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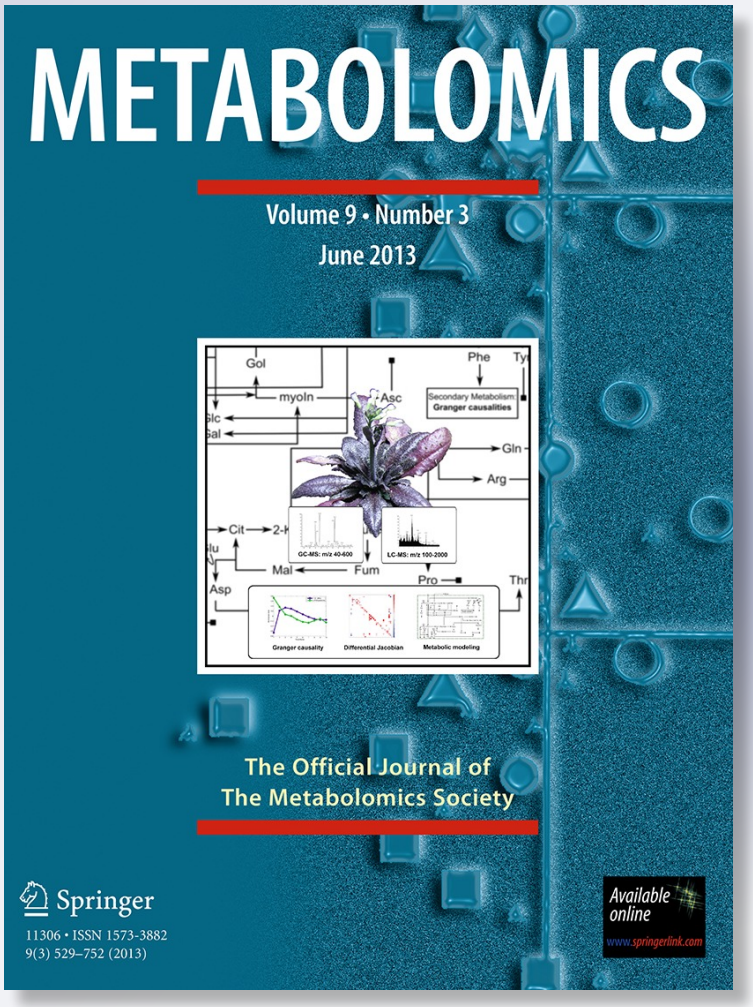


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ORIGINAL ARTICLE

# Dietary sugars affect cold tolerance of *Drosophila melanogaster*

**Hervé Colinet · Vanessa Larvor · Raphaël Bical · David Renault**

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**Abstract** In spite of the extensive knowledge of the biology and the genetics of *Drosophila melanogaster*, the mechanisms by which this fly builds up cold tolerance remain poorly understood. Recent studies have reported that acclimation-mediated acquisition of cold tolerance is associated with moderate accumulation of sugars in drosophilids. However, it is not known whether there is a genuine causative link between cold tolerance and body sugar accumulation in *Drosophila* flies. We thus tested whether increasing body sugars levels, via dietary enrichment, will promote the cold tolerance of *D. melanogaster* adults. We gradually augmented the concentration of four different sugars (sucrose, fructose, glucose and trehalose) in rearing diets and tested the basal cold tolerance (acute and chronic). Using SIM-GC/MS approach, we verified whether feeding of larvae and adults on sugar-enriched diets was associated with increasing body sugars. We also tested whether development, body mass, fat stores, metabolites composition and metabolic pathways were altered by these dietary manipulations. The data confirm an effective incorporation of all sugars. Contrary to the expectation, cold tolerance was negatively affected by exogenous sugars, especially when supplemented at high concentrations. Rearing on high-sugar doses induced system-wide metabolic alteration associated with carbohydrate metabolism imbalance, a developmental delay and a fresh mass reduction. Our data show that high dietary sugars create a metabolic imbalance and negatively affect cold tolerance. This study provides an intriguing connection between nutritional conditions and thermal trait. It also underlines that careful attention should be given to dietary factors when studying thermal traits.

