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RESEARCH ARTICLE

Hemolymph metabolites and osmolality are tightly linked to cold tolerance of *Drosophila* species: a comparative study

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ABSTRACT

Drosophila, like most insects, are susceptible to low temperatures, and will succumb to temperatures above the freezing point of their hemolymph. For these insects, cold exposure causes a loss of extracellular ion and water homeostasis, leading to chill injury and eventually death. Chill-tolerant species are characterized by lower hemolymph [Na^+] than chill-susceptible species and this lowered hemolymph [Na^+] is suggested to improve ion and water homeostasis during cold exposure. It has therefore also been hypothesized that hemolymph Na^+ is replaced by other 'cryoprotective' osmolytes in cold-tolerant species. Here, we compared the hemolymph metabolite profiles of five drosophilid species with marked differences in chill tolerance. All species were examined under 'normal' thermal conditions (i.e. 20°C) and following cold exposure (4 h at 0°C). Under benign conditions, total hemolymph osmolality was similar among all species despite chill-tolerant species having lower hemolymph [Na^+]. Using NMR spectroscopy, we found that chill-tolerant species instead have higher levels of sugars and free amino acids in their hemolymph, including classical 'cryoprotectants' such as trehalose and proline. In addition, we found that chill-tolerant species maintain a relatively stable hemolymph osmolality and metabolite profile when exposed to cold stress while sensitive species suffer from large increases in osmolality and massive changes in their metabolic profiles during a cold stress. We suggest that the larger contribution of classical cryoprotectants in chill-tolerant *Drosophila* plays a non-colligative role for cold tolerance that contributes to osmotic and ion homeostasis during cold exposure and, in addition, we discuss how these comparative differences may represent an evolutionary pathway toward more extreme cold tolerance of insects.

KEY WORDS: Insect, Chill tolerance, Osmotic balance, Membrane potential, Chill injury, NMR, Metabolomics

INTRODUCTION

Some cold-adapted insect species have evolved the ability to either tolerate or avoid ice crystal formation in the hemolymph and thus survive at sub-zero temperatures (Zachariassen, 1985; Bale, 1996; Ramløv, 2000). Most insects are chill susceptible, however, meaning they succumb to low temperature exposures that are unrelated to the risk of their hemolymph freezing (Zachariassen, 1985; Bale, 1996; Sinclair, 1999; MacMillan and Sinclair, 2011a; Andersen et al., 2015). The thermal sensitivity of these 'chill-susceptible' species is therefore not linked to damage caused by the water–ice transformation or recrystallization but instead to physiological problems induced by the low temperature, per se.

Increasing evidence indicates that chill injuries in insects accumulate in association with a loss of ion and water homeostasis at low temperatures (e.g. Koštal et al., 2004; MacMillan and Sinclair, 2011b; MacMillan et al., 2014, 2015a,c,d). Under normal conditions, extracellular ion balance is maintained by active transporters in cell membranes and epithelia, where hemolymph [Na^+] is kept high and [K^+] is kept low by a continuous active transport that balances the passive leak of these ions. Low temperature impairs the activity of ion pumps, causing Na^+ to leak away from, and K^+ toward, the hemolymph (Koštal et al., 2004; MacMillan and Sinclair, 2011a,b; MacMillan et al., 2015a,b). A number of studies have further shown that hemolymph volume is reduced during cold exposures. This reduction in volume, which is probably caused by water following Na^+ osmotically, has the end result that hemolymph [K^+] increases in the reduced hemolymph volume (Koštal et al., 2004; MacMillan and Sinclair, 2011b; MacMillan et al., 2015a,d). Membrane resting potential is highly dependent on the cellular [K^+] gradient (Hoyle, 1954; Wareham et al., 1974; Fitzgerald et al., 1996) and hemolymph hyperkalemia therefore causes cellular depolarization that leaves the neuromuscular system unexcitable and slows chill coma recovery time; ultimately, hyperkalemia may cause cold-induced injury and death (Boutilier, 2001; Koštal et al., 2004; Findsen et al., 2013; MacMillan et al., 2015c).

This cascade of events, starting with Na^+ leaving the hemolymph and ending with a depolarization of the cell membranes due to hyperkalemia, is mechanistically linked to the initial electrochemical gradient of Na^+ from the hemolymph to the gut and other tissues (see discussion in MacMillan and Sinclair, 2011b and MacMillan et al., 2015a,d). In support of this hypothesis, a lower [Na^+] gradient (driven by a lower hemolymph [Na^+]) is associated with increased cold hardiness following different forms of cold acclimation in *Drosophila melanogaster* (MacMillan et al., 2015a) and strikingly similar observations have been made recently when comparing multiple *Drosophila* species with different cold tolerances (MacMillan et al., 2015b,d). What remains unknown, however, is whether these observed differences in [Na^+] contribute to general differences in hemolymph osmolality (such that chill-tolerant species generally have lower hemolymph osmolality) or whether this 'missing' Na^+ is 'replaced' with other compatible osmolytes in chill-tolerant species. Measurements on *D. melanogaster* from different acclimation treatments suggest that the hemolymph osmolality is similar (or slightly larger) in cold-acclimated animals, and therefore reductions in [Na^+] are matched or even exceeded by accumulation of other compatible osmolytes in the

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hemolymph (MacMillan et al., 2015a). Here, we examined whether a similar pattern is found among species of the same genus, and further we examined which osmolytes may replace Na^+ in the hemolymph of cold-tolerant *Drosophila* species.

A number of previous studies have examined the metabolite profile of chill-susceptible insect species before and after cold exposure. Overall, these results suggest that both cold acclimation and cold exposure cause an increase in the relative abundance of some sugars (trehalose, maltose and glucose), polyols (glycerol and sorbitol) and free amino acids (proline and β-alanine) (Chen et al., 1987; Yoder et al., 2006; Michaud and Denlinger, 2007; Lalouette et al., 2007; Overgaard et al., 2007; Koštal et al., 2011a; Colinet et al., 2012; Purać et al., 2016). While these results might offer clues as to which metabolites could replace Na^+ in the hemolymph of the cold-tolerant *Drosophila* species they are all based on whole-body samples and therefore do not provide any information on the metabolite profile of the hemolymph specifically. The main goals for the present study were: (1) to investigate the osmolality of the hemolymph in five *Drosophila* species: *D. birchii*, *D. equinoxialis*, *D. melanogaster*, *D. persimilis* and *D. montana*, before and after a cold exposure; and (2) to analyze the composition of small organic molecules in the hemolymph of the same species using NMR-spectroscopy. As the accumulation of chill injuries seems to be linked to a general failure to maintain ion gradients across membranes, we hypothesized that the cold-sensitive species suffer from larger shifts in ion and water balance and hence experience larger increases in osmolality than the more cold-tolerant species. Further, we hypothesized that chill-tolerant *Drosophila* species will constitutively maintain higher concentrations of compatible osmolytes (sugars, polyols and/or amino acids) compared with chill-sensitive species, in effect 'replacing' [Na^+].

MATERIALS AND METHODS

Animal stock

The *Drosophila* species used in the present study were *D. birchii*, *D. equinoxialis*, *D. melanogaster*, *D. persimilis* and *D. montana*. These species were selected from different species subgroups (*obscura*, *melanogaster*, *virilis*, *montium* and *willistoni*) within the *Drosophila* phylogeny, with four species from the subgenus *Sophophora* and one species from the subgenus *Drosophila*. The choice of species was therefore an attempt to avoid strong phylogenetic bias, but was also based on the fact that we had already generated considerable physiological and ecological information on the cold biology of these five species. The five species differ greatly in their chill tolerance, with *D. birchii* being very chill susceptible (LTe_{50}=−3.3°C, where LTe_{50} is the temperature that causes mortality in 50% of individuals after a 2 h exposure) and *D. montana* being more chill tolerant (LTe_{50}= −13.2°C; Andersen et al., 2015). The five species of *Drosophila* also maintain very different hemolymph [Na^+] when reared under the same conditions, such that *D. birchii* maintain much higher [Na^+] than *D. montana* (94 and 63 mmol l^{-1},respectively), and the hemolymph [Na^+] correlates very tightly with cold tolerance (MacMillan et al., 2015d).

