© 2016. Published by The Company of Biologists Ltd | Journal of Experimental Biology (2016) 219, 2358-2367 doi:10.1242/jeb.142158

RESEARCH ARTICLE

Arginine and proline applied as food additives stimulate high freeze tolerance in larvae of *Drosophila melanogaster*

Vladimír Koštál‡, Jaroslava Korbelová, Rodolphe Poupardin*, Martin Moos and Petr Šimek

ABSTRACT

The fruit fly *Drosophila melanogaster* is an insect of tropical origin. Its larval stage is evolutionarily adapted for rapid growth and development under warm conditions and shows high sensitivity to cold. In this study, we further developed an optimal acclimation and freezing protocol that significantly improves larval freeze tolerance (an ability to survive at −5°C when most of the freezable fraction of water is converted to ice). Using the optimal protocol, freeze survival to adult stage increased from 0.7% to 12.6% in the larvae fed standard diet (agar, sugar, yeast, cornmeal). Next, we fed the larvae diets augmented with 31 different amino compounds, administered in different concentrations, and observed their effects on larval metabolomic composition, viability, rate of development and freeze tolerance. While some diet additives were toxic, others showed positive effects on freeze tolerance. Statistical correlation revealed tight association between high freeze tolerance and high levels of amino compounds involved in arginine and proline metabolism. Proline- and arginine-augmented diets showed the highest potential, improving freeze survival to 42.1% and 50.6%, respectively. Two plausible mechanisms by which high concentrations of proline and arginine might stimulate high freeze tolerance are discussed: (i) proline, probably in combination with trehalose, could reduce partial unfolding of proteins and prevent membrane fusions in the larvae exposed to thermal stress (prior to freezing) or during freeze dehydration; (ii) both arginine and proline are exceptional among amino compounds in their ability to form supramolecular aggregates which probably bind partially unfolded proteins and inhibit their aggregation under increasing freeze dehydration.

KEY WORDS: Fruit fly, Diet augmentation, Cold tolerance, Metabolomics, Cryoprotectant, Compatible solute, Preferential exclusion, Protein aggregation

INTRODUCTION

Organisms as diverse as bacteria, plants and animals are known to naturally accumulate small protective molecules in response to various environmental stressors (recently reviewed in Yancey and Siebenaller, 2015). These low molecular weight metabolic products are often referred to as organic compatible osmolytes, as they were first described in organisms in high-salinity environments. It was later recognized that similar compounds convergently evolved in most forms of life, and have a variety of protective functions (reviewed in Yancey et al., 1982). The mechanisms by which protection is achieved might be different for different molecules and under different environmental contexts such as osmotic stress, dehydration, thermal stress and barometric stress. Therefore, the generic term 'cytoprotective molecules' is preferable to 'compatible osmolytes' (Yancey and Siebenaller, 2015). According to Yancey (2005), the cytoprotective properties fall into two broad categories: (1) metabolic protection (antioxidation, redox balance maintenance, detoxification) and (2) stabilization of macromolecules, which is based on specific and diverse interactions between the molecules of solvent (water), solute (cytoprotectant) and a protected structure (protein, nucleic acid, lipid bilayer). Chemically, cytoprotectants fall into a few classes: polyols and sugars, free amino acids and their derivatives, methylamines and urea (Yancey et al., 1982).

The accumulation of small cytoprotective molecules is also one of the hallmarks of insect adaptation to cold (Salt, 1961; Somme, 1982; Zachariassen, 1985; Lee, 2010). In the insect cold-tolerance literature, these molecules are called 'cryoprotectants' and are represented by sugars and polyols (Storey and Storey, 1988, 1991), and free amino acids (Koštál et al., 2011a). The specific roles of cryoprotectants may vary with cold-tolerance strategy: supercooling, freezing or cryoprotective dehydration (Storey and Storey, 1988). In this paper, we will focus specifically on freeze tolerance (i.e. the capacity for survival after formation of ice crystals in extracellular fluids). Extracellular freezing is inevitably accompanied by osmotically driven cell dehydration. The general role of accumulated cryoprotectants is to reduce the osmotic outflow of water from cells, i.e. reduce the cell shrinkage (Meryman, 1971; Storey and Storey, 1988). Dehydration, however, also results in increased acidity, increased metal ion concentrations, increased viscosity and tight packing of macromolecules, which, together with thermal stress, threaten the chemical and conformational stability of proteins and other macromolecules (Wang, 1999; Brovchenko and Oleinikova, 2008). Another role for cryoprotectants is to assist in protection/recovery of the functional native state of macromolecules or biological membranes during freezing stress (Timasheff, 1992; Wang, 1999, 2005; Bolen and Baskakov, 2001).

Diet augmentation is a simple method that allows the levels of certain cryoprotectants in the insect body to be artificially increased. The augmentation of artificial larval diet with the amino acid proline proved highly successful in stimulation of freeze tolerance in the larvae of the fruit fly *Drosophila melanogaster* in our previous experiments (Koštál et al., 2012). Acquisition of freeze tolerance came as a surprise in this insect species considering its tropical evolutionary origin, ecological adaptation to warm climate and relatively high susceptibility to chill injury (Strachan et al., 2011; Koštál et al., 2011b). Natural accumulation of proline has been observed in various insects during cold acclimation (Storey et al., 1981; Morgan and Chippendale, 1983; Fields et al., 1998; Koštál

Biology Centre CAS, Institute of Entomology, České Budějovice 37005, Czech Republic.
*Present address: Institut für Populationsgenetik, Vetmeduni Vienna, Vienna, Austria.

‡Author for correspondence (kostal@entu.cas.cz)

V.K., 0000-0002-4994-5123

Received 20 April 2016; Accepted 25 May 2016

Journal of Experimental Biology

Journal of Experimental Biology (2016) 219, 2358-2367 doi:10.1242/jeb.142158

et al., 2011b). We have also demonstrated a critical role of proline accumulation in the acquisition of high freeze tolerance, including the capacity for cryopreservation in liquid nitrogen, in the larvae of the drosophilid fly *Chymomyza costata* (Koštál et al., 2011a). The positive effect of proline augmentation on insect cold tolerance has since also been confirmed in the wasp parasitoid *Nasonia vitripennis* that was developing in a fly host, *Sarcophaga crassipalpis*, the larvae of which were reared on a proline-augmented diet (Li et al., 2014). In this case, however, metabolomic analysis revealed that the effect of the proline-augmented diet was mediated indirectly by the metabolic perturbations associated with diet supplementation rather than by directly changing proline levels in the *N. vitripennis* body (Li et al., 2014). The study on *N. vitripennis* exemplifies our insufficient knowledge on the mechanisms by which diet augmentations influence insect cold tolerance or, more broadly, environmental stress tolerance.

The fruit fly model has been used to examine how diet composition affects environmental stress tolerance in several studies. For instance, diet augmentation with cholesterol increased the amount of cholesterol in the cell membranes of adult *D. melanogaster* flies and significantly increased survival after a 2 h cold shock at −5°C (Shreve et al., 2007). In other studies, adult fruit flies that developed on a casein-enriched diet had increased heat and desiccation tolerance, while those supplied at the larval stage with a sucrose-enriched diet showed faster recovery from chill coma (Andersen et al., 2010; Sisodia and Singh, 2012). Other authors (Colinet et al., 2013) tried to build on these observations and gradually augmented the concentrations of four different sugars (sucrose, fructose, glucose and trehalose) in the fruit fly larval diet. These diet augmentations, however, induced metabolic alterations that had no or negative effects on adult fruit fly cold tolerance. Supplementation of the adult diet with live yeast elevated the amount of several amino acids (valine, isoleucine, leucine, threonine, glycine, phenylalanine and glutamate) in adult fruit flies, while the amount of several sugars (fructose, glucose and trehalose) decreased (Colinet and Renault, 2014). Importantly, the supplementation of the adult diet with live yeast greatly promoted cold tolerance: chill coma recovery time decreased, while survival after acute and chronic cold stress increased (Colinet and Renault, 2014). Collectively, the results of earlier studies convincingly show that augmenting the diet with various compounds is a powerful tool to alter insect cold tolerance. Nevertheless, the observations collected so far are rather anecdotal. The translation of dietary augmentation to an altered metabolomic composition of the organism, and the mechanisms by which such a change would affect the insect's cold tolerance, remain poorly understood.

The main aim of this study was to build on our earlier experiments on the effect of diet augmentation with proline on larval freeze tolerance in *D. melanogaster* (Koštál et al., 2012) and construct an extended data matrix to provide insight into correlations between diet augmentation with various compounds, metabolomic profiles of larvae and larval freeze tolerance. We fed larvae diets augmented with 31 different amino compounds in different concentrations, and screened their effects on larval viability, rate of development and, mainly, freeze tolerance (ability to survive at −5°C when most of the fraction of osmotically active, freezable water is converted to ice). We identified 14 compounds with a positive effect on larval freeze tolerance. Other compounds exhibited toxic effects. Analysis of metabolomic composition was conducted in larvae that were fed 17 different compounds (showing both positive and toxic effects). The combined results of extensive diet augmentation experiments and the correlation analysis of metabolomic profiles point towards a prominent role of arginine and proline metabolism in promoting high freeze tolerance. The potential mechanisms by which arginine, proline and other compounds might stimulate high freeze tolerance are discussed.