**Keywords** *Drosophila* · Carbohydrate · Diet · Cold tolerance · Metabolic fingerprinting

## 1 Introduction

Thermal tolerance of ectotherms and its plasticity have long been a central theme in the field of ecology, physiology and evolutionary biology. In many insect species, acquisition of cold tolerance involves the accumulation of large quantities of cryoprotective solutes, such as polyols, sugars and free amino acids (Lee 2010; Storey and Storey 2012). Polyols, such as glycerol, are by far the most common cryoprotectants found in insects (Storey and Storey 2005). In spite of the extensive knowledge of the genetics of *Drosophila melanogaster* (Diptera: Drosophilidae) and the long experimental experience with this model organism, a good picture of how this fly builds up cold tolerance has not yet been clearly established (Korsloot et al. 2004; Doucet et al. 2009). There is no evidence of the role of polyols in promoting cold tolerance of *D. melanogaster* (Kelty and Lee 2001; Overgaard et al. 2007; Colinet et al. 2012a), but *myo*-inositol seems related to overwintering in the northern species, *Drosophila*

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*montana* (Vesala et al. 2012). Other compatible solutes, with assumed thermoprotective functions, might contribute to the cold tolerance of fruit flies. Several studies have reported that acclimation-mediated acquisition of cold tolerance was associated with accumulation of sugars, such as sucrose, fructose, glucose and trehalose in drosophilids (Kimura 1982; Overgaard et al. 2007; Koštál et al. 2011a, 2012; Colinet et al. 2012a; Vesala et al. 2012). While these studies suggest a growing consensus regarding the potential implication of sugars in the cold tolerance of *D. melanogaster*, so far, it is not known whether there is a genuine causative link between cold tolerance and sugar accumulation in this species.

Variation of dietary composition has proved to be an efficient method for manipulating body concentration of targeted compounds, and thus addressing their functional role(s) in various biological traits, including thermal response (e.g. Shreve et al. 2007, Smith et al. 2007; Rzezniczak et al. 2011; Koštál et al. 2011b). There is a vast literature describing how modifications of dietary sugars (via restriction or excess) affect fitness- and physiological traits of *D. melanogaster* (Wang and Clark 1995; Partridge et al. 2005; Skorupa et al. 2008; Reed et al. 2010; Matzkin et al. 2011; Musselman et al. 2011). However, the impact of dietary sugars on stress-related traits is much less known (Lushchak et al. 2011; Rzezniczak et al. 2011), and this is particularly true for thermal stress. So far, only a few studies have examined whether dietary manipulation of sugars affects thermal traits of *D. melanogaster*. Koštál et al. (2012) found that feeding on trehalose-augmented diet marginally affected the freezing tolerance of *D. melanogaster* larvae. Burger et al. (2007) reported that dietary enrichment of both sucrose and yeast simultaneously did not affect chill coma recovery (CCR) of young flies (i.e. 4- and 22-days-old), but marginally promoted CCR of senescent adults (i.e. 33-and 47-days-old). By contrast, Andersen et al. (2010) found that CCR of 3-days-old flies reared on a sucrose-enriched diet was faster than CCR of flies developed on a protein-enriched diet. However, they did not report the effect of sucrose-enrichment *per se*, as there was no control diet (i.e. with no enrichment).

