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

Vladimír Koštál[a,b,1], Helena Zahradníčková[a], and Petr Šimek[a]

[a]Institute of Entomology, Biology Centre, Academy of Sciences of the Czech Republic, Branišovská 31, 370 05 České Budějovice, Czech Republic; and [b]Faculty of Science, University of South Bohemia, Branišovská 31, 370 05 České Budějovice, Czech Republic

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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-augmented 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 length 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 (N_2) (−196 °C) for 1 h (9). To our knowledge, larva of *C. costata* represents the most complex metazoan organism that has reportedly survived after submergence in liquid N_2 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 N_2. 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 N_2.

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 N_2. 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. 1 *A* 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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[1]To whom correspondence should be addressed. E-mail: kostal@entu.cas.cz.

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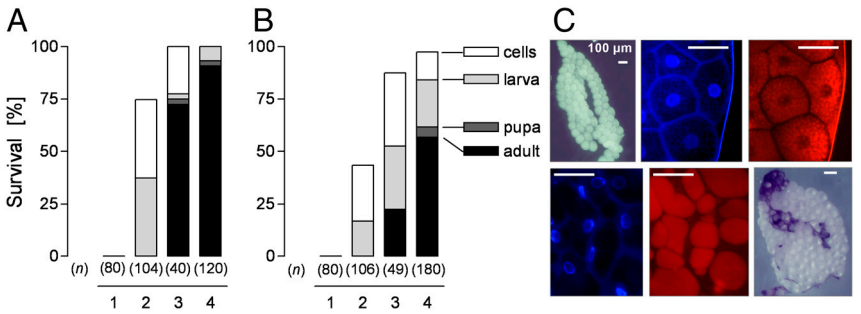

Fig. 1. Survival of differently acclimated larvae of *Chymomyza costata* (*A*) at −32 °C or (*B*) in liquid N_2. The total heights of columns depict survival at the cellular level. Proportions of larval, pupal, and adult survivors (of the total) are depicted using different column-filling styles, as specified. The acclimation treatments were: 1, nondiapause, warm-acclimated larvae; 2, diapause-destined, warm-acclimated larvae; 3, diapause, warm-acclimated larvae; and 4, diapause, cold-acclimated larvae. Total numbers of larvae (*n*) in each experiment are given in parentheses. (*C*) Fat body tissue photographed under a Zeiss Axioplan 2 fluorescence microscope. Upper row: control larvae, not exposed to freezing temperatures: (*Left*) no staining, (*Center*) DAPI, (*Right*) Nile red. Lower row: larvae exposed to liquid N_2 were stained with (*Left*) DAPI, (*Center*) Nile red, and (*Right*) trypan blue during recovery. The scale bar is 100 µm in all photographs.

Control larvae (not exposed to freezing temperatures) were negative for trypan blue staining and exhibited numerous small lipid droplets in fat body cells (Fig. 1*C*, 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. 1*C*, 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. 1*C*, 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 N_2 in only a few cases [e.g., insect embryos (17)], trochophore larvae of bivalve mollusks (18), and larvae of marine annelid (19). In contrast, desiccated anhydrobiotic 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. 2*A* 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 N_2. Fig. 2*B* 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 hemocel. 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, T_g, which depends on the chemical composition of a solution and its water content. Vitrification is widely used in cryopreservation protocols (14–16, 27) because

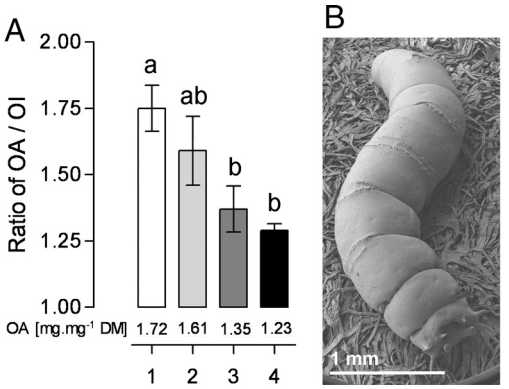

Fig. 2. (*A*) Gradual decrease in the amount of osmotically active (OA) water and the OA/OI ratio (OI, osmotically inactive water) during the transition to diapause and cold acclimation in the larvae of *Chymomyza costata*. Each column represents mean ± SEM. Columns flanked by different letters are statistically different (ANOVA: $F = 10.34$, $P < 0.0001$; followed by Tukey's post hoc test, $P = 0.05$). For details, see Dataset S1. Acclimation treatments (1–4) are described in the legend of Fig. 1. (*B*) Cryo-SEM (JEOL 7401F) micrograph of *C. costata* larva (variant 4) that was slowly frozen to −32 °C and then plunged into liquid N_2.

