Conversion of the chill susceptible fruit fly larva (*Drosophila melanogaster*) to a freeze tolerant organism

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Among vertebrates, only a few species of amphibians and reptiles tolerate the formation of ice crystals in their body fluids. Freeze tolerance is much more widespread in invertebrates, especially in overwintering insects. Evolutionary adaptations for freeze tolerance are considered to be highly complex. Here we show that surprisingly simple laboratory manipulations can change the chill susceptible insect to the freeze tolerant one. Larvae of *Drosophila melanogaster*, a fruit fly of tropical origin with a weak innate capacity to tolerate mild chilling, can survive when approximately 50% of their body water freezes. To achieve this goal, synergy of two fundamental prerequisites is required: (*i*) shutdown of larval development by exposing larvae to low temperatures (dormancy) and (*ii*) incorporating the free amino acid proline in tissues by feeding larvae a proline-augmented diet (cryopreservation).

insect cold tolerance | long-term storage | metabolomics | cryoprotection | quiescence

The vast majority of insect lineages and species evolved, diversified, and recently live in warm lowland tropics, where seasonal and daily temperatures fluctuate little. This scenario is also true for the genus *Drosophila*, which contains almost 1,500 described species (1) including a common model of modern biology, the fruit fly *Drosophila (Sophophora) melanogaster* (Meigen, 1830) (fruit fly in further text). The ancestral members of this genus were adapted to warm temperatures (2), and most of the extant species still have tropical and/or subtropical distributions and are chill susceptible (3). The immature development of *D. melanogaster* halts at temperatures below 10 °C (4), chill injury occurs below 6 °C (5), and all developmental stages die when chilled (supercooled) below −5 °C for just a few hours (6). The ability to tolerate freezing; i.e., formation of ice crystals in body fluids, is unknown in any *Drosophila* species (2, 5).

Numerous insect lineages, including some drosophilid flies, colonized colder environments in higher altitudes or temperate and polar regions. Such lineages had to overcome selection pressures of shorter growing season, exaggerated daily temperature fluctuations, and seasonal drop of temperatures below the physiological thresholds for activity, growth, and development (7). It is now widely accepted that cold adaptation in insects is highly complex and requires adjustments at all levels of biological organization (8–16). Supercooling and freeze tolerance are two main strategies that help insects to cope with the risk of water freezing in a cryothermic state. While the capacity for supercooling seems to be basal in cold-adapted arthropod lineages, freeze tolerance probably converged in several groups in response to either severe Arctic winters or unpredictable subzero temperature events typical of cold habitats in the southern hemisphere (17, 18). For example, larvae of subarctic drosophilid fly, *Chymomyza costata* (Zetterstedt, 1838) are extremely freeze tolerant and may even survive after submergence in liquid nitrogen (−196 °C) (19). We have previously shown that high freeze tolerance in these larvae is based on two principal prerequisites. First, larvae enter into a state of diapause and second, during the cold acclimation process, they accumulate high levels of the free amino acid L-proline—an innate cryoprotectant (proline in further text) (20).

In this study, we investigated whether the principles underlying high freeze tolerance in *C. costata* could apply to chill-susceptible *D. melanogaster*. We acclimated the fruit fly larvae at low temperatures including the fluctuating thermal regime (FTR) inducing dormancy—a low temperature quiescence state. In addition, larvae were fed diets augmented with proline, trehalose, and glycerol. The treatment combining the effects of FTR-quiescence and cryopreservation by high tissue levels of proline resulted in acquisition of freeze tolerance in the fruit fly larvae. Treated larvae survived when half of their body water froze at −5 °C. Upon melting, these larvae were able to continue development, metamorphosed into adults, and produced viable offspring.