On the basis of the earlier findings that variations of body sugar concentrations, even moderate, seem associated with the acquisition of cold tolerance in *D. melanogaster* (Overgaard et al. 2007; Koštál et al. 2011a, 2012; Colinet et al. 2012a) and in other *Drosophila* species (Kimura 1982, Vesala et al. 2012), we hypothesized that manipulating body level of sugars via dietary supplementation may positively affect cold tolerance. We gradually augmented dietary concentration of four different sugars (sucrose, fructose, glucose and trehalose) and examined if the basal cold tolerance of adults (acute and chronic stress) was promoted. Using a targeted SIM-GC/MS approach, we first verified whether feeding of larvae and adults on sugar-augmented diets was associated with a corresponding increase in sugars in whole-body extracts. Our experimental approach, based on metabolic fingerprinting, also permitted us to check whether metabolites' composition and metabolic pathways were altered by diet manipulations. Finally, in addition to cold tolerance, we also tested whether rearing on sugar-enriched diets affected the body mass and the fat body stores of adults. The data show that sugar and triglyceride concentrations increased in sugar-augmented phenotypes, confirming an effective incorporation of sugars. However, contrary to our expectation, cold tolerance was negatively affected by exogenous sugars, especially when supplemented at high concentrations. All together, metabolic profiles, developmental delay and fresh mass reduction suggest that high dietary sugars are detrimental (pathological) for flies.

## 2 Materials and methods

### 2.1 Fly culture

We conducted our experiments on a mass-bred *D. melanogaster* line derived from the mix of two wild populations collected in October 2010 at Plancoët and Rennes (Brittany, France). Prior to the experiment, flies were maintained in laboratory in 200 mL bottles at 25 ± 1 °C (16L:8D) on standard fly medium consisting of brewer yeast (80 g/L), sucrose (50 g/L), agar (15 g/L), kalmus (9 g/L) and Nipagin® (8 mL/L).

### 2.2 Experimental design

Four different types of sugar were tested separately in rearing diets: sucrose (Suc), fructose (Fru), glucose (Glc) and trehalose (Tre). Sucrose is a disaccharide composed of Glc and Fru, and Tre is disaccharide composed of two Glc units. The concentration of each targeted sugar was gradually augmented in diets to generate four different concentration levels: control (C0) with no sugar (0 mM), C1, C2 and C3 where the concentration was 10, 400 and 1,000 mM respectively. The concentrations of the other constituents of the diet (i.e. yeast, agar, kalmus and Nipagin®) remained the same as in the standard fly medium (see above). All recipes are detailed in Table S1.

To generate flies for the experiments, groups of 15 mated females were allowed to lay eggs in 200 mL bo ttles containing the different sugar concentrations during a restricted period of 6 h. This controlled procedure allowed larvae to develop under uncrowded conditions at 25 ± 1 °C (16L:8D). At emergence, adult flies were collected and development times were noted. Adults were

sexed visually (with an aspirator) without $CO_2$ to avoid any confusing metabolic effects due to $CO_2$ anesthesia (Colinet and Renault 2012), and only females were kept for subsequent trials. Females were then transferred to fresh diets with the same sugar concentration as that experienced during larval development. Females were left to age on their specific diet for 5 days before being used for the experiments. Diets were changed every day. For each type of sugar, the rearing with the four different concentrations was initiated simultaneously. At the end of the rearing period, pools of females from each nutritional group were used for the assays or snap-frozen in liquid nitrogen and stored at −80 °C until metabolite and triglyceride assays.

### 2.3 Cold tolerance assays

Different metrics were used to assess cold tolerance of the adults. Recovery time following a nonlethal chronic cold stress was measured as previously described (Colinet et al. 2010). Briefly, for each sugar x concentration combination (i.e. 4 sugars × 4 concentrations), 50 females were exposed to 0 °C for 16 h by placing a vial in a cold incubator (Model MIR-153, SANYO Electric Co. Ltd, Japan). Flies were then allowed to recover at 25 ± 1 °C (16L:8D) and recovery times were individually recorded. Data were used to generate temporal recovery curves which were compared with Mantel-Cox analysis (Colinet et al. 2010). After scoring the recovery times, the same females were returned to 25 ± 1 °C (16L:8D) on their respective diet and the mortality was scored 24 h after the exposure to the chronic cold stress (0 °C for 16 h).