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 °C). In contrast, clear T_g-like transitions were observed in all analyzed diapause larvae, irrespective of the acclimation treatment (Fig. 3*A* 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 °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 °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 μL) 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; $n = 5$; $T_g = -25.24 \pm 1.98$ °C; $\Delta Cp = 0.919 \pm 0.390$ J/g·°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*) 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. 4*B*). 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 N_2 (Pearson's $r = 0.9843$, $P = 0.0157$).

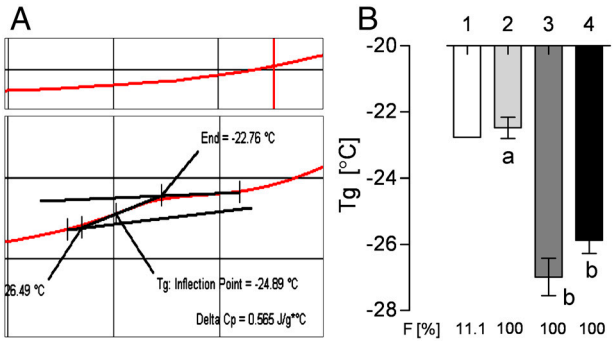

Fig. 3. Results of DSC thermal analysis in differently acclimated larvae of *Chymomyza costata*.(*A*) The examples of DSC thermal analyses in (*Upper*) nondiapause and (*Lower*) diapause larvae. Only the parts of thermal curves where T_g-like transitions typically occur are shown. The original outputs 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. Acclimation treatments (1–4) are described in the legend of Fig. 1.

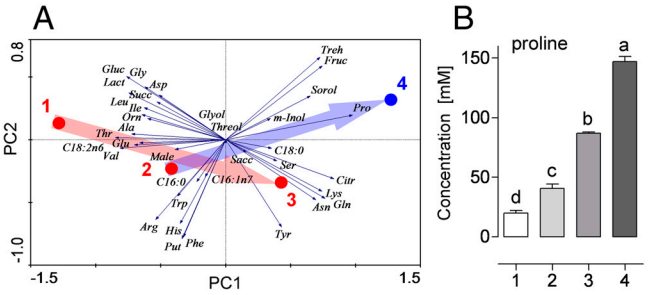

Fig. 4. Principal component analysis of metabolomic changes in differently acclimated larvae of *Chymomyza costata*. (*A*) The PC1 axis explains 95.7% of the variance and the PC2 axis adds 3.6%. Red and blue points show positions of acclimation variants (1–4, described in the legend of Fig. 1).The directions of changes associated with transition to diapause and cold acclimation are depicted by red and blue arrows, respectively. Thin arrows (eigenvectors) depict changes in the concentrations of individual metabolites (for details, see Dataset S2). The longer the eigenvector and the closer its orientation to acclimation variant-point, the more characteristic is this metabolite for this respective acclimation variant. (*B*) Changes in proline levels. Each column represents mean ± SEM. Columns flanked by different letters are statistically different (ANOVA: $F = 1257$, $P < 0.0001$; followed by Tukey's post hoc test, $P = 0.05$).

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.

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 °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 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 °C and in liquid N_2 increased from 0% (variant 2) to 50.0% and 35.7%,

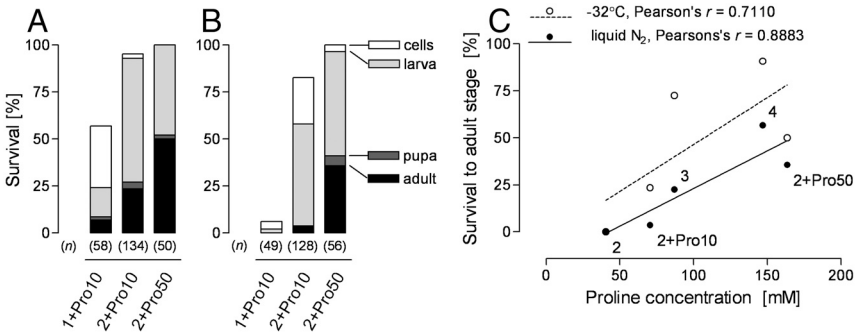

Fig. 5. Freeze tolerance of the larvae of *Chymomyza costata* that were fed proline-augmented diets (Pro10 and Pro50, see text for details). Survival at (*A*) −32 °C or (*B*) in liquid N_2. The total heights of the columns depict survival at the cellular level. Proportions of larval, pupal, and adult 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 °C or in liquid N_2 in diapause larvae. Acclimation treatments (1–4) are described in the legend of Fig. 1.