Laboratory populations of these species were kept at a constant temperature of 20°C and a 12 h:12 h light:dark cycle. Flies were maintained in 250 ml plastic bottles (ca. 50–250 per bottle) containing 50 ml of Leeds *Drosophila* medium (oatmeal–sugar–yeast–agar). Groups of parental flies were given an appropriate amount of time (from 4 h to 4 days) to lay eggs in the medium, after which they were tipped into a new bottle. New adult flies were collected on the day of emergence into smaller vials containing 4–7 ml of *Drosophila* medium; 3–5 day old adult flies were then sexed using a light CO_2 anesthesia (less than 5 min) and females were kept for 3 days to recover before they were used in the experiments. Accordingly, all flies were 6–8 day old females at the time of the experiments.

Experimental protocol

Separate experiments were conducted to investigate the osmolality before and after cold exposure in the five *Drosophila* species, and to characterize the interspecific variance in metabolomic profile before and after cold exposure in these species. These findings were analyzed and are discussed in relation to a number of other ecological and physiological traits that we have recently characterized (within the same year) on the same fly stocks. For example, we associate the physiological parameters investigated here in relation to recent measures of species cold tolerance (Andersen et al., 2015) and furthermore we discuss the observed differences in osmotic balance in the light of recent measurements of ion content and hemolymph volume on the same fly lines (MacMillan et al., 2015d).

A common experimental protocol was used for the experiments, characterizing the osmotic and metabolomic status before and after cold exposure. Flies were either taken directly from standard conditions (20°C; control group) or exposed to a cold treatment (4 h at 0°C) by submerging them in an ice/water slurry (in plastic vials sealed with screw caps). Osmolality could be measured on hemolymph samples from individual flies (see below). For assessment of osmolality, between 9 and 16 flies were sampled for each species and treatment combination. To achieve sufficient hemolymph volume for measurements of hemolymph metabolite profile, flies were again randomly distributed to one of the two treatments (control or cold exposed) but here we pooled hemolymph from 13–15 flies for each sample to be used in the subsequent NMR spectroscopy. Six samples were prepared for each species and treatment [i.e. 90 flies (6×15) for each species and treatment combination].

Hemolymph extraction

Hemolymph was extracted using antennal ablation as described by MacMillan and Hughson (2014). Here, the fly was pushed head first into a 10 µl pipette tip using positive air pressure in a system composed of rubber tubing and three-way stopcocks. When the fly was positioned in the pipette tip, the pipette was cut directly in front of the fly's head to expose the antennae. One antenna was removed using sharp forceps and the positive air pressure then caused a clear droplet of hemolymph to emerge from the wound. The hemolymph was immediately collected for either osmolality measurements or NMR spectroscopy (see 'Sample preparation for NMR measurements', below, for details). Flies exposed to the cold treatment were preloaded individually into the pipette tips before cold exposure to allow for rapid sampling after cold exposure. The pipette tips containing the flies were then put in larger bottles, which were sealed before they were placed in the ice/water slurry.

Hemolymph osmolality

Osmolality measurements were obtained using a Clifton nanoliter osmometer (Clifton Technical Physics, Hartford, NY, USA) through measurements of melting point. A pulled borosilicate glass capillary was used to inject approximately 5 nl of hemolymph from individual flies into immersion oil suspended in a platinum disc. When all samples were loaded, the controller box was set to 'freeze' causing all sample droplets to flash freeze. The droplets

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were then slowly warmed while observing the ice crystals and osmolality was determined from the melting point (i.e. the temperature at which the last tiny ice crystal disappeared). We did not observe any signs of thermal hysteresis and as the melting point of an aqueous solution is decreased by 1.86°C with each mole of solute, the melting point was used to determine the hemolymph osmolality through reference to standards of known osmolality.

Sample preparation for NMR measurements

In an attempt to obtain quantitative measurements of hemolymph composition by NMR-spectroscopy, we used a modified method following the procedures developed by Ragan et al. (2013), who recently described a method for NMR-based quantification of metabolites in larval *Drosophila* hemolymph. However, it is possible to sample considerably more hemolymph from larvae (>200 nl per larva) while antennal ablation only renders 10–50 nl of hemolymph because the adult flies have less hemolymph volume available (depending on species and treatment). To obtain sufficient hemolymph volume, each experimental sample was composed of hemolymph pooled from 13–15 flies. A Petri dish with wax in the bottom was filled to the top with paraffin oil, and the dish was placed on a cooling plate at 5°C. A 20 µl drop of cold saline with 100 µmol l^{-1} sodium ^{13}C-formate was placed in the dish using a Hamilton syringe, so that it was completely covered in oil to prevent evaporation. Hemolymph samples were prepared as described above and after antennal ablation the hemolymph droplets were immediately dipped into the oil bath and merged with the 20 µl drop of saline. Sampling of the 13–15 flies needed for each sample took maximally 90 min and after the last fly was sampled 17.5 µl of the saline/hemolymph droplet was removed using a Hamilton syringe. This sample was then added to 200 µl of deionized water containing 80 µmol l^{-1} trimethylsilyl propionate [TSP; note, the original experiments by Ragan et al. (2013) used 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS)]. The sample was then centrifuged through a 0.22 µm Millipore filter at ∼2800 ***g*** for 2 min in order to remove any cells. To control for any evaporation or contamination during the procedure, a 20 µl drop of saline was left under the oil for 40–90 min, after which 17.5 µl was removed and treated as described for the rest of the samples. These samples are henceforth referred to as the blank samples. After the centrifugation, a methanol–chloroform metabolite extraction was performed using the method of Bligh and Dyer (1959) in order to purify to the hydrophilic metabolites and separate them from the lipophilic metabolites. The solution was transferred to an Eppendorf tube containing 750 µl of 2:1 methanol:chloroform and vortexed thoroughly. Then, 250 µl of water and 200 µl of chloroform were added to obtain phase separation. The aqueous layer (upper layer) containing the hydrophilic metabolites was isolated and evaporated to dryness using N_2 gas, after which the samples were kept in the freezer until they were run on the NMR spectrometer.

NMR measurements

Immediately before NMR measurements, samples were rehydrated in 50 µl of 50 mmol l^{-1} phosphate buffer (pH 7.4) in D_2O, and 30 µl was transferred to 1.7 mm NMR tubes. Two samples were lost during sample transfer so the final dataset consisted of 64 samples (58 biological samples and 6 blank samples). NMR measurements were performed at 300 K on a Bruker Avance III 600 spectrometer (Bruker Biospin, Rheinstetten, Germany), operating at a ^{1}H frequency of 600.13 MHz, equipped with a 1.7 mm TXI cold probe. ^{1}H NMR spectra were acquired using a 1D NOESY-sequence with water signal suppression by pre-saturation during relaxation delay (4.0 s) and mixing time (100 ms). A total of 256 transients of 32k data points spanning a spectral width of 20 ppm were collected (acquisition time 1.36 s), resulting in a total acquisition time of 26 min per sample.

Data analysis

Osmolality data

Two-way ANOVA was used to test for effects of species and treatment (before/after cold) on hemolymph osmolality. Because initial analysis showed that the data were not normally distributed, the osmolality data were log-transformed. Further, a number of correlation analyses using Pearson's correlation tests were done to examine whether the osmotic differences among species were correlated to cold tolerance. In these correlations, we referenced the newly obtained data on osmolality with recent data on hemolymph Na^+ and K^+ concentrations (MacMillan et al., 2015d) and LTe_{50} (Andersen et al., 2015).

NMR data

Data reduction and statistical analysis

All spectra were referenced to the TSP signal at −0.017 ppm and baseline corrected. Unfortunately, the signal intensity of TSP showed very large variations (presumably due to interactions with larger molecules), such that we could not obtain absolute metabolite concentrations as in Ragan et al. (2013). Before analysis, we removed the remaining water signal (5.1–4.7 ppm) as well as the high- and low-field ends of the spectrum (including TSP at −0.017 ppm). The blank samples (containing no hemolymph) showed a fairly high degree of contamination (presumably from the oil), including very strong signals from an unidentified polyol. The contaminated regions (8.60–8.27, 5.63–5.59, 5.34–5.27, 4.90–4.27, 4.12–4.05, 3.90–3.56, 1.91–1.27 ppm) were removed from the entire dataset. The remaining signals in the spectra were normalized to total intensity to allow for analysis of relative concentrations.