Tolerance to acute cold stress was scored by measuring mortality 24 h after an exposure to −3.5 °C for 2 h. Most mortality in *D. melanogaster* adults happens within 24 h after the cold stress (Rako and Hoffmann 2006), and we therefore did not consider a longer period. For each treatment combination, a total of 100 females (5 × 20) were placed in 42 mL glass vials immersed in a glycol solution cooled to −3.5 °C for 2 h. After the acute cold stress, the flies were returned to 25 °C on their respective diet, and the mortality was scored after 24 h. Chi square contingency tests were used to compare mortality rates between concentration levels.

### 2.4 Metabolic fingerprinting

For each nutritional group, six biological replicates, each consisting of a pool of 15 females, were used for metabolic fingerprinting. Each sample was weighed (Mettler Toledo UMX2, accurate to 0.001 mg) before metabolite extractions. Sample preparation and derivatization were performed as previously described (Colinet et al. 2012b) with minor modifications. Briefly, after homogenization in methanol-chloroform solution (2:1) and phase separation with 400 µL of ultrapure water, a 120 µL aliquot containing polar metabolites was vacuum-dried. The dry residue was resuspended in 30 µL of 20 mg $mL^{-1}$ methoxyaminehydrochloride in pyridine before incubation under automatic orbital shaking at 40 °C for 60 min. Then, 30 µL of MSTFA was added to make a total volume of 60 µL and the derivatization was conducted at 40 °C for 60 min under agitation (see Colinet et al. 2012b). A CTC CombiPal autosampler (GERSTEL GmbH and Co.KG, Mülheim an der Ruhr, Germany) was used, ensuring standardized sample preparation and timing.

Metabolites were separated, identified and quantified using a GC–MS platform consisting of a Trace GC Ultra chromatograph and a Trace DSQII quadrupole mass spectrometer (Thermo Fischer Scientific Inc, Waltham, MA, USA). We used the analytical method previously described by Colinet et al. (2012b), with minor temperature ramping changes. The oven temperature ranged from 70 to 170 °C at 5 °C $min^{-1}$, from 170 to 280 °C at 7 °C $min^{-1}$, from 280 to 320 °C at 15 °C $min^{-1}$, and then the oven remained 4 min at 320 °C. We completely randomized the injection order of the samples. All samples were run under the SIM mode rather than the full-scan mode. We therefore only screened for the 60 pure reference compounds included in our custom spectral database. Calibration curves for 60 pure reference compounds at 10, 20, 50, 100, 200, 500, 700, 1000, 1500 and 2,000 µM concentrations were run concurrently. Chromatograms were deconvoluted using XCalibur 2.0.7, and metabolite levels were quantified based on the quadratic calibration curves for each reference compound and concentration. Arabinose was used as the internal standard (see Colinet et al. 2012b). For every type of sugar, examples of SIM-GC/MS chromatograms at the concentrations C0 and C3 are provided in Figure S1; the retention times for all detected compounds are listed in Table S2.

Among the 60 metabolites included in our spectral library, 37 were detected in our samples. We found 14 free amino acids, nine sugars, five polyols, three metabolic intermediates and six other metabolites (see Table 1 for compounds' list and abbreviations). For each metabolite, the variations of concentration (log-transformed) were first analyzed individually using two-way ANOVAs with 'type of sugar' and 'concentration dose' as the main factors. ANOVA's outcomes are summarized in Table S3. For each type of sugar, the whole-system metabolic changes among the four sugar concentration levels were also investigated using Partial-Least Squares Discriminant Analysis (PLS-DA). Scaled data (i.e. mean-centered and divided by $\sqrt{SD}$) were used in multivariate analyses. The statistical significance of the PLS-DA was checked with Monte-Carlo permutation tests (1000 permutations).

