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RESEARCH ARTICLE

Thermal analysis of ice and glass transitions in insects that do and do not survive freezing

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ABSTRACT

Some insects rely on the strategy of freeze tolerance for winter survival. During freezing, extracellular body water transitions from the liquid to the solid phase and cells undergo freeze-induced dehydration. Here, we present results of a thermal analysis (from differential scanning calorimetry) of ice fraction dynamics during gradual cooling after inoculative freezing in variously acclimated larvae of two drosophilid flies, *Drosophila melanogaster* and *Chymomyza costata*. Although the species and variants ranged broadly between 0 and close to 100% survival of freezing, there were relatively small differences in ice fraction dynamics. For instance, the maximum ice fraction (IF_{max}) ranged between 67.9% and 77.7% total body water (TBW). *Chymomyza costata* larvae showed statistically significant phenotypic shifts in parameters of ice fraction dynamics (melting point and IF_{max}) upon entry into diapause, cold acclimation and feeding on a proline-augmented diet. These differences were mostly driven by colligative effects of accumulated proline (ranging between 6 and 487 mmol kg^{-1} TBW) and other metabolites. Our data suggest that these colligative effects per se do not represent a sufficient mechanistic explanation for high freeze tolerance observed in diapausing, cold-acclimated *C. costata* larvae. Instead, we hypothesize that accumulated proline exerts its protective role via a combination of mechanisms. Specifically, we found a tight association between proline-induced stimulation of glass transition in partially frozen body liquids (vitrification) and survival of cryopreservation in liquid nitrogen.

KEY WORDS: Freeze tolerance, Ice fraction, Osmotically inactive water, Proline, Vitrification

INTRODUCTION

Insects overwintering in temperate and polar habitats have evolved different cold-tolerance strategies to cope with situations when their body temperature decreases below the equilibrium melting point (Salt, 1961; Lee, 2010). Some insect species show only limited capacity for cold tolerance and are often classified as chill susceptible (Bale, 1993, 1996; Koštál et al., 2011a). The overwintering strategies of truly cold-tolerant insects are categorized as freeze avoidance, i.e. reliance on supercooling of body liquids (Zachariassen, 1985; Renault et al., 2002), or freeze tolerance, i.e. survival after formation of ice crystals inside the body (Sinclair et al., 2003; Sinclair and Renault, 2010). Some insects use the strategy of cryoprotective dehydration, i.e. they lose most of their body water by evaporation and deposition to surrounding ice crystals in their overwintering microhabitat (Holmstrup and Westh, 1994; Holmstrup et al., 2002). Under specific conditions, insect body solutions may also undergo phase transition into a biological glass via the process of vitrification (Sformo et al., 2010; Koštál et al., 2011b).

In this paper, we focused on the strategy of freeze tolerance. The classical view (Lovelock, 1954; Asahina, 1969) is that freeze-tolerant organisms rely on the formation of ice crystal nuclei in the extracellular space. As the ice nuclei grow with decreasing temperatures, the extracellular solutions become more concentrated, which osmotically drives water out of cells. It remains under debate whether and how often intracellular ice formation occurs *in vivo* and whether it is always lethal (Wharton and Ferns, 1995; Sinclair and Renault, 2010). In this study on dipteran larvae, we adhered to the classical model of extracellular ice formation that is also supported by our direct observations of extracellular ice masses in frozen larvae of *Chymomyza costata* by scanning electron microscopy of cryo-fractured specimens (Koštál et al., 2011b). Considering the classical model, a freeze-tolerant insect must cope with not only deep sub-zero body temperature but also the multiple deleterious effects linked to freeze-induced cellular dehydration (Muldrew et al., 2004; Sinclair and Renault, 2010). All of these effects threaten the chemical and conformational stability of proteins and other macromolecules (Wang, 1999; Brovchenko and Oleinikova, 2008). In order to alleviate the stresses linked with extracellular freezing, freeze-tolerant insects often synthesize and accumulate a variety of small cryoprotective molecules (Sømme, 1982; Storey and Storey, 1991; Koštál et al., 2016a) and/or macromolecular compounds that regulate the process of ice formation (Zachariassen and Kristiansen, 2000; Duman, 2001, 2015). Under eco-physiological conditions, only a certain fraction of the insect's total body water is freezable (i.e. osmotically active water, OAW; corresponding to maximum ice fraction, IF_{max}), while the rest is unfreezable (i.e. osmotically inactive water, OIW) because it is non-covalently bound in the hydration shells of charged molecules and ions and, therefore, is not sufficiently mobile to join the growing ice crystals (Franks, 1986; Wolfe et al., 2002; Block, 2003). Although the phenomenon of insect freeze tolerance is widely recognized, the current knowledge about whether and how the relative size of the ice fraction (IF) limits survival remains poor. Available data (summarized in Table S1) are mostly descriptive, although the relationship between IF and freeze tolerance was specifically addressed in some studies (Zachariassen et al., 1979; Ramlov and Westh, 1993; Gehrken and Southon, 1997; Patricio Silva et al., 2013). Collectively, the literature suggests that seasonal change in the IF_{max} may be one of the adaptive facets of the insect freeze-tolerance strategy (Block, 2003). However, a comprehensive test of this hypothesis is missing.

In addition to the transition of liquid water into a crystalline phase, i.e. ice, we address in this study the transition into an amorphous non-crystalline solid, i.e. glass. The formation of amorphous glass 'traps' the structures and/or macromolecules and

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List of symbols and abbreviations

DM	dry mass
DSC	differential scanning calorimetry
Equi-melt	equilibrium melting
FTR	fluctuating thermal regime
IF	relative ice fraction
IF_{max}	maximum ice fraction
Ino-freeze	inoculative freezing
LD	long day length (16 h/8 h light/dark cycle)
MP	melting point
OAW	osmotically active body water
OIW	osmotically inactive body water
Pro50	proline-augmented diet (50 mg proline per g standard diet)
SD	short day length (12 h/12 h light/dark cycle)
SDA	short day length with cold acclimation
T	temperature
TBW	total body water
T_{dg}	temperature of de-glassing transition
T_{EM}	equilibrium melting temperature
T_g	temperature of vitrification
T_{INO}	temperature of inoculation of body fluids by external ice crystals
T_{SCP}	temperature of spontaneous freezing (supercooling point)
ΔC_p	change in specific heat capacity

'locks' them in place and conformation as they are at the moment of solidification. To date, vitrification has been observed in only two cases in insects. The first evidence (Sformo et al., 2010) was obtained in larvae of the beetle *Cucujus clavipes puniceus*. These larvae avoid freezing by partial dehydration and concomitant accumulation of glycerol, which decreases their supercooling point to as low as −58°C. Some individuals, however, do not freeze at all (to at least −150°C), but vitrify. The second observation comes from partially frozen larvae of *Chymomyza costata* (Koštál et al., 2011b). It is known that the crystalline phase can coexist with the vitreous phase in partially frozen systems. At very low temperatures, and high concentrations of solutes in the unfrozen phase, the residual solution may vitrify in presence of ice (Taylor et al., 2004). It should be noted that in medicinal cryobiology, the term vitrification is more often used to refer to a cryopreservation technique that attempts to vitrify the fully hydrated system without any ice formation (Fahy and Wowk, 2015), while dehydration or freeze concentration of body fluids played an important role in the two insect examples.

Here, we present results of thermal analysis of IF dynamics during freezing of the larvae of two fly species using differential scanning calorimetry. In addition, we verified the occurrence and characterized the parameters of vitrification. The two fly models, *Chymomyza costata* and *Drosophila melanogaster*, belong to the same family (Drosophilidae), their larvae are morphologically similar, and we sampled them at a relatively well-defined and comparable ontogenetic stage. The larvae are ecologically similar during the warm season: growing and developing rapidly on decaying plant material. However, the two species inhabit different geographical regions; *D. melanogaster* originated in the tropics and has spread to mild temperate regions during the last century (Throckmorton, 1975), while *C. costata* lives in cool temperate and subarctic regions (Hackmann et al., 1970). Larvae of *D. melanogaster* do not overwinter, are highly chill susceptible, and exhibit only a mild capacity for cold acclimation (Strachan et al., 2011; Koštál et al., 2011a, 2012). In contrast, *C. costata* larvae are highly seasonal; these larvae overwinter in diapause and exhibit extreme plasticity in freeze tolerance (Koštál et al., 2011b). By acclimating larvae of the two species under different photoperiods, temperatures and dietary conditions, we were able to produce experimental variants that covered the whole conceivable range of insect freeze tolerances. Having these variants in hand, we asked the following specific questions: (i) do IF dynamics differ between the two species?; (ii) does acclimation affect IF dynamics?; (iii) is there a maximum IF that an insect can withstand?; (iv) what is the effect of accumulated cryoprotectants, specifically proline, on IF dynamics?; (v) do we see glass transition in both species and, if yes, under what conditions?; and (vi) does vitrification correlate with survival of freezing?

MATERIALS AND METHODS

Insects and acclimation treatments

We compared two dipterans with contrasting freeze-tolerance capacities: the vinegar fly, *Drosophila* (*Sophophora*) *melanogaster* Meigen 1830, and the malt fly, *Chymomyza costata* (Zetterstedt 1838) (Diptera: Drosophilidae). Cultures of vinegar flies (Oregon-R strain) and malt flies (Sapporo strain) were maintained in MIR 154 incubators (Sanyo Electric, Osaka, Japan). Vinegar flies were reared as described previously (Koštál et al., 2011a) on a standard cornmeal–yeast–agar diet, and malt flies were reared on a similar cornmeal–yeast–agar diet supplemented with ground malted barley (Lakovaara, 1969; Koštál et al., 2016b). All experiments were conducted with fully grown third instar larvae that were sampled from the diet prior to the onset of wandering behaviour. This ontogenetic stage was chosen because pre-wandering larvae can survive freezing of extracellular body fluids (Koštál et al., 2011b, 2012). To modulate the level of larval freeze tolerance, we applied different photoperiodic and thermal acclimation regimes, and augmented the diet with L-proline (Sigma-Aldrich, St Louis, MO, USA; abbreviated hereafter as proline) as listed in Table 1.

In brief, all *D. melanogaster* larvae were grown under the same photoperiodic conditions: 12 h/12 h light/dark cycle (as larvae are photoperiodically insensitive) and one of three different thermal regimes (Table 1): constant 25°C, constant 15°C, and constant 15°C followed by 3 days at a fluctuating thermal regime (FTR) of 6°C for 20 h/11°C for 4 h. Under FTR conditions, *D. melanogaster* larvae enter quiescence (developmental arrest induced directly by low temperature) and increase their cold tolerance (Koštál et al., 2011a, 2016c). The larvae of *C. costata* reared under constant 18°C are photoperiodically sensitive; they continue direct development (to pupariation) under long day length (16 h/8 h light/dark cycle, LD) but enter diapause (hormonally regulated developmental arrest) under short day length (12 h/12 h light/dark cycle, SD; Koštál et al., 2000, 2016b). Gradual cold acclimation of diapausing (SD) larvae was performed by transferring them to 11°C and constant darkness at 6 weeks of age and, 1 week later, transferring them to 4°C for another 4 weeks. This cold acclimation (SDA) increases proline concentrations in the larvae and enhances freeze tolerance such that larvae survive cryopreservation in liquid nitrogen (Koštál et al., 2011b). We reared some larvae of both species on a proline-augmented diet (50 mg proline per g standard diet, Pro50), which further increases freeze tolerance according to our previous studies (Koštál et al., 2011b, 2012, 2016a).

Freezing and cryopreservation protocols

The inoculation of larval body fluids with external ice crystals at relatively high sub-zero temperatures (close to 0°C) and slow cooling/freezing rates are two essential factors underlying survival in freezing assays (Shimada and Riihimaa, 1988). In our experiments, inoculative freezing was ensured by wrapping the

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Table 1. Photoperiodic, thermal and dietary conditions used to induce different levels of freeze tolerance in larvae of *Drosophila melanogaster* and *Chymomyza costata*

Species	Treatment	Photoperiod (light/dark)	Temperature (time)	Diet
D. melanogaster	25°C	12 h/12 h	25°C (5 days)	Standard
	15°C	12 h/12 h	15°C (11 days)	Standard
	15°C FTR	12 h/12 h	15°C (11 days)→6°C/11°C (3 days)	Standard
	15°C FTR Pro50	12 h/12 h	15°C (15 days)→6°C/11°C (3 days)	Proline augmented
C. costata	LD	16 h/8 h	18°C (3 weeks)	Standard
	LD Pro50	16 h/8 h	18°C (5 weeks)	Proline augmented
	SD	12 h/12 h	18°C (6 weeks)	Standard
	SDA	12 h/12 h→0/24 h*	18°C (6 weeks)→11°C (1 week)→4°C (4 weeks)	Standard

FTR, fluctuating thermal regime; Pro50, proline-augmented diet (50 mg proline per g standard diet); LD, long day length (16 h/8 h light/dark cycle); SD, short day length (12 h/12 h light/dark cycle); SDA, short day length with cold acclimation.
*Larvae were shifted to constant darkness on the day of their transfer to 11°C.

larvae between two layers of moist cellulose, overlain with a small ice crystal (for a more detailed description, see Fig. S1 and Koštál et al., 2016a). The standardization of all steps, including the cooling and warming rates, was achieved by performing all experiments in programmable Ministat 240 cooling circulators (Huber, Offenburg, Germany). Temperature inside the cellulose wrapping was monitored using K-type thermocouples connected to a PicoLog TC-08 datalogger (Pico Technology, St Neots, UK).

The larvae of *D. melanogaster* show only limited ability to survive freezing and their freezing protocol was optimized previously (Koštál et al., 2012, 2016a). The optimal protocol (Fig. S2) consists of six steps: (i) 20 min of manipulation with larvae at 0°C (washing larvae out of the diet, counting and placing them into tubes); (ii) 10 min of pre-incubation at −0.5°C (with ice crystal added); (iii) slow cooling to −2°C for 180 min (cooling rate, 0.008°C min^{-1}); (iv) rapid cooling to −5°C for 30 min; (v) heating to +5°C for 40 min (heating rate, 0.25°C min^{-1}); (vi) melting at +5°C for 10 min.

