Hyperprolinemic larvae of the drosophilid fly, *Chymomyza costata*, survive cryopreservation in liquid nitrogen

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The larva of the drosophilid fly, *Chymomyza costata*, is probably the most complex metazoan organism that can survive submergence in liquid nitrogen (−196 °C) in a fully hydrated state. We examined the associations between the physiological and biochemical parameters of differently acclimated larvae and their freeze tolerance. Entering diapause is an essential and sufficient prerequisite for attaining high levels of survival in liquid nitrogen (23% survival to adult stage), although cold acclimation further improves this capacity (62% survival). Profiling of 61 different metabolites identified proline as a prominent compound whose concentration increased from 20 to 147 mM during diapause transition and subsequent cold acclimation. This study provides direct evidence for the essential role of proline in high freeze tolerance. We increased the levels of proline in the larval tissues by feeding larvae proline-adopted diets and found that this simple treatment dramatically improved their freeze tolerance. Cell and tissue survival following exposure to liquid nitrogen was evident in proline-fed nondiapause larvae, and survival to adult stage increased from 0% to 36% in proline-fed diapause destined larvae. A significant statistical correlation was found between the whole-body concentration of proline, either natural or artificial, and survival to the adult stage in liquid nitrogen for diapause larvae. Differential scanning calorimetry analysis suggested that high proline levels, in combination with a relatively low content of osmotically active water and freeze dehydration, increased the propensity of the remaining unfrozen water to undergo a glass-like transition (vitrification) and thus facilitated the prevention of cryoinjury.

Insect freeze tolerance | cold hardiness | glass transition | metabolomics

Temperate and polar insects overwinter at subzero body temperatures (1–3), which makes them suitable models for studying the principles of survival in animal cells, tissues, and complex organisms at cryothermic conditions. The drosophilid fly, *Chymomyza costata* (Zetterstedt) (Diptera: Drosophilidae), appears to be a highly promising model. It is distributed over a cool-temperate, Holarctic area (4) and fully grown third instar larvae enter facultative winter diapause in response to long night lengths and/or low temperature (5). Diapause larvae supercool to between −15°C and −25°C (6) but can survive freezing to −100°C provided they undergo cold acclimation (1 mo at 4°C), are inoculated by external ice at −2°C, and are cooled at a slow rate of 0.1°C/min (7, 8). Diapause, cold-acclimated larvae of *C. costata* were successfully cryopreserved at the temperature of liquid nitrogen (N2) (−196°C) for 1 h (9). To our knowledge, larva of *C. costata* represents the most complex metazoan organism that has reportedly survived submergence in liquid N2 in a fully hydrated state. Physiological mechanisms underlying this unique capacity remain poorly understood. Inoculative freezing and a slow cooling rate allow time for osmotic equilibration during extracellular freezing and prevent formation of intracellular ice crystals (9). The process of cold acclimation involves remarkable biochemical changes such as the accumulation of proline and trehalose (8) and the increase in relative proportions of phospholipids that pair palmitic and linoleic fatty acids in cell membranes (10), which may contribute to the preservation of protein and membrane structures at low temperatures (11, 12).

In this paper, we examined the associations between physiological and biochemical parameters of differently acclimated larvae of *C. costata* and their freeze tolerance; i.e., the ability to survive when frozen at −32°C and subsequent submersion in liquid N2. We showed that larvae acquire high freeze tolerance without cold acclimation during their transition to diapause at a relatively high temperature of 18°C, although their freeze tolerance is further improved by cold acclimation. Metabolomic profiling of 61 different compounds identified proline as a prominent metabolite, as the increase in the molar concentration of proline explains most of the change in the overall osmolality of body fluids during diapause transition and cold acclimation. Next, we artificially elevated proline concentrations in larval tissues by feeding the larvae diets augmented with proline, which resulted in a significant improvement of larval freeze tolerance. On the basis of the results of differential scanning calorimetry (DSC), we suggest that high proline levels, and relatively low content of osmotically active water resulting from the freeze dehydration process during slow cooling to −32°C, increased the propensity of unfrozen water to undergo glass-like transition (vitrification), which probably contributed to high larval survival in liquid N2.