respectively (variant 2+Pro50) (Fig. 5 *A* and *B*). Fig. 5*C* 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 °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 to 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 biophysical 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. 6*A* 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. 6*B* 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

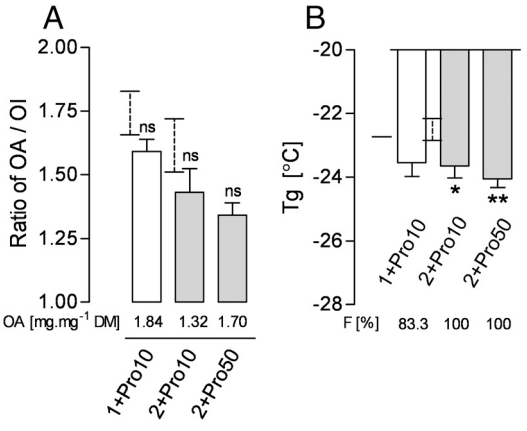

Fig. 6. Results of DSC thermal analyses in the larvae of *Chymomyza costata* that were fed proline-augmented diets (Pro10 and Pro50, see text for details). (*A*) The amount of OA water and the OA/OI ratio. (*B*) The temperatures of T_g-like transitions [°C] and the frequencies (*F*) of T_g-like transition occurrence. The dashed abscissas show relevant comparative values in standard-diet-fed counterparts redrawn from Fig. 2 (Student's *t* tests: ns, nonsignificant 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 T_g-like 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 linkange 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 their imaginal discs cease developing (5);
3. diapause, warm-acclimated larvae, 10-wk-old, which do not grow and develop, and show maximum diapause intensity (5); 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 level of freeze tolerance was estimated as a proportion of larvae that survived after exposure to −32 °C for 2 h inside the programmable thermostat F32-ME. The temperature program consisted of 4 steps: (*i*) a hold for 20 min at −1 °C, during which ice crystals were added, resulting in an almost immediate ice nucleation of larvae; (*ii*) cooling to −32 °C at a rate of 0.1 °C·min^{-1}; (*iii*) a hold for 2 h at −32 °C; and (*iv*) heating to +5 °C at a rate of 0.1 °C·min^{-1}. After exposure, the larvae were allowed to recover for 12 h at 18 °C and then transferred to a fresh diet. During transfer, survival was checked by distinguishing dead larvae (no movements) from living larvae (spontaneous, coordinated crawling) and those showing signs of survival at the cellular/tissue levels (uncoordinated movements). Next, the larvae were kept at +18 °C and long-day regimen (16 h light : 8 h dark) for 45 d and their abilities to pupariate and emerge as adults were scored.

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 under a Zeiss Axioplan 2 fluorescence microscope (Carl Zeiss) 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*) and 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 (ΔCp) with no exchange of latent heat involved (second order transition) and the glass transition temperature (T_g) was calculated as an inflection point of this transition.

Metabolomic Profiling. The metabolomic profiles were extensively investigated in the whole body, dissected tissues, and hemolymph by a set of targeted 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).

Low molecular weight sugars and polyols were quantitatively determined in ethanolic extracts after *o*-methyloxime trimethylsilyl derivatization and subsequent analysis by gas chromatography coupled to mass spectrometry (GC/MS) as described earlier (52). Nontargeted metabolite profilings were accomplished by a combination of GC/MS and LC/MS (liquid chromatography/MS) techniques in the same ethanolic extracts after their treatment with ethyl chloroformate under pyridine catalysis and simultaneous extraction into chloroform (53, 54).