The larvae of *C. costata* survive freezing relatively well and, when appropriately acclimated, can survive cryopreservation in liquid N_2 (Koštál et al., 2011b). In this study, we optimized the conditions of freezing and cryopreservation in SDA larvae by exposing them to protocols with modified rates and/or durations of cooling, heating and incubation. A detailed description of all protocols is given in Table S2. The final optimum freezing/cryopreservation protocol for *C. costata* (Figs S2 and S3A) consisted of six steps: (i) 20 min of larval manipulation at 0°C (washing larvae out of the diet, counting and placing them into tubes); (ii) slow cooling to −30°C (T_1; with ice crystal added) for 300 min (cooling rate, r_1=0.1°C min^{-1}); (iii) plunging them into liquid N_2 for 60 min (or, alternatively, maintaining at T_1 for 60 min); (iv) transfer from liquid N_2 to −30°C (T_2); (v) heating to +5°C for 60 min (heating rate, r_4=0.6°C min^{-1}); (vi) melting at +5°C for 10 min.

Freeze-tolerance assays

The larvae of both species and all experimental variants (see Table 1) were exposed to optimal freezing protocols where the rates of cooling and heating were kept constant but the target temperatures (the minimum temperatures) varied. The data on freeze tolerance of *D. melanogaster* larvae were taken from our previous paper where the target temperatures varied between −2.5°C and −10°C (see table S3A of Koštál et al., 2016a). Results for the freeze tolerance of *C. costata* larvae were obtained from the present study. The target temperatures for *C. costata* varied between −5 and −75°C. Because our Ministats were not able to cool the larvae below −40°C, the freeze-tolerance assay at −75°C was conducted by pre-freezing the larvae to −40°C in the Ministat and then transferring them to a freezer (Platinum 370H, Angelantoni, Massa Martana, Italy) where they gradually cooled to a temperature of −75°C (see the temperature record in Fig. S3B). At the end of the freeze-tolerance assay, the unpacked cellulose balls were transferred to fresh standard diet in a tube maintained at constant 18°C. Alive/dead larvae were scored after 12 h recovery. All living larvae were maintained at 18°C for the subsequent 14 days (*D. melanogaster*) or 2 months (*C. costata*) and successful pupariation and emergence of adult flies were scored as criterions of survival. Exact numbers of larvae used for each specific experiment are shown in Results. Survival of cryopreservation in liquid N_2 was assessed in the SDA variant larvae of *C. costata* during the optimization of the freezing and cryopreservation protocol (see above).

Differential scanning calorimetry

The dynamics of gradual IF formation in a biological system is dictated by decreasing temperature according to the formula (Wang and Weller, 2011):

$$\mathrm{IF} = \mathrm{OAW} \times [1 - (\mathrm{MP}/T)],$$

meaning that the IF is in equilibrium with osmolality (melting point, MP, decreases 1.86°C per osmole of dissolved osmotically active particles) at any given temperature (T), and the maximum IF is limited by the amount of osmotically active water (OAW). In a real situation, this simple mathematical relationship is complicated by: (a) the supercooling capacity: a difference between the MP and the actual temperature of ice crystallization (supercooling point, T_{SCP}); (b) the activity of ice nucleators and/or anti-freeze proteins; and (c) the vulnerability of an insect to inoculation with external ice crystals (Zachariassen, 1985; Williams and Hirsch, 1986; Block, 1995).

We conducted the thermal analyses of whole larvae in 50 µl aluminium pans using a differential scanning calorimeter (DSC4000, Perkin Elmer, Waltham, MA, USA), with some modifications (see below) to previously described methods (Block, 1994; Koštál et al., 2011b). The temperature scale of the DSC4000 was calibrated using indium and mercury according to the manufacturer's instructions. The heat flow was calibrated by measuring the areas under the melt endotherms of known masses of ice (Fig. S4). We developed two different DSC protocols, abbreviated as Equi-melt (equilibrium melting) and Ino-freeze (inoculative freezing), to measure the latent heat absorbed/released during the first-order phase transitions (melting and freezing). Using these protocols, we analysed the relative IF size occurring in a partially frozen larva at a given sub-zero temperature.

In the Equi-melt protocol (Fig. S5), the larva was hermetically sealed in an aluminium pan and inserted into the DSC4000 together with an empty reference aluminium pan. The larva was then rapidly

cooled, and freezing occurred spontaneously at the larva's innate T_{SCP}. Maximum ice fraction (IF_{max}) was allowed to form as the larva reached a sufficiently deep sub-zero temperature (−30°C). Next, a portion of body ice was melted by heating the larva to a specific Equi-melt temperature (T_{EM}). The larva was equilibrated at T_{EM} for 30 min, after which the remaining ice was melted. The equilibrium IF for each specific T_{EM} was derived from the melt endotherm and related to total body water (TBW). TBW was measured as the difference between the total mass of the freshly sealed larva inside the aluminium pan and the total mass of the same pan, punctured after DSC analysis and dried for 3 days at 60°C.

In the Ino-freeze protocol (Fig. S6), the pre-weighed larva was put into the instrument (placed in the aluminium pan that contained external ice crystals) during the run of a temperature program, exactly at −1°C. The larva was inoculated with external ice crystals at a specific temperature (T_{INO}) and the IF gradually increased during slow cooling/freezing at a rate of 0.1°C min^{-1} (simulating the conditions in freeze-tolerance assays) to a specific target temperature (used to analyse the presence/absence of vitrification; see below). The amount of ice formed during gradual freezing was estimated from the area under the freeze exotherm and related to TBW.

The thermal curves obtained by running the Equi-melt and Ino-freeze DSC protocols were analysed using Pyris Software (Perkin Elmer, Waltham, MA, USA) (see Figs S5 and S6 for examples of analysis and more details). The fraction of freezable ('free'), osmotically active water (g OAW g^{-1} TBW) was calculated from the Boltzmann sigmoid curves fitted to summarized Equi-melt data. The fraction of unfreezable ('bound'), osmotically inactive water [g OIW g^{-1} dry mass (DM)] was calculated analogously (Figs S7 and S8).

The results of Ino-freeze protocols that were run with different target temperatures were used not only to derive the IF from inoculative freeze exotherms but also to observe (upon rapid heating) the occurrence and parameters of de-vitrification (de-glassing) phase transition (a second-order phase transition without absorbing/releasing any latent heat). Vitrification typically happens over a ca. 10°C temperature interval centred on a glass transition temperature (T_g) when the viscosity rises by a factor of 1000, and heat capacity, thermal expansivity and compressibility suddenly fall from liquid values to near those of a crystal (Wowk, 2010). Vitrified matter maintains the structure, energy and volume of a liquid, but the changes in energy and volume with temperature are similar in magnitude to those of a crystalline solid (Kauzmann, 1948). In this study, an inflection point of the de-glassing transition was read as the temperature of de-vitrification (T_{dg}). The change in specific heat capacity (ΔC_p) was derived from a difference in heat flow between the onset and the end of the de-glassing transition. The temperature of vitrification (T_g) during slow freezing (at a rate of 0.1°C min^{-1}) was estimated based on the presence/absence of the T_{dg} transition after reaching each specific target temperature of slow freezing (see Fig. S6 for more details).

Metabolomic analysis

Metabolomic analysis was performed for *C. costata* larvae only, while the comparative data for *D. melanogaster* were taken from our previous studies (Koštál et al., 2012, 2016a,c). Whole larvae of *C. costata* (pools of 5 larvae in each of 4 replicates) were sampled from the diet, weighed to obtain fresh mass, plunged into liquid nitrogen and stored at −80°C until analysis. The dry mass and water mass were measured in a parallel sample of 20 larvae weighed individually and dried at 65°C for 3 days. The pools of larvae were homogenized in 400 µl of methanol:acetonitrile:water mixture (volumetric ratio, 2:2:1) containing internal standards (*p*-fluoro-DL-phenylalanine, methyl α-D-glucopyranoside; both at a final concentration of 200 nmol ml^{-1}; both from Sigma-Aldrich). The TissueLyser LT (Qiagen, Hilden, Germany) was set to 50 Hz for 5 min (with a rotor pre-chilled to −20°C). Homogenization was repeated twice and the two supernatants from centrifugation at 20,000 ***g*** for 5 min at 4°C were combined. The extracts were subjected to targeted analysis of major metabolites using a combination of mass spectrometry-based analytical methods described previously (Koštál et al., 2016a,c).

Low molecular weight sugars and polyols were determined after *O*-methyloxime trimethylsilyl derivatization using a gas chromatograph with flame ionization detector (GC-FID-2014) equipped with an AOC-20i autosampler (both from Shimadzu Corporation, Kyoto, Japan). Profiling of acidic metabolites was done after treatment with ethyl chloroformate under pyridine catalysis and simultaneous extraction in chloroform (Hušek and Šimek, 2001). The analyses were conducted using a Trace 1300 gas chromatograph combined with single quadrupole mass spectrometry and a Dionex Ultimate 3000 liquid chromatograph coupled with a high-resolution mass spectrometer (Q Exactive Plus; all from Thermo Fisher Scientific, San Jose, CA, USA). All metabolites were identified against relevant standards and subjected to quantitative analysis using a standard calibration curve method. All standards were purchased from Sigma-Aldrich. The analytical methods were validated by simultaneously running blanks (no larvae in the sample), standard biological quality-control samples (the periodic analysis of a standardized larval sample – the pool of all samples), and quality-control mixtures of amino acids (AAS18, Sigma-Aldrich).

RESULTS

Experimental variants cover the whole range of insect freeze tolerance

By exposing larvae of two drosophilid species to various acclimation conditions, we produced experimental variants that cover the broadest range of insect freeze tolerance: from intolerance (100% mortality) of mild freezing at −2.5°C for a few minutes in *D. melanogaster* larvae (25°C variant) to almost 100% survival of larvae and high production of adults in *C. costata* (SDA variant) exposed to −75°C for a few hours (Fig. 1) or cryopreserved in liquid N_2 for 18 months (Table S2). The two species differed in the overall ability to tolerate freezing but, more importantly, we found that freeze tolerance is a highly plastic trait in both species. The actual level of freeze tolerance was strongly influenced by acclimation conditions (Fig. 1).

IF dynamics are similar among species and acclimation variants

We measured the IF inside the larval body at different temperatures using two DSC-based methods. First, we measured the IF that remains in the larval body after heating the frozen larva to T_{EM} (Equi-melt method). Boltzmann sigmoid curves fitted well to the empirical data (Figs S7 and S8), which allowed us to interpolate the IF at any given temperature. The final curves describing the dynamics of IF formation at decreasing temperature for all experimental variants are presented in Fig. 2A,E. In the Equi-melt method, however, the ice crystals grow inside the larval body very rapidly at relatively low temperatures (at the larva's T_{SCP}, ranging between approximately −16 and −20°C). Freezing at the T_{SCP} is lethal in many insects including *C. costata* and *D. melanogaster* larvae. Therefore, we additionally used the Ino-freeze method to measure the IF that gradually grows in the larval body after

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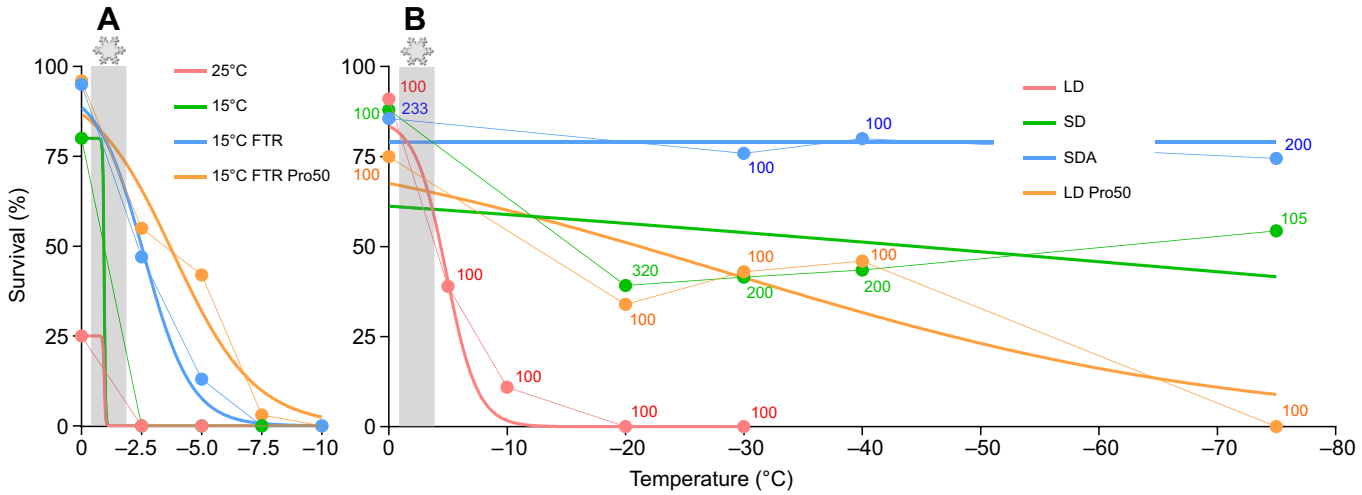

Fig. 1. Larval freeze tolerance in two drosophilid fly species. Prior to freeze-tolerance assays, larvae were maintained under different acclimation conditions (see Table 1: FTR, fluctuating thermal regime; Pro50, proline-augmented diet; LD, long day; SD, short day; SDA, short day cold acclimation) in order to induce variation in freeze tolerance. During the assays, larval body fluids were seeded by external ice crystals and gradually cooled to a target temperature (*x*-axis) at a slow rate (see Figs S1–S3 for more details). After thawing, the larvae were returned to artificial diets and their ability to metamorphose into the adult stage was used as a criterion for survival. (A) *Drosophila melanogaster*: data are taken from our previous paper (see table S3A of Koštál et al., 2016a). (B) *Chymomyza costata*: original data obtained in the present study. Numbers flanking the data points are the number of larvae exposed to each temperature. Sigmoid curves were fitted to all data: y=bottom+(top−bottom)/(1+10$^{(\log EC_{50}-x)\times \text{Hill slope}}$), where 'bottom' is constrained to 0, and 'top' is constrained to survival of controls exposed to 0°C for 20 min (manipulation time).

inoculative freezing at mild sub-zero temperatures (T_{INO}) (Figs S9–S11). The two methods gave similar estimations of IF (see overlap of Equi-melt lines and Ino-freeze ellipses in Fig. 2A,E). The two fly species and different experimental variants differed relatively slightly (when compared with large differences in freeze tolerance) in various parameters of the IF dynamics. Thus, the mean MP varied from −0.4°C to −1.0°C in *C. costata* variants, and from −0.4°C to −0.6°C in *D. melanogaster* variants. The IF_{max} varied between 67.9% and 76.1% of TBW in *C. costata* variants, and between 75.0% and 77.7% of TBW in *D. melanogaster* variants. The temperature at which 99% of IF_{max} was reached varied from −5.9°C to −10.5°C in *C. costata* variants, and from −3.9°C to −6.3°C in *D. melanogaster* variants (for details, see Figs S7 and S8).