Results and Discussion

Larvae Acquire a High Freeze Tolerance with Entrance to Diapause, which is Further Improved by Cold Acclimation. In our experiments, nondiapause larvae of *C. costata* (variant 1) showed no ability to survive in a frozen state at −32°C or in liquid N2. No signs of cellular or tissue survival were recorded 12 h after melting. Diapause destined larvae (variant 2) showed relatively high survival at the cellular and larval levels but were not able to pupariate and metamorphose to adult flies. High survival into the adult stage was observed in the diapause, warm-acclimated larvae (variant 3), and this was further improved by cold acclimation (variant 4) (Fig. 1A and B). These results verify previous reports (7–9) and extend their findings by showing that cold acclimation does not appear to be an essential prerequisite for attaining high freeze tolerance. We found that reaching the appropriate stage of diapause development [i.e., transition from initiation to maintenance phase (5, 13)], at a constant high temperature of 18°C is sufficient for the development of high freeze tolerance.

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Control larvae (not exposed to freezing temperatures) were negative for trypan blue staining and exhibited numerous small lipid droplets in fat body cells (Fig. 1C, upper row). In contrast, all recovering larvae after freezing treatments, irrespective of temperature and acclimation variant, displayed massive coalescence of lipid droplets in fat body cells. Small lipid droplets merged and formed a few large droplets or a single lipid droplet (Fig. 1C, lower row). Trypan blue staining was observed in approximately 10–30% of fat body cells 12 h after melting irrespective of temperature and acclimation variant. The large lipid droplets and islands of damaged fat body tissue persisted throughout the rest of larval life and were also observed during the wandering stage; i.e., shortly before pupariation (Fig. 1C, lower right corner). We detected no changes in gross morphology or trypan blue staining in post-freeze-recovering larvae in tissues other than the fat body.

The Amount of Osmotically Active Water Decreases During Diapause Transition and Cold Acclimation. Water-ice phase transitions, especially the lethal formation of intracellular ice crystals and physical damage to the delicate structure of the extracellular matrix caused by ice, are great challenges for the cryopreservation of hydrated complex tissues or whole organisms (14–16). Fully hydrated complex metazoans were successfully cryopreserved in liquid N₂ in only a few cases [e.g., insect embryos (17)], trophophore larvae of bivalve mollusks (18), and larvae of marine anelid (19). In contrast, desiccated anabolic invertebrates exhibit relatively easy survival at temperatures as low as −273°C; i.e., within a fraction of a degree of absolute zero (20–23).

Fully hydrated larvae of C. costata were frozen in our experiments. The larvae of different acclimation variants differed, however, in their hydration state (Dataset S1). Our DSC thermal analyses of whole C. costata larvae revealed that the amount of osmotically active water (OA) decreased from 1.72 mg·mg⁻¹ dry mass (DM) in nondiapause larvae (variant 1) to 1.23 mg·mg⁻¹ DM in the diapause, cold-acclimated larvae (variant 4); i.e., by 28.4%. As the amount of osmotically inactive water (OI) remained almost constant (ranging from 0.96 to 1.03 mg·mg⁻¹ DM), the OA/OI ratio showed a significantly decreasing trend with transition to diapause and cold acclimation (from 1.75 in nondiapause larvae to 1.29 in diapause, cold-acclimated larvae). This trend significantly correlated with increased survival at −32°C (Pearson’s r = 0.9501, P = 0.0499) (Fig. 2A and Dataset S1).

Freeze-tolerant animals commonly rely on freeze dehydration of their cells (24). The principles of this process are well known (2, 25): hemolymph ice nucleators, or external ice crystals, induce extracellular freezing at high subzero temperatures. Growing ice crystals concentrate the solutes in hemolymph and slow cooling rates allow osmotic equilibration, which results in progressive dehydration of cells, thereby inhibiting lethal intracellular freezing.

C. costata is no exception to this scenario (7–9), in which decreasing the amount of unfrozen (OA) water diminishes the amount of ice formed at any given temperature and, consequently, reduces the freeze concentration of intracellular fluids below potentially damaging levels (26–28).

The survival capacity of C. costata larvae, however, goes beyond the ecological limits of freeze tolerance. After being slowly frozen to −32°C, these larvae can survive plunging in liquid N₂. Fig. 2B is a cryo-SEM micrograph of one such larva (note that the larva shows no signs of body water loss). Fig. S1 shows cryofractured larvae with dehydrated tissues surrounded by a mass of frozen extracellular water in the hemocoel. Interestingly, these cryo-SEM-exposed larvae actively wriggled, including those that were cryofractured, when they were melted.

