Feeding on Proline-Augmented Diet. Developmentally synchronized larvae (age 11–13 d after egg laying) of the variants 1 and 2 were transferred to a proline-augmented diet in a 5 mL glass vial (25 larvae per 1 g of diet). Larvae were maintained in proline-augmented diet until they reached the age of 3 wk (variant 1) or 4 wk (variant 2).

Our preliminary experiments revealed that feeding Pro1 diet (1 mg proline/1 g diet) had no significant effect on larval freeze tolerance, and feeding Pro100 diet (100 mg proline/1 g diet) resulted in high larval mortality (approximately 80%). That is why we decided to conduct our experiments with Pro10 and Pro50 diets only, which exhibited significant positive effects on freeze tolerance and caused low mortality (approximately 1% in Pro10, and 8% in Pro50 diet). Pro10-feeding had no significant influence on larval growth (Dataset S1), and developmental destiny (2); i.e. nondiapause larvae pupariated and diapause larvae ceased development and remained sensitive to photoperiod. Pro50-fed larvae, however, were smaller than their standard-diet-fed counterparts (Dataset S1), not able to finish development (pupariate) and all died when left in this diet continuously.

Supporting Information

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SI Materials and Methods

Insects and Acclimation. Insects were cultured on an artificial diet (1) under a constant temperature of 18 °C. Larvae were developmentally synchronized during their second to third larval instar transition on days 11–13 after egg laying.

Developmental destiny of experimental larvae was programmed using two different photoperiodic regimes: a long-day regime (16 h light : 8 h dark), at which all larvae continue direct development (pupariate); and a short-day regime (12 h light : 12 h dark) that induces diapause in all larvae (2). Warning-cocoon formation means that the larvae were maintained at constant 18 °C. Cold acclimation started in 4-wk-old diapause larvae that were transferred to 15 °C (short-day) for 1 wk, followed by 10 °C (short-day) for 1 wk, followed by 5 °C (constant darkness) for remaining 4 wk.

Mass, Hydration, Osmolality, and Thermal Hysteresis. Larval fresh mass (FM) was measured using Sartorius electronic balance (precision 0.01 mg) in a sample of 5 larvae (6 replications) in each variant. Weighed specimens were dried at 60 °C for 3 days, their dry mass (DM) was taken and water mass (WM, in mg) and hydration (H, in mg water/mg dry mass (DM) were calculated from gravimetric data.

For osmolality measurement, a small droplet of hemolymph was collected into calibrated capillary after puncturing the larval integument. Known volume of hemolymph was then mixed with 10 μL of 100 mM NaCl (Invitrogen) and 0.1% triphenyltetrazolium chloride (TTC) and 10 μL of the mixture was applied to the instrument. The osmolality of hemolymph (X) was calculated using a formula:

\[ X = \frac{[\text{OSMOL} \times (10 + \text{VOLUME})]}{1.000} \]

where OSMOL means the reading of the instrument and VOLUME is the volume of hemolymph.

Presence/absence and magnitude of thermal hysteresis (difference between equilibrium freezing point and melting point) was determined in a sample of hemolymph using Clifton Nanoliter Osmometer (Clifton Technical Physic) as previously described (5, 6).

Differential Scanning Calorimetry. Heat flow of our DSC4000 instrument was calibrated by measuring the areas under the melting endotherms of known masses of ice. The temperature scale was calibrated using indium, distilled water, and cyclohexane standards.