Results and Discussion

Using Three Different Methods, We Verified That *D. melanogaster* Larvae do Freeze in Our Freeze Tolerance Assays. Hence, we can rule out a theoretical alternative that larvae remain supercooled when surrounded with ice. First, infrared camera recordings of the body temperatures in individual larvae showed that all larvae ($n = 25$) were inoculated by external ice and froze at −1.82 °C ± 0.92 °C (mean ± SD, range: −0.8 °C to −4.3 °C) (Fig. 1*A* and *B*). Second, no typical individual supercooling points (SCPs) were recorded in *wet* situation because all larvae were inoculated by external ice and froze at much higher temperatures (between −1 °C and −5 °C) together with surrounding water (Fig. 1*C*; Fig. S1). Third, differential scanning calorimetry (DSC) thermal analyses revealed that all larvae ($n = 30$) were inoculated by external ice when placed on a piece of cellulose containing ice crystals and gradually cooled from −3 °C to −5 °C (Fig. 1*D*; Fig. S2). Using DSC, we found that approximately half of the total body water was converted to ice crystals at −5 °C. The amounts of ice ranged between 46.7% and 51.0% and did not differ significantly among treatments (Dataset S1).

Entering Quiescent State and Long-Term Cold Acclimation Is Not Sufficient to Induce Freeze Tolerance. Because diapause in the fruit fly is weakly expressed in the adult stage, and no capacity for diapause exists in the larval stage (21, 22), we exposed larvae to low temperatures in order to achieve two aims at once: (*i*) slowing down larval development and/or bringing larvae into a state of dormancy (quiescence), and (*ii*) allowing the process of long-term cold acclimation (5). The development from the egg stage to

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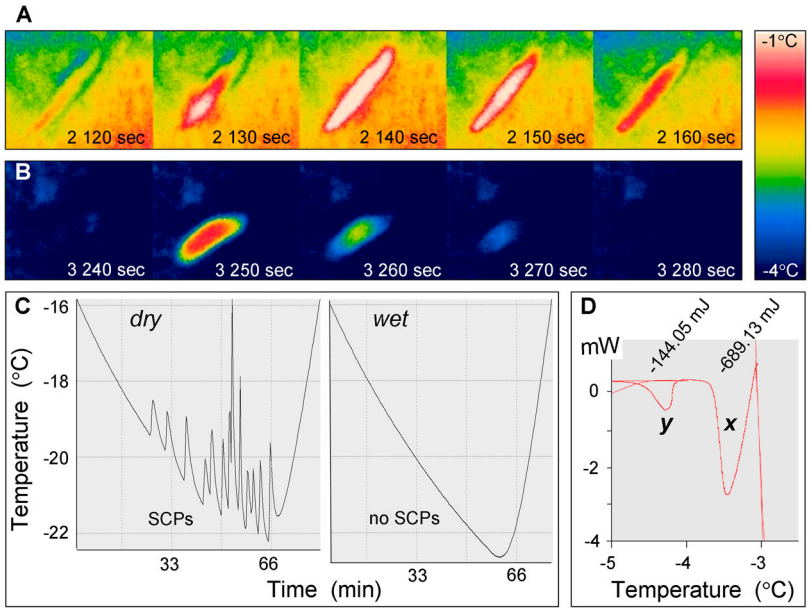

Fig. 1. Proof of the occurrence of ice crystals in body fluids of *D. melanogaster* 3rd instar larvae. (*A*), (*B*), Infrared camera time-lapse recordings of larvae during cooling to −5 °C when in contact with ice crystals (on the wet piece of cellulose). Two examples of freezing events in larval body fluids are shown at −2.7 °C (*A*) and −4.3 °C (*B*). (*C*), Freeze exotherms ($n = 13$) corresponding to spontaneous freezing at SCP temperatures in larvae gradually cooled to −25 °C in a *dry* situation (no external ice crystals); in contrast, no SCP-related freeze exotherms for larvae ($n = 15$) ice-inoculated at above −5 °C in a *wet* situation (surrounded with external ice crystals in a piece of wet cellulose) (for details, see Fig. S1). (*D*), An example of differential scanning calorimetry thermal analysis of one larva inoculated by external ice on a piece of wet cellulose containing ice crystals and gradually cooled from −3 °C to −5 °C. Freeze exotherm labeled as ***x*** belongs to external water in the DSC test-pan. The area of −144.05 mJ under the freeze exotherm labeled as ***y*** corresponds to 0.43 μL of water, which represents 53.1% of total body water in this particular larva (for details, see Fig. S2).