MATERIALS AND METHODS

Insects

All experiments were conducted with Oregon R strain of *Drosophila* (*Sophophora*) *melanogaster* (Lindsley and Grell, 1968). The stock insects were maintained in glass tubes (12 cm high, 2.5 cm in diameter) at a constant 18°C on a 12 h:12 h light: dark cycle in MIR 154 incubators (Sanyo Electric, Osaka, Japan). Each tube contained 5–10 g of a standard diet composed of agar (1%), sugar (5%), yeast (4%), cornmeal (8%) and methylparaben (0.2%). A more detailed composition of the major amino acids, organic acids, sugars and polyols in the standard diet is published elsewhere (Koštál et al., 2012).

In order to obtain synchronously developing cohorts of larvae for experiments, approximately 50 pairs of flies (25 females) were transferred to tubes for egg laying for 24 h at the conditions specified above. Females oviposited onto standard or augmented diets. Embryos were then transferred to cold-acclimation conditions (constant 15°C with a 12 h:12 h light:dark cycle) and reared until the appearance of the first wandering larvae. Next, wandering larvae were removed and the tube with remaining larvae was transferred to the fluctuating thermal regime (FTR; 20 h at 6°C:4 h at 11°C under constant darkness) for 3 days, which induced a low-temperature quiescence (see Fig. S1 and Koštál et al., 2011b, for more details). These procedures ensured, first, that all larvae were sampled at a similar ontogenetic stage (fully grown third instars prior to wandering) and, second, that the larvae attained a maximum level of cold tolerance via cold acclimation and FTR-induced quiescence (Koštál et al., 2012).

Optimal freezing protocol and freeze-tolerance assay

In order to optimize the freezing protocol prior to performing the extensive diet-augmentation experiment, we used larvae reared on two different diets: standard diet and Pro50 (see below for an explanation of the abbreviation). The larvae were exposed to various freezing protocols where rates and durations of cooling/warming/incubation steps were modified using a programmable Ministat 240 cooling circulator (Huber, Offenburg, Germany). The complete list and description of all protocols are given in Table S1. All manipulations (washing out of diet, transferring to test tubes) with larvae prior to the start of the freezing protocol were standardized in terms of time (20 min) and temperature (0°C) in order to minimize any potential effects on larval freeze tolerance. Groups of approximately 20 larvae were wrapped between two layers of cellulose (75 mg) that was moistened with 300 µl of distilled water. The moist cellulose 'ball' with larvae inside was placed in the plastic tube (diameter, 1 cm; length, 5 cm), and placed in the cooling circulator. The temperature inside the cellulose ball was continuously monitored in control tubes (containing no larvae) by using the K-type thermocouples connected to a PicoLog TC-08 datalogger (Pico Technology, St Neots, UK). At the start of each protocol, a small ice crystal was added to the surface of the moist cellulose, which resulted in early freezing of water and stimulated ice penetration and inoculative freezing of larval body fluids (Koštál et al., 2012). At the end of the protocol, the unpacked cellulose balls were transferred on to fresh standard diet in a tube maintained at a constant 18°C. Live/dead larvae were scored after 12 h recovery. All

Journal of Experimental Biology (2016) 219, 2358-2367 doi:10.1242/jeb.142158

living larvae were maintained at 18°C for the subsequent 14 days and successful pupariation and emergence of adult flies were scored as ultimate criterions of survival. Exact numbers of larvae used for each specific experiment are shown in Results. Next, the optimal freezing protocol was applied routinely in screening experiments with diet augmentations.

Diet augmentations

Amino compounds were added to standard diet in concentrations ranging from 1 to 200 mg compound g^{-1} diet. All isomeric amino acids were applied in their L-form, except proline, where both L- and D-forms were assayed (no isomerism exists in glycine). Therefore, the isomeric form will be specifically mentioned only in case of D-proline. All augmentations are abbreviated as the amino compound and associated concentration in the diet, e.g. Arg25 is the abbreviation for arginine-augmented diet at a dose of 25 mg g^{-1} standard diet.

First, we tested whether diet augmentation with proline causes significant improvement of larval freeze tolerance when the optimized freezing protocol is applied (see Table S2 for details). Next, larvae were fed diets augmented with 31 different compounds. All compounds were purchased from Sigma (St Louis, MO, USA) and applied in different doses ranging from 1 to 200 mg g^{-1} of standard diet (see Table S3 for a complete list of compounds and doses). Pre-weighed standard diet was liquefied in the insect-rearing tube by using several brief microwave pulses (5 s at 700 W, 3–4 times). The augmented compound in powder form was applied on top of the liquefied diet and mixed very thoroughly using a glass spatula. The tubes with augmented diets were allowed to cool overnight at room temperature before adults were added to the tubes (see above). Different diets/doses caused developmental delays and larval mortality. These two effects were roughly categorized as: (i) no delay (12–13 days until wandering) associated with no or low mortality; (ii) slight delay (14–19 days until wandering) associated with low or medium mortality; (iii) long delay (≥20 days until wandering) associated with high mortality; (iv) inability to proceed to wandering stage associated with 100% mortality.

Fully grown third instar larvae were sampled either for freeze-tolerance assays (see above) or for metabolomic analysis. Exact numbers of larvae used for each specific assay are given in Results.

Metabolomic analysis

We first performed a non-targeted metabolomic experiment using larvae that were fed six selected compounds (proline, arginine, glutamine, glutamate, ornithine and putrescine) involved in the metabolism of proline and arginine – two amino acids that emerged as the most potent enhancers of freeze tolerance. We fed larvae the diets augmented with specific non-toxic doses of the compounds which produced highest survival in our screening assay of diet augmentations. Standard diet-fed larvae were used as the control. The non-targeted analysis of major metabolites employed a combination of three mass spectrometry (MS)-based analytical methods as described previously (Koštál et al., 2011b). Whole larvae (pool of five larvae in five replications from each treatment) were homogenized and extracted in 70% ethanol. Low molecular weight sugars and polyols were quantitatively determined in the ethanolic extracts after *o*-methyloxime trimethylsilyl derivatization using a GC-FID-2014 gas chromatograph (GC) with flame ionization detector equipped with an AOC-20i autosampler (both from Shimadzu Corporation, Kyoto, Japan). Profiling of acidic metabolites was done in the same ethanolic extracts after treatment with ethyl chloroformate under pyridine catalysis and simultaneous extraction in chloroform (Hušek and Šimek, 2001; Koštál et al., 2011b) using Trace 1300 GC with single quadrupole mass spectrometry (ISQ-MS) (both from Thermo Fisher Scientific, San Jose, CA, USA) and liquid chromatography performed on an Accela LTQ XL with linear ion trap combined with Q Exactive Plus high-resolution mass spectrometer coupled with Dionex Ultimate 3000 (all from Thermo Fisher Scientific). The metabolites were identified against relevant standards and subjected to quantitative analysis by using an internal standard calibration method. All standards were purchased from Sigma-Aldrich.

In a second metabolomic experiment, we fed larvae diets enriched with 17 different amino acids supplied in non-toxic doses (see Table S4 for details) and sampled fully grown third instars for targeted analysis of basic animal amino acids using the methods described above. We used pools of five larvae in three replications from most treatments except Arg25, Ser25, His25 and Lys25, where only one replicate of five larvae was analysed because of high larval mortality. The metabolomic dataset resulting from targeted analysis was subjected to statistical analysis. First, we performed an unconstrained principal component analysis (PCA) using Canoco v5.04 for Windows (Biometris-Plant Research International, The Netherlands, and Petr Šmilauer, Czech Republic). For PCA analysis, the concentrations of metabolites were log-transformed [using a $\ln(100\times y+1)$ formula] and then centred and standardized. PCA identifies metabolite clustering effects according to the experimental treatments (diet augmentations). Next, we applied Pearson correlation analysis conducted using CorrPlot and Hmisc libraries on R software (https://www.R-project.org/) in order to identify metabolites and their concentrations that are positively or negatively correlated with larval freeze tolerance and to identify groups of associated metabolites exhibiting correlated changes in concentration in response to diet augmentations.

RESULTS

Optimized protocol ensures high survival in freeze-tolerance assay

We assessed freeze tolerance in FTR-acclimated *D. melanogaster* larvae that were subjected to 14 new variants of freezing protocol derived from the original freezing protocol described in Koštál et al. (2012). A detailed summary of the results is presented in Table S1. Adding two new steps, namely pre-incubation for 10 min at −0.5°C (step i) and melting for 10 min at +5°C (step v), had no significant influence on survival rates in comparison to the original protocol. Survival to adult stage following the original protocol was 0.7% in standard diet-fed larvae or 5.1% in Pro50-fed larvae; this increased considerably to 13.0% and 19.0%, respectively, when the pre-incubation step (i) was extended to 180 min; similarly, survival increased to 12.6% and 42.1%, respectively, after extending the slow cooling step (ii) to 180 min. Neither additional extension of step ii to 360 min nor other manipulations with the later steps (iii and iv) resulted in significant improvement of survival. Because it produced the highest adult survival, we considered the protocol with step ii extended to 180 min as 'optimal'.