The two fly species differed in TBW, which ranged between 2.26 and 2.98 mg mg^{-1} DM in *C. costata* variants (Fig. 2B), and between 3.63 and 4.10 mg mg^{-1} DM in *D. melanogaster* variants (Fig. 2F). The OIW was relatively low in *C. costata* LD, SD and SDA variants (ranging between 0.67 and 0.85 mg mg^{-1} DM), while it was slightly higher in the *C. costata* LD Pro50 variant and all variants of *D. melanogaster* (ranging between 0.90 and 0.96 mg mg^{-1} DM; Fig. 2B,F). The inter-species difference in TBW was also reflected in the maximum ice content, which ranged between 1.5 and 2.3 mg mg^{-1} DM in *C. costata* variants (Fig. 2C) and between 2.7 and 3.2 mg mg^{-1} DM in *D. melanogaster* variants (Fig. 2G).

Association between IF and freeze tolerance

Fig. 3 shows the relationships between IF (derived from Fig. 2A,E; see also Figs S7 and S8) and survival at a given sub-zero temperature (taken from Fig. 1). Three basic patterns were observed: (i) the freezing event was lethal (*D. melanogaster*, 25°C and 15°C acclimation variants); (ii) lethal effects of freezing occurred when the IF reached a specific threshold (*D. melanogaster*, 15°C FTR and 15°C FTR Pro50 acclimation variants; *C. costata*, LD and LD Pro50 acclimation variants); the specific threshold for tolerable IF lay close to the IF_{max}, which corresponds to the OAW fraction (or, vice versa, which is limited by the OIW fraction); (iii) the freezing event was always survived irrespective of the size of the IF (*C. costata*, SD and SDA acclimation variants).

Chymomyza costata larvae accumulate high concentrations of proline

We analysed concentrations of 23 amino compounds, 6 polyols, 3 sugars, 4 intermediates of the TCA cycle, and lactate (37 metabolites in total) in the larvae of *C. costata* (for details, see Table S3). The concentrations of the three most abundant metabolites (proline, glutamine and trehalose), plus the sum concentration of all other quantified metabolites, are presented in Fig. 2D. In order to make a direct comparison between the two species, similar data for *D. melanogaster* are shown in Fig. 2H (Koštál et al., 2012, 2016a,c). The two species dramatically differed in their ability to accumulate proline. The larvae of *C. costata* naturally accumulated 339 mmol kg^{-1} TBW of proline during cold acclimation (SDA variant). Feeding the non-diapause larvae of *C. costata* a proline-augmented diet (LD Pro50 variant) increased the concentration of proline up to 487 mmol kg^{-1} TBW. In contrast, *D. melanogaster* larvae naturally accumulated only 9 mmol kg^{-1} TBW of proline (15°C FTR variant) and feeding them a proline-augmented diet increased the concentration of proline to only 57 mmol kg^{-1} TBW (15°C FTR Pro50 variant).

Glass transition occurs only in *C. costata*

Vitrification (revealed as the presence of a de-glassing transition) was never registered in any DSC thermal analysis of *D. melanogaster* larvae (all experimental variants), irrespective of whether larvae were frozen rapidly (at the innate T_{SCP}) or gradually (after inoculation by external ice at T_{INO}), and irrespective of the target temperature (ranging between −5 and −70°C). When heating frozen larvae back to 20°C at a fast rate of 10°C min^{-1}, typical melt endotherms were always observed, but the characteristic de-glassing transition was absent in all cases.

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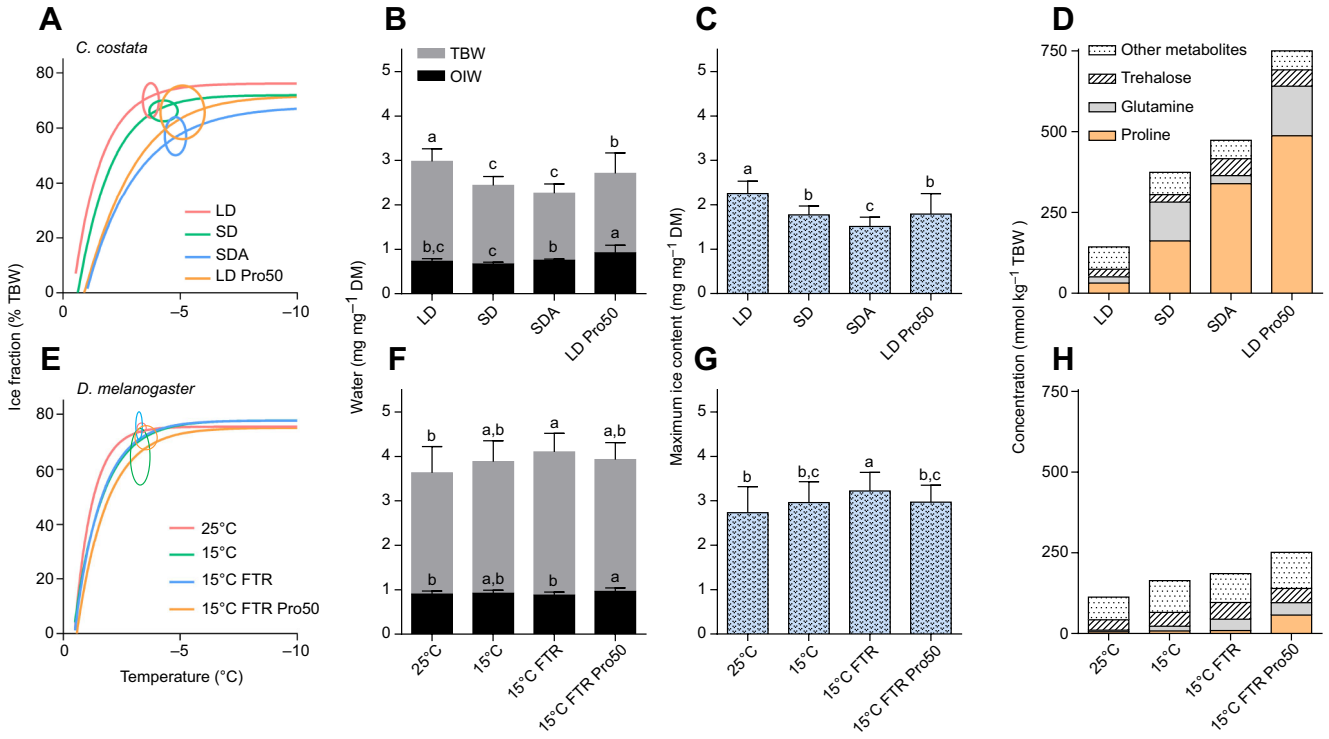

Fig. 2. Ice fraction dynamics and related parameters in larvae of two drosophilid fly species. (A–D) *Chymomyza costata*, (E–H) *Drosophila melanogaster*. The larvae were maintained under different acclimation conditions and exhibited different levels of freeze tolerance (see Fig. 1). (A,E) The gradual increase of the ice fraction (IF) with decreasing temperature in *C. costata* (A) and *D. melanogaster* (E) was measured using two variants of the differential scanning calorimetry (DSC) technique: Boltzmann sigmoids were fitted to Equi-melt data and ellipses were based on mean *x* (±s.d.) and mean *y* (±s.d.) values of Ino-freeze data (see Materials and methods and Figs S5 and S6 for more details). (B,F) Total body water (TBW) content and the amount of unfreezable, osmotically inactive water (OIW). (C,G) The maximum ice content calculated as the difference between TBW and OIW. (D,H) Summary of metabolomic analyses. The three most abundant metabolites are shown: proline, glutamine and trehalose. The remaining 34 quantified metabolites are shown only as a sum (other metabolites). The data for *C. costata* were obtained in the present study (see Table S3 for more details), while the data for *D. melanogaster* are taken from our previous studies (Koštál et al., 2012, 2016a,c). The means (columns) in B, C, F and G flanked by different letters are significantly different according to ANOVA followed by Bonferroni's *post hoc* test. See Figs S7–S10 for complete datasets.

In contrast, the de-glassing transition was detected in DSC Ino-freeze thermal analyses of *C. costata* larvae for all experimental variants (see an example in Fig. S6). However, the presence/absence and parameters of de-glassing were strongly affected by acclimation and freezing conditions (Fig. 4). De-glassing never occurred in larvae that were frozen to target temperatures higher than −30°C. At the target temperature of −30°C, de-glassing transitions were observed only in SDA larvae (50% of cases) and LD Pro50 larvae (20% of cases) but not in LD or SD larvae. With decreasing target temperature, the frequency of occurrence of the de-glassing transition rapidly increased in all experimental variants. Nevertheless, the rates of this increase differed among experimental variants in the order: SDA>LD Pro50>SD>LD (Fig. 4A). Similar ordering of experimental variants was also observed in the other two

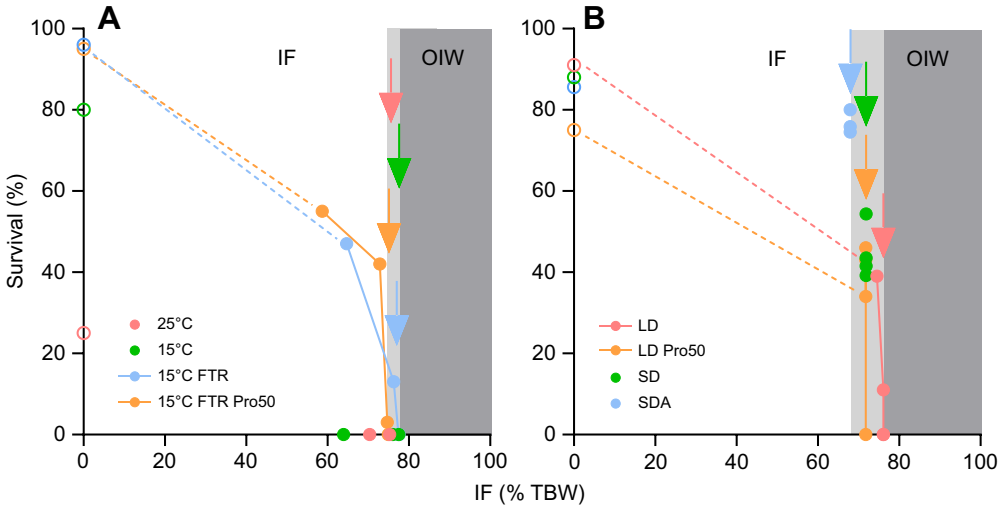

Fig. 3. Association between IF and freeze tolerance. Data on survival (Fig. 1) plotted against data on IF (Fig. 2A,E) for (A) *D. melanogaster* and (B) *C. costata* larvae. The grey areas show the fraction of unfreezable OIW. The pale grey part delimits the range of OIW differences among acclimation variants. Coloured arrows show the exact position of the boundary between OIW and the fraction of osmotically active water (here shown as maximum ice fraction, IF_{max}) for individual acclimation variants. The dashed lines are used to visually connect the data points to the respective initial values (survival after exposure to 0°C for 20 min).

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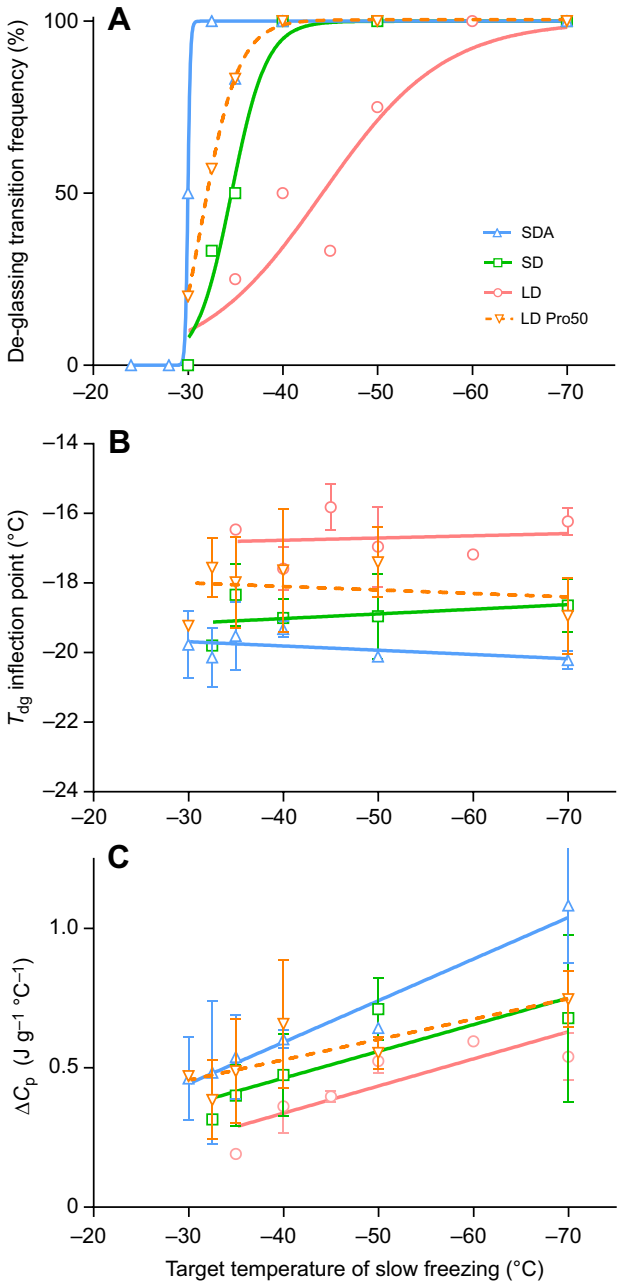

Fig. 4. De-glassing transition analysis in *C. costata*. Larvae were maintained under different acclimation conditions and exhibited different levels of freeze tolerance (see Fig. 1). (A) The frequency of occurrence of the de-glassing transition. (B) The inflection point of the de-glassing transition was read as the temperature of de-vitrification (T_{dg}). (C) The change in specific heat capacity (ΔC_p) was derived from the difference in heat flow between the onset and the end of the de-glassing transition. In A, each point is the percentage of larvae showing the de-vitrification transition after freezing to different target temperatures (see Fig. S6). In B and C, each point is the mean±s.d. of data for several larvae (see Materials and methods and Fig. S11A for details).

parameters of de-glassing: T_{dg} and ΔC_p. The mean T_{dg} varied from −15.8°C to −20.1°C and was variant specific (SDA<LD Pro50<SD<LD), but independent of target freezing temperature within each variant (slopes of linear regressions did not deviate from zero; Fig. 4B). The ΔC_p was variant specific (SDA>LD Pro50>SD>LD) and it increased as the target temperature decreased (slopes of linear regressions deviated from zero; Fig. 4C).