In the initial analysis of the metabolomics data, we wanted to identify metabolic 'fingerprints' specific to species and/or treatment. This was done using principal component analysis (PCA), where variation in large and complex datasets can be reduced to a few components explaining a large fraction of the variation. For the PCA, the data were centered by subtracting the mean intensity of each signal, and scaled using Pareto scaling (Craig et al., 2006) such that the intensity at each frequency was divided by the square root of the standard deviation at that frequency. This analytical approach is a compromise between giving low-concentration metabolites reasonable weight and not putting too much weight on noise. The initial PCA identified one sample, an extract from *D. equinoxialis* kept at room temperature, as an outlier, and this was removed from all further analyses.

To further investigate species differences in the metabolite profiles, we performed an O2PLS (orthogonal partial least squares) discriminant analysis (O2PLS-DA). This analysis resembles PCA, but components are structured to maximize their correlation with group membership (i.e. species and/or treatment) rather than explaining maximal variation. The O2PLS-DA models were validated by cross validation where models were made with randomly chosen groups of samples left out one at a time, and group membership was predicted for the left out samples. All O2PLS-DA and orthogonal partial least squares (OPLS) plots show cross-validated values. As the O2PLS-DA analysis gave a clear separation of species and because this separation correlated very well with cold tolerance, we interpreted the loadings of this analysis to identify the metabolites responsible for the variation in PC1 and

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PC2 (and hence also cold tolerance). Metabolites were identified based on earlier assignments in hemolymph from *D. melanogaster* larvae (Ragan et al., 2013), whole flies (Malmendal et al., 2006; Pedersen et al., 2008) and metabolomics databases (Cui et al., 2008; Wishart et al., 2007) (see Fig. S1 and Table S1).

To characterize the variation among species in the response to the cold treatment, the median spectrum from the control (unexposed) samples from that species were subtracted from each of the cold-exposed spectra [exposed−median(control)]. This yielded a new set of spectra, which were then submitted to PCA modeling, in order to analyze the variation among species. Here, the data were not centered; hence, zero in the PC scores and loadings plots corresponds to no change in the metabolomics profile after cold exposure.

To test whether the metabolite profiles of the five *Drosophila* species showed correlations with their cold tolerance, two OPLS models were made, the first using the metabolite profiles from the untreated group, and the second using the response to cold exposure [exposed−median(control)]. When cross-validating the OPLS models, all samples for each species were left out one at a time, so that the cold tolerance for that 'left out' species could be predicted only based on the relationship between cold tolerance and metabolite concentration for the other four species. This analysis was repeated for all five species and predictability of the model (Q^2), which is the correlation between predicted and actual cold tolerance, was used as a measure of the quality of the model. In this analysis, we also made loading plots in order to identify specific metabolites responsible for the change in metabolite profile associated with cold. The loadings and the correlation coefficient (R^2) between intensities at the individual frequencies and the predictive component were calculated for the entire spectrum, including the regions that were not included in the model. A cutoff value for R^2 corresponding to $P<0.05$ with Bonferroni correction for an assumed number of 30 metabolites was used for identification of significant metabolites. All multivariate analysis was performed using Simca software (Umetrics, Umeå, Sweden).

RESULTS

Cold tolerance, ion balance and hemolymph volume

Cold tolerance of the five *Drosophila* species used here was recently characterized in our laboratory (Andersen et al., 2015). Because these previously published measurements were performed on the same populations and within the same year as the present study, we can draw from these observations when relating differences in osmotic balance and metabolites to cold tolerance. We also recently characterized the ion concentrations and hemolymph volume before and after a cold exposure identical to that used in the present study (MacMillan et al., 2015d). These values are also presented here for reference to the discussion of osmotic balance and metabolite changes (Table 1). In the present study, we focused on the cold-tolerance trait LTe_{50}, which represents the temperature that causes mortality in 50% of individuals after a 2 h cold exposure. LTe_{50} was chosen because it represents an ecologically important measure of cold-induced injury, which we found to be a very powerful correlate of drosophilid latitudinal distribution (Andersen et al., 2015). As seen in Table 1, LTe_{50} varied considerably among the five species: LTe_{50} was much lower for the two temperate species (*D. montana* and *D. persimilis*), intermediate for the widespread species (*D. melanogaster*) and high for the two tropical species (*D. birchii* and *D. equinoxialis*). Table 1 also shows that cold-tolerant species were characterized by a lower initial [Na^+] in the hemolymph and a smaller perturbation of hemolymph volume and [K^+] after cold exposure (see also MacMillan et al., 2015a,d, for further discussion).

Osmolality and ion concentration

When species were measured under benign conditions (20°C), hemolymph osmolality of the five *Drosophila* species ranged from 369.5±10.2 to 426.3±28.8 mOsm (Fig. 1A, Table 1) and in all species except *D. persimilis* the osmolality of the hemolymph increased significantly following cold treatment (two-way ANOVA: $F=63.5$, $P<0.001$). There was no significant correlation between cold tolerance and hemolymph osmolality before cold exposure (Pearson's correlation test; $R^2=0.50$, $P=0.14$; Fig. 1A), but there was a non-significant tendency for the osmolality to be higher in the less cold-tolerant species after cold exposure (Pearson's correlation test: $R^2=0.64$, $P=0.07$; Fig. 1A). This tendency was confirmed by two-way ANOVA; a significant interaction between species and treatment suggests that the osmotic response to cold varied among species ($F=2.9$, $P=0.025$).

The chill-sensitive species had higher hemolymph [Na^+] than the chill-tolerant species and, assuming that each 1 mmol l^{-1} of ions corresponds to 2 mOsm of hemolymph osmolality (i.e. each cation

Table 1. Cold tolerance and hemolymph characteristics of five temperate and tropical *Drosophila* species

	D. birchii	*D. equinoxialis*	*D. melanogaster*	*D. persimilis*	*D. montana*	References
LTe_{50} (2 h) (°C)	−3.3	−4.6	−8.1	−12.1	−13.2	Andersen et al., 2015
K^+ initial (mmol l^{-1})	23	21	15	16	21	MacMillan et al., 2015d
K^+ cold (mmol l^{-1})	51	39	25	12	18	MacMillan et al., 2015d
Na^+ initial (mmol l^{-1})	94	90	76	63	63	MacMillan et al., 2015d
Na^+ cold (mmol l^{-1})	67	69	77	73	78	MacMillan et al., 2015d
Estimated ion osmolality (mOsm)*	234	222	183	159	167	
Osmolality before cold (mOsm)	426	400	374	397	370	Present study
Osmolality after cold (mOsm)	626	505	458	433	462	Present study
Proportion of osmolality accounted for by ions (mOsm)**	0.55	0.56	0.49	0.40	0.45	
Hemolymph volume before (% of wet mass)	7.0	8.0	7.7	7.6	5.3	MacMillan et al., 2015d
Hemolymph volume after (% of wet mass)	4.0	5.8	6.2	7.1	5.0	MacMillan et al., 2015d

Cold tolerance was assayed from the temperature causing 50% mortality (LTe_{50}). Hemolymph [Na^+], [K^+], osmolality and volume were measured before and after a 4 h cold exposure at 0°C. Species are listed in the order of increasing cold resistance (decreasing LTe_{50}).
*Estimated ion osmolality was calculated assuming that each 1 mmol l^{-1} of ions corresponds to 1 mOsm of the hemolymph osmolality. From this, the osmolality of K^+ and Na^+ together with a corresponding anion was calculated. **The proportion of osmolality accounted for by ions was calculated as the ratio between the ion osmolality estimate and the measured hemolymph osmolality before cold exposure.