puparium formation takes approximately 5 d at 25 °C (treatment no. *i*., Dataset S2 contains detailed data on development and physiology of larvae in all treatments) but 19 d at 15 °C (treatment *ii*.). In addition, we transferred fully grown 15 °C-acclimated larvae to a FTR where temperature oscillated daily: 6 °C/11 °C (12 h/12 h) under constant darkness for 3 d (treatment *iii*.). Under an FTR, most larvae were in a dormant state (quiescence) with their development halted and chill injury and mortality of larvae was prevented (23–25). We examined the capacity for freeze tolerance in *D. melanogaster* larvae under various treatments, by assessing their ability to survive after being gradually frozen to −5 °C in the presence of surrounding ice and then gradually melted (see *Materials and Methods* for details). Fig. 2 shows that no larva survived when acclimated at constant 25 °C or 15 °C on a standard diet. Thus, absolutely no capacity for freeze tolerance at −5 °C was detected, although these larvae can survive when supercooled at the same temperature (2, 5, 6). FTR-quiescence resulted in the occurrence of freeze tolerance in some individuals; 6.3% of larvae (18 of 284) showed signs of activity at the cellular level, and one (0.35% of total) could continue developing to the adult stage.

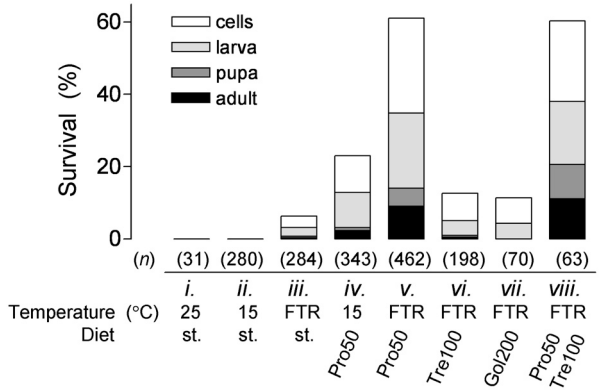

Fig. 2. Survival of 3rd instar larvae of *D. melanogaster* after freezing at −5 °C. The total heights of columns depict survival at the cellular level. Proportions of larvae, pupae, and adult survivors (of the total) are depicted using different column-filling styles. Total numbers of larvae (*n*) are in parentheses. The larvae were acclimated at different temperatures and fed different diets as specified. FTR, fluctuating thermal regime; st., standard diet; Pro50, Tre100, and Gol200, diets augmented with proline, trehalose, and glycerol, respectively. For details, see text and Dataset S2.

Synergy Between Quiescence and High Levels of Proline Is Required to Induce Freeze Tolerance. We tested proline and trehalose as potential cryoprotectants, on the basis of our earlier findings that moderate metabolic potential exists in *D. melanogaster* larvae that accumulate these compounds during cold acclimation (5). Glycerol, a typical cryoprotectant in many other insect species (26), was tested for comparison. The larvae were fed throughout larval development (first instar to fully grown third instar larvae) on diets augmented with these compounds at 15 °C or 15 °C followed by 3 d in the FTR (treatments *iv*–*viii*.). Feeding a proline-rich diet improved freeze tolerance (Fig. 2) at the cellular level (23.0% survival), but the survivors had little ability to continue developing; i.e., to pupariate (3.2%) and to metamorphose into adults (2.3%, or 8 of 343). This observation corresponds well to the situation in nondiapausing larvae of *C. costata*, where artificially increased levels of proline promote the occurrence of freeze tolerance but at the cellular and tissue levels only (20). A strong synergism between FTR-quiescence and cryopreservation by proline was observed in treatment *v*. These larvae showed high survival at the cellular level (61.0%), 14.1% of them pupariated, and 9.1% (42 of 462) metamorphosed into adults. After mating, survivors produced viable offspring. This result closely resembles the situation in *C. costata* larvae, where combination of diapause and elevated concentrations of proline support the highest survival to the adult stage after freezing (20). Feeding the trehalose-or glycerol-augmented diets had no significant effect on larval freeze

tolerance. Feeding the diet containing a mixture of proline and trehalose (treatment *viii*.) did show a clear positive effect on larval freeze tolerance; however, the magnitude of this effect did not exceed the effect of the proline diet alone.