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1. Salt RW (1961) Principles of insect cold-hardiness. *Annu Rev Entomol* 6:55–74.
2. Zachariassen KE (1985) Physiology of cold tolerance in insects. *Physiol Rev* 65:799–832.
3. Danks HV (1991) Winter habits and ecological adaptations for winter survival. *Insects at Low Temperature*, eds RE Lee, Jr and DL Denlinger (Chapman and Hall, New York), pp 318–359.
4. Hackman W, Lakovaara S, Saura A, Sorsa M, Vepsalainen K (1970) On the biology and karyology of *Chymomyza costata* Zetterstedt, with reference to the taxonomy and distribution of various species of *Chymomyza* (Dipt, Drosophilidae). *Ann Entomol Fenn* 36:1–9.
5. Koštál V, Shimada K, Hayakawa Y (2000) Induction and development of winter larval diapause in a drosophilid fly, *Chymomyza costata*. *J Insect Physiol* 46:417–428.
6. Enomoto O (1981) Larval diapause in *Chymomyza costata* (Diptera: Drosophilidae) II. Frost avoidance. *Low Temp Sci, Ser B* 39:31–39.
7. Shimada K, Riihimaa A (1988) Cold acclimation, inoculative freezing and slow cooling: Essential factors contributing to the freezing-tolerance in diapausing larvae of *Chymomyza costata* (Diptera: Drosophilidae). *Cryo Letters* 9:5–10.
8. Shimada K, Riihimaa A (1990) Cold-induced freezing tolerance in diapausing and non-diapausing larvae of *Chymomyza costata* (Diptera: Drosophilidae) with accumulation of trehalose and proline. *Cryo Letters* 11:243–250.
9. Moon I, Fujikawa S, Shimada K (1996) Cryopreservation of *Chymomyza* larvae (Diptera: Drosophilidae) at −196 °C with extracellular freezing. *Cryo Letters* 17:105–110.
10. Koštál V, Berková P, Šimek P (2003) Remodelling of membrane phospholipids during transition to diapause and cold-acclimation in the larvae of *Chymomyza costata* (Drosophilidae). *Comp Biochem Physiol B Biochem Mol Biol* 135:407–419.
11. Storey KB, Storey JM (1991) Biochemistry of cryoprotectants. *Insects at Low Temperature*, eds RE Lee, Jr and DL Denlinger (Chapman and Hall, New York), pp 64–93.
12. Hazel JR (1995) Thermal adaptation in biological membranes: Is homeoviscous adaptation the explanation? *Annu Rev Physiol* 57:19–42.
13. Koštál V (2006) Eco-physiological phases of insect diapause. *J Insect Physiol* 52:113–127.
14. Mazur P (1990) Equilibrium, quasi-equilibrium, and non-equilibrium freezing of mammalian embryos. *Cell Biophys* 17:53–92.