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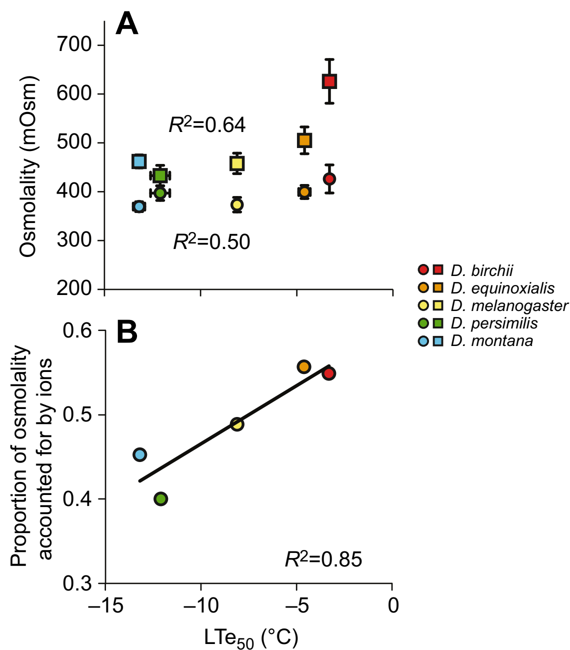

Fig. 1. Osmolality and ion concentration in relation to cold tolerance of five *Drosophila* species. (A) Hemolymph osmolality in untreated controls (circles) and after a 4 h exposure to 0°C (squares) for the five *Drosophila* species (*D. birchii*, *D. equinoxialis*, *D. melanogaster*, *D. persimilis* and *D. montana*) as a function of LTe_{50} (the temperature causing 50% mortality). Values are plotted as means±s.e.m. (error bars that are not visible are obscured by the symbols). (B) Proportion of osmolality accounted for by ions (before cold exposure) plotted as a function of LTe_{50} (see Table 1). Species are color coded according to their cold tolerance (warm colors indicate chill-susceptible species and cool colors indicate chill-tolerant species).

has a corresponding anion), we calculated the proportion of total osmolality accounted for by K^+ and Na^+ (and their accompanying anions; Table 1). As shown in Fig. 1B, chill-tolerant species have less of their osmolality accounted for by the ions than the chill-susceptible species (Fig. 1B, Pearson's correlation test; R^2=0.85 P=0.001).

Metabolomic profile of adult *Drosophila* hemolymph

Fig. 2 depicts a representative ^{1}H NMR spectrum of a control group *D. melanogaster* hemolymph sample. The areas that were removed because of contamination from the oil (see Materials and methods) are marked in gray (see Fig. S1 for an example of the buffer sample without hemolymph). We initially analyzed the spectral variation among all samples using principal component analysis (PCA; N=57, 5–6 samples per species and treatment). This analysis separates the samples based on the variation in metabolite signal intensity only and as shown in Fig. 3 the PCA scores revealed a clear separation of the species, particularly along the first principal component (PC1). Interestingly, the untreated flies of the most cold-tolerant species (*D. montana* and *D. persimilis*) were characterized by low PC1 scores, while the most chill-sensitive species (*D. birchii*) was characterized by high scores, and *D. equinoxialis* and *D. melanogaster* had intermediate PC1 scores.

This initial PCA also reveals that cold exposure shifts the metabolite profile of all species. However, the metabolite response after cold exposure varies among species and the magnitude and direction of the metabolite response change with the cold tolerance (Fig. 3). Thus, cold exposure results in slightly lower PC1 and unaltered PC2 values for the cold-tolerant species (*D. montana*, *D. persimilis*) and intermediate species (*D. melanogaster*) while the more cold sensitive species (*D. birchii* and *D. equinoxialis*) shift toward higher PC1 and lower PC2 scores (Fig. 3).

In order to identify the metabolites that differ between untreated flies from the different species, we used O2PLS-DA, a supervised regression and prediction method that identify the differences in the multivariate dataset responsible for the group variation (importantly, this analysis is also blind to species cold tolerance). In accordance with the initial PCA analysis, this O2PLS-DA analysis effectively separated the different species (Fig. 4A). Again, we found that the species separated according to their cold tolerance such that PC1 scores correlated tightly with LTe_{50} (Pearson's correlation, P=0.001, R^2=0.93; Fig. 4B). A high PC1 score (and a low cold tolerance) corresponds to an increase in phosphocholine and maltose, and a metabolite signal at 7.67 ppm that is likely to represent guanine or xanthine, and is henceforth called 'possible purine'. In contrast, a low PC1 score (and high cold tolerance) corresponds to an increase in trehalose, phosphoethanolamine, β-alanine, succinate, proline and methionine sulfoxide or sarcosine (Fig. 4C). There is also a separation of the species along PC2 that is not immediately related to cold tolerance (Fig. 4A,D).

Given the strong species separation in the O2PLS-DA analysis, we could further demonstrate strong positive relationships between

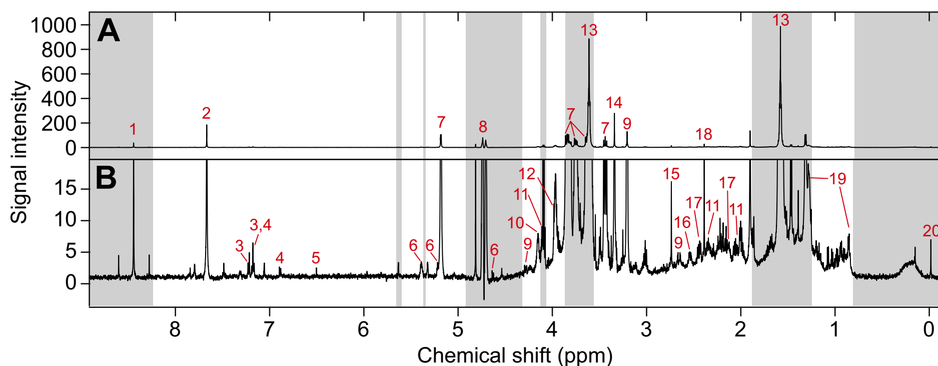

Fig. 2. General ^{1}H NMR spectrum from hemolymph of *D. melanogaster*. Gray shading indicates the areas that were removed in the data reduction process (see Materials and methods). A and B are from the same sample but emphasize strong (A) and weak (B) intensity signals. The most dominant metabolite signals have been assigned numbers: (1) formate, 8.44 ppm; (2) possibly guanine or xanthine, 7.67 ppm; (3) *O*-phosphotyrosine, 7.22, 7.17 ppm; (4) tyrosine, 7.18, 6.89 ppm; (5) fumarate, 6.50 ppm; (6) maltose, 5.39, 5.22, 3.92, 3.89, 3.68, 3.40, 3.26 ppm; (7) trehalose, 5.18, 3.84, 3.81, 3.75, 3.44 ppm; (8) residual water, 4.72 ppm; (9) malate, 4.28, 2.66 ppm; (10) *O*-phosphocholine, 4.15, 3.21 ppm; (11) proline, 4.12, 2.34, 2.06, 1.99 ppm; (12) *O*-phosphoethanolamine, 3.97, 3.21 ppm; (13) polyol, 3.61, 1.58 ppm; (14) methanol, 3.38 ppm; (15) methionine sulfoxide or sarcosine, 2.74 ppm; (16) β-alanine, 2.54 ppm; (17) glutamine, 2.44, 2.13 ppm; (18) succinate, 2.39 ppm; (19) fatty acid, 1.28, 0.85 ppm; (20) trimethylsilyl propionate, −0.017 ppm.

