www.researchgate.net/publication/211353600_PROTOCOL_Extraction_and_determination_of_proline2011 [28 June 2018].
4. Tennessen JM, Barry WE, Cox J, Thummel CS 2014 Methods for studying metabolism in *Drosophila*. *Methods*. **68**, 105-115.

SI Tables

Table S1. Generalised linear models testing the effect of elevated cryoprotectant concentrations on survival of freeze-tolerant (FT) and freeze-intolerant (FI) crickets and their fat body cells. Separate models were run for each group (FI, FT-LLT, and FT-Lt) within each sample type (whole crickets, and fat body frozen *ex vivo*). Freeze-intolerant crickets were frozen to -8 °C for 1.5 h (FI); FT crickets were frozen to the LLT (-12 °C for 1.5 h; FT-LLT), or for the Lt (1 week at -8 °C; FT- Lt). Fat body from FI crickets was frozen to -8 °C for 10 min (FI); fat body from FT crickets was frozen to the cellular LLT (-16 °C for 10 min; FT-LLT), or for the cellular lethal time (24 h at -8 °C; FT- Lt). LLT, lower lethal temperature; Lt, lethal time. Significant effect of cryoprotectant on survival denoted by bold *P*-values.

Sample	FI		FT-LLT		FT-Lt	
Cryoprotectant	χ^2	*P*	χ^2	*P*	χ^2	*P*
Whole crickets						
myo-Inositol	0.000	1.000	0.847	0.397	0.007	0.994
Proline	0.000	1.000	0.899	0.369	2.558	**0.011**
Trehalose	0.000	1.000	3.162	**0.002**	1.922	**0.045**
Combination[a]	0.000	1.000	1.913	**0.045**	2.516	**0.013**
Glycerol	0.002	0.998	3.237	**0.001**	2.391	**0.017**
PEG	0.000	1.000	0.011	0.991	0.007	0.994
Glucose	0.000	1.000	0.305	0.761	0.008	0.994
Fat body frozen *ex vivo*						
myo-Inositol	0.788	0.431	2.170	**0.030**	1.921	**0.045**
Proline	0.652	0.515	0.268	0.789	0.376	0.707
Trehalose	0.878	0.379	0.635	0.526	1.981	**0.048**
Combination[a]	1.290	0.197	2.611	**0.009**	2.329	**0.020**
Glycerol	3.517	**<0.001**	2.611	**0.009**	0.872	0.383
PEG	0.901	0.368	0.322	0.747	0.856	0.392
Glucose	0.299	0.765	0.215	0.829	0.588	0.556

[a] 'Combination' is a combination of *myo*-inositol, proline, and trehalose.