The Propensity of Body Water to Undergo Glass Transition Increases with Diapause Programming. In the presence of cryoprotectant agents (nontoxic and membrane-penetrable compatible osmolytes), the process of freeze dehydration may reach a point when the concentration of cryoprotectants is such that it will stimulate vitrification. Vitrified solution exists in an amorphous glassy state that resembles solid material but retains the physical properties of a liquid (29). The vitrified state is characterized by the glass-liquid transition temperature, Tg, which depends on the chemical composition of a solution and its water content. Vitrification is widely used in cryopreservation protocols (14–16, 27) because...
it prevents lethal formation of intracellular ice crystals and provides molecular stability to macromolecules and cell structures. Recently, it was suggested that vitrification is an essential part of the survival strategy in anhydrobiotic invertebrates (30, 31) and deeply supercooled arctic beetles (32).

In our experiments, a $T_g$-like transition was detected in only one of nine DSC-analyzed nondiapause larvae (at $-22.8\, ^\circ\text{C}$). In contrast, clear $T_g$-like transitions were observed in all analyzed diapause larvae, irrespective of the acclimation treatment (Fig. 3A and Dataset S1). The temperature of the $T_g$-like transition decreased with transition to diapause and the negative correlation of this decrease with survival at $-32\, ^\circ\text{C}$ was close to significant (Pearson’s $r = -0.9338$, $P = 0.0662$ (Fig. 3 B and C and Dataset S1)).

During our DSC thermal analyses, larvae typically froze at relatively low subzero temperatures corresponding to their supercooling point. We assessed whether stimulation of freezing at higher temperatures by external ice nucleation, which is important for survival (7), influenced the parameters of thermal analysis. Freezing exotherms occurred at approximately $-3\, ^\circ\text{C}$ when the larva was in contact with the ice crystals in the bottom of analytical pan. The OA and OI parameters could not be reliably resolved because we added large amounts of water ($10\, \mu\text{L}$) into each analytical pan. Similar to the DSC analysis without ice nucleation, $T_g$-like transitions were not detected in nondiapause larvae (variant 1; $n = 3$), but they were clearly detectable in all diapause, cold-acclimated larvae (variant 4; $n = 5$; $T_g = -25.24 \pm 1.98\, ^\circ\text{C}$; $\Delta C_p = 0.919 \pm 0.390\ J/\text{g}^{\circ}\text{C}$). Because high concentrations of cryoprotectants are usually present in naturally vitrified systems (33, 34) and are utilized in cryopreservation protocols to facilitate vitrification of cryopreserved materials (16), we performed metabolomic analysis of C. costata larvae to identify which cryoprotective compounds it accumulates.

The Accumulation of Proline Is the Most Prominent Biochemical Change Associated with Transition to Diapause and Cold Acclimation.

Of the 61 common metabolites included in our analysis, 34 occurred at detectable levels and most of their concentrations were significantly influenced by the acclimation treatments (Dataset S2). Principal component analysis (Fig. 4 A and B) showed that the metabolomic profile was affected by both the transition to diapause and cold acclimation but the orientations of the two respective axes slightly differed (arrows in Fig. 4 A: red, transition to diapause; blue, cold acclimation). Proline concentrations exhibited the most conspicuous changes (Fig. 4B). Proline levels increased from 19.8 mM in nondiapause larvae (variant 1) to 147.0 mM in diapause, cold-acclimated larvae (variant 4), and this increase significantly correlated with survival to adult stage in liquid $\text{N}_2$ (Pearson’s $r = 0.9843$, $P = 0.0157$).

The increase in proline levels explains most of the change in overall osmolality from 465 mOsm·kg$^{-1}$ in nondiapause larvae to 699 mOsm·kg$^{-1}$ in diapause, cold-acclimated larvae (a difference of 234 mOsm·kg$^{-1}$; Dataset S1). Because partial dehydration (loss of 15% of body water; Dataset S1) increases osmolality by approximately 70 mOsm·kg$^{-1}$, the remaining difference is 164 mOsm·kg$^{-1}$. This value closely corresponds to the increase in the sum concentration of all detected metabolites [i.e., 160.3 mM (Dataset S2)], to which proline contributes approximately 80%. Accumulation of proline in response to cold acclimation was previously reported in some other insects leading to the suggestion that proline could have a cryoprotective function (35–37). In plants, proline often accumulates during cold acclimation and direct genetic evidence demonstrates that it improves their freeze tolerance (38, 39). Therefore, we artificially modified the levels of proline in C. costata larvae by feeding them a diet augmented with proline and assessed the influence of elevated proline concentrations on larval freeze tolerance.