A graphical comparison of the original and new (optimal) freezing protocols is shown in Fig. 1A. In most experiments, we applied the thermocouples only to separate temperature-control tubes containing no larvae as the thermocouples complicated manipulations with larvae. In a preliminary experiment, however, we inserted eight thermocouples into eight tubes, each containing six standard diet-fed larvae positioned in close proximity to the thermocouple. Fig. 1B depicts an example temperature record in one

Journal of Experimental Biology

Journal of Experimental Biology (2016) 219, 2358-2367 doi:10.1242/jeb.142158

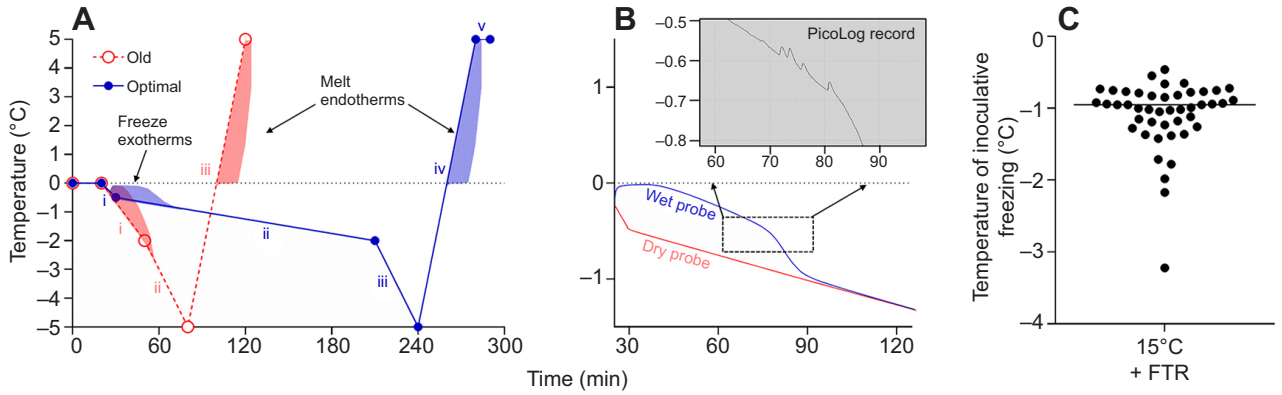

Fig. 1. Optimal protocol for *Drosophila melanogaster* larval freeze-tolerance assay. (A) Schematic representation of the original (Koštál et al., 2012) and optimal freezing protocols. Protocols consist of steps i–v that are specified in terms of temperature, ramping time and duration in Table S1. The coloured areas represent changes of temperature linked to the release or absorption of heat during freezing (freeze exotherms) or melting (melt endotherms) of water or ice, respectively, in the cellulose ball surrounding the larvae (see Materials and methods for details). (B) Schematic view of the optimal protocol during the early part of step ii, and an example of a real temperature record from the PicoLog TC-08 datalogger (inset, taken from the boxed region) showing four small exotherms probably representing larval ice nucleation and body fluid-freezing events. (C) Distribution of larval freeze exotherms (temperatures of larval body water crystallization) observed during freeze-tolerance assays. FTR, fluctuating thermal regime. The horizontal line shows the median value of −0.95°C; the spread of the data points along the horizontal axis is used only to enhance their resolution.

of these tubes, showing detectable freeze exotherms of individual larvae inside the frozen cellulose ball. Running the optimal freezing protocol in this preliminary experiment, we recorded 45 (of 48 possible) larval freeze exotherms with a median of −0.95°C and range from −0.5 to −3.2°C. Only two exotherms occurred below −2.0°C (−2.2 and −3.22°C; Fig. 1C). These results suggest that larval body fluids start freezing upon inoculation by external ice crystals at temperatures ranging between −0.5 and −2.0°C in most individuals.

Next, we verified that a synergy between FTR-induced quiescence and diet augmentation with proline is required in order to stimulate the highest levels of larval freeze tolerance (Fig. 2; Table S2A). Control larvae had no ability to pupariate and reach adult stage after being frozen using the optimized protocol even to a moderate target temperature of −2.5°C. Either quiescence or proline augmentation alone induced only partial improvements of freeze tolerance. Synergy of quiescence and proline augmentation ensured 90% larval survival, 61% pupariation and 42% emergence of adults after freezing to −5°C (Table S2A).

Some diet augmentations, especially arginine and proline, significantly improve freeze tolerance

The detailed results of diet augmentation experiments using 31 different compounds are summarized in Table S3 and the most important data are presented in Fig. 3. We considered the 1.5-fold (and 3-fold) increases of adult survival as an arbitrary threshold for a mild positive (and highly positive) effect in our freeze-tolerance assays. Only two diets, Arg25 and Pro50, showed highly positive effects, increasing adult survival in the freeze-tolerance assay from 12.6% (control, standard diet) to 50.6% and 42.1%, respectively. A number of compounds showed mild positive effects on freeze tolerance when administered at a specific dose: ectoine50 (37.0%), Val25 (33.1%), Ile50 (33.0%), Asp50 (33.0%), Ala50 (31.7%), Leu25 (30.0%), Asn100 (24.0%), Thr10 (24.7%), Gln100 (23.8%), Glu50 (21.0%), 6-oxo-pipecolinic acid (20.0%) and Tyr50 (19.0%). In most cases, the effect was hormetic-like: relatively low doses had a positive influence on freeze tolerance, while higher doses were toxic (Table S3). Other compounds showed prevailingly toxic effects, manifested as long delays in larval development, usually accompanied by significant developmental desynchronization of individual larvae, and high or absolute larval mortality on augmented diets (Table S3; Fig. 3).

For two diet augmentations, Pro50 and Arg25, we assessed their capacity to decrease the lower temperature limit for freeze tolerance. Although limited adult survival was observed at −7.5°C (2.5% in Pro50; 5.9% in Arg25), no adults were formed after freezing the larvae to −10.0°C (Table S2A,B).

Metabolomic analysis reveals positive correlation between freeze tolerance and body concentration of proline, arginine and metabolically related amino acids

Detailed results of non-targeted metabolomic analysis (45 major metabolites) and targeted amino acid analysis (20 animal protein amino acids plus ornithine, Orn) are summarized in Table S4. Generally, and not surprisingly, the compounds used for diet

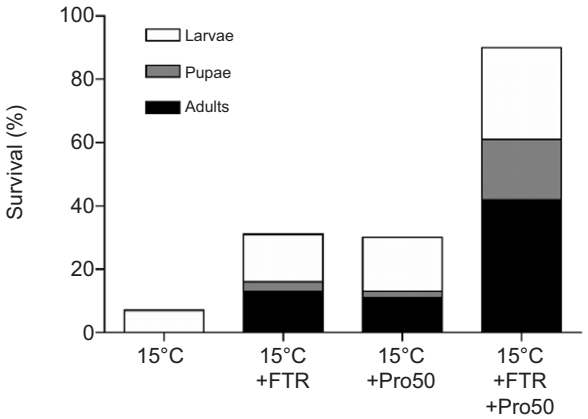

Fig. 2. Synergy between acclimation to quiescence and diet augmentation with proline. Survival of *D. melanogaster* larvae subjected to a freeze-tolerance assay using the optimal freezing protocol (see Fig. 1) with a target temperature of −5°C. Survival was scored at three developmental stages – larva, pupa and adult (only a fraction of surviving larvae formed puparia and, subsequently, a fraction of pupae emerged as fit adults) – for cold acclimation (15°C), quiescence (FTR), diet augmentation (Pro50, 50 mg proline g^{-1} standard diet) and the synergy of quiescence and proline augmentation. A detailed dataset showing survival at other target temperatures (−2.5, −7.5, −10.0°C) is presented in Table S2.