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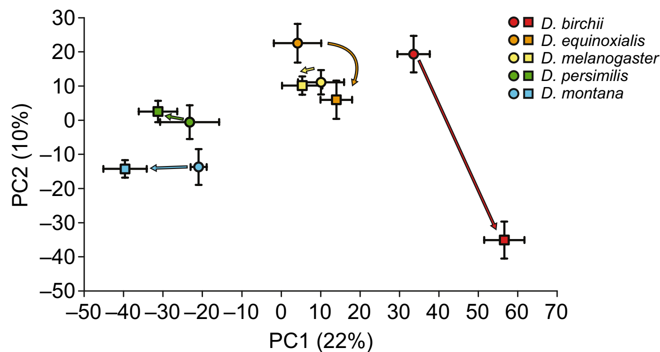

Fig. 3. Principal component analysis (PCA) scatter plot for the five species before (circles) and after (squares) exposure to 0°C for 4 h. Arrows indicate the metabolomic response to cold exposure. Each point represents the mean±s.e.m. PCA score of a given species and treatment (*N*=5–6 from each species and treatment combination). The two first components account for 22% and 10% of the variation, respectively.

cold tolerance and individual metabolites (especially trehalose and methionine sulfoxide or sarcosine) and negative correlations to other substances such as maltose and phosphocholine (see Fig. S2 for further details).

Metabolite response to cold exposure

To further characterize the metabolite response to cold exposure, we performed PCA on the metabolite changes, defined as the metabolite spectra of cold-exposed flies minus the median of all (*N*=5–6) unexposed (control) samples of that species. The data were not centered before PCA, and therefore the distance to zero in the scores plot reflects the relative magnitude of the cold response.

As shown in Fig. 5A, the metabolite response to cold exposure was largest in the chill-susceptible species (*D. birchii* and *D. equinoxialis*). The chill-susceptible species are characterized by negative scores along PC1, implying an increase in maltose and the probable purine, and simultaneous decreases in trehalose, phosphotyrosine, phosphocholine, proline and succinate (Fig. 5A,B). The cold-tolerant *D. persimilis* and *D. montana*, in contrast, show positive PC2 scores, indicating increases in the relative abundance of trehalose, glutamine and the possible purine and decreases in phosphotyrosine, phosphoethanolamine, fumarate, proline, β-alanine and succinate (Fig. 5A,C). *Drosophila melanogaster*, which have an intermediate cold tolerance, shows responses that are intermediate between the two extremes.

Prediction of cold tolerance from the metabolite profile

OPLS models were made to test the direct relationship between hemolymph metabolite profiles and cold tolerance (the model included only two components, one predictive and one orthogonal). Values were predicted with leave-one-out cross-validation, where the cold tolerance for each species was predicted using a model based on data from the metabolite profiles of the other four species. The loading plots for the control samples show that high levels of trehalose, proline, malate, succinate, fumarate and methionine sulfoxide or sarcosine and low levels of phosphocholine, formate

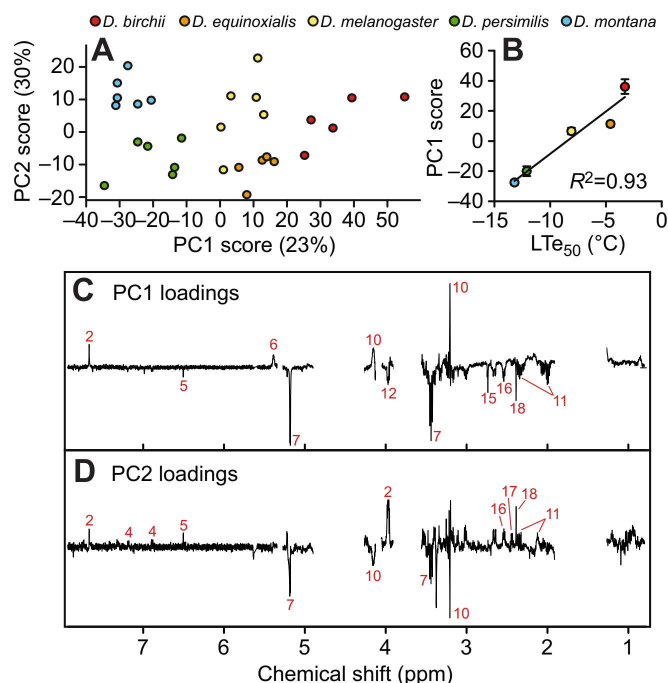

Fig. 4. Hemolymph metabolite variations between five *Drosophila* species with different cold tolerance. (A) Orthogonal partial least squares discriminant analysis (OPLS-DA) scatter plot of individual samples of untreated flies of the five different species. (B) Correlation plot for LTe_{50} and PC1 score. (C) Loading plot for PC1 that identifies the individual metabolites in the spectral signals associated with PC1. The signal intensity provides information on the amplitude of the metabolite changes and whether the metabolite is present in higher or lower concentration (positive and negative signal intensity, respectively). (D) Loading plot for PC2. Numbers in both loading plots correspond to the metabolites given in Fig. 2.

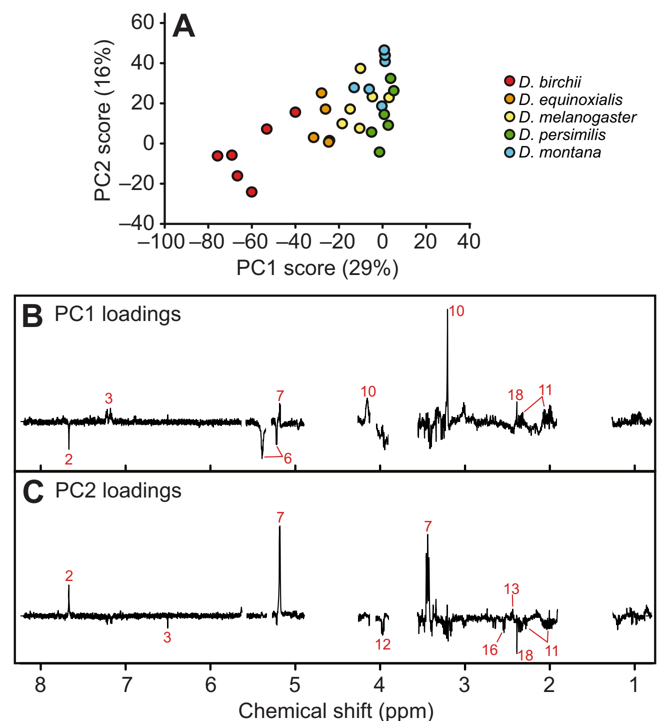

Fig. 5. Changes in hemolymph metabolites after cold exposure in five *Drosophila* species with different cold tolerance. (A) PCA scatter plot for the response to cold exposure for the five species. Each point represents a cold-exposed sample minus the median(control) of the same species. The origin (0, 0) corresponds to no response relative to the control. (B) Loading plot for PC1. The loading plot identifies the individual metabolites in the spectral signals associated with PC1. The signal intensity provides information on the amplitude of the metabolite changes and whether the metabolite is present in higher or lower concentration (positive and negative signal intensity, respectively). (C) Loading plot for PC2. Numbers in both loading plots correspond to the metabolites given in Fig. 2.

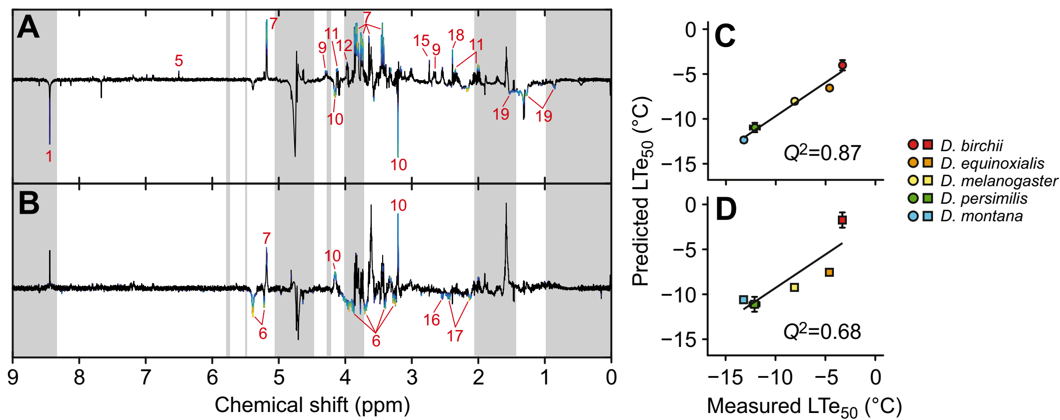

Fig. 6. Model based on metabolomics data, to identify metabolite profiles that are predictive of *Drosophila* cold tolerance. See Materials and methods and Results for details. (A) Loading plot identifying the individual metabolites predicting cold tolerance from the metabolite profile of untreated *Drosophila*. The signal intensity provides information on whether the metabolite is present in higher or lower concentration (positive and negative signal intensity, respectively). Significant alterations were color coded from blue to red, with red representing the highest correlation between the metabolite and the cold-tolerance model. (B) Loading plot for predicting cold sensitivity from the metabolite response to cold stress. (C) Correlation between LTe_{50} values predicted from untreated metabolite levels using cross-validation and measured values. (D) Correlation between LTe_{50} values predicted from the cold response and measured values.

and fatty acid are significantly and positively correlated to cold tolerance (Fig. 6A). The predictability of the model (Q^2), which is the correlation between the actual cold tolerance (LTe_{50}) and that predicted from the metabolite profile, was 0.87 (P=0.0026).