Feeding Larvae Proline-Augmented Diet Results in Accumulation of Proline in Tissues. Using mass spectrometry-based methods, we examined the metabolomic profiles in the whole body, haemolymph, and larval tissues of *D. melanogaster*. We confirmed our previous results (5) that proline and trehalose are the two most abundant metabolites accumulating during cold acclimation (Fig. 3). Rearing larvae on proline-augmented diet significantly increased proline concentrations in whole-body extracts on the background of relatively stable concentrations in remaining 36 analytes (Dataset S3 contains detailed results). For example, proline increased from 8.1 mM in 15 °C-acclimated larvae fed a standard diet (treatment *ii*.) to 60.6 mM (i.e., 7.5-fold) in 15 °C-acclimated larvae fed a Pro50 diet (treatment *iv*.) (Fig. 3*B*). Exogenous proline penetrates through the gut wall into the haemolymph, where it probably accumulates and increases to 130.1 mM (treatment *iv*.). Body wall and fat body tissues of larvae fed a proline-augmented diet (treatment *iv*.) exhibited a 7.5-fold higher proline content than in larvae fed a standard diet (treatment *ii*.). Feeding larvae a diet augmented with $^{13}C_5$ stable isotope-labeled proline confirmed that proline penetrates into tissues (Dataset S3).

Rearing of larvae on trehalose-and glycerol-augmented diets resulted in approximately 1.3-fold and 1.6-fold increases, respectively, of these compounds in the whole-body extracts (Fig. 3*B*, compare treatments *iv*. to *vi*. for trehalose, and *iv*. to *vii*. for glycerol). This result shows that the biochemical profile of larvae is under efficient homeostatic control, which is further supported by our observation that feeding larvae proline-augmented diet had no statistically significant effects on the larval osmotic balance (Dataset S1). For instance, concentration of glycerol in the glycerol-augmented diet (1.48 M) was more than 2,600-fold higher than that in the standard diet (0.56 mM) and glycerol is expected to diffuse easily across cell membranes. Nevertheless, whole-body concentrations of glycerol increased only moderately (from 1.58 mM to 2.50 mM) in the larvae that were fed a glycerol-augmented diet. As a side effect of glycerol feeding, the concentration of trehalose in larvae increased to a maximum of 66.7 mM. These results suggest that a strategy to increase the concentration of exogenous cryprotectant via feeding on augmented diet can be successful only in the compounds for which metabolic control either does not exist or is specifically set to the "accumulation mode."

Proline as a Cryoprotectant. Proline fulfils the criteria for suitable exogenous cryoprotective compound, at least in drosophilid larvae (20). First, proline can penetrate into tissues when added to the diet (20, Dataset S3). Second, proline is under metabolic control of a distinct family of enzymes, different from generic amino acid aminotransferases and decarboxylases (27), that can be specifically regulated in response to special environmental or physiological demands (28) and, consequently, proline accumulates naturally in response to cold or drought in various organisms (20, 29, 30). Third, proline belongs to a group of compatible organic osmolytes that can be accumulated in high concentrations without exerting perturbing effects on cellular macromolecules. Proline can be up-or down-regulated to prevent changes in cellular water content in response to changing osmotic concentration in the cell environment (29, 30). Thus, high proline levels may reduce the freeze concentration of intracellular fluids during extracellular freezing (11, 31). We found that feeding the fruit fly larvae proline-augmented diet had no statistically significant