Journal of Experimental Biology (2016) 219, 2358-2367 doi:10.1242/jeb.142158

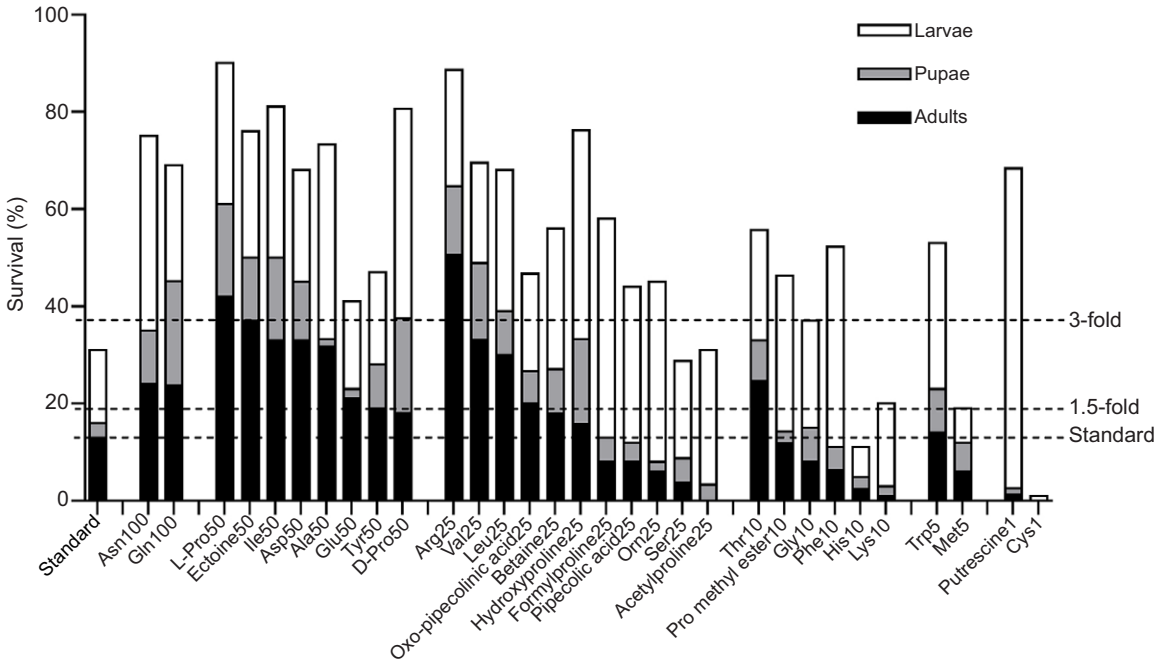

Fig. 3. The effect of diet augmentation on *D. melanogaster* larval freeze tolerance. Thirty one different amino compounds were used to augment the larval diet. Larval freeze tolerance was assessed using the optimal freezing protocol (see Fig. 1). The figure shows the most important data; for each column, survival was scored at three developmental stages (larva, pupa and adult) when the larvae were fed the optimum dose of augmented compound, i.e. the dose showing the highest stimulation of freeze tolerance (responses to other doses, numbers of larvae and all other details are given in Table S3). The three dashed horizontal lines mark three levels of survival (to adult stage): standard level found in the larvae fed standard (control, non-augmented) diet; 1.5-fold higher than control; and 3-fold higher than control.

augmentation were often detected in higher concentrations in larval bodies (see red-highlighted values in Table S4). For instance, the concentration of valine was as high as 73.9 nmol mg^{-1} fresh mass (FM) in the larvae fed Val25 diet, which is a 67-fold higher concentration those fed the standard diet (1.1 nmol mg^{-1} FM). Most of our diet augmentations resulted in similar 'direct effects', the magnitude of which ranged from up to 2-fold (tyrosine, histidine, glutamate) to 2- to 5-fold (glycine, serine, arginine, asparagine, lysine, aspartate), 8-fold (proline), 10- to 20-fold (leucine, isoleucine) and 67-fold (valine) (Table S4). We only observed no response in the larval body levels of an amino acid following its augmentation in one case: alanine augmentation (non-significantly) reduced larval alanine concentrations 0.9-fold. Alanine, however, showed a strong 'indirect effect', elevating the concentration of glutamine in larval bodies more than 3-fold, from 21.0 nmol mg^{-1} FM to 64.3 nmol mg^{-1} FM. Other compounds showed a similar indirect effect, increasing the concentration of glutamine in a range from 1.5- to 2.5-fold. Among the other remarkable indirect effects, Arg25 diet caused more than a 26-fold elevation of ornithine and a 2- to 4-fold elevation of proline; and the Orn25 diet caused a 2-fold increase of proline concentration in the larval body.

Fig. 4 presents results of statistical analysis of the changes in metabolomic composition caused by augmented larval diets. PCA analysis (Fig. 4A) clustered together the Arg25, Pro50 and Orn25 diets (dark blue ellipse), which are loaded by eigenvectors of arginine, proline, ornithine and also cysteine and glycine (the long eigenvectors are associated with high levels of metabolites in the larval body). These three diets are further characterized by negative eigenvectors (low levels) for aspartate. Several diets that caused mild positive effects on freeze tolerance in the feeding experiment tended to form a loose cluster characterized by a negative PC1 score. These diets are associated with high levels of glutamine and aspartate and also with low levels of toxic compounds such as tryptophan and phenylalanine in the larval body. The toxic diets His25, Lys25, Ser25, Orn25 and Putrescine1 form another loose cluster associated with high levels of toxic metabolites countered with low levels of glutamine and, with the exception of Orn25 diet, also with relatively low levels of highly positive metabolites (arginine and proline).

Fig. 4B presents a data matrix resulting from Pearson correlation analysis. Statistically significant positive correlations (blue) were found between survival in the freeze tolerance assay and high larval body concentrations of arginine, proline, glutamine and ornithine. In addition, survival positively correlated with the total sum of concentrations of those amino acids that showed a positive (hormetic) effect on freeze tolerance in our feeding experiment. In contrast, negative correlations (red) were found between survival and concentrations of tyrosine, histidine, lysine, tryptophan and the sum concentration of amino acids with toxic effects in our feeding experiment.

DISCUSSION

In this study, we confirmed our earlier observation (Koštál et al., 2012) that synergy between FTR-induced quiescence and diet augmentation with proline is required in order to stimulate the highest levels of freeze tolerance in *D. melanogaster* larvae (Fig. 2; Table S2). In contrast to our earlier study, however, we found that a proline-augmented diet is not a necessary pre-requisite for acquisition of freeze tolerance. Using the optimized freezing protocol, we found that the induction of quiescence was sufficient to stimulate moderate freeze tolerance even in the fruit fly larvae fed on standard diet. Our results thus demonstrate the critical importance of the optimization of the freezing protocol for correct

Journal of Experimental Biology

Journal of Experimental Biology (2016) 219, 2358-2367 doi:10.1242/jeb.142158

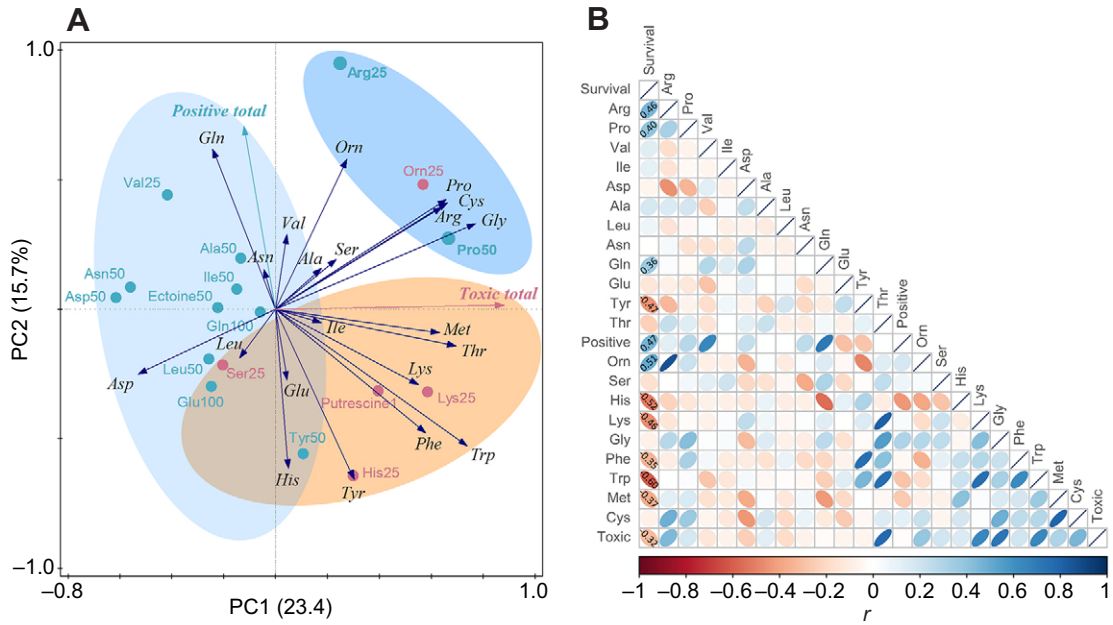

Fig. 4. Statistical analysis. Data from targeted metabolomic analysis of amino acids were subjected to principal component analysis (PCA) and Pearson correlation analysis. (A) PCA shows clustering of treatments (diet augmentations) in a 2D space delimited by the first two principal components (PC1 and PC2), which together explained 39.1% of variation in the dataset. Diets stimulating larval freeze tolerance in feeding experiments are blue, while toxic diets are red. Eigenvectors (arrows) representing individual amino acids are colour-coded analogously. The vectors 'Positive total' and 'Toxic total' represent the sum concentrations of all amino acids with positive and toxic effects, respectively, in our feeding experiments. Three loose clusters are highlighted by arbitrarily driven ellipses: the dark blue ellipse depicts the cluster of Arg25, Pro50 and Orn25 diets; the pale blue ellipse surrounds diets causing mild stimulation of freeze tolerance; and the orange ellipse is drawn to highlight mostly toxic diets. (B) The coloured scale codes for the value of the Pearson correlation coefficient *r*. Positive correlations are blue while negative correlations are red (selected *r* coefficients are shown in the first column for clarity and all details are summarized in Table S4). Survival to adult stage in the freeze-tolerance assay is used as one of the correlates. 'Positive' represents the sum of concentrations of all amino acids (from Arg to Thr) that stimulated larval freeze tolerance when used as diet additives. 'Toxic' represents the sum of concentrations of all amino acids (from Orn to Cys) that caused toxicity when used as diet additives. Detailed results of targeted and non-targeted metabolomic analyses (together with the original Pearson correlation matrix) are presented in Table S4.

categorization between freeze tolerance and intolerance. A slow freezing rate was previously found to be essential for freeze tolerance in several insect species (Miller, 1978; Lee and Lewis, 1985; Shimada and Riihimaa, 1988; Bale et al., 1989). Slow freezing in extracellular compartments is believed to limit the osmotic stress on cells as it allows sufficient time for the redistribution of ions and soluble metabolites including potential cryoprotectants and permits controlled shrinkage of the cell membranes during the gradual freeze dehydration of the cell (Storey and Storey, 1988). A slow rate of cooling was also essential in our experiments. The larvae were highly sensitive to cooling rate, especially during the phases of ice nucleation and initial ice crystal growth inside the larval body, which occurred at temperatures between −0.5 and −2.0°C (step ii). We varied the cooling rate during step ii from 0.05°C min^{-1} (30 min) to 0.004°C min^{-1} (360 min), which produced a dramatic difference in survival to adult stage from 0.7% to 14.6% with the standard diet and from 5.1% to 41.1% with the Pro50 diet. A medium–slow cooling rate of 0.008°C min^{-1} (180 min) also produced high survival (12.6% and 42.1% on the standard and Pro50 diet, respectively), and therefore we decided to use this cooling rate routinely in our optimized freezing protocol (Table S2).