The same analysis was repeated for the metabolite changes following cold exposure. Here, the cold-tolerant species responded to cold by a higher increase or lower decrease in trehalose and phosphocholine and the possible purine, and a lower increase or higher decrease in maltose, glucose, β-alanine and glutamine (Fig. 6B). For this analysis, we found the Q^2 for LTe_{50} was 0.68 (P=0.0015). The strong link between the changes in metabolome and chill tolerance is further emphasized by an even better prediction (Q^2=0.86) when we repeated the analysis using the time to stand following the particular cold treatment used here, as an alternative cold-tolerance measure (data not shown). The metabolite changes following cold were thus also closely related to variation in other cold-tolerance traits.

DISCUSSION

Hemolymph osmolality before and after cold exposure

Previous studies have found that the cold tolerance of *Drosophila* spp. is tightly linked to the hemolymph [Na^+], so that the more chill-tolerant species depend less on Na^+ as an osmolyte in the hemolymph (MacMillan et al., 2015b,d). This suggests that either hemolymph osmolality is lower in cold-tolerant species or cold-tolerant species have higher concentrations of other osmolytes in their hemolymph. In the present study, we found no differences in osmolality among species in benign conditions (Fig. 1A), suggesting that cold-tolerant species maintain higher hemolymph concentrations of other compatible osmolytes. This finding is further supported by a significant negative correlation between cold tolerance and the proportion of osmolality that can be accounted for by ions (i.e. the correlation between LTe_{50} and ion contribution is positive; Fig. 1B). We also examined hemolymph osmolality after cold exposure and found that osmolality increased in four of the five species. In this case, we found a (non-significant) trend suggesting that the cold-tolerant species had smaller increases in osmolality following cold stress, which supports the hypothesis that cold-tolerant insects may better maintain water balance in the cold (Koštal et al., 2004; MacMillan and Sinclair, 2011b; MacMillan et al., 2015a,d).

The present study cannot directly reveal why osmolality increases with cold, but we speculate that three mechanisms could be involved. Firstly, different species may experience different degrees of respiratory, evaporative or excretory water loss during cold exposure. Indeed, tropical (and cold-sensitive) *Drosophila* species are generally also more susceptible to dehydration than xeric or cold-adapted species (Kellermann et al., 2013). Further, it has previously been shown that dehydration exacerbates the development of chill injury in *D. melanogaster*, particularly at moderate cold exposure temperatures (Kobey and Montooth, 2013). It should be mentioned, however, that the water vapor deficit is very low during the cold exposure and the flies are therefore unlikely to be severely stressed by dehydration during short-term exposures (Kobey and Montooth, 2013). Secondly, it is possible that dissipation of osmotic gradients occurs within the animal as a consequence of reduced active transport at low temperatures. While it is normally recognized that there cannot be any osmotic gradients between intracellular and extracellular spaces (Hill et al., 2008), it is possible to maintain osmotic gradients in different body compartments through continuous active transport. It has been shown, for example, that the insect hindgut is markedly hyperosmotic relative to the hemolymph and somatic cells (Edney, 1977). Hyperosmolality of the gut is dependent on continuous active ion transport in the rectal wall driving reabsorption of water from the gut to the hemocoel and we therefore speculate that cold-induced impairment of active transport in the hindgut could lead to an increase in hemolymph osmolality. Finally, it is possible that increases in hemolymph osmolality are a consequence of (adaptive or maladaptive) breakdown of protein or glycogen sources or transport to/from the hemocoel in response to cold (Edney, 1977; Hochachka and Somero, 2002). Regardless of the proximate cause of these osmotic perturbations, our data clearly indicate that cold exposure is associated with larger osmotic disturbance in the most sensitive species. For all species, we observed that the total cation concentration (sum of [Na^+] and [K^+]) remained fairly constant before and after cold exposure, while osmolality increased in most species (Table 1). Assuming that the concentrations of anions are also relatively constant after the volume change, we conclude that the osmolality increase must be associated with increases in other osmolytes and that these increases are more dramatic in the cold-sensitive species.

General metabolite profiles

Based on previously published data (MacMillan et al., 2015d), we knew that cold-tolerant *Drosophila* experience smaller disturbances in ion homeostasis during cold exposure (Table 1). This homeostatic capacity is partially attributed to smaller Na^+ gradients between the gut lumen and hemolymph, which help to preserve hemolymph water balance (see MacMillan and Sinclair, 2011b; MacMillan et al., 2015a,b,d, for further discussion; Table 1). Because cold-tolerant species have lower [Na^+] but similar osmolality, we suggest that these species must replace Na^+ with other osmolytes in the hemolymph (Fig. 1A,B). To investigate this hypothesis, we characterized the metabolomic profile of hemolymph samples using ^{1}H NMR spectroscopy and found clear interspecific differences in the hemolymph metabolite profile (note, these differences represent qualitative differences in signal intensity that are not necessarily directly proportional to concentration differences). Importantly, the metabolic profiles of species segregated in very close association with their cold tolerance (Figs 3 and 4). Specifically, the characteristics of the cold-tolerant species before cold exposure were larger relative signals of sugars (trehalose and glucose) and free amino acids (β-alanine, proline and methionine sulfoxide or sarcosine) and lower signals of maltose, phosphocholine and the possible purine (Fig. 4C).

The spectra showed that trehalose is by far the most abundant sugar molecule in the hemolymph and that the greater abundance of trehalose in tolerant species more than outweighs the lower levels of maltose found in these species. Chill-tolerant species are therefore generally characterized by a higher content of sugars in their hemolymph. Trehalose is a known cryoprotectant which can contribute to cold tolerance colligatively by suppressing the super cooling point (Zachariassen, 1985). However, given the modest (normal) osmolality found in the *Drosophila* hemolymph, it is unlikely that the increased relative signal for trehalose found in either *D. persimilis* or *D. montana* exerts any important colligative effect. Nevertheless, trehalose (in low concentrations) has previously been found to facilitate other non-colligative functions in stressed organisms. These functions include protection of native proteins and biological membranes by preferential exclusion from their hydration shells (Arakawa and Timasheff, 1983,1985), assistance in refolding unfolded proteins by molecular chaperones (Lee and Goldberg, 1998; Viner and Clegg, 2001), scavenging for oxygen radicals (Benaroudj et al., 2001) and replacement of missing water molecules in hydration shells of proteins and phospholipid membranes during desiccation (Yancey et al., 1982; Crowe et al., 1987, 1988; Crowe, 2002). We were unfortunately unable to directly quantify trehalose content in the present study and cannot therefore conclude whether the concentrations were high enough to offer increased cold tolerance by any of these mechanisms. However, small increases in trehalose are found on a whole-organism level after both rapid cold hardening and long-term cold acclimation in larval and adult *D. melanogaster* (Overgaard et al., 2007; Koštal et al., 2011a; Colinet et al., 2012). Further, whole-organism levels of trehalose (and glucose) were found to increase during autumn and winter in *D. montana* (Vesala et al., 2012). However, interestingly, feeding with dietary sugars does not increase cold tolerance of *D. melanogaster* even though it increases the general level of sugars (Colinet et al., 2013).