The examples of DSC thermal analyses in (Upper) nondiapause and (Lower) diapause larvae. Only the parts of thermal curves where $T_g$-like transitions typically occur are shown. The original outputs of Pyris software analysis of DSC4000 are shown, including the $T_g$-like transition calculation by Pyris software. (B) The temperature of the $T_g$-like transition decreased and the frequency ($f$) of its occurrence increased with the transition to diapause. Each column represents mean ± SEM. Columns flanked by different letters are statistically different (ANOVA: $F = 15.76$, $P < 0.0001$; followed by Tukey’s post hoc test, $P = 0.05$). For details, see Dataset S1.

**Feeding the Larvae with Proline-Augmented Diets Resulted in Considerable Improvement of their Freeze Tolerance.** Rearing the larvae on proline-augmented diets significantly increased the concentrations of proline in their hemolymph, gut, fat body, body wall including muscles, and whole-body extracts (Dataset S3). For example, the whole-body concentration of proline increased from 40.5 mM in diapause-destined, warm-acclimated larvae (variant 2) fed a standard diet to 163.6 mM in similar larvae that were fed a Pro50 diet. Feeding the larvae proline-augmented diets considerably improved their freeze tolerance (Fig. 5). In fact, nondiapause larvae acquired the capacity for freeze tolerance completely de novo after feeding on the Pro10 diet (variant 1 + Pro10). These larvae exhibited relatively high survival at $-32\, ^\circ\text{C}$ at the cellular level (32.8%) and some even survived to the adult stage (6.9%). We also observed signs of their survival in liquid $\text{N}_2$, although only at the cellular and larval levels. The survival of diapause-destined larvae also greatly increased at the cellular and larval levels after being fed proline-augmented diets. Most importantly, their survival to adult stage at $-32\, ^\circ\text{C}$ and in liquid $\text{N}_2$ increased from 0% (variant 2) to 50.0% and 35.7%, respectively.
respectively (variant 2+Pro50) (Fig. 5A and B). Fig. 5C shows that a significant statistical correlation exists (Pearson’s $r = 0.8883$, $P = 0.0441$) between the whole-body concentration of proline and survival to the adult stage in liquid $N_2$ for diapause larvae. All acclimation and feeding variants of diapause larvae were considered in this calculation. A similarly significant correlation was not observed for exposure to $-32^\circ$C (Pearson’s $r = 0.7110$, $P = 0.1780$).

Our results show that a single factor [i.e., increased proline levels] was sufficient to improve the freeze tolerance of $C.\ costata$ larvae, including de novo development of the capacity to survive at the temperature of liquid $N_2$. This strong effect of proline was unexpected because the cold tolerance of insects is considered a highly complex adaptive trait that is composed of numerous mechanisms (40). Individual mechanisms may interact and affect each other, making it difficult to assess their separate contributions (41). In our experiments, the entrance into diapause was a second factor that markedly improved larval freeze tolerance. Diapause represents the regulated switching off of potentially cold-sensitive processes such as progression through the cell cycle (42), morphogenesis, and development (43, 44). Increasing proline levels as high as 229.9 mM in the nondiapause larvae (variant 1) was not sufficient for attaining freeze tolerance at levels comparable to those observed in diapause larvae. However, high proline levels in nondiapause animals were sufficient to ensure high survival at the cellular and tissue levels. A third factor, cold acclimation, may indirectly contribute to high levels of freeze tolerance by further suppressing cold-sensitive processes or may directly contribute by stimulating biochemical and physiological adjustments such as membrane restructuring (10) or proline accumulation (8).

Three nonspecific mechanisms by which high concentrations of proline improve the freeze tolerance of $C.\ costata$ larvae can be considered. First, proline belongs to a diverse group of solutes that are preferentially excluded from contact with the surface of proteins and phospholipid bilayers in aqueous solutions. According to the preferential exclusion hypothesis (45), the addition of proline to a solution stabilizes the native structure of protein monomers and protects oligomeric protein complexes from denaturation and dissociation (34, 46, 47). For example, it has been empirically demonstrated that increasing the concentration of proline from 0 to 200 mM in 10 mM potassium phosphate buffer results in a linear increase in the protection of lactate dehydrogenase from loss of activity during freeze-thawing (48). Second, amphipathic proline molecules can intercalate between the headgroups of membrane phospholipids (PLs) during freeze dehydration and alleviate mechanical stresses in the membranes or can disturb the membranes, making them less prone to the liquid crystalline-to-gel transition (34). At very low water activity, which is an improbable situation in freeze-dried $C.\ costata$ when >40% is nonfrozen water, proline molecules can directly replace missing water molecules between the PL headgroups (49). A third potential mechanism of cryoprotection exerted by high concentrations of proline; i.e., induction of glass transition, was addressed in this study.

