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

Vladimír Koštál\(^{a,b}\), Helena Zahradničková,\(^{a}\), 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.

**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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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)], trocho- phore larvae of bivalve mollusks (18), and larvae of marine arthropods (19). In contrast, desiccated anhydrobiotic invertebrates exhibit relatively easy survival at temperatures as low as −237°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.

Control larvae, not exposed to freezing temperatures, were negatively stained for trypan blue 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.

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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 $-22.8^\circ C$). 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 $-32^\circ C$ 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 $-3^\circ C$ when the larva was in contact with the ice crystals in the bottom of the 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; $n = 5$; $T_g = -25.24 \pm 1.98^\circ C$; $\Delta C_p = 0.919 \pm 0.390$ 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. 4A) 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. 4A: 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 $N_2$ (Pearson’s $r = 0.9843$, $P = 0.0157$).

The increase in proline levels explains most of the change in overall osmolality from 465 mosmol/kg$^{-1}$ in nondiapause larvae to 699 mosmol/kg$^{-1}$ in diapause, cold-acclimated larvae (a difference of 234 mosmol/kg$^{-1}$; Dataset S1). Because partial dehydration (loss of 35% of body water; Dataset S1) increases osmolality by approximately 70 mosmol/kg$^{-1}$, the remaining difference is 164 mosmol/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 (varially 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 $-32^\circ C$ 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 $-32^\circ C$ and in liquid $N_2$ increased from 0% (variant 2) to 50.0% and 35.7%,
respectively (variant 2+Pro50) (Fig. 5A and B). Fig. 5C shows that a single 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 N₂ 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 N₂. 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 in nondiapause animals were sufficient to achieve 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 biological 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

![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 N₂. 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 N₂ in diapause larvae. Acclimation treatments (1–4) are described in the legend of Fig. 1.](image-url)

![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. 2B (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.](image-url)
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 \( T_\text{f} \)-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 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 prewarming 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 (55);
3. diapause, warm-acclimated larvae, 10-wk-old, which do not grow and develop, and show maximum diapause intensity (56); 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 eutomer’s linked to water/rice 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 \(-32^\circ\text{C}\) for 2 h inside the programmable thermostat F32–ME. The temperature program consisted of 4 steps: (i) a hold for 20 min at \(-1^\circ\text{C}\), during which ice crystals were added, resulting in an almost immediate ice nucleation of larvae; (ii) cooling to \(-32^\circ\text{C}\) at a rate of \(0.1\ ^\circ\text{C} \cdot \text{min}^{-1}\); (iii) heating to \(-5^\circ\text{C}\) at a rate of \(1\ ^\circ\text{C} \cdot \text{min}^{-1}\) after exposure, the larvae were allowed to recover for 12 h at \(18^\circ\text{C}\) 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 \(-18^\circ\text{C}\) 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 \(-32^\circ\text{C}\) for 1 h (step (ii)) were plunged into liquid \( N_2 \), maintained there for 1 h, and returned to a thermostat hold at \(-32^\circ\text{C}\).

### Microscopy.

The status of larval tissues before and after freezing was checked using an AxioLab 2 fluorescence microscope (Carl Zeiss) equipped with a 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 caloriometer (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 \(30^\circ\text{C}\); (ii) cooling to \(0^\circ\text{C}\) at a rate of \(5\ ^\circ\text{C} \cdot \text{min}^{-1}\); (iii) cooling to \(-30^\circ\text{C}\) at a rate of \(0.1\ ^\circ\text{C} \cdot \text{min}^{-1}\); (iv) cooling to \(-70^\circ\text{C}\) at a rate of \(40\ ^\circ\text{C} \cdot \text{min}^{-1}\) (the maximum rate and minimum temperature available in DSC4000); (v) a hold for 60 min at \(-70^\circ\text{C}\); and (vi) heating to \(+30^\circ\text{C}\) at a rate of \(10\ ^\circ\text{C} \cdot \text{min}^{-1}\). 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.

### Metabolic 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 within the 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-methylxime trimethylsilyl derivatization and subsequent analysis by gas chromatography coupled to mass spectrometry (GC/MS) as described earlier (52). Nontargeted metabolite profiles 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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