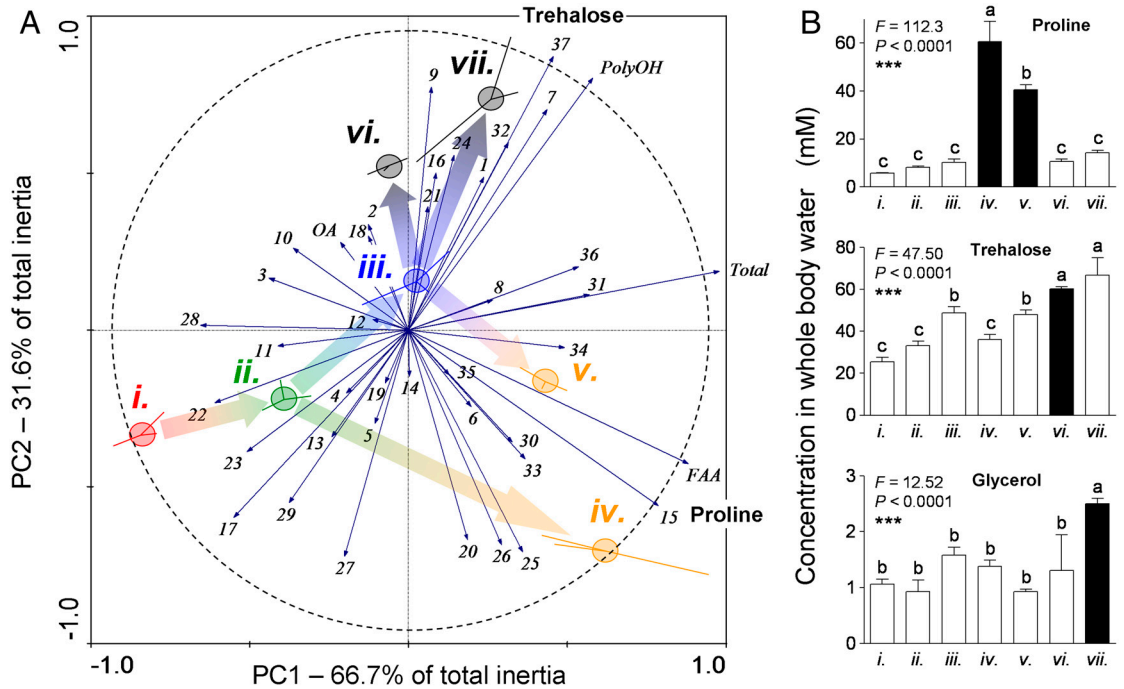

Fig. 3. Results of metabolomic analysis in 3rd instar larvae of *D. melanogaster* acclimated at different temperatures and fed different diets (treatments *i*.–*vii*.; for details, see text and Dataset S2). (*A*), Principal component analysis shows the association between treatments and the concentrations of metabolites (eigenvectors). The short lines link three independent replications to their respective centroids: colored circles. The numbers of metabolites are decoded in Dataset S3. The eigenvectors extending beyond the dashed correlation circle represent the compounds that fit the model by more than 90% (only two such compounds were found: no. 15, proline; no. 37, trehalose) (Monte Carlo test:$F = 14.898$;$P = 0.0010$). OA, total organic acids; FAA, free amino acids; PolyOH, sugars and polyols. The colored arrows depict transitions between treatments. (*B*), Three examples of metabolites. Each column represents mean ± SD of three independent replications. Influence of treatment on metabolite concentration was tested by analysis of variance followed by Bonferroni post hoc test (means indicated with different letters are significantly different). The black columns indicate that the compound under analysis was added to the respective diet: Pro50 diet was used in treatments *iv*. and *v*.; Tre100 diet in treatment *vi*., and Gol200 diet in treatment *vii*.

effects on the larval whole-body osmotic balance. The ratio of osmotically active to osmotically inactive water was constant over different treatments and the amounts of ice formed at −5 °C were similar in all treatments (Dataset 1). Further experiments are needed, with the resolution level enhanced to tissues and cells, to assess whether exogenous proline can act as compatible solute and reduce the osmotic shrinkage of larval cells below potentially damaging levels. Proline can also stabilize the native structure of protein monomers in aquaeous solutions and protect oligomeric protein complexes from denaturation and dissociation caused by low temperatures and freeze/thaw cycles (32, 33). In addition, proline molecules can intercalate between the headgroups of membrane phospholipids 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, 35).