### The Relative Proportion of Osmotically Active Water Decreases and the Propensity of Body Water to Undergo Glass Transition Increases with Proline Feeding.

Our DSC analyses revealed a tendency toward decreasing the OA/OI ratio in the larvae that were fed proline-augmented diets in comparison to their standard-diet-fed counterparts. The differences, however, were not statistically significant (Fig. 6A and Dataset S1). The frequency of occurrence of $T_g$-like transitions increased from 11.1% in the nondiapause larvae (variant 1) fed a standard diet to 83.3% in similar larvae fed a Pro10 diet (5 of 6 larvae showed clear $T_g$-like transitions). The temperatures of $T_g$-like transitions were significantly lower in the larvae fed proline-augmented diets than in their standard-diet-fed counterparts (Fig. 6B and Dataset S1). It means that proline-augmented diets influenced the larval osmotic state and the phase behavior of body water by shifting them in a direction similar to the shifts observed during the transition to diapause and cold acclimation. Such results suggest that accumulation of proline and glass transition are linked. Glass transition probably

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**Fig. 5.** Freeze tolerance of the larvae of $C.\ costata$ that were fed proline-augmented diets (Pro10 and Pro50, see text for details). Survival at (A) $-32^\circ$C or (B) in liquid $N_2$. The total heights of the columns depict survival at the cellular level. Proportions of larval, pupal, and adult survivors (of the total) are depicted using different column-filling styles, as specified. The total numbers of larvae ($n$) in each experiment are given in parentheses. (C) Pearson’s correlation analysis of the association between the whole-body proline concentration and survival to the adult stage at $-32^\circ$C or in liquid $N_2$ in diapause larvae. Acclimation treatments (1–4) are described in the legend of Fig. 1.

**Fig. 6.** Results of DSC thermal analyses in the larvae of $C.\ costata$ that were fed proline-augmented diets (Pro10 and Pro50, see text for details). (A) The amount of OA water and the OA/OI ratio. (B) The temperatures of $T_g$-like transitions [°C] and the frequencies (f) of $T_g$-like transition occurrence. The dashed abscissas show relevant comparative values in standard-diet-fed counterparts redrawn from Fig. 2 (Student’s t tests: ns, non-significant difference; *, $P < 0.05$; **, $P < 0.01$). Each column (or abscissa) represents mean ± SEM. For details, see Dataset S1. Acclimation treatments (1 and 2) are described in the legend of Fig. 1.
increases the viability of cells and tissues at deeply subzero temperatures but can not itself assure survival at the organismal level. Thus, the proline-augmented-diet-fed nondiapause larvae (variant 1 + Pro10), and the standard-diet-fed diapause-destined larvae (variant 2), both showed high frequencies of 

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三期 transition occurrence and both exhibited high levels of survival in liquid \( N_2 \) at cellular and tissue levels but had no capacity to survive to adult stage.

Collectively, this study brings evidence for causal linkage between the high concentrations of proline and the high capacity for survival in liquid \( N_2 \) in the larvae of \( C. \) costata. High proline levels accumulate during transition to diapause and cold acclimation and increase the propensity of osmotically inactive (unfrozen) water to undergo a glass-like transition (vitrification), which, probably, helps in preventing of cryoinjury. A simple method of artificial elevation of proline levels in larval tissues by feeding the larvae proline-augmented diets appeared as useful tool to further examine the mechanisms of this phenomenon.

**Materials and Methods**

For a detailed description of the materials and methods used, see SI Text.

**Insects, Acclimation, and Feeding.** The laboratory strain Sapporo of Chymomyza costata (Zetterstedt) (Diptera: Drosophilidae) was established from wild flies collected from nearby Sapporo, Hokkaido, Japan, in 1983 (50). Four acclimation treatments (variants) were compared:

1. nondiapause, direct development-destined larvae of prewandering stage, warm-acclimated, 3-wk-old;
2. diapause-destined, warm-acclimated larvae, 4-wk-old, which continue feeding and growing but with their imaginal discs cease developing (S1);
3. diapause, warm-acclimated larvae, 10-wk-old, which do not grow and develop, and show maximum diapause intensity (S2); and
4. diapause, cold-acclimated larvae, 10-wk-old.