Arginine, proline and their metabolic associates show the highest potential for improvement of larval freeze tolerance

In this study, we did not assess whether and how much the augmented compound is stable in the diet, and whether and how much it moves from the larval gut to haemolymph, organs and cells. Instead, we applied an extensive screening approach assaying high numbers of larvae, in many replicates, exposed to a high number of compounds, administered in different doses. Most compounds, however, exerted pronounced effects on larval development, mortality and freeze tolerance (Table S3), which itself was taken as an indication of the ability of the assayed compounds (or their degradation products) to penetrate into the larval body and to interfere with the insect metabolic system. In our earlier study, we directly proved that ^{13}C-labelled proline molecules move from diet to tissues (Koštál et al., 2012). In the current study, analysis of the metabolomic composition of larvae supplied another piece of evidence supporting the movement from diet to the body: most of our diet augmentations caused either direct effects (increasing body concentrations of the specific compound that was used for augmentation) or indirect effects (perturbations affecting the concentrations of the other compounds).

The statistical analysis of the correlation matrix consisting of survival data and metabolomic data (Fig. 4B) does not consider which compound was augmented in the diet, only the resulting body concentrations of different amino acids. The results of freeze-tolerance assays (Fig. 3) and the results of statistical correlation analysis (Fig. 4B) were broadly similar: the amino acids proline and arginine showed the highest potential for improving larval freeze tolerance. The metabolic pathways of proline and arginine are interlocked in animals (Wu and Morris, 1998; Phang et al., 2015). Briefly, both amino acids are synthesized from glutamine and glutamate precursors via a short-lived metabolic intermediate, pyrroline-5-carboxylate (P5C). Via P5C, arginine can also be

Journal of Experimental Biology (2016) 219, 2358-2367 doi:10.1242/jeb.142158

converted to proline, but not vice versa. Both proline and arginine can be either reconverted back to glutamate and glutamine or degraded via ornithine to various polyamines such as putrescine (Wu and Morris, 1998; Phang et al., 2015). We used the above-mentioned metabolic associates of proline and arginine (except P5C) in our diet augmentation experiments. We also conducted relevant metabolomic analyses and observed various direct and indirect effects (see Results) documenting that these metabolites are intimately interlocked. For instance, proline and arginine, the two diet additives with the highest potential to increase larval freeze tolerance, showed the following direct and indirect effects: the Pro50 diet resulted in an 8-fold increase of proline concentration in the larval body and also slightly affected glutamine levels, increasing them 1.4-fold; the Arg25 diet caused a 2.2- to 3.7-fold elevation of arginine levels and also increased the levels of proline (2.0- to 3.3-fold), glutamine (2.0- to 2.2-fold) and ornithine (26.2- to 29.3-fold).

In addition to arginine and proline, correlation analysis identified glutamine and ornithine as other tight statistical associates of high freeze tolerance. In the case of glutamine, this result of the correlation analysis is in good agreement with the result of the freeze-tolerance assay on the larvae fed a glutamine-augmented diet. The body concentration of glutamine was directly sensitive to diet augmentation with glutamine (increased 2.5- to 2.8-fold). However, glutamine was also increased indirectly via diet augmentations with almost any other amino compound except tyrosine, histidine and putrescine. For instance, diet augmentation with alanine had no effect on alanine body concentration but it increased the concentration of glutamine 3-fold (Table S4). The positive statistical correlation between high freeze tolerance and high levels of ornithine appears more problematic. No positive effect (rather, a weak toxicity) was seen when we directly (via Orn25 diet) increased the concentration of ornithine from 0.6 to 6.3 nmol mg^{-1} FM. The positive statistical correlation between high ornithine levels and high freeze tolerance seems to be mainly driven by very high ornithine levels (14.6–16.7 nmol mg^{-1} FM) induced by the Arg25 diet, the positive influence of which (probably mediated by high body levels of arginine, proline and glutamine) slightly outweighed the toxic effects of ornithine.

The indirect effects occurred as an inevitable consequence of metabolic activity of the augmented compound and were observed previously in other studies (Li et al., 2015; Colinet et al., 2013; Colinet and Renault, 2014). Similarly complex perturbation of metabolic pathways and metabolomic composition was also documented in response to a change of temperature (Williams et al., 2014). Such complexity complicates interpretation of the results. Many metabolites change their levels simultaneously, and their individual effects (positive, neutral, negative, toxic) on the trait under investigation (in our case, freeze tolerance) variously combine. Certain combinations of metabolites might exert effects that will be different from a simple sum of the effects of individual metabolites (e.g. synergy, counterbalancing). Our results, for instance, suggest that high concentrations of arginine and proline (caused by Arg25 and Pro50 diets) may alleviate the toxic effects of ornithine, glycine or cysteine (as all these metabolites clustered together in PCA analysis and also the Pearson correlation analysis revealed their linkage). In nature, cytoprotectants typically occur in combinations, and often in strict proportions (Yancey et al., 1982; Koštál et al., 2007). The mechanisms by which cytoprotectants in a mixture either cooperate or counterbalance each other are insufficiently understood (Yancey, 2001). For instance, it was observed that addition of non-ionic chaotropes only, or kosmotropes only, may inhibit the activity of enzymes in simple *in vitro* systems, whereas the combination of chaotropes and kosmotropes restores optimum activity (Wiggins, 2001). It was also shown that known chaotropes (such as urea or guanidine) can paradoxically behave as kosmotropes when present at very low concentrations (Sen et al., 2013). We discuss below how the chaotropic/kosmotropic properties of augmented compounds might relate to larval freeze tolerance. The perturbations of balanced combinations, or producing new combinations, via augmented diets might bring unexpected results. We realize that our diet augmentation experiments have limited power in revealing the causal relationships and mechanisms behind cryoprotection. Moreover, the mechanisms by which small, biologically relevant compounds could protect macromolecules have been studied in only a few cases, such as for trehalose, proline, arginine and urea, but remain insufficiently explored for most other compounds (Ball, 2008; Yancey and Siebenaller, 2015). Taking into account such limitations, we would like to avoid far-reaching speculations on each single metabolite that showed some effect in our study. Instead, we will focus on discussing the strongest indications pointing toward the linkage of proline and arginine metabolism, and, specifically, of high body concentrations of the free amino acids proline and arginine, to larval freeze tolerance in *D. melanogaster*.

Potential mechanisms behind proline- and arginine-stimulated freeze tolerance

Proline and arginine are structurally unique among the animal amino acids. Proline contains a non-polar cyclic pyrrolidine side chain with an imino group. Arginine is the only amino acid containing a complex guanidinium group on the distal end of its aliphatic side chain. Such structural features dictate the physico-chemical properties of proline and arginine molecules and determine the nature of their interactions with molecules of solvent (water) on one side and macromolecules and biocomplexes (proteins, nucleic acids, nucleoprotein complexes, biological membranes) on the other. Four potential interactions are discussed below: preferential exclusion and protection of native protein structure, binding of partially unfolded proteins, stabilization of membrane structure and vitrification.

Preferential exclusion and protection of native protein structure

In aquatic solutions, proline behaves as a kosmotrope, i.e. a compound that is highly soluble in water and prefers interactions with water molecules over interactions with proteins. Consequently, proline is preferentially excluded from the protein vicinity, rendering the protein molecule preferentially hydrated (Timasheff, 1992; Arakawa and Timasheff, 1985). Many other compatible solutes, including sugars (trehalose, sucrose), polyols (glycerol, sorbitol) and amino acids (glycine, serine, alanine) also behave as kosmotropes. Empirical observations confirmed that these compatible solutes enhance the stability of thermally stressed proteins in aqueous solutions (Jensen et al., 1997; Xie and Timasheff, 1997a,b; Timasheff, 1998). The theoretical thermodynamic mechanism of preferential exclusion has been postulated and is now broadly accepted (Bolen and Baskakov, 2001; Arakawa and Timasheff, 1985; Timasheff, 2002). The theory of preferential exclusion probably constitutes a plausible explanation for the positive effects of proline, and perhaps of some other amino compounds and trehalose, on high freeze tolerance in our experiments. The highest observed concentration of proline in *D. melanogaster* was 45.6 nmol mg^{-1} FM (larvae fed on Pro50 diet, Table S4), which corresponds to 57.3 mmol l^{-1} in

Journal of Experimental Biology (2016) 219, 2358-2367 doi:10.1242/jeb.142158

larval body water (larval body water constitutes 79.6% of FM). Even such moderate levels of proline, in combination with other kosmotropes, might theoretically exert protective functions via preferential exclusion. Therefore, proline and other kosmotropes could help prevent or reduce partial unfolding of proteins in larvae exposed to thermal stress (prior to freezing).