DISCUSSION

Differences in IF dynamics among species and acclimation variants

We found that the IF dynamics are very similar in two drosophilid species [a response to our Introduction question (i)]. This close similarity contrasts with the profound differences observed in freeze tolerance between the two species. Temperature-dependent IF dynamics in a larva is mathematically described by two partially interlocked parameters, the MP and the OAW (Wang and Weller, 2011). As the MP ranged only moderately between −0.4 and −1.0°C in our study, its effect on IF dynamics was relatively small (Figs S7 and S8). The OAW affects the overall shape of IF dynamics more profoundly, as it directly sets the IF_{max}. It is known that the total volume of OAW may undergo relatively massive and rapid changes in response to changing ambient conditions (Wharton and Worland, 2001; Block, 2002; Hadley, 1994), especially in small soil invertebrates that rely on the overwintering strategy of cryoprotective dehydration (Holmstrup and Westh, 1994). However, we did not see any dramatic changes in OAW in our model species. The OAW (IF_{max}) values measured in *D. melanogaster* and *C. costata* larvae, ranging between 67.9% and 77.7% TBW, fall within the scope of published records for other insects, which range between 64% and 84.5% TBW (Table S1).

The two model species differed mainly in their ability to phenotypically modulate IF_{max} in response to acclimation. [Introduction question (ii)]. The *C. costata* larvae showed decreases of IF_{max} upon entry into diapause, cold acclimation and feeding on a proline-augmented diet. As discussed above, these shifts in IF_{max} were driven by concomitant decreases in MP and OAW fraction, both metrics intimately related to increasing the osmolality of body fluids, which was driven by accumulation of proline. We will discuss later whether this phenotypic modulation of IF_{max} driven by proline may have some adaptive meaning. In contrast, the IF_{max} slightly increased in *D. melanogaster* larvae upon cold acclimation and entry into quiescence, while feeding on a proline-augmented diet had no effect on IF_{max}.

Increasing the OIW fraction has been proposed by Storey et al. (1981) as a potentially important adaptive mechanism for insect freeze tolerance. They observed that warm-acclimated larvae of *Eurosta solidaginis* had 0.193 mg OIW mg^{-1} DM, which considerably increased to 0.633 mg OIW mg^{-1} DM after a 6 week stepwise acclimation to −30°C. The hypothesis about the adaptive meaning of increasing OIW rests fundamentally on a concomitant reduction in OAW that causes a reduction of IF at any given temperature and, consequently, mitigates the deleterious effects linked with freeze-induced cell dehydration. In this study, the OIW fraction was relatively constant in all acclimation variants in both species ranging between 0.67 and 0.96 mg mg^{-1} DM. This amount of bound water was safely above the anhydrobiotic threshold of 0.2–0.4 mg mg^{-1} DM that limits normal functionality of cellular structures and enzymes (Brovchenko and Oleinikova, 2008; Ball, 2008). Moreover, we have seen that the more freeze-tolerant larvae of *C. costata* exhibit a lower amount of OIW (expressed per mg DM) than the less freeze-tolerant larvae of *D. melanogaster*. These results lead us to question whether relatively small differences in OIW fraction explain such large variation in freeze tolerance in *C. costata* and *D. melanogaster* larvae.

Association between IF, unfreezable water and freeze tolerance

The seemingly trivial question (iii) posed in the Introduction – ‘is there a maximum IF that an insect can withstand?’ – appears as

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difficult to address empirically as it is to answer generally. Most likely, it is not the IF itself but rather the detrimental effects linked to freeze-induced dehydration that limit freeze tolerance. These effects include mechanical stress caused by growing extracellular ice crystals and shrinking of the cell, decreased activity of water molecules, increased ionic strength, acidity and concentrations of potentially toxic intermediates of metabolism, increased viscosity and increased packing of macromolecules (Muldrew et al., 2004). The association between IF_{max}, OAW/OIW and larval survival is depicted in Fig. 3. Our data suggest that strongly freeze-tolerant insects (such as cold-acclimated diapausing larvae of *C. costata*) survive after the formation of IF_{max} inside their body. The maximum IF that an insect can withstand thus obviously exists only for the insects falling into the categories of partial or moderate freeze tolerance (*sensu* Sinclair, 1999; such as warm-acclimated active larvae of *C. costata* or quiescent and proline-fed larvae of *D. melanogaster*). The existence of maximum tolerable IF was previously estimated in other invertebrates (Zachariassen et al., 1979; Ramlov and Westh, 1993; Gehrken and Southon, 1997; Patricio Silva et al., 2013). However, the follow-up question on what mechanism sets this maximum tolerable IF remains open. We can only speculate why the maximum tolerable IF occurs relatively close to the IF_{max} not only in our study but also in the other studies: in adult tenebrionid beetles, *Eleodes blanchardi*, the IF_{max} represented approximately 75% TBW in both cold- and warm-acclimated specimens, which died when reaching a threshold IF of 62% and 65% TBW, respectively (Zachariassen et al., 1979). In New Zealand wetas, *Hemideina maori*, the IF_{max} was 82% TBW but they died when exposed to temperatures below −7°C, which corresponds to approximately 81% TBW (Ramlov and Westh, 1993). In adult chrysomelid beetles, *Melasoma collaris*, the IF_{max} varied between 77% and 84.5% TBW (cold- and warm-acclimated specimens, respectively), and the lower limit of freeze tolerance was associated with an IF of 73–75% TBW (Gehrken and Southon, 1997). In a freeze-tolerant potworm, *Enchytraeus albidus*, the IF was manipulated by exposing them to various sub-zero temperatures and environmental salinities. For two different populations of potworms, it was found that lethal effects of freezing occurred when IF reached a sharp threshold between 56% and 57% TBW, while IF_{max} was similar in the two populations at 58.8% and 61.4% TBW, respectively (Patricio Silva et al., 2013). One potential hypothetical explanation for such commonality (proximity of maximum tolerable IF to IF_{max}) could be that some (moderately) freeze-tolerant insects can tolerate the loss of most of their OAW relatively well, while greater losses impair the integrity of the OIW pool and, consequently, lead to irreversible changes in macromolecular conformation (Wang, 1999; Ball, 2008; Brovchenko and Oleinikova, 2008) and ultimately mortality. Other (partially) freeze-tolerant insects can be sensitive to even relatively small losses of their OAW associated with relatively mild cell dehydration.

Cryoprotective role of accumulated proline

In response to our Introduction question (iv) regarding the effects of proline on IF, we can say that in both fly species, the accumulated proline affected the empirically measured parameters of IF dynamics according to its theoretically expected colligative effects on biological systems. The MP, the relative OAW fraction and the IF_{max} all decreased with increasing proline concentration. The classically proposed mechanistic model of cryoprotection is based just on these colligative effects. This model posits that at any given cryogenic temperature T, the amount of extracellular ice and, consequently, the magnitude of deleterious cellular freeze-dehydration, is lower in the system with accumulated cryoprotectant than in the system without cryoprotectant (Lovelock, 1954; Salt, 1961; Meryman, 1971; Zachariassen, 1985; Storey and Storey, 1988; Lee, 2010). Our results suggest that the colligative effects linked to proline accumulation are detectable in fly larvae and may thus theoretically contribute to larval freeze tolerance. However, these colligative effects should not be used as sole and straightforward predictors of freeze tolerance. The insect cold-tolerance literature agrees on a view that a single molecule, such as proline, may play more than one (colligative) mechanistic role in building the insect's freeze tolerance. Moreover, a whole complex of other mechanisms, in addition to accumulation of cryoprotectants, needs to be taken into account in order to fully explain the resulting freeze tolerance (for review, see Storey and Storey, 1988, 1991; Sinclair, 1999; Lee, 2010).

We are of the view that proline exerts its protective role in *C. costata* by a combination of mechanisms, and that the importance of individual mechanisms may gradually change with the acclimation state of the insect during the course of its entry into dormancy, cold acclimation, cooling and freezing. Thus, proline might be actively involved in entry into diapause via its diverse regulatory functions in sensing the energy status and production of reactive oxygen species (Phang et al., 2010; Liang et al., 2013). Proline can also scavenge free radicals (Kaul et al., 2008). Proline accumulation continues as the ambient temperature decreases. Finally, proline levels elevate to 339 mmol kg^{-1} TBW in cold-acclimated, diapausing larvae (*C. costata*, SDA variant), which represents as much as 499 mmol kg^{-1} OAW. Our previous analysis (Koštál et al., 2011b) most probably underestimated the concentration of proline in cold-acclimated *C. costata* larvae (showing only 147 mmol kg^{-1} TBW). The higher value (339 mmol kg^{-1} TBW) is correct, as we have verified in several generations of larvae since the original publication. It is plausible to propose that high concentrations of proline help to reduce partial unfolding of proteins in chilled and supercooled larvae (prior to freezing) via the mechanism of preferential exclusion (Arakawa and Timasheff, 1985; Timasheff, 1992, 2002; Bolen and Baskakov, 2001). Upon freezing, proline can serve as a molecular shield and prevent aggregation of partially unfolded proteins or fusion of membranes in tightly packed organelles (Bryant et al., 2001; Hoekstra et al., 2001; Hoekstra and Golovina, 2002; Ball, 2008). At high levels of freeze-dehydration, proline concentrations exceed>1 mol kg^{-1} TBW. At such extremely high concentrations, proline is known to form specific supramolecular aggregates (Rudolph and Crowe, 1986; Samuel et al., 2000) which may interact with the hydrophobic surfaces presented by partially unfolded proteins, thereby stabilizing the folding intermediates and preventing their aggregation (Samuel et al., 1997; Ignatova and Gierasch, 2006; Das et al., 2007). In addition, the supramolecular structures of proline may promote the formation of amorphous biological glass – vitrification (Rudolph and Crowe, 1986).

Association between glass transition and freeze tolerance

We observed glass transition in all acclimation variants of *C. costata* but found no indication of glass transition in *D. melanogaster* larvae [Introduction question (v)]. Our study provides no direct mechanistic explanation for why this difference between the two species exists. Although proline is known to stimulate glass transition (Rudolph and Crowe, 1986), we assume that some additional mechanisms should be involved in the stimulation of glass transition in *C. costata* [compare: no glass transition in *D. melanogaster*, 15°C FTR Pro50 variant (proline, 57.2 mmol kg^{-1} TBW) versus T_{dg}≈−17°C in *C. costata*, LD variant (proline, 31.4 mmol kg^{-1} TBW)].

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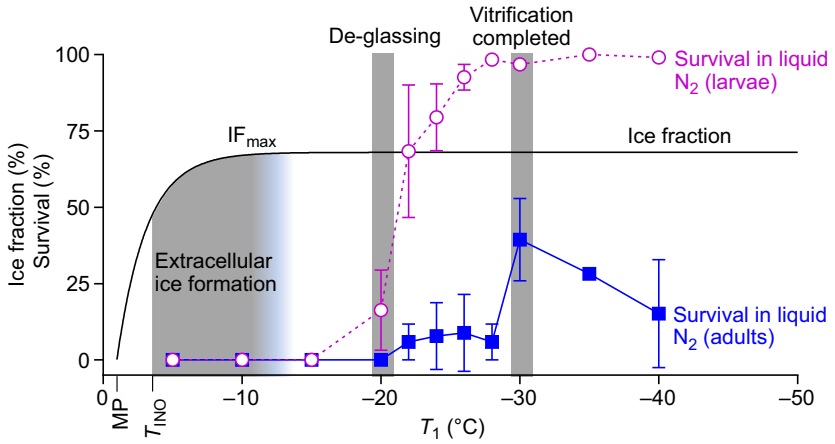

Fig. 5. Summary of data for cold-acclimated, diapausing larvae of *C. costata*. The graph integrates results on gradually growing body IF with decreasing temperature (black solid line, the same Boltzmann curve as shown in Fig. S7C) and survival after cryopreservation in liquid N_2 (see below for explanation), and depicts several important transitions: the melting point (MP) derived from the Boltzmann curve is −1°C; the larvae were inoculated with external ice crystals at −2.6°C on average (T_{INO}; Fig. S1F) and 99% of IF_{max} was formed at −10.5°C (Fig. S7C). The larvae exhibit the de-vitrification transition (upon warming) at approximately −20°C (Fig. 3B) but their vitrification is not completed (not observable by de-vitrification signal) at temperatures higher than −30°C (Fig. 3A). Note the association between the vitrification transition (occurring between de-glassing and vitrification completed temperatures) and the steep increase in survival of larvae and adults in the cryopreservation conditions (liquid N_2). Survival lines show the same data as presented in Table S2, where the target temperature of slow freezing (T_1, just prior to plunging into liquid N_2) is variable.