To assess the influence of ice nucleation at a temperature close to 0 °C on glass/liquid transition, we made following modifications of our experimental setup: (i), the same temperature setting but no test-pan was present in instrument (only the reference-pan); (ii) cool to −2 °C at a rate of 5 °C min−1. When the temperature reached −2 °C, we inserted into instrument the bottom of test-pan. The bottom was lined with filter paper disc (SS-033, Wescor) to which 10 μL of distilled water was applied and then submerged in liquid nitrogen to freeze it; (iii) after the bottom of test-pan equilibrates back to program temperature of ca. −2 °C.
Biochemistry and Metabolomic Profiling. Five larvae in four replications were typically processed from each experimental variant. Total lipid content was measured by spectrophotometric analysis using H₂PO₄:v-anilin solution (7) after extraction of total lipids using chloroform:methanol solution (2:1, v/v) (8). The solvents were evaporated under a stream of nitrogen, and lipids were reconstituted in 1 mL of chloroform. Glycogen content was measured by colorimetric determination using phenol and concentrated sulfuric acid (9) after extraction of glycogen in hot alkali (10). Total proteins were measured by the bicinchoninic acid protein assay (11) after extraction of total water-soluble proteins using 50 mM Tris, pH 6.8 followed by reextraction of detergent-soluble proteins from a centrifugation pellet in the same buffer with addition of 0.5% deoxycholate and 0.1% SDS. GC/MS metabolite profiles were obtained on a VF-17 capillary column (Agilent) coupled to a Trace DSQ mass spectrometer (Thermo Finnigan) equipped with an electron impact ion source and operated in the full scan mode from 40 to 500 amu. A Trace Ultra gas chromatograph (Thermo Finnigan) with a programmable injector and interface held at 230 °C was directly coupled to the spectrometer via an interface held at 250 °C. A 0.5 μL aliquot of chloroform extract was injected using the splitless mode into the GC/MS. Oven temperature was initially kept at 50 °C for 1 min. Thereafter the temperature was raised at 12 °C/min until 302 °C and held for 2 min. Helium was used as carrier gas and delivered at a constant flow rate of 1.2 mL/min. LC/MS metabolite profiles were measured after evaporation to dryness of 30 μL aliquot of the chloroform extract using a mild stream of nitrogen. After dissolution in the 200 μL of the LC mobile phase, a 5 μL aliquot was injected to and separated on a Kinex C18, 2.6 μm, 150 × 2 mm column (Phenomenex) at 35 °C, flow rate 200 μL/min, using a gradient elution with a mobile phase consisting of (a) 5 mM ammonium formate in methanol and (b) 5 mM ammonium formate in water. The gradient program was linear from 30% to 100% A in 12 min, then held at 100% A to 14 min and finally equilibrated for 5 min. The nontargeted GC/MS and LC/MS data were processed by means of Thermo Scientific XCalibur 2.1 software and in-house developed Metabolite Mapper platform providing automated peak picking and metabolite deconvolution employing retention time, mass spectral and detector response features followed by time alignment of each particular analysis within a defined experimental sample set and data matrix formation that was exported automatically into a predefined Excel spread sheet for further statistical processing. The 61 major metabolites were identified against relevant standards and further subjected to quantitative analysis by using an internal standard calibration method (see Dataset S2 for the metabolite list).

All chemicals used were purchased from Sigma–Aldrich Co. except isotope-labeled metabolites used as internal calibration standards that were obtained from Cambridge Isotope Laboratories.

Statistical Analyses. One-way ANOVAs were used to analyze whether there is any influence of acclimation treatment on physiological and biochemical parameters, and Tukey’s post hoc tests (p = 0.05) were applied to find the differences among treatments. Pearson’s correlation analysis was used to test the associations between the parameters and survival at −32 °C or in liquid N₂. Unpaired two-tailed Student t tests (p = 0.05) were used to assess the differences in parameters between the standard-diet-fed and proline-augmented-diet-fed larvae of the same acclimation treatment. All these analyses were performed using Prism, v. 4 (GraphPad Software). The complex association of metabolomic changes with acclimation treatments was assessed by principal component analysis (PCA) using Canoco for Windows, v. 4.52 (Biometris-Plant Research International).

Fig. S1. Cryo-SEM (JEOL 7401F) micrographs of cryofractured larvae of *Chymomyza costata* (variant 4) that were slowly frozen to −32°C and then plunged into liquid N$_2$. (A) Transversal section (cryofracture) cuts the larva approximately in 2/3 of its length from the front tip. Large masses of continuous ice in the hemocel surround dehydrated tissues situated in the center (fat body) and periphery (muscle layers). Arrows point to main tracheae. White rectangles delimit the areas enlarged in micrographs B and C, which show freeze dehydrated fat body cells containing coalesced lipid droplets. (D) Transversal section of another larva shows freeze dehydrated tissues in the periphery of larval body, layers of striated muscle, epidermis, and two cuticle layers.

Other Supporting Information Files
Dataset S1 (XLS)
Dataset S2 (XLS)
Dataset S3 (XLS)