In contrast to proline, arginine behaves as a weak chaotrope in aquatic solution. It directly binds the proteins in their native state (Arakawa and Timasheff, 1985). Arginine favourably interacts with proteins via hydrogen bonding between the positively charged guanidinium group and the negatively charged amino acid side chains of proteins (Arakawa et al., 2007). In fact, the guanidinium ion is commonly used in laboratory practice as a powerful protein denaturant, usually as a chloride or thiocyanate salt (Vanzi et al., 1998); guanidinium salts interact too strongly with the protein and thereby unfold the protein. The presence of the carboxylate group in the arginine molecule limits the interaction of the guanidinium group with the protein surface. Therefore, the direct interaction of arginine with the protein neither stabilizes nor destabilizes the protein in its native state (Shukla and Trout, 2010) but provides a potential mechanism for stabilization of partially unfolded states.

Binding partially unfolded proteins

In our experiments, most larvae were inoculated with external ice crystals at temperatures between −0.5 and −2.0°C (Fig. 1). Differential scanning calorimetry (DSC) analyses of ice formation in *D. melanogaster* larvae showed that most of the osmotically active water fraction is frozen at −5°C, which means that the ice crystals represent 55–61% of larval fresh mass [Koštál et al., 2012; V.K., unpublished data]. Gradual freezing dramatically affects the conditions in biological solutions by decreasing water activity (water molecules are trapped in an ice crystal lattice), thereby altering viscosity, osmolality, pH and ion concentrations. Under such conditions, proteins are highly prone to denaturation. It is probable that partially unfolded proteins (molten globule stage) will occur more frequently and may directly interact with proline and arginine molecules, the concentrations of which will also be elevated as a result of freeze-induced dehydration. Indeed, at relatively high concentrations, both proline and arginine were shown to directly interact with partially unfolded proteins, stabilizing them and preventing their further denaturation/aggregation and/or assisting them in regaining their functional conformation. However, the theoretical mechanisms by which these stabilization effects are mediated are not yet fully understood and empirically confirmed (Lange and Rudolph, 2009; Schneider et al., 2011).

Arginine is well known as a versatile additive that is highly effective in inhibiting protein aggregation (Arakawa and Tsumoto, 2003; Lange and Rudolph, 2009). The theoretical explanation for this effect is based on the striking tendency of arginine molecules to self-associate and form clusters in aqueous solutions. These clusters display hydrophobic surfaces (alignment of the three methylene groups), which interact with the hydrophobic surfaces presented by partially unfolded proteins (Das et al., 2007; Shukla and Trout, 2010). Masking of the hydrophobic surfaces of partially unfolded proteins is believed to inhibit protein–protein aggregation (Schneider et al., 2011). Interestingly, arginine and proline are the only two amino acids that display a high propensity to bind hydrophobic proteins and solubilize them (Samuel et al., 1997; Das et al., 2007). Experimental evidence suggests that proline, similar to arginine, inhibits protein aggregation by binding to folding intermediates. Moreover, proline molecules probably also form aggregates (stacked columns) in aqueous solutions with methylene groups of the pyrrolidine rings constituting the hydrophobic surface (Rudolph and Crowe, 1986; Samuel et al., 2000). It has been shown that proline abrogates protein aggregation not only *in vitro* but also *in vivo* in an *Escherichia coli* expression system (Ignatova and Gierasch, 2006).

Importantly, sugars, polyols, many amino acids (other than arginine and proline), racemic mixtures of L- and D-proline, and hydroxyproline have only little or no ability to form supramolecular aggregates and to inhibit protein aggregation. These same compounds, however, are known to stabilize native structure of proteins by preferential exclusion (Paleg et al., 1981; Samuel et al., 2000; Myung et al., 2006; Das et al., 2007; Hamada et al., 2009). We observed little or no effect of D-proline and hydroxyproline on larval freeze tolerance in our experiments (Table S3), which suggests that the formation of supramolecular aggregates of proline and arginine and inhibition of aggregation of partially unfolded proteins might represent another mechanism, in addition to preferential exclusion, that explains the high freeze tolerance in *D. melanogaster* larvae reared on diets augmented specifically with arginine and proline. However, the formation of supramolecular aggregates requires relatively high concentrations (>1 mol l^{-1}) of proline or arginine. Such concentrations are higher than those we observed in *D. melanogaster* larvae in our experiments. Perhaps tight association of proline and arginine molecules with proteins creates microenvironments where the formation of these aggregations could take place (Rudolph and Crowe, 1986).

Stabilization of membrane structure

Upon water loss during freeze dehydration, the cell contents become more viscous, cytoplasmic components are crowded and organelles come into closer contact, which can cause unregulated membrane fusions. It has been proposed that a wide selection of compatible solutes, including proline and trehalose, can serve as a molecular shield between macromolecules or membranes, and/or cause preferential hydration of membrane surfaces based on the principle of preferential exclusion (Bryant et al., 2001; Hoekstra et al., 2001; Ball, 2008). Trehalose, the disaccharide of glucose, is the principal sugar circulating in the insect haemolymph (Thompson, 2003). It is present at relatively high concentrations (30–50 nmol mg^{-1} FM) in *D. melanogaster* larvae (Table S4). Thus, proline in combination with trehalose (and some other amino acids) might help prevent membrane fusions via a preferential exclusion/hydration mechanism in thermally stressed and freeze-dehydrated cells of *D. melanogaster*.

When the hydration drops below a threshold of approximately 0.3 g water g^{-1} dry mass, most bulk water is removed (Brovchenko and Oleinikova, 2008; Ball, 2008), and the mechanism of preferential hydration thus fails to work. Most compatible solutes are unable to protect proteins and membranes beyond this level of dehydration (Hoekstra et al., 2001). Trehalose and some other sugars (di- and oligo-saccharides), however, may function as potent 'lyoprotectants'. They have been shown to possess a remarkable ability to stabilize membranes during almost complete desiccation (Crowe et al., 1984; Thompson, 2003). These sugars can replace missing water molecules by forming hydrogen bonds with polar residues on membrane phospholipid headgroups, thereby stabilizing the structure in the absence of water. In addition, sugars can concentrate water molecules near membrane surfaces and can induce formation of amorphous glasses (Crowe et al., 2001; Crowe, 2007). It is important to note that the extent of freeze dehydration in the larvae of *D. melanogaster* probably does not fall below the

critical threshold of 0.3 g water g^{-1} dry mass. At −5°C, when most freezable water is already frozen, larvae still maintain the unfreezable water fraction at approximately 1 mg mg^{-1} DM (Koštál et al., 2012; V.K., unpublished data).

Vitrification

Vitrification (formation of amorphous glasses) is promoted not only by trehalose and many other sugars but also by supramolecular structures of proline. In the *in vitro* experiments, high concentrations (2–5 mol l^{-1}) of proline in aqueous solution significantly reduced, or even completely abolished, the formation of ice crystals, while increasing the propensity of the system to undergo the water–glass transition (Rudolph and Crowe, 1986). Indeed, we observed that the propensity to undergo glass transition greatly increases in hyperprolinemic body fluids of the larvae of the drosophilid fly *Chymomyza costata* (Koštál et al., 2011a). Vitrification has also been documented in the deeply supercooled, partially dehydrated larvae of *Cucujus clavipes* (Sformo et al., 2010). Vitrification probably could not occur in *D. melanogaster* because of the relatively high temperatures in our freezing assays. Indeed, we never observed vitrification in *D. melanogaster* larvae that were subjected to DSC analyses conducted at temperatures down to −70°C (V.K., unpublished data). In insect species other than *D. melanogaster*, however, vitrification appears to be a plausible mechanism of freeze tolerance, provided that specific conditions are met: significant loss of water and/or a high level of freeze dehydration, very high concentrations of glass transition-promoting substances and very low sub-zero temperatures.

Conclusions

In this study, we show that the free amino acids arginine and proline have the highest potential to stimulate freeze tolerance in larvae of *D. melanogaster* among 31 different amino compounds assayed as additives to the larval diet. We would like to stress that *D. melanogaster* is a fly of tropical origin and its larval stage is evolutionarily adapted for rapid growth and development under warm conditions, showing very high sensitivity to cold. The diet augmentations with arginine and proline, however, help to ensure larval survival in a situation where most of its freezable water fraction is converted to ice. It is important to clarify here that we are dealing with survival at −5°C. So far, we have not been able to extend these results into a widely applicable technique for long-term storage or cryopreservation.