15. Pegg DE (2001) The current status of tissue cryopreservation. *Cryo Letters* 22:105–114.
16. Taylor MJ, Song YC, Brockbank KGM (2004) Vitrification in tissue preservation: New developments. *Life in the Frozen State*, eds BJ Fuller, N Lane, and EE Benson (CRC Press, Boca Raton), pp 603–641.
17. Leopold RA, Rinehart JP (2010) A template for insect cryopreservation. *Low Temperature Biology of Insects*, eds DL Denlinger and RE Lee, Jr (Cambridge University Press, Cambridge), pp 325–341.
18. McFadzen I, Utting S (1990) The viability and growth of cryopreserved bivalve larvae. *World Aquac* T-13:65.
19. Olive PJW, Wang WB (1997) Cryopreservation of *Nereis virens* (Polychaeta, Annelida) larvae: The mechanism of cryopreservation of a differentiated metazoan. *Cryobiology* 34:284–294.
20. Keilin D (1959) The problem of anabiosis or latent life: history and current concept. *P Roy Soc B-Biol Sci* 150:149–191.
21. Wright JC (2001) Cryptobiosis 300 years on from van Leuwenhoek: What have we learned about tardigrades? *Zool Anz* 240:563–582.
22. Clegg JS (2001) Cryptobiosis—a peculiar state of biological organization. *Comp Biochem Physiol B Biochem Mol Biol* 128:613–624.
23. Jönsson KI, Rabbow E, Schill RO, Harms-Ringdahl M, Rettberg P (2008) Tardigrades survive exposure to space in low Earth orbit. *Curr Biol* 18:R729–R731.
24. Storey KB, Storey JM (1988) Freeze tolerance in animals. *Physiol Rev* 68:27–84.
25. Duman JG (2001) Antifreeze and ice nucleator proteins in terrestrial arthropods. *Annu Rev Physiol* 63:327–357.
26. Storey KB, Baust JG, Buescher P (1981) Determination of water "bound" by soluble subcellular components during low temperature acclimation of the overwintering gall fly larva, *Eurosta solidaginis*. *Cryobiology* 18:315–321.
27. Franks F (1986) Unfrozen water: yes; unfreezable water: hardly; bound water: certainly not. An editorial note. *Cryo Letters* 7:207.
28. Block W (2003) Water or ice?—the challenge for invertebrate cold survival. *Sci Prog* 86:77–101.
29. MacFarlane DR (1987) Physical aspects of vitrification in aquaeous solution. *Cryobiology* 24:181–195.
30. Sakurai M, et al. (2008) Vitrification is essential for anhydrobiosis in an African chironomid, *Polypedilum vanderplanckі*. *Proc Natl Acad Sci USA* 105:5093–5098.
31. Hengherr S, Worland MR, Reuner A, Brümmer F, Schill RO (2009) High temperature tolerance in anhydrobiotic tardigrades is limited by glass transition. *Physiol Biochem Zool* 82:749–755.
32. Sformo T, et al. (2010) Deep supercooling, vitrification and limited survival to −100 °C in the Alaskan beetle *Cucujus clavipes puniceus* (Coleoptera: Cucujidae) larvae. *J Exp Biol* 213:502–509.
33. Buitink J, Hemminga MA, Hoekstra FA (2000) Is there a role for oligosacharides in seed longevity? An assessment of intracellular glass stability. *Plant Physiol* 122:1217–1224.
34. Hoekstra FA, Golovina EA, Buitink J (2001) Mechanisms of plant desiccation tolerance. *Trends Plant Sci* 6:431–438.
35. Storey KB, Baust JG, Storey JM (1981) Intermediary metabolism during low temperature acclimation in the overwintering gall fly larva, *Eurosta solidaginis*. *J Comp Physiol B* 144:183–190.
36. Morgan TD, Chippendale GM (1983) Free amino acids of the haemolymph of the southwestern corn borer and the european corn borer in relation to their diapause. *J Insect Physiol* 29:735–740.
37. Fields PG, et al. (1998) The effect of cold acclimation and deacclimation on cold tolerance, trehalose and free amino acid levels in *Sitophilus granarius* and *Cryptolestes ferugineus* (Coleoptera). *J Insect Physiol* 44:955–968.
38. Dörfling K, et al. (1997) Heritable improvement of frost tolerance in winter wheat by in vitro selection of hydroxyproline-resistant proline overproducing mutants. *Euphytica* 93:1–10.
39. Nanjo T, et al. (1999) Antisense suppression of proline degradation improves tolerance to freezing and salinity in *Arabidopsis thaliana*. *FEBS Lett* 461:205–210.
40. Lee RE, Jr (2010) A primer on insect cold-tolerance. *Low Temperature Biology of Insects*, eds DL Denlinger and RE Lee, Jr (Cambridge University Press, Cambridge), pp 3–34.
41. Murray P, Hayward SAL, Govan GG, Gracey AY, Cossins AR (2007) An explicit test of the phospholipid saturation hypothesis of acquired cold tolerance in *Caenorhabditis elegans*. *Proc Natl Acad Sci USA* 104:5489–5494.
42. Koštál V, Šimunková P, Kobelková A, Shimada K (2009) Cell cycle arrest as a hallmark of insect diapause: Changes in gene transcription during diapause induction in the drosophilid fly, *Chymomyza costata*. *Insect Biochem Mol Biol* 39:875–883.
43. Denlinger DL (2002) Regulation of diapause. *Annu Rev Entomol* 47:93–122.
44. MacRae TH (2010) Gene expression, metabolic regulation and stress tolerance during diapause. *Cell Mol Life Sci* 67:2405–2424.
45. Arakawa T, Timasheff SN (1985) The stabilization of proteins by osmolytes. *Biophys J* 47:411–414.
46. Arakawa T, Timasheff SN (1983) Preferential interactions of proteins with solvent components in aquaeous amino acid solutions. *Arch Biochem Biophys* 224:169–177.
47. Carpenter JF, Hand SC, Crowe LM, Crowe JH (1986) Cryoprotection of phosphofructokinase with organic solutes: Characterization of enhanced protection in the presence of divalent cations. *Arch Biochem Biophys* 250:505–512.
48. Carpenter JF, Crowe JH (1988) The mechanisms of cryoprotection of proteins by solutes. *Cryobiology* 25:244–255.
49. Rudolph AS, Crowe JH, Crowe LM (1986) Effects of three stabilizing agents—proline, betaine, and trehalose—on membrane phospholipids. *Arch Biochem Biophys* 245:134–143.
50. Riihimaa AJ, Kimura MT (1988) A mutant strain of *Chymomyza costata* (Diptera: Drosophilidae) insensitive to diapause-inducing action of photoperiod. *Physiol Entomol* 13:441–445.
51. Block W (1994) Differential scanning calorimetry in ecophysiological research. *Acta Oecol* 15:13–22.
52. Koštál V, Zahradníčková H, Šimek P, Zelený J (2007) Multiple component system of sugars and polyols in the overwintering spruce bark beetle, *Ips typographus*. *J Insect Physiol* 53:580–586.
53. Hušek P (1997) Urine organic acid profiling by capillary gas chromatography after a simple sample pretreatment. *Clin Chem* 43:1999–2001.
54. Hušek P, Šimek P (2001) Advances in amino acid analysis. *LC-GC North America* 19:986–999.