We found that de-vitrification occurred at around −20°C, while vitrification was completed in all larvae of the SDA variant of *C. costata* at approximately −30°C (Fig. 5). Interestingly, we found that these larvae can survive plunging into liquid N_2 only when slowly pre-frozen to below −20°C (corresponding to T_{dg}), and that they can survive and metamorphose into adults only when slowly pre-frozen to below −30°C (corresponding to a completion of glass transition). Assuming that T_g must lie somewhere in the interval between T_{dg} and the completion of glass transition, our results reveal a strikingly tight association between glass transition and the dramatic increase of survival of cryopreserved SDA *C. costata* larvae (Fig. 5). Based on these results, we formulate the answer to our last Introduction question (vi): correlative evidence was obtained showing that vitrification of the residual solution after formation of IF_{max} is associated with the survival of SDA *C. costata* larvae cryopreserved in liquid N_2. We suggest that vitrification may further stabilize the structures of macromolecular complexes and protect them against thermomechanical stress (Rubinsky et al., 1980) linked to rapid changes of temperature during plunging into liquid N_2 and re-warming.

Conclusions

Using DSC thermal analysis, we found that temperature-dependent IF dynamics are very similar in the larvae of *D. melanogaster* and *C. costata* (each species analysed in four different acclimation variants). The two species differed mainly in their ability to phenotypically modulate the parameters of IF dynamics in response to acclimation. The *C. costata* larvae decreased MP, OAW and IF_{max} upon entry into diapause, cold acclimation and feeding on a proline-augmented diet. The maximum tolerable IF changed according to the acclimation state in both species: from freeze intolerance to a specific tolerable threshold in *D. melanogaster*, or from specific tolerable threshold to independence of IF in *C. costata*. The specific threshold (maximum tolerable IF) was situated very close to IF_{max}. The phenotypic shifts in IF dynamics were associated with colligative effects caused by accumulated proline. In addition to colligative effects, accumulated proline probably affected freeze tolerance of *C. costata* larvae by a combination of other mechanisms. We have not detected any glass transitions in *D. melanogaster* larvae exposed to temperatures as low as −70°C. In contrast, the glass transitions of the residual solution after formation of IF_{max} occurred at temperatures below −30°C in all acclimation variants of *C. costata*. We found a tight correlation between the occurrence of glass transition and the dramatic increase of survival in liquid N_2-cryopreserved *C. costata* larvae of the SDA acclimation variant.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: J.R., V.K.; Methodology: J.R., M.M., P.S., V.K.; Validation: V.K.; Formal analysis: J.R., M.M., P.S., V.K.; Investigation: J.R., M.M., P.S., V.K.; Writing - original draft: V.K.; Writing - review & editing: J.R., M.M., P.S., V.K.; Supervision: V.K.; Project administration: V.K.; Funding acquisition: V.K.

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Supplementary information

Supplementary information available online at
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Table S1. Summary of literature data on maximum ice fraction in various invertebrates.

Species and taxonomic affiliation	Geographic origin and acclimation status	IF_{max} * (% TBW)	Reference
Insecta			
Eleodes blanchardi (Coleoptera)	Norway, field-collected, overwintering adult	75	Zachariassen et al., 1979
Eurosta solidaginis (Diptera)	Ohio, New York, field-collected, overwintering larva	64	Lee and Lewis, 1985
Hemideina maori (Orthoptera)	New Zealand, field-collected, cold acclimated	82	Ramlov and Westh, 1993
Melasoma collaris (Coleoptera)	Norway, field-collected, cold acclimated	77	Gehrken and Southon, 1997
	ibid., warm-acclimated in lab.	84.5	
Celatoblatta quinquemaculata (Blattodea)	New Zealand, field-collected, overwintering adult	74	Block et al., 1998
Heleomyza borealis (Diptera)	Arctics, field-collected, overwintering larva	81	Worland et al., 2000
Tardigrada			
Adorybiotus coronifer	Greenland, field-collected in winter	79	Westh and Kristensen, 1992
	Greenland, field-collected in summer	89	
	ibid., cold-acclimated in lab.	83	
Amphibolus nebulosus	Greenland, field-collected in summer	88	
Halobiotus crispae	Denmark, field collected, active stage	69	Halberg et al., 2009
	ibid., diapause P1 stage	59	
Macrobiotus sapiens	Croatia, lab culture, active, starved	84.5	Hengherr et al., 2009
	ibid., cold-acclimated	81.1	
Paramacrobiotus richtersi	Germany, lab culture, active, starved	85.7	
	ibid., cold-acclimated	82.5	
Macrobiotus tonollii	Oregon, lab culture, active, starved	86.4	
	ibid., cold-acclimated	81.2	
Milnesium tardigradum	Germany, lab culture, active, starved	85.1	
	ibid., cold-acclimated	80.5	
Echinscus granulatus	Germany, field-collected, active, fed	83.8	
	ibid., cold-acclimated	78.0	
Echinscus testudo	Germany, field-collected, active, fed	84.3	
	ibid., cold-acclimated	80.5	
Nematoda			
Panagrolaimus davidi	Antarctics, lab culture, warm-acclimated,	82	Wharton and Block, 1997
Anelida			
Buchholzia apendiculata	Austria, field-collected, spring active	18.1	Block and Bauer, 2000
Buchholzia simplex		60.1	
Enchytraeus buchholzi		17.0	
Enchytraeus albidus		64.3	
Henlea ventriculosa		40.1	
Fridericia tubulosa		65.6	
Enchytraeus albidus	Germany, lab culture, acclimated to 5°C	61.4	Patricio Silva et al., 2013
	Greenland, lab culture, acclimated to 5°C	58.8	

* The IF_{max} was measured by gradual layer calorimetry (Zachariassen et al., 1979), or by a custom-made calorimeter registering heat absorbed during melting of frozen sample (Lee and Lewis, 1985), or by differental scanning calorimetry (DSC, most other studies). The ^{1}H nuclear magnetic resonance was used to determine the amount of unfrozen water in one study (Gehrken and Southon, 1997).

Table S2. Optimization of cryopreservation protocol for *Chymomyza costata* larvae.

Assay	Parameter value	n (replicates)	Survival (%)		
			larvae	puparia	adults
Control 1:	manipulation only	233 (4)	100	92,0	85,6
Control 2:	freezing only: -30°C / 1h	100 (2)	100	87,0	76,0
Cryopreservation protocols with variable values of assessed parameters:					
Rate r_1	1,5°C / min	80 (1)	55,0	0	0
	1°C /min	60 (1)	35,0	0	0
	0,5°C / min	60 (1)	73,4	13,3	6,7
	0,25°C / min	60 (1)	76,7	13,3	11,7
	0,17°C / min	60 (1)	95,0	28,3	23,3
	0,10°C / min	440 (7)	96,8	53,6	39,3
	0,05°C / min	60 (1)	83,3	28,3	13,3
Rate r_2	2 - 4°C / sec	440 (7)	96,8	53,6	39,3
	10 - 20°C / sec*	50 (1)	56,0	22,0	10,0
	20 - 60°C / sec**	20 (1)	45,0	0	0
Rate r_3	not assessed, maintained invariably between 1 - 2°C / sec				
Rate r_4	0,1°C /min	300 (3)	97,4	55,7	39,8
	0,2°C / min	60 (1)	98,4	43,3	30,0
	0,6°C / min	440 (7)	96,8	53,6	39,3
	1,2°C / min	60 (1)	92,4	15,0	0
Temperature T_1	-5°C	60 (1)	0	0	0
	-5°C*	30 (1)	0	0	0
	-10°C	60 (1)	0	0	0
	-15°C	80 (1)	0	0	0
	-20°C	269 (4)	16,3	0	0
	-22°C	120 (2)	68,4	7,5	5,9
	-24°C	220 (3)	79,5	10,6	7,8
	-26°C	220 (3)	92,6	13,4	8,9
	-28°C	120 (2)	98,4	10,0	5,9
	-30°C	440 (7)	96,8	53,6	39,3
	-35°C	160 (2)	100,0	45,7	28,2
	-40°C	340 (4)	98,1	23,7	15,2
Temperature T_2	-30°C	440 (7)	96,8	53,6	39,3
	-20°C	60 (1)	100,0	31,7	23,3
	-10°C	280 (3)	95,4	63,8	39,9
	-5°C	60 (1)	96,7	5,0	1,7
	0°C	60 (1)	78,3	0	0
	r.t.	40 (1)	65,0	0	0
Storage of *C. costata* larvae in LN_2 (cryopreserved using the optimal protocol) for various periods of time:					
Assay	Storage time	n (replicates)	larvae	puparia	adults
Optimal protocol	1 hour	440 (7)	96,8	53,6	39,3
	2 months	40 (1)	97,5	57,5	42,5
	4 months	80 (1)	95,0	22,5	10,0
	6 months	40 (1)	97,5	55,0	42,5
	9 months	180 (1)	97,2	20,6	13,3
	18 months	160 (1)	92,5	13,8	5,6

n, total number of larvae assessed. Number of biological replicates is shown in parentheses. Generally, variation in adult survival was relatively high not only between generations (biological replicates) abut also between replicated tubes of the same generation (technical replicates). For instance, the optimal protocol assay was replicated in (7) different generations with the following results (mean±S.D., range): larvae, 96.8±1.9, 93.3-98.8; puparia, 53.5±12.3, 41.7-70.0; adults, 39.4±13.5, 11.7-58.3. Because most other treatments were replicated only once or few times, we show final survival as a percentage of survivors from all individuals (generations pooled).

***,** larvae placed into copper capillary (5 cm long, 1.2 mm inner diameter, filled with distilled water), plunged to LN_2

****,** larvae placed into copper capillary, plunged to liquid propane held at -196°C

All parameters were assessed one by one. The values of the assessed parameter varied as shown in the Table (left column), while all other parameters were kept optimal. The optimal protocol is highlighted in blue.

Table S3. Metabolomic profiles in *Chymomyza costata* larvae of four acclimation variants.

	LD		SD		SDA		LD Pro50	
3-Alanine	1,19	0,00	t		t		t	
Alanine	6,69	0,54	6,98	0,48	3,42	0,12	4,26	0,67
Arginine	6,68	0,75	6,43	0,14	7,84	0,62	6,19	0,81
Asparagine	4,91	0,24	20,92	1,97	17,38	1,70	15,52	1,15
Aspartate	0,27	0,03	0,24	0,01	t		0,71	0,10
Cysteine	0,34	0,05	t		t		t	
Glutamine	19,43	1,24	120,61	10,05	24,96	5,12	153,89	57,83
Glutamate	8,63	0,47	5,47	0,35	6,26	0,26	7,55	0,76
Glycine	1,69	0,17	0,60	0,10	1,35	0,09	0,49	0,07
Histidine	7,23	0,79	1,53	0,23	2,07	0,32	t	
Isoleucine	1,10	0,18	0,64	0,03	0,59	0,05	0,74	0,04
Leucine	1,21	0,17	0,76	0,08	0,67	0,06	0,76	0,06
Lysine	1,72	0,30	3,50	0,46	1,57	0,14	1,06	0,24
Methionine	0,36	0,05	t		t		t	
Phenylalanine	0,88	0,02	0,57	0,05	0,63	0,04	0,75	0,08
Proline	31,41	2,11	161,75	12,75	339,08	19,31	487,02	22,95
Serine	1,91	0,25	2,12	0,31	1,48	0,15	1,22	0,19
Threonine	3,31	0,48	0,62	0,13	0,64	0,05	1,17	0,15
Trytophan	0,62	0,07	1,32	0,16	0,72	0,06	0,52	0,08
Tyrosine	5,55	0,93	3,17	0,04	2,58	0,20	2,11	0,37
Valine	2,18	0,26	1,01	0,04	0,84	0,13	1,08	0,23
Ornithine	0,26	0,02	0,31	0,06	t		t	
Sarcosine	0,34	0,03	0,25	0,05	0,38	0,04	0,00	0,00
Citrate	3,87	0,36	6,96	0,29	4,46	0,50	6,31	1,16
Ketoglutarate	t		0,73	0,14	t		t	
Malate	2,17	0,19	1,60	0,06	0,99	0,11	2,51	0,16
Succinate	1,23	0,35	t		t		t	
Lactate	2,95	0,18	2,29	2,24	0,58	0,10	t	
Glycerol	0,19	0,01	0,16	0,02	0,23	0,04	0,32	0,13
Erythritol	0,04	0,00	0,14	0,01	0,18	0,04	0,29	0,08
Mannitol	t		0,05	0,01	0,04	0,00	0,18	0,08
Sorbitol	t		0,47	0,04	0,60	0,03	2,28	0,81
chiro-Inositol	0,21	0,02	0,16	0,01	0,30	0,02	0,23	0,05
myo-Inositol	0,13	0,01	0,09	0,01	0,12	0,01	0,22	0,02
Fructose	0,37	0,02	0,68	0,02	0,77	0,08	1,43	0,37
Glucose	2,05	0,32	0,40	0,06	0,37	0,04	0,61	0,10
Trehalose	23,54	3,25	22,87	1,31	52,36	3,61	50,55	1,01
Total	143,62	9,76	374,29	27,73	473,48	24,85	749,98	80,54

Each value represents mean ± S.D. of four biological replicates (each consisting of a pool of 5 larvae).
All data are expressed as mmol.kg^{-1} TBW. Three most abundant metabolites are highlighted in yellow fields.
t, traces of metabolite present (below the threshold for reliable quantitation).