Metabolomic analysis confirmed that diet augmentation with arginine and proline is reflected in significant increases of their levels in the larval body. In addition, diet augmentations typically caused complex disturbances of metabolomic composition, which complicates interpretation of the results. Nevertheless, two other compounds identified as tight statistical associates of high freeze tolerance were glutamine and ornithine, again pointing towards a prominent role of proline and arginine metabolism in freeze tolerance. Based on the available literature, we have listed several potential mechanisms by which high concentrations of proline and arginine might stimulate high freeze tolerance. Among them, two mechanisms seem the most plausible: (1) proline, probably in combination with trehalose and some other amino acids, could help prevent or reduce partial unfolding of proteins and membrane fusions in the larvae exposed to thermal stress (prior to freezing) or even during freeze dehydration; (2) both arginine and proline are probably exceptional among amino compounds in their ability to form supramolecular aggregates that can bind partially unfolded proteins and inhibit their aggregation. This mechanism requires relatively high concentrations of arginine and proline and, therefore, could come into play with increasing levels of freeze dehydration. The probability of actual employment of this mechanism is indirectly supported by exclusively high freeze-tolerance stimulation linked specifically to arginine and proline among 31 different amino compounds tested (including those with closely similar molecular structures, such as D-proline or hydroxyproline).

Acknowledgements

We thank Irena Vacková, Anna Heydová and Helena Zahradníčková (all from Biology Centre CAS) for assistance with insect rearing, sample preparation and analyses.

Competing interests

The authors declare no competing or financial interests.

Author contributions

V.K. designed the research. V.K., J.K., R.P., M.M. and P.Š. performed the research. V.K. wrote the manuscript. All authors read and approved the paper.

Funding

This work was supported by Grantová agentura České republiky grant 13-01057S (to V.K.). R.P. received his salary from Modbiolin grant no. 316304 sponsored by the European Union Seventh Framework Programme (FP7/2007-2013).

Supplementary information

Supplementary information available online at
http://jeb.biologists.org/lookup/doi/10.1242/jeb.142158.supplemental

References

Andersen, L. H., Kristensen, T. N., Loeschcke, V., Toft, S. and Mayntz, D. (2010). Protein and carbohydrate composition of larval food affects tolerance to thermal stress and desiccation in adult *Drosophila melanogaster*. *J. Insect Physiol.* **56**, 336-340.

Arakawa, T. and Timasheff, S. N. (1985). The stabilization of proteins by osmolytes. *Biophys. J.* **47**, 411-414.

Arakawa, T. and Tsumoto, K. (2003). The effects of arginine on refolding of aggregated proteins: not facilitate refolding, but suppress aggregation. *Biochem. Biophys. Res. Comm.* **304**, 148-152.

Arakawa, T., Ejima, D., Tsumoto, K., Obeyama, N., Tanaka, Y., Kita, Y. and Timasheff, S. N. (2007). Suppression of protein interactions by arginine: a proposed mechanism of the arginine effects. *Biophys. Chem.* **127**, 1-8.

Bale, J. S., Hansen, T. N., Nishino, M. and Baust, J. G. (1989). Effect of cooling rate on the survival of larvae, pupariation, and adult emergence of the gallfly *Eurosta solidaginis*. *Cryobiology* **26**, 285-289.

Ball, P. (2008). Water as an active constituent in cell biology. *Chem. Rev.* **108**, 74-108.

Bolen, D. W. and Baskakov, I. V. (2001). The osmophobic effect: natural selection of a thermodynamic force in protein folding. *J. Mol. Biol.* **310**, 955-963.

Brovchenko, I. and Oleinikova, A. (2008). Which properties of a spanning network of hydration water enable biological functions? *Chem. Phys. Chem.* **9**, 2695-2702.

Bryant, G., Koster, K. L. and Wolfe, J. (2001). Membrane behaviour in seeds and other systems at low water content: the various effects of solutes. *Seed Sci. Res.* **11**, 17-25.

Colinet, H. and Renault, D. (2014). Dietary live yeast alters metabolic profiles, protein biosynthesis and thermal stress of *Drosophila melanogaster*. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **170**, 6-14.

Colinet, H., Larvor, V., Bical, R. and Renault, D. (2013). Dietary sugars affect cold tolerance of *Drosophila melanogaster*. *Metabolomics* **9**, 608-622.

Crowe, J. H. (2007). Trehalose as a chemical chaperone: fact and fantasy. *Adv. Exp. Med. Biol.* **594**, 143-158.

Crowe, L. M., Mouradian, R., Crowe, J. H., Jackson, S. A. and Womersley, C. (1984). Effects of carbohydrates on membrane stability at low water activities. *Biochim. Biophys. Acta* **769**, 141-150.

Crowe, J. H., Crowe, L. M., Oliver, A. E., Tsvetkova, N., Wolkers, W. and Tablin, F. (2001). The trehalose myth revisited: introduction to a symposium on stabilization of cells in the dry state. *Cryobiology* **43**, 89-105.

Das, U., Hariprasad, G., Ethayathulla, A. S., Manral, P., Das, T. K., Pasha, S., Mann, A., Ganguli, M., Verma, A. K., Bhat, R. et al. (2007). Inhibition of protein aggregation: supramolecular assemblies of arginine hold the key. *PLoS ONE* **2**, e1176.

Fields, P. G., Fleurat-Lessard, F., Lavenseau, L., Febvay, L. G., Peypelut, L. and Bonnot, G. (1998). The effect of cold acclimation and deacclimation on cold tolerance, trehalose and free amino acid levels in *Sitophilus granarius* and *Cryptolestes ferrugineus* (Coleoptera). *J. Insect Physiol.* **44**, 955-965.