Proline-augmented diets were prepared by mixing l-proline powder (Sigma-Aldrich) with the standard diet according to the following recipes: Pro10, 10 mg proline/1 g diet; Pro50, 50 mg proline/1 g diet.

**Supercooling, Freezing, and Survival in Liquid Nitrogen (\( N_2 \)).** Supercooling point was recorded as the exotherms linked to water/ice phase transition using the programmable thermostat F32-ME (Julabo) in combination with temperature data logger TC-08 (Pico Technology). The temperature program consisted of 4 steps: (i) a hold for 1 min at 30 °C; (ii) cooling to 0 °C at a rate of 5 °C·min⁻¹; (iii) heating to +30 °C at a rate of 0.1 °C·min⁻¹; (iv) cooling to −70 °C at a rate of 40 °C·min⁻¹ (the maximum rate and minimum temperature available in DSC4000); (v) a hold for 60 min at −70 °C; and (vi) heating to +30 °C at a rate of 10 °C·min⁻¹. The thermal curves were analyzed using Pyris Software (v. 10.1.0.0412; Perkin Elmer). The amount of osmotically active (OA) water was calculated from the area under the melt endotherm using the value of 334.5 J·g⁻¹ for the enthalpy of water. The amount of osmotically inactive (OI) water was derived by subtracting the OA water from the total water mass. The phase transition from the vitrified to liquid state was characterized by the change in heat capacity (\( \Delta C_P \)) with no exchange of latent heat involved (second order transition) and the glass transition temperature (\( T_g \)) as calculated an inflection point of this transition.

**Metabolic Profiling.** The metabolic profiles were extensively investigated in the whole body, dissected tissues, and hemolymph by a set of targetted and nontargeted mass spectrometry-based analytical methods. In the case of dissection, hemolymph was collected in a calibrated capillary and the following tissues were taken: fat body (approximately 90% of fat body cells were collected), gut (whole alimentary canal including Malpighian tubules), and body wall (epidermis with cuticle, muscles, and nerves, including the CNS). The whole larvae and tissues were homogenized and extracted in 70% ethanol. To be able to compare metabolite levels in whole body and hemolymph, the whole-body concentrations were recalculated to mmol·L⁻¹ of whole-body water (i.e., mM).

The survival after submergence in liquid \( N_2 \) was assessed using a similar experimental setup as described above but the larvae exposed at −32 °C for 1 h (step iii) were plunged into liquid \( N_2 \), maintained there for 1 h, and returned to a thermostat hold at −32 °C.

**Microscopy.** The status of larval tissues (before and after freezing) was checked using a Nikon Optiphot microscope (Carl Zeiss Jena, Germany) equipped with CCD camera. A field emission scanning electron microscope (JEOL 7401F (JEOL Ltd.) equipped with cryoattachment Alto 2500 (Gatan) was used to examine frozen specimens of \( C. \) costata.

**Osmolality, Thermal Hysteresis, and Differential Scanning Calorimetry.** Osmolality of hemolymph was measured using vapour pressure osmometer (Vapro 5520 (Wescor)). Thermal hysteresis was determined in a sample of hemolymph using Clifton Nanoliter Osmometer (Clifton Technical Physics).

Thermal analyses of whole larvae were conducted on a DSC4000 calorimeter (Perkin Elmer) as described (28, 32, 51). Each experimental larva was hermetically sealed in a 50 μL aluminum pan and subjected to a program consisting of 6 steps: (i) a hold for 1 min at 30 °C; (ii) cooling to 0 °C at a rate of 5 °C·min⁻¹; (iii) cooling to −30 °C at a rate of 0.1 °C·min⁻¹; (iv) cooling to −70 °C at a rate of 40 °C·min⁻¹ (the maximum rate and minimum temperature available in DSC4000); (v) a hold for 60 min at −70 °C; and (vi) heating to +30 °C at a rate of 10 °C·min⁻¹. The thermal curves were analyzed using Pyris Software (v. 10.1.0.0412; Perkin Elmer). The amount of osmotically active (OA) water was calculated from the area under the melt endotherm using the value of 334.5 J·g⁻¹ for the enthalpy of water. The amount of osmotically inactive (OI) water was derived by subtracting the OA water from the total water mass. The phase transition from the vitrified to liquid state was characterized by the change in heat capacity (\( \Delta C_P \)) with no exchange of latent heat involved (second order transition) and the glass transition temperature (\( T_g \)) as calculated an inflection point of this transition.

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