Of the amino acids, we found proline to be higher in cold-tolerant species. Proline has also been found to have non-colligative effects that increase insect cold tolerance. Like trehalose, it is unclear from this study whether the relatively large abundance of proline in cold-tolerant species has any direct cryoprotective role but proline may also help to protect proteins and cell membranes by preferential exclusion from hydration shells (Arakawa and Timasheff, 1983, 1985). Several other studies have found increases in free amino acid levels in relation to different cold-acclimation treatments. In cold-acclimated adult *D. melanogaster*, several amino acids increased (Colinet et al., 2012) and in *D. melanogaster* larvae, long-term cold acclimation increased levels of proline in particular (Koštal et al., 2011a). Furthermore, in *D. montana*, proline varies seasonally, showing a maximum in the winter (Vesala et al., 2012), while fluctuating thermal regimes and diapause lead to increased levels of many amino acids (e.g. lysine, isoleucine and leucine) in the tropical beetle *Alphitobius diaperinus* and codling moth *Cydia pomonella*, respectively (Lalouette et al., 2007; Rozsypal et al., 2013). Finally, proline seems to be a very potent (and dominant) cryoprotectant in the freeze-tolerant drosophilid *Chymomyza costata* (Koštal et al., 2011b). Most convincingly, increased dietary proline allows *C. costata* to survive freezing in liquid nitrogen (Koštal et al., 2011b), and (when combined with cold acclimation) a proline-augmented diet even allows *D. melanogaster* larvae to survive freezing of almost 50% of their body water (Koštal et al., 2012).

The small sample volumes and sensitive techniques used in this study caused a fairly high degree of contamination in the samples. As a result of this contamination, we excluded several areas of the spectra from the analysis (Fig. 2). These removed areas could potentially contain important information on other important metabolites such as glycerol and other polyols whose chemical shifts lie in these areas. Further, NMR spectroscopy generally has lower sensitivity than, for example, mass spectroscopy-based techniques and it is therefore quite possible that our analysis has missed compounds that are in low concentration or are obscured by the contamination found in some regions of the spectra. Glycerol is a very important cryoprotectant in many freeze-avoiding insect species (Zachariassen, 1985) and has also been found to be associated with increased cold tolerance in cold-sensitive species like the flesh fly and the tropical beetle *A. diaperinus* (Chen et al., 1987; Yoder et al., 2006; Michaud and Denlinger, 2007; Lalouette et al., 2007; Teets et al., 2012). Glycerol has not previously been associated with cold tolerance in *Drosophila* (Kelty and Lee, 1999; Overgaard et al., 2007; Koštal et al., 2011a; Colinet et al., 2012), but *D. montana* does accumulate large amounts of another polyol, myo-inositol, as a cryoprotectant (Vesala et al., 2012). However, analysis of the entire spectra without removal of the contaminated areas did not show any strong signals for these compounds.

In summary, the metabolite profiles of cold-tolerant species of *Drosophila* show relatively high levels of sugars and amino acids compared with more sensitive species. These compounds (including trehalose and proline) are likely to replace Na^+ to preserve osmolality, but they could also potentially contribute to the higher cold tolerance of these species. Although absolute concentrations were not measured in the present study, the magnitude of the differences in the metabolite profile are unlikely to offer cryoprotection by means of colligative effects (Zachariassen, 1985) and instead we suggest that these compounds offer cryoprotection by means of non-colligative effects. In support of this, we found that the metabolite profiles were very exact in their prediction of the cold tolerance of the species (Fig. 6C). This indicates that the metabolomic profile is highly important (and predictive) for species-specific cold tolerance. Cryoprotectants are classically associated with adaptations to avoid or tolerate ice formation (Zachariassen, 1985; Ramløv, 2000) but in these chill-sensitive species we demonstrate that classical cryoprotectants also play an important role for chill-susceptible insects. Thus, we propose that these compatible sugars and amino acids are important for osmotic balance and non-colligative cellular protection, and suggest that this mechanistic role of cryoprotectants (in low concentrations)

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might represent a new/alternative view on the pathway that has led to the evolution of freeze tolerance and freeze avoidance.

Metabolite response to cold exposure

The metabolome of the very chill-sensitive species *D. birchii* responded vigorously to cold exposure (Fig. 5A) with massive increases in maltose levels and smaller decreases in phosphocholine and trehalose. *Drosophila equinoxialis* and *D. melanogaster* showed smaller but qualitatively similar responses with the addition of an increase in the possible purine. Increases in maltose levels have previously been found in response to cold shock treatments in *D. melanogaster* (Overgaard et al., 2007; Pedersen et al., 2008) and maltose has also been found to increase in *D. melanogaster* selected for high chill susceptibility (Williams et al., 2014). Maltose has been linked to the protection and function of proteins and membranes during temperature stress (Kaplan and Guy, 2004; Pereira and Hünenberger, 2006), and maltose accumulation has been linked to a stress-induced increase in amylase activity in plants (Kaplan and Guy, 2004). The accumulation of maltose in the chill-susceptible species could therefore function to reduce the likelihood of chill injury following cold exposure, but may also be a more general and non-specific response to stressful conditions. Increased levels of maltose have previously been linked to a wide range of different stressors like inbreeding, thermal stress, starvation and artificial expression of the amyloid β-peptide (Pedersen et al., 2008; Malmendal et al., 2013; Ott et al., 2016).

Following cold exposure, *D. persimilis* and *D. montana* showed very little change along the trajectory of the more chill-sensitive species, while both showed increases in the relative abundance of trehalose and the possible purine (Fig. 5B,C). The relatively small response to cold exposure suggests that these species maintain their metabolite homeostasis far better than the chill-susceptible species. Similarly, the metabolomes of cold-selected, cold-acclimated and rapidly cold-hardened *D. melanogaster* are less responsive to cold exposure than warm-acclimated flies or flies directly exposed to a cold shock (Overgaard et al., 2007; Colinet et al., 2012; Williams et al., 2014). The increases in trehalose and the possible purine found in response to cold in *D. persimilis* and *D. montana* seem to be adaptive for coping with the cold stress. Indeed, in a parallel study, we found that these two species were able to reverse the cold-induced drift of ions down their gradients and began to recover neuromuscular function and stand while still being held at 0°C (MacMillan et al., 2015d), which suggests that these species are capable of acclimating at this temperature.

Conclusions

This study has provided the first examination of hemolymph-specific metabolite profiles and osmolality measurements both before and after cold exposure in five *Drosophila* species with different cold tolerances. We found that there were no osmolality differences among species before cold exposure but that a larger proportion of the hemolymph osmolality was accounted for by organic osmolytes in chill-tolerant species. ^{1}H NMR spectroscopy on the hemolymph revealed that the chill-tolerant species had higher levels of trehalose and proline and that their metabolite profiles correlated remarkably well with cold tolerance. The response to cold exposure was increased osmolality in all species, suggesting that all species had an accumulation of osmolytes during cold exposure. However, during cold exposure, the chill-sensitive species lost hemolymph water and accumulated the stress-related metabolite maltose, while the chill-tolerant species accumulated primarily trehalose (which may be a more adaptive response). High levels of trehalose and proline in the hemocoel may offer cryoprotection through non-colligative and osmotic effects in chill-sensitive insects. Further, we suggest that the small increases found in the more tolerant species might represent a step in an evolutionary pathway to true cold tolerance where these compounds are accumulated to such high levels that they offer cryoprotection from both colligative and non-colligative effects. In this respect, it would also be interesting to examine the somewhat larger increases in cryoprotectants found in diapausing drosophilids such as *D. montana* as metabolite changes reported under these conditions (Vesala et al., 2012) are considerably larger, albeit still too low to confer significant colligative effects.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
T.O., H.M.A., A.M. and J.O. contributed to the design of the experiments. T.O, N.N. and D.S. performed the experiments. T.O. H.M.A. and A.M analyzed the data. T.O, A.M. and J.O. wrote the manuscript, and all of the authors revised the manuscript.