Hamada, H., Arakawa, T. and Shiraki, K. (2009). Effect of additives on protein aggregation. *Curr. Pharm. Biotechnol.* **10**, 400-407.
Hoekstra, F. A., Golovina, E. A. and Buitink, J. (2001). Mechanisms of plant desiccation tolerance. *Trends Plant Sci.* **6**, 431-438.
Hušek, P. and Šimek, P. (2001). Advances in amino acid analysis. *LC-GC North Am.* **19**, 986-999.
Ignatova, Z. and Gierasch, L. M. (2006). Inhibition of protein aggregation in vitro and in vivo by a natural osmoprotectant. *Proc. Natl. Acad. Sci. USA* **103**, 13357-13361.
Jensen, W. A., Armstrong, J. M., De Giorgio, J. and Hearn, M. T. W. (1997). Thermodynamic analysis of the stabilisation of pig heart mitochondrial malate dehydrogenase and maize leaf phosphoenolpyruvate carboxylase by different salts, amino acids and polyols. *Biochim. Biophys. Acta* **1338**, 186-198.
Koštál, V., Zahradníčková, H., Šimek, P. and Zelený, J. (2007). Multiple component system of sugars and polyols in the overwintering spruce bark beetle, *Ips typographus*. *J. Insect Physiol.* **53**, 580-586.
Koštál, V., Zahradníčková, H. and Šimek, P. (2011a). Hyperprolinemic larvae of the drosophilid fly, *Chymomyza costata*, survive cryopreservation in liquid nitrogen. *Proc. Natl. Acad. Sci. USA* **108**, 13035-13040.
Koštál, V., Korbelová, J., Rozsypal, J., Zahradníčková, H., Cimlová, J., Tomčala, A. and Šimek, P. (2011b). Long-term cold acclimation extends survival time at 0°C and modifies the metabolomic profiles of the larvae of the fruit fly *Drosophila melanogaster*. *PLoS ONE* **6**, e25025.
Koštál, V., Šimek, P., Zahradníčková, H., Cimlová, J. and Štětina, T. (2012). Conversion of the chill susceptible fruit fly larva (*Drosophila melanogaster*) to a freeze tolerant organism. *Proc. Natl. Acad. Sci. USA* **109**, 3270-3274.
Lange, C. and Rudolph, R. (2009). Suppression of protein aggregation by L-arginine. *Current Pharm. Biotechnol.* **10**, 408-414.
Lee, R. E., Jr. (2010). A primer on insect cold-tolerance. In *Low Temperature Biology of Insects* (ed. D. L. Denlinger and R. E. Lee), pp. 3-34. Cambridge: Cambridge University Press.
Lee, R. E. and Lewis, E. A. (1985). Effect of temperature and duration of exposure on tissue ice formation in the gall fly, Eurosta solidaginis (Diptera: Tephritidae). *Cryo-Lett.* **6**, 25-34.
Li, Y., Zhang, L., Zhang, Q., Chen, H. and Denlinger, D. L. (2014). Host diapause status and host diets augmented with cryoprotectants enhance cold hardiness in the parasitoid *Nasonia vitripennis*. *J. Insect Physiol.* **70**, 8-14.
Li, Y., Zhang, L., Chen, H., Koštál, V., Šimek, P., Moos, M. and Denlinger, D. L. (2015). Shifts in metabolomic profiles of the parasitoid *Nasonia vitripennis* associated with elevated cold tolerance induced by the parasitoid's diapause, host diapause and host diet augmented with proline. *Insect Biochem. Mol. Biol.* **63**, 34-46.
Lindsley, D. L. and Grell, E. H. (1968). Genetic variations of *Drosophila melanogaster*. *Publs. Carnegie Instn.* **627**, 469.
Meryman, H. T. (1971). Osmotic stress as a mechanism of freezing injury. *Cryobiology* **8**, 489-500.
Miller, L. K. (1978). Freezing tolerance in relation to cooling rate in an adult insect. *Cryobiology* **15**, 345-349.
Morgan, T. D. and Chippendale, G. M. (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.
Myung, S., Fioroni, M., Julian, R. R., Koeniger, S. L., Baik, M.-H. and Clemmer, D. E. (2006). Chirally directed formation of nanometer-scale proline clusters. *J. Am. Chem. Soc.* **128**, 10833-10839.
Paleg, L. G., Douglas, T. J., van Daal, A. and Keech, D. B. (1981). Proline, betaine and other organic solutes protect enzymes against heat inactivation. *Aust. J. Plant Physiol.* **8**, 107-114.
Phang, J. M., Liu, W., Hancock, C. N. and Fischer, J. W. (2015). Proline metabolism and cancer: emerging links to glutamine and collagen. *Curr. Opin. Clin. Nutr. Metab. Care* **18**, 71-77.
Rudolph, A. S. and Crowe, J. H. (1986). A calorimetric and infrared spectroscopic study of the stabilizing solute proline. *Biophys. J.* **50**, 423-430.
Salt, R. W. (1961). Principles of insect cold-hardiness. *Annu. Rev. Entomol.* **6**, 55-74.
Samuel, D., Kumar, T. K. S., Jayaraman, G., Yang, P. W. and Yu, C. (1997). Proline is a protein solubilizing solute. *Biochem. Mol. Biol. Int.* **41**, 235-242.
Samuel, D., Kumar, T. K. S., Ganesh, G., Jayaraman, G., Yang, P. W., Chang, M. M., Trivedi, W. D., Wang, S. L., Hwang, K. C., Chang, D. K. et al. (2000). Proline inhibits aggregation during protein refolding. *Prot. Sci.* **9**, 344-352.
Schneider, C. P., Shukla, D. and Trout, B. L. (2011). Arginine and the Hofmeister series: the role of ion–ion interactions in protein aggregation suppression. *J. Phys. Chem. B* **115**, 7447-7458.
Sen, P., Khan, M. M., Equbal, A., Ahmad, E. and Khan, R. H. (2013). At very low concentrations known chaotropes act as kosmotropes for the N and B isoforms of human serum albumin. *Biochem. Cell Biol.* **91**, 72-78.
Sformo, T., Walters, K., Jeannet, K., Wowk, B., Fahy, G. M., Barnes, B. M. and Duman, J. G. (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.
Shimada, K. and Riihimaa, A. (1988). Cold acclimation, inoculative freezing and slow cooling: essential factors contributing to the freeze-tolerance in diapausing larvae of *Chymomyza costata* (Diptera: Drosophilidae). *Cryo-Lett.* **9**, 5-10.
Shreve, S. M., Yi, S. X. and Lee, R. E., Jr. (2007). Increased dietary cholesterol enhances cold tolerance in *Drosophila melanogaster*. *Cryo-Lett.* **28**, 33-37.
Shukla, D. and Trout, B. L. (2010). Interaction of arginine with proteins and the mechanism by which it inhibits aggregation. *J. Phys. Chem. B* **114**, 13426-13438.
Sisodia, S. and Singh, B. N. (2012). Experimental Evidence for Nutrition Regulated Stress Resistance in Drosophila ananassae. *PLoS ONE* **7**, e46131.
Somme, L. (1982). Supercooling and winter survival in terrestrial arthropods. *Comp. Biochem. Physiol. A Physiol.* **73**, 519-543.
Storey, K. B. and Storey, J. M. (1988). Freeze tolerance in animals. *Physiol. Rev.* **68**, 27-84.
Storey, K. B. and Storey, J. M. (1991). Biochemistry of cryoprotectants. In *Insects at Low Temperature* (ed. R. E. Lee and D. L. Denlinger), pp. 64-93. New York and London: Chapman and Hall.
Storey, K. B., Baust, J. G. and Storey, J. M. (1981). Intermediary metabolism during low temperature acclimation in the overwintering gall fly larva, *Eurosta solidaginis*. *J. Comp. Physiol. B* **144**, 183-190.
Strachan, L. A., Tarnowski-Garner, H. E., Marshall, K. E. and Sinclair, B. J. (2011). The evolution of cold tolerance in *Drosophila* larvae. *Physiol. Biochem. Zool.* **84**, 43-53.
Thompson, S. N. (2003). Trehalose–the insect blood sugar. *Adv. Insect Physiol.* **31**, 205-285.
Timasheff, S. N. (1992). A physicochemical basis for the selection of osmolytes by nature. In *Water and Life: A Comparative Analysis of Water Relationships at the Organismic, Cellular, and Molecular Levels* (ed. G. N. Somero, C. B. Osmond and C. L. Bolis), pp. 70-84. Berlin: Springer Verlag.
Timasheff, S. N. (1998). Control of protein stability and reactions by weakly interacting cosolvents: the simplicity of the complicated. *Adv. Protein Chem.* **51**, 355-432.
Timasheff, S. N. (2002). Protein-solvent preferential interactions, protein hydration, and the modulation of biochemical reactions by solvent components. *Proc. Natl. Acad. Sci. USA* **99**, 9721-9726.
Vanzi, F., Madan, B. and Sharp, K. (1998). Effect of the protein denaturants urea and guanidinium on water structure: a structural and thermodynamic study. *J. Am. Chem. Soc.* **120**, 10748-10753.
Wang, W. (1999). Instability, stabilization, and formulation of liquid protein pharmaceuticals. *Internatl. J. Pharm.* **185**, 129-188.
Wang, W. (2005). Protein aggregation and its inhibition in biopharmaceutics. *Internatl. J. Pharm.* **289**, 1-30.
Wiggins, P. M. (2001). High and low density intracellular water. *Cell. Mol. Biol.* **47**, 735-744.
Williams, C. M., Watanabe, M., Guarracino, M. R., Ferraro, M. B., Edison, A. S., Morgan, T. J., Boroujerdi, A. F. B. and Hahn, D. A. (2014). Cold adaptation shapes the robustness of metabolic networks in *Drosophila melanogaster*. *Evolution* **68**, 3505-3523.
Wu, G. and Morris, S. M. (1998). Arginine metabolism: nitric oxide and beyond. *Biochem. J.* **336**, 1-17.
Xie, G. and Timasheff, S. N. (1997a). Mechanism of the stabilization of ribonuclease A by sorbitol: preferential hydration is greater for the denatured than for the native protein. *Protein Sci.* **6**, 211-221.
Xie, G. and Timasheff, S. N. (1997b). The thermodynamic mechanism of protein stabilization by trehalose. *Biophys. Chem.* **64**, 25-43.
Yancey, P. H. (2001). Water stress, osmolytes and Protein. *Am. Zool.* **41**, 699-709.
Yancey, P. H. (2005). Organic osmolytes as compatible, metabolic and counteracting cytoprotectants in high osmolarity and other stresses. *J. Exp. Biol.* **208**, 2819-2830.
Yancey, P. H. and Siebenaller, J. F. (2015). Co-evolution of proteins and solutions: protein adaptation versus cytoprotective micromolecules and their roles in marine organisms. *J. Exp. Biol.* **218**, 1880-1896.
Yancey, P. H., Clark, M. E., Hand, S. C., Bowlus, R. D. and Somero, G. N. (1982). Living with water stress: evolution of osmolyte systems. *Science* **217**, 1214-1222.
Zachariassen, K. E. (1985). Physiology of cold tolerance in insects. *Physiol. Rev.* **65**, 799-832.

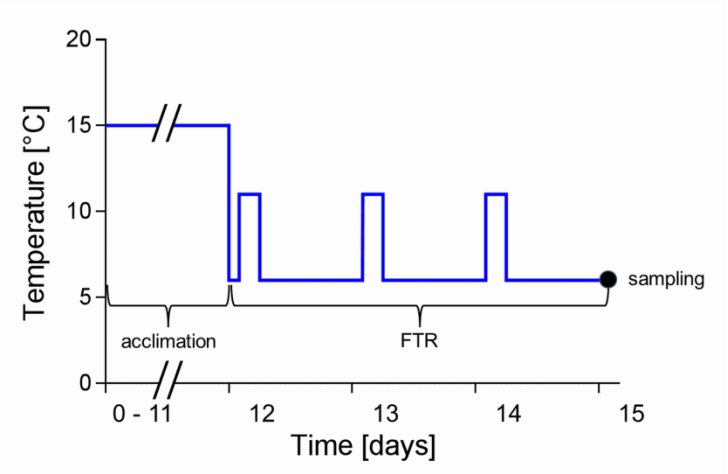

Fig. S1: Acclimation protocol for *Drosophila melanogaster* larvae

From the early embryonic stage until the stage of pre-wandering larva, the insects were reared under the temperature conditions that ensured proper cold acclimation and finally induced a low temperature quiescence state.

Constant temperature of 15°C with 12-h/12-h L/D cycle was applied until the first wandering larvae occurred (approximately 11 days on standard diet, usually longer on augmented diets). Next, all wandering larvae were removed and the tube with remaining larvae was transferred to the fluctuating thermal regime (FTR; 20 h of 6°C/4 h of 11°C under constant darkness) for 3 days, which resulted in the arrestment of larval development and induction of low temperature quiescence state. Such larvae were shown to acquire highest levels of cold tolerance (Koštál et al., 2011b, 2012).

Table S1.

Click here to Download Table S1

Table S2.

Click here to Download Table S2

Table S3.

Click here to Download Table S3

Table S4.

Click here to Download Table S4