In summary, this paper provides evidence that a complex metazoan organism with tropical origin and high sensitivity to low temperature can be converted to a freeze tolerant organism and survive after conversion of approximately half of its body water into ice at subzero temperatures. We achieved this goal in larvae of fruit fly *D. melanogaster* by using laboratory manipulations that mimicked the principles underlying high freeze tolerance in a related subarctic species, *C. costata* (20). Synergy between two fundamental principles was required: first, all potentially cold-sensitive processes linked to development were interrupted in quiescence via subjecting larvae to low temperature; and second, the tissue concentrations of the free amino acid proline were elevated by feeding larvae a proline-augmented diet. Although we cannot currently provide detailed mechanisms for this phenomenon, the most salient outcome of this study is that simple manipulations are sufficient to induce freeze tolerance in a chill-susceptible insect.

Materials and Methods

Flies, Treatments, and Freeze Tolerance Assay. We maintained a laboratory stock of fruit flies, *D. melanogaster*, Oregon strain, at 18 °C with 12 h light /12 h dark (L/D) cycle on a standard diet as described earlier (5). Experimental larvae were under treatment conditions specified in the Dataset S2, and prewandering larvae of similar physiological age (but different ages in days) were used in freeze tolerance assays.

We assayed freeze tolerance using a programmable thermostat F32-ME (Julabo) coupled with a temperature data logger TC-08 (Pico Technology). We placed 15–20 larvae inside a plastic tube (1 cm diameter, 5 cm long) on a small piece of cellulose (75 mg) moistened with 150 μL of distilled water. A similar piece of moistened cellulose was used to cover larvae and was slightly pressed, which ensured that all larvae were in a tight contact with moisture. We mounted the thermocouple between the two pieces of cellulose. The tubes were inserted into holes drilled in an aluminum block, which was situated inside the programmable thermostat. The temperature program started at −1 °C and comprised 3 steps: (*i.*) cooling to −2 °C at a rate of 0.033 °C · min^{-1} (30 min); (*ii.*) cooling to −5 °C at a rate of 0.1 °C · min^{-1} (30 min); and (*iii.*) heating to 5 °C at a rate of 0.33 °C · min^{-1} (30 min). Thus, larvae spent a total of 75 min at subzero temperatures. At the start of the program, a small ice crystal was added on the surface of the moist cellulose, which resulted in an almost immediate freezing of water and stimulated ice penetration and freezing of body fluids. At the end of freeze tolerance assay, we unwrapped the cellulose and larvae were allowed to recover for 18 h at 18 °C and were then transferred to a fresh diet. Four different levels of survival were distinguished. During transfer, the first two levels were scored: (*i.*) survival at the cellular level (uncoordinated movements and/or responsiveness to mechanical stimulation, which indicated that at least some neurons and muscle cells survived); (*ii.*) survival at the larval level (fully coordinated crawling). Next, larvae were kept at 18 °C and 12 h light/12 h dark (L/D) cycle until the age of 40 d, and their abilities to (*iii.*) pupariate and (*iv.*) emerge as adults were scored.

Osmolality, Supercooling, and Freezing. Gravimetric data (larval fresh mass, dry mass) were obtained using a Sartorius electronic balance (precision, 0.01 mg). Osmolality of haemolymph was measured using a vapor pressure osmometer Vapro 5520 (Wescor). SCP refers to the temperature at which spontaneous freezing of body water occurs during gradual cooling of an insect specimen. Osmolality and SCPs were measured as described earlier (5). The SCPs were recorded in: (*i*) the *dry* state (larvae were directly attached to thermocouples using thermally conductive paste or surrounded with dry cellulose); and (*ii*) the *wet* state (a similar arrangement as in our freeze-tolerance assays) in order to verify the occurrence of ice-nucleation of larval body fluids with surrounding ice crystals between −1 °C and −5 °C. The existence of ice in body fluids was confirmed when we recorded no typical SCPs in the *wet* state (because all larvae freeze at a much higher temperature together with surrounding water). We obtained further direct proof of ice penetration by recording freeze exotherms of individual larvae using a ThermoCam Infrared Camera FLIR P660 (Flir Systems). Five larvae of each treatment (only treatments nos. *i.–v.* were used; i.e., 25 larvae in total) were placed on the surface of wet cellulose (4 cm × 4 cm) at −0.5 °C. Next, ice crystals were added to the corners of the cellulose and the whole specimen was cooled gradually from −0.5 °C to −5 °C at a rate of 0.01 °C · min^{-1} in the programmable thermostat F32-ME (Julabo) (slow rate simulates our freeze tolerance assays). Final proof of ice-nucleation was obtained using differential scanning calorimetry (DSC) thermal analysis.