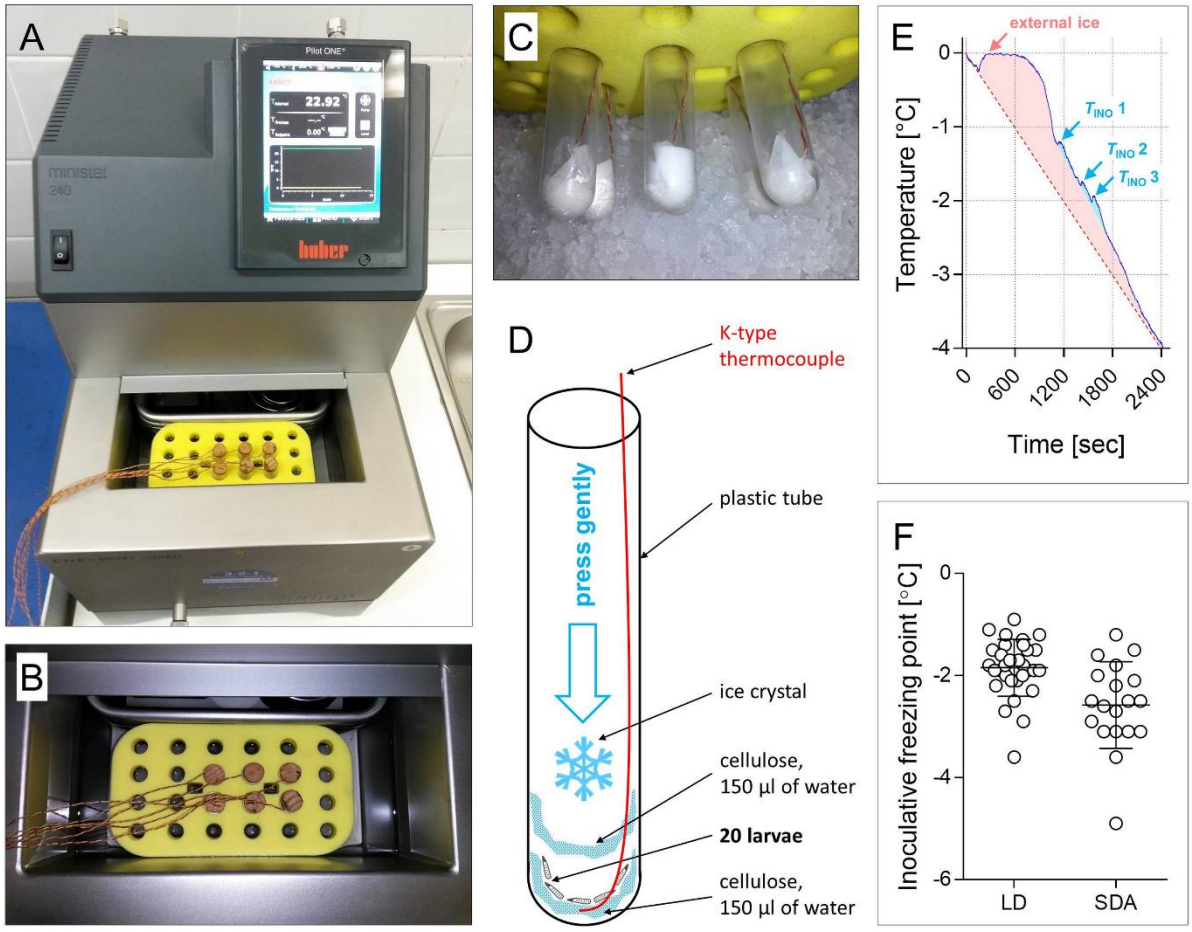

Fig. S1. Ice nucleation of larval body fluids with external ice crystals in Ministat 240 cooling circulator, Huber.

(A-C) Photographs show placement of six plastic test tubes (1 cm in diam., 5 cm long), each containing different number of larvae (5 – 20) depending on experiment, inside the Huber Ministat. The tubes were inserted into holes of a floating island and submerged to cooling medium (Thermofluid SilOil, M60 115/200.05, Huber). (D) Schematic view of the arrangements inside each plastic test tube. Larvae were placed on a small piece of cellulose (75 mg) that was moistened with 150 uL of distilled water. Similar piece of moistened cellulose was placed over the larvae and slightly pressed, which ensured that all larvae were in a tight contact with moisture. The thermocouple was mounted in between the two pieces of cellulose. A small ice crystal was added on top of wet cellulose, the tube was closed using cork plug and temperature program was started in Huber Ministat. (E) An example of temperature record (PicoLog TC-08 datalogger) in an experiment where five larvae of *Chymomyza costata* (acclimation variant LD) were slowly cooled (cooling rate 0.1ºC min^{-1}) in the tube arranged as described on D). Large freeze exotherm (red area) belongs to external water in cellulose. Three small freeze exotherms (blue areas) were detected, which belong to three larvae seeded by external ice crystals. (F) Results of replicated experiments as described in E). The *Chymomyza costata* larvae of two acclimation variants were measured: LD (n = 40), SDA (n = 25) (see Table 1 for detailed description of acclimation variants). All recorded larval freeze exotherms (inoculation freezing points, T_{INO}) are shown: LD (n = 31; mean = -1.85; S.D. = 0.56), SDA (n = 19; mean = -2.58; S.D. = 0.85), the means of LD and SDA group are statistically different (t-test, t = 3.680, P = 0.0006).

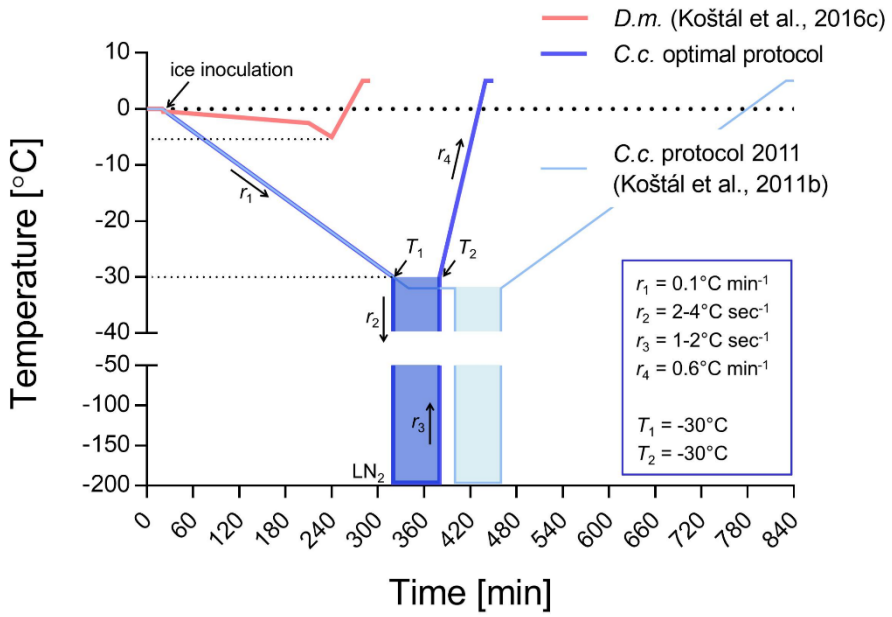

Fig. S2. Freezing and cryopreservation protocols.

In all protocols, larval freezing was initiated by contact with external ice crystals (ice inoculation) at relatively high sub-zero temperatures (see Fig. S1). The protocol used for larvae of *Drosophila melanogaster* (*D.m.*, red line) was previously optimized and published in Koštál et al. (2016). The protocol used previously for cryopreservation of larvae of *Chymomyza costata* (*C.c.*, pale blue line, Koštál et al., 2011) was further optimized in this study (Table S1) and the optimum parameters (ensuring the highest survival of adults) are listed in the blue frame and shown graphically as dark blue line (the rates of cooling/heating are shown as r_1, r_2, r_3 and r_4. At temperature T_1, frozen larvae are plunged into liquid nitrogen (LN$_2$), and, later, returned to Huber Ministat pre-set to T_2 temperature).

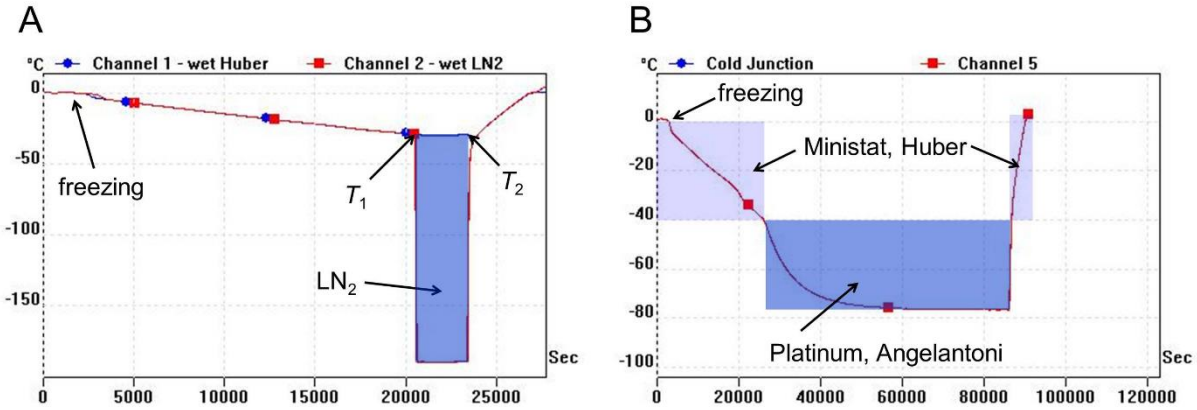

Fig. S3. Examples of temperature record from PicoLog TC-08 datalogger.

(A) Freezing to -30°C / 1h (Channel 1, blue line) and freezing to a T_1 = -30°C followed by 1h in liquid nitrogen (LN$_2$, Channel 2, red line). Note that both channels show freeze exotherms (water in the cellulose wrapping) extending over approximately 1st hour of slow cooling (rate 0.1°C min^{-1}). External ice crystals inoculate larvae inside the wrapping (see Fig. S1 E). (B) Slow freezing to -40°C in Ministat (Huber) followed by transfer to Platinum freezer (Angelantoni), where the sample gradually cooled to -75°C (Channel 5, red line).

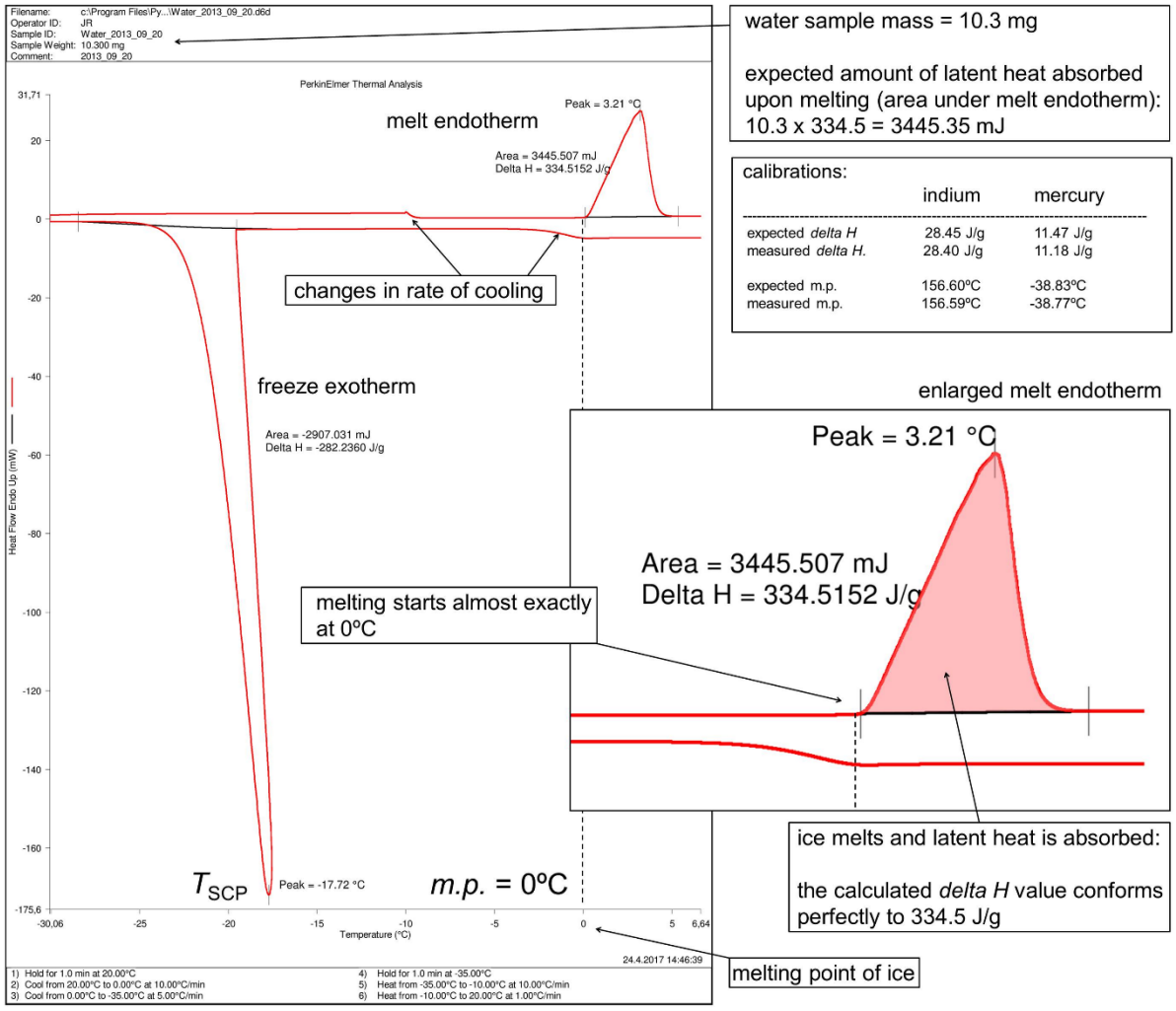

Fig. S4. DSC calibration: example protocol (distilled water).

The temperature scale and the heat flow of DSC4000 instrument were calibrated using indium, mercury and distilled water standards. Calibration parameters for indium and mercury are shown in the frame (right). An example protocol (left) shows that running the thermal analysis of distilled water sample (10.3 mg) returns expected values of melting point (m.p., 0ºC) and specific enthalpy of ice/water transition (*delta H*, 334.5 J g^{-1}) (Wang and Weller, 2011).