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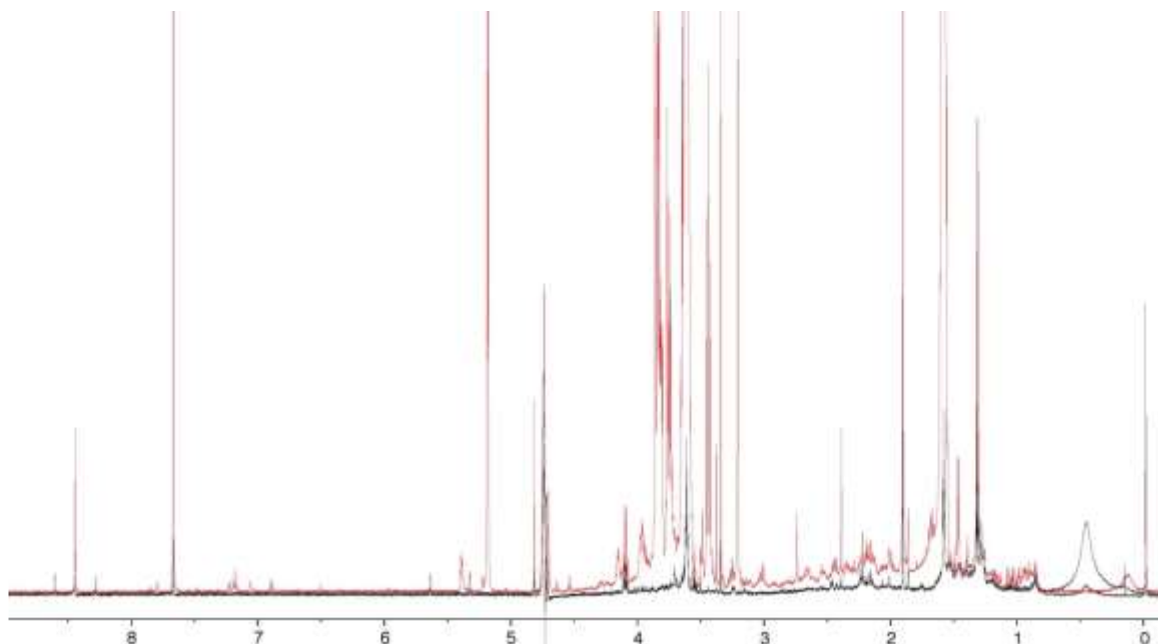

Fig. S1: Two examples of NMR-spectra from blank samples (black lines) compared to a biological sample (red line). The blank samples were obtained by sampling the buffer droplet that had been placed for up to 45 min. The biological sample is from a cold exposed *D. melanogaster*. On the basis of such comparisons we identified regions of the NMR spectra that were contaminated and these were omitted from the analysis (See Fig. 1 in the main manuscript).

The individual metabolites found in the spectra are listed in table S1. The variation in metabolite content can also be seen from a more simple correlation analysis between the relative intensities of the individual metabolites and the cold tolerance measure LTe_{50} (Fig. S1A-D). The metabolite intensities have first been normalized to the total intensity in each spectrum which allows us to compare the intensities regardless of how many flies were used in each sample. However it is important to remember that the spectra allow us to "see" H-atoms on different molecules. This means that if there are more of "the same" H-atoms on a given molecules the intensity of its signal will increase accordingly.

Therefore the metabolite intensities shown in Fig. S1 are also normalized to the number of hydrogen atoms that were present in the signals used for each metabolite (given in table S1). This allows the y-axes in figure S1 to be directly compared even though the intensities themselves are still only relative (to the total signal intensity). This is important since it gives us the ability to see that trehalose is by far the most abundant metabolite of the four (appr. 40 times more abundant than sarcosine) which means that it is also far more important as an osmolyte.

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Table S1: Identified metabolites in the NMR spectra. No. corresponds to the numbers given in the spectrum in Fig. 2.

No	Name	Chemical shift	No. of hydrogen atoms
1	Guanine/Xanthine	7.67 ppm	1
2	Formate	6.50 ppm	1
3	O-phosphotyrosine	7.22, 7.17 ppm	2
4	Tyrosine	7.18, 7.89 ppm	2
5	Maltose	5.39, 5.22 ppm	1
6	Glucose	5.21, 3.97 ppm	1
7	Trehalose	5.18, 3.44 ppm	2
8	O-phosphocholine	4.15, 3.21 ppm	9
9	Methanol	3.38 ppm	3
10	unidentified metabolite	3.01, 1.71 ppm	?
11	methionine sulfoxide or sarcosine	2.74 ppm	3
12	β-alanine	2.54 ppm	2
13	Glutamine	2.44 ppm	2
14	Sarcosine	2.39 ppm	2
15	Proline	2.34, 2.06, 1.99 ppm	2
16	Unidentified metabolite	1.09 ppm	?
17	Unidentified fatty acid	0.85 ppm	3

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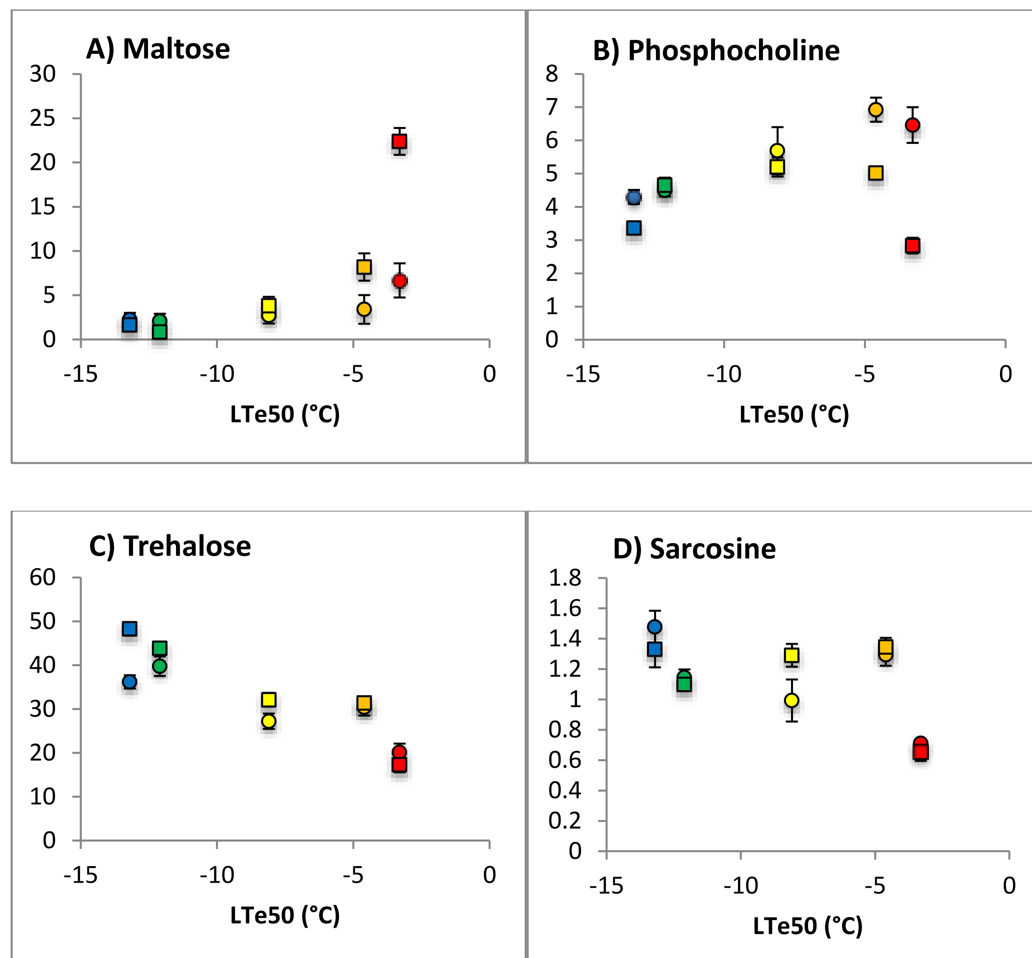

Fig. S2: The relative intensities of four specific metabolites identified in the five species before (circle) and after cold treatment (square). The metabolites are A) Maltose B) Phosphocoline C) Trehalose and D) Sarcosine