Differential Scanning Calorimetry. Thermal analyses of whole larvae were conducted on a DSC4000 calorimeter (Perkin Elmer) as described earlier (5). Two different analyses, *I.* and *II.* were run. In the analysis *I.*, hermetically sealed larva in a 500-μL aluminum test-pan was subjected to a temperature program: (*i.*) hold for 1 min at 20 °C; (*ii.*) cool to −25 °C at a rate of 1 °C · min^{-1}; (*iii.*) hold for 1 min at −25 °C; (*iv.*) and heat to 20 °C at a rate of 10 °C · min^{-1}. The analysis *II.* begins with only the reference-pan in the instrument: (*i.*) hold for 1 min at 20 °C; (*ii.*) cool to −3 °C at a rate of 5 °C · min^{-1}; (*iii.*) hold for 3 min at −3 °C; during this step, as soon as the temperature reaches −3 °C, insert into the test chamber the bottom of test-pan lined with a filter paper disc (SS-033, Wescor), to which 10 μL distilled water was previously applied, and then submerged in liquid nitrogen to freeze it. After the bottom of test-pan equilibrates back to the program temperature of −3 °C (approximately within 10 s), add larva, cover the bottom of test-pan with lid and continue running the program. Next, (*iv.*) cool to −5 °C at a rate of 0.01 °C · min^{-1} (slow rate simulates our freeze tolerance assays); (*v.*) cool to −25 °C at a rate of 5 °C · min^{-1}; (*vi.*) hold for 1 min at −25 °C; (*vii.*) and heat to 20 °C at a rate of 10 °C · min^{-1}. In the analysis *I.*, all larvae froze spontaneously at a temperature corresponding to their SCP, while in the analysis *II.*, freezing of larval body fluids occurred by contact with ice crystals in the bottom of test-pan at temperatures slightly below −3 °C. The amount of frozen, osmotically *active* (OA) water was calculated from the area under the melt endotherm (*I.*) or freeze exotherm (*II.*) using the value of 334.5 J · g^{-1} for the enthalpy of water. The amount of unfrozen, osmotically *inactive* (OI) water was determined by subtracting the OA water from the total water mass (calculated from gravimetric data).

Metabolomic Profiling. For metabolomic analyses, we collected haemolymph in a calibrated capillary and dissected the following tissues: fat body (approximately 90% of fat body tissue was 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 compare metabolite levels in whole body and haemolymph, we recalculated the whole-body concentrations to mmol · L^{-1} of whole-body water (i.e., mM). The analyses were performed as described earlier (5, 20). Briefly, 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). We obtained additional metabolite profiles using a combination of GC/MS and liquid chromatography/MS techniques in the same ethanolic extracts after their treatment with ethyl chloroformate under pyridine catalysis and simultaneous extraction in chloroform.

Statistical Analyses. A nonparametric Kruskal-Wallis test or parametric one-way analysis of variance (ANOVA) was used to determine if the treatments influenced physiological and biochemical parameters. A Dunn or Bonferroni post hoc test separated the treatment means. The analyses were performed using Prism v. 4 (GraphPad Software). The complex association of metabolomic changes with treatments was determined by principal component analysis (PCA) using Canoco v. 4.52 for Windows (Biometris-Plant Research International).

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