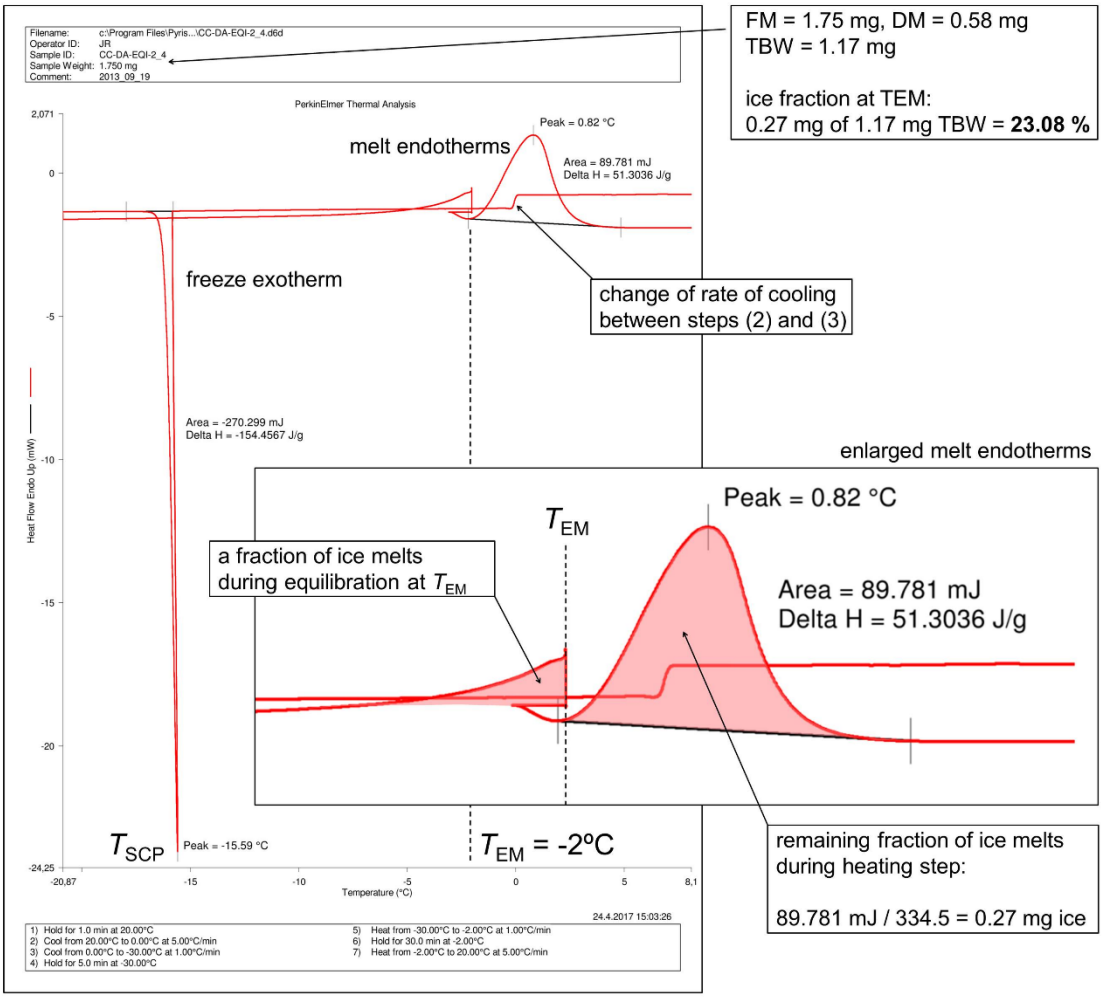

Fig. S5. *Equi-melt* thermal analysis: example protocol (*C. costata*, SDA).

The *Equi-melt* temperature programme: (1) hold for 1 min at 20ºC; (2) cool to 0ºC at a rate 5ºC min^{-1}; (3) cool to -30ºC at a rate 1ºC min^{-1}; (4) hold for 5 min at -30ºC; (5) heat to *Equi-melt* temperature (T_{EM}) at a rate 1ºC min^{-1}; (6) hold for 30 min at T_{EM}; (7) heat to 20ºC at a rate 5ºC min^{-1}.

Notes:

steps (3 and 4): the temperature of -30ºC was sufficient to reach maximum ice fraction in all treatments. We verified in preliminary experiments that neither exposing the larvae to lower temperature (down to -70ºC), nor extending the time of exposure at -30ºC (up to 48 hours) did further increase the ice fraction;

steps (5 and 6): the ice fraction equilibrates to specific T_{EM} that varied from -30ºC to -0.5ºC (in addition, T_{EM} of -50ºC was analyzed for *C. costata* SDA variant, see Fig. S7 C);

step (7): the ice fraction was calculated from the area under melt endotherm using the value of *delta H* = 334.5 J g^{-1} as the enthalpy of ice/water transition.

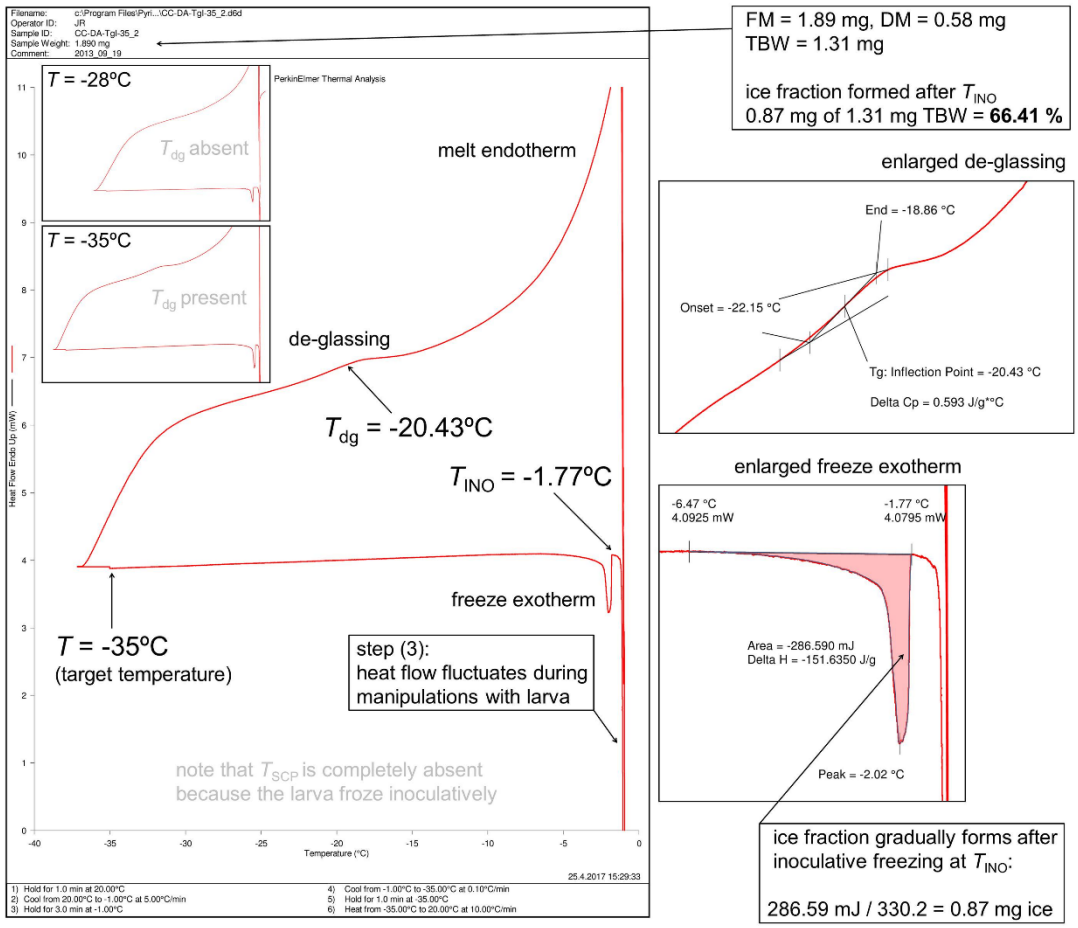

Fig. S6. *Ino-freeze* thermal analysis: example protocol (*C. costata*, SDA).

The *Ino-freeze* temperature programme: (1) hold for 1 min at 20°C; (2) cool to -1°C at a rate 5°C min^{-1}; (3) hold for 3 min -1°C and, meanwhile, open the instrument and insert aluminum pan bottom lined with filter paper disc (SS-033, Wescor) to which 10 uL of distilled water was applied and then submerged in liquid nitrogen to freeze it. Next, after the bottom of test-pan equilibrates to programmed temperature of -1°C (approximately within 10 sec), add larva, cover the bottom loosely with lid and close the instrument; (4) cool to target temperature at a rate 0.1°C min^{-1}; (5) hold for 1 min at target temperature; (6) heat to 20°C at a fast rate 10°C min^{-1}.

Notes:

steps (1 and 2): performed with the reference pan only inside instrument;

step (3): the test pan and larva were added. When performed carefully, this critical step ensures that all larvae are exposed to external ice crystals at exactly -1°C;

steps (4 and 5): the target temperature can be varied in order to see whether or not the de-glassing transition (T_{dg}) occurs upon rapid heating back to 20°C [when it occurs, it indicates that the vitrification transition (T_g) must have occurred during previous slow cooling to target temperature. Otherwise, glass trasition is not observable by DSC method at slow cooling rates]. However, observing only the specific aim of inoculative ice fraction analysis, the target temperature of -10°C would be sufficient as all larval freeze exotherms ended between -3°C and -5°C (see data in Fig. S11). The ice fraction was calculated from freeze exotherms and the enthalpy of water/ice transition (*delta H*) was modified according to the exact peak temperature of inoculative freezing (T_{INO} = -2.02°C in this example) using the formula:

$delta\ H = 334.5 + 2.12\ T_{INO} + 0.0042\ (T_{INO})^2 = 330.2$ (Wang and Weller, 2011)

step (6): the fast rate of heating (10°C min^{-1}) facilitates the observation of de-glassing (T_{dg}) transition.

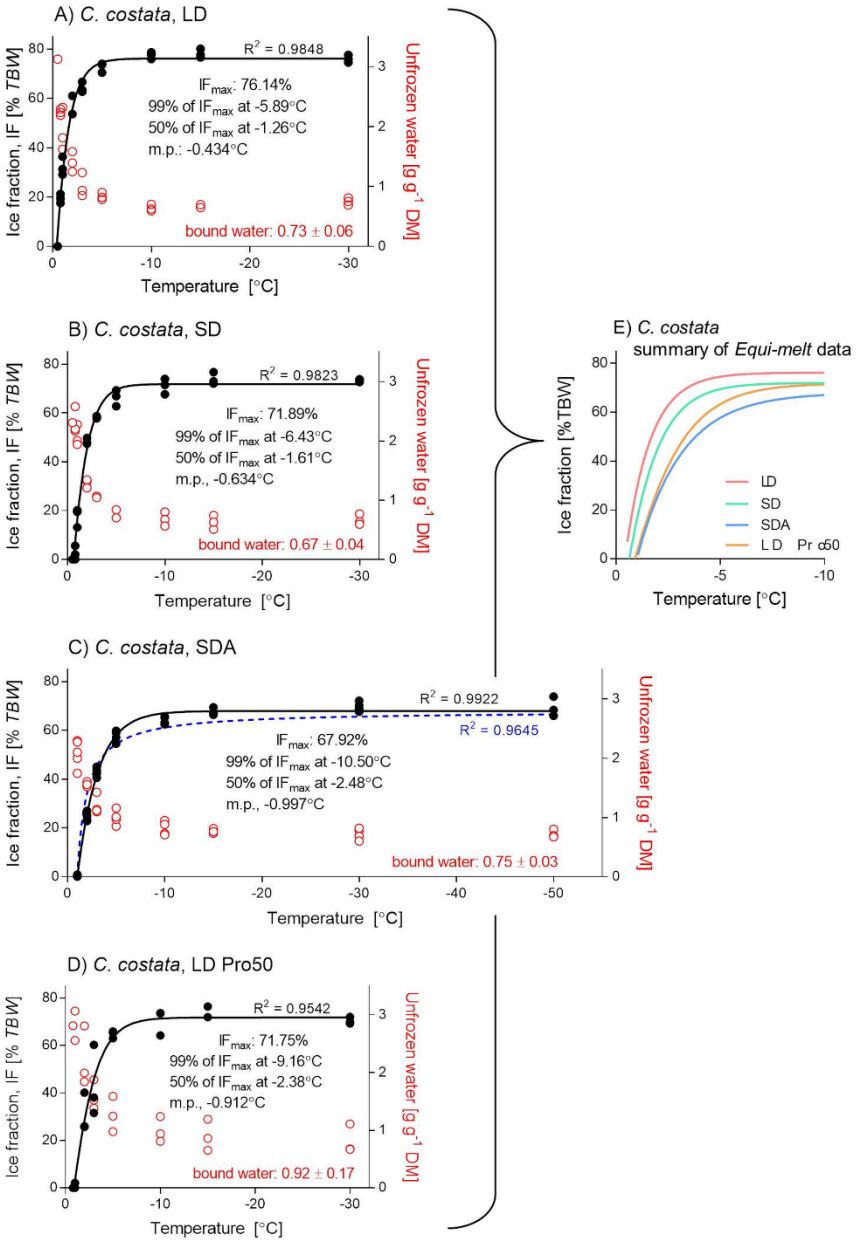

Fig. S7. Summary of *Equi-melt* data for *Chymomyza costata*

The *Equi-melt* thermal analyses were run with different target temperatures (*T*) ranging between -0.5°C and -30°C (or -50°C in SDA variant). At least three larvae were analyzed for each experimental variant (A-D) and target temperature combination (three data-points). The Boltzmann sigmoids were fitted to empirical data (black solid lines). For comparison, an example of a theoretical freezing curve (dashed blue line) *vs.* Boltzmann sigmoid fitting is shown in (C):

theoretical freezing curve (Wang and Weller., 2011): $IF = OAW*(1 - (m.p./T)$

Boltzmann sigmoid: $IF = OAW + (top – OAW)/(1 + \exp((V50-x)/slope))$

All other parameters (IF_{max}; 99% of IF_{max}; 50% of IF_{max}; m.p.) were derived from Boltzmann sigmoids. The amount of unfrozen water per mg DM for each larva is shown as red circle. Bound water (OIW) is calculated as a bottom of a Boltzmann sigmoid fitted to unfrozen water data.

(E) A summary figure showing only the *Equi-melt* curves for each treatment (these lines are used in Fig. 2A).

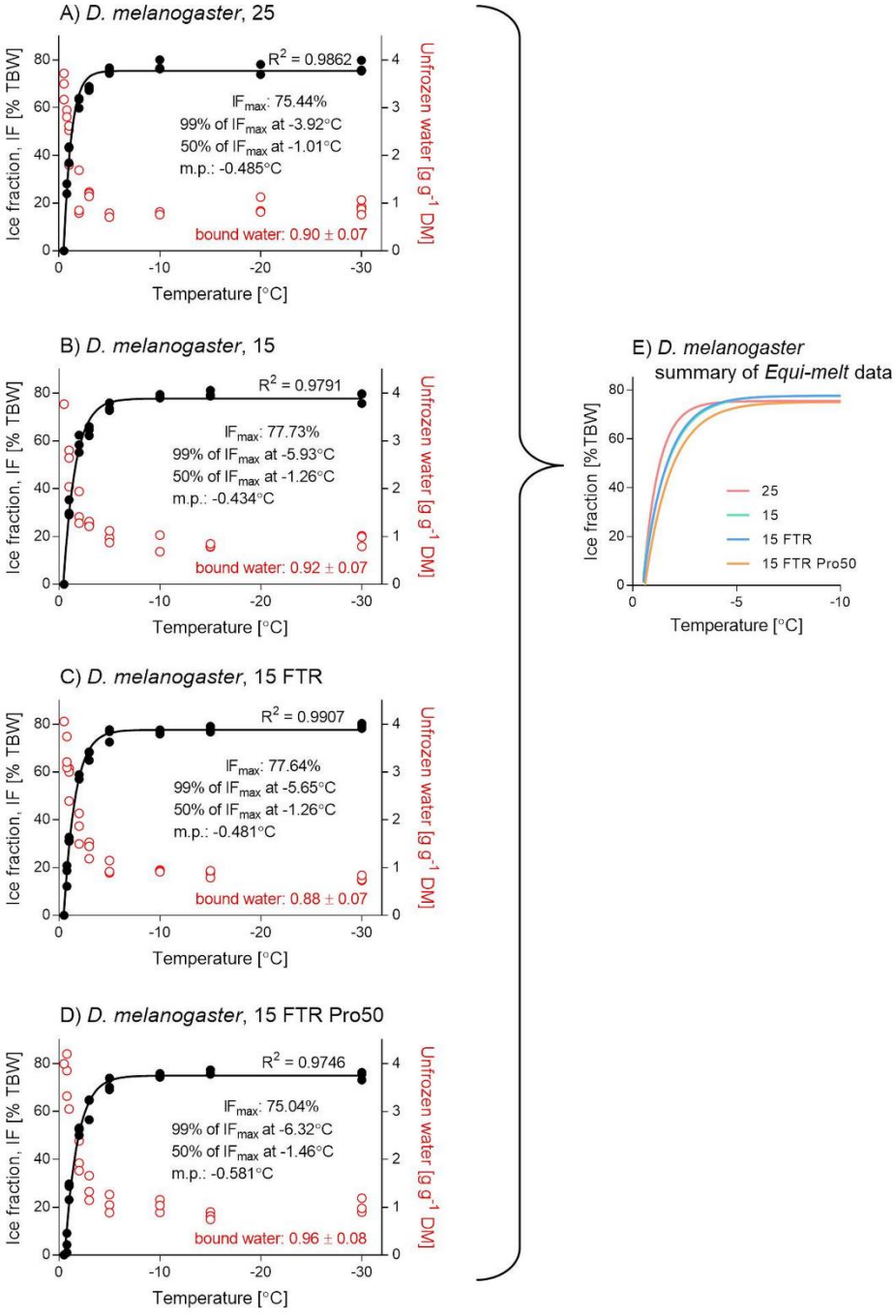

Fig. S8. Summary of *Equi-melt* data for *Drosophila melanogaster*.

All descriptions as in Fig. S7**.**
(E) A summary figure showing only the *Equi-melt* curves for each treatment (these lines are used in Fig. 2E).

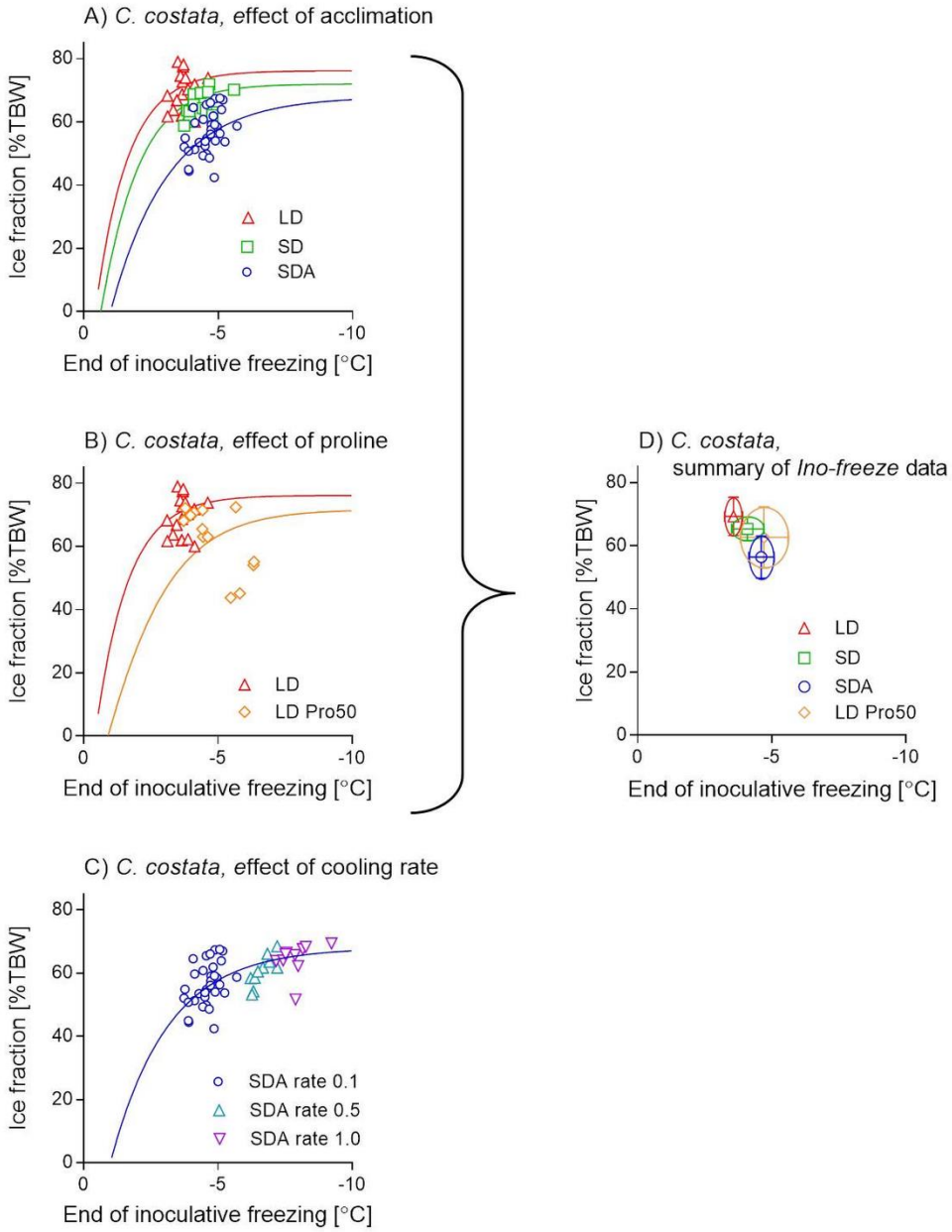

Fig. S9. Summary of *Ino-freeze* data for *Chymomyza costata*.

The *Ino-freeze* thermal analyses were run for individual larvae (points). Detailed results of *Ino-freeze* analyses are summarised in Fig. S11. *Equi-melt* curves (taken from Fig. S7) are also shown in order to allow direct comparison of the ice fraction analyzed by two methods. Note that *Ino-freeze* points and *Equi-melt* lines match very well. (A) Three acclimation variants (treatments LD, SD, SDA). (B) The effect of proline augmented diet (LD *vs.* LD Pro50). (C) The effect of cooling rate during the step (ii.) of freezing protocol. Three different cooling rates were compared: 0.1°C min^{-1}; 0.5°C min^{-1}; 1°C min^{-1}. The points express the ice fraction calculated from area under inoculative freeze exotherm at a temperature corresponding to the end of inoculative freezing (see Fig. S6).
(D) A summary figure showing ellipses based on mean x ± S.D. and mean y ± S.D. values of all *Ino-freeze* data points for each treatment (these ellipses are used in Fig. 2A).

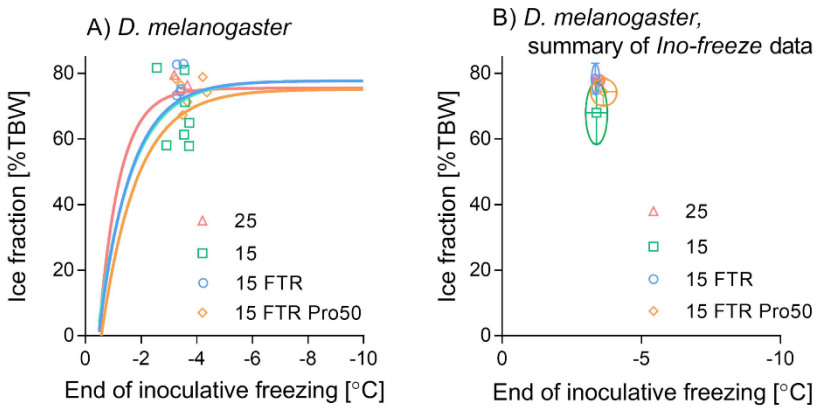

Fig. S10. Summary all *Ino-freeze* data for *Drosophila melanogaster*

All descriptions as in Fig. S9**.**
(B) A summary figure showing ellipses based on mean x ± S.D. and mean y ± S.D. values of all *Ino-freeze* data points for each treatment (these ellipses are used in Fig. 2E).

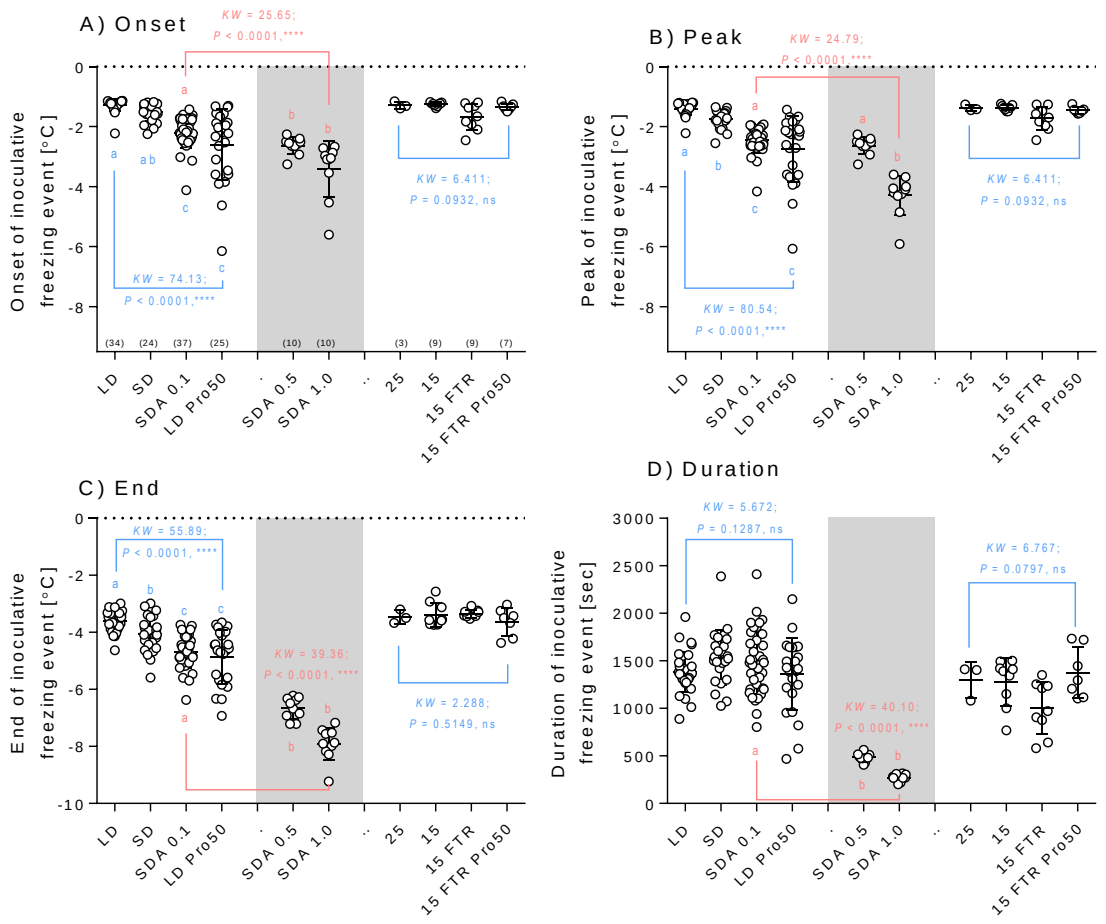

Figure S11. Summary of data obtained by *Ino-freeze* thermal analyses.

The parameters of inoculative freezing event [onset (= T_{INO}), peak, end, and duration; see Fig. S6 for more explanations] in variously treated larvae of *C. costata* (LD, SD, SDA, LD Pro50) and *D. melanogaster* (25, 15, 15 FTR, 15 FTR Pro50). In addition, the effect of cooling rate during the step (ii.) of freezing protocol was analyzed in *C. costata*. Three different cooling rates were compared: 0.1°C min^{-1}; 0.5°C min^{-1}; 1°C min^{-1}. Each point represents single thermal analysis (single larva). Number of larvae analyzed in each treatment (n) is shown in parentheses in (A). Note that there is a good match between the data on onset of inoculative freezing analyzed by DSC (Fig. S11 A) and similar data recorded directly by thermocouples in freeze-tolerance assays (Fig. S1 F). The differences between treatments (shown by blue and red lines) were assessed using Kruskal-Wallis nonparametric test (*KW* statistics is shown) followed by Dunn's multiple comparison test. Treatments flanked by different letter were statistically different according to the Dunn's test.

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