**Table 1** List of metabolites detected in females of *Drosophila melanogaster*

| Compounds abbreviations in brackets |
|---|
| Free amino acids |
| Alanine (Ala) |
| Valine (Val) |
| Serine (Ser) |
| Leucine (Leu) |
| Threonine (Thr) |
| Proline (Pro) |
| Methionine (Met) |
| Ornithine (Orn) |
| Glycine (Gly) |
| Isoleucine (Ile) |
| Glutamate (Glu) |
| Lysine (Lys) |
| Phenylalanine (Phe) |
| Tyrosine (Tyr) |
| Sugars |
| Sucrose (Suc) |
| Fructose (Fru) |
| Glucose (Glc) |
| Trehalose (Tre) |
| Mannose (Man) |
| Galactose (Gal) |
| Ribose (Rib) |
| Maltose (Mal) |
| Glucose-6-phosphate (G6P) |
| Polyols |
| Sorbitol |
| Glycerol |
| Glycerol-3-phosphate |
| Inositol |
| Xylitol |
| Intermediate metabolites |
| Succinate |
| Malate |
| Citrate |
| Other metabolites |
| Lactate |
| Ethanolamine (ETA) |
| Free phosphate (PO4) |
| Gamma-aminobutyric acid (GABA) |
| Glucono delta-lactone (GDL) |
| Spermine |

Variable Importance in Projection (VIP) scores were obtained from the PLS-DAs. VIP scores are weighted sum of squares of the PLS loadings. In addition, to look for evidence of enriched metabolic pathways in response to sugar dose, for each type of sugar, metabolite pathway enrichment analysis (MPEA) was conducted using MetPA online package, with *D. melanogaster* specific library (Xia and Wishart 2010). All analyses were conducted using both the statistical software 'R 2.13.0' (R Development Core Team 2008) and MetaboAnalyst (Xia et al. 2012).

### 2.5 Triglyceride assessment

For each nutritional group, we assessed whether triglycerides (TGs) accumulated concomitantly with increasing dietary sugars. Five biological replicates, each consisting of a pool of 10 females, were used. Each sample was weighed (Mettler Toledo UMX2, accurate to 0.001 mg). Then, the concentrations of TGs were measured in the whole insect body as previously described (Laparie et al. 2011). Briefly, samples were homogenized in a total volume of 1,000 µL of methanol–chloroform–water solution (1:2:1) using bead-beating at 25 Hz for 1.5 min. After phase separation, 400 µL of the lower phase (chloroform + lipids), was dried under nitrogen stream and the residual lipid droplet was redissolved in 200 µL of Triton X100 (0.2 %) and BSA (3 %). We used a triglyceride colorimetric assay kit (Cayman Chemical Company, Ann Arbor, MI, USA) to quantify TGs following manufacturer's instructions. For each type of sugar, TGs levels were compared among the four concentration levels using one-way ANOVA followed by Student–Newman–Keuls (SNK) comparison tests.

## 3 Results

### 3.1 Sucrose-augmented diets

Flies reared on all the Suc-augmented diets successfully developed to adult stage; however, there was an asynchrony in development times. Flies reared on Suc-C0 and Suc-C1 took 10 days to emerge at 25 °C, while those on Suc-C2 and Suc-C3 took respectively 11 and 13 days to emerge. The fresh mass of flies varied with Suc dose ($F = 20.19$, $P < 0.001$), with intermediate concentration (i.e. Suc-C2) producing the biggest flies (Fig. 1a). The TGs content also varied with Suc dose ($F = 16.34$, $P < 0.001$), with flies reared on Suc-C2 and Suc-C3 being fatter than the others (Fig. 1e).

The GC/MS data showed that Suc was undetectable in adult flies reared on Suc-C0 and also on the other diets with no Suc. By contrast, body concentration of Suc increased significantly with dietary Suc-enrichment (Fig. 2 and Table S3). A number of other metabolites had their concentrations affected by dietary Suc-enrichment (Fig. 2 and Table S3), which resulted in different metabotypes. The PLS-DA revealed a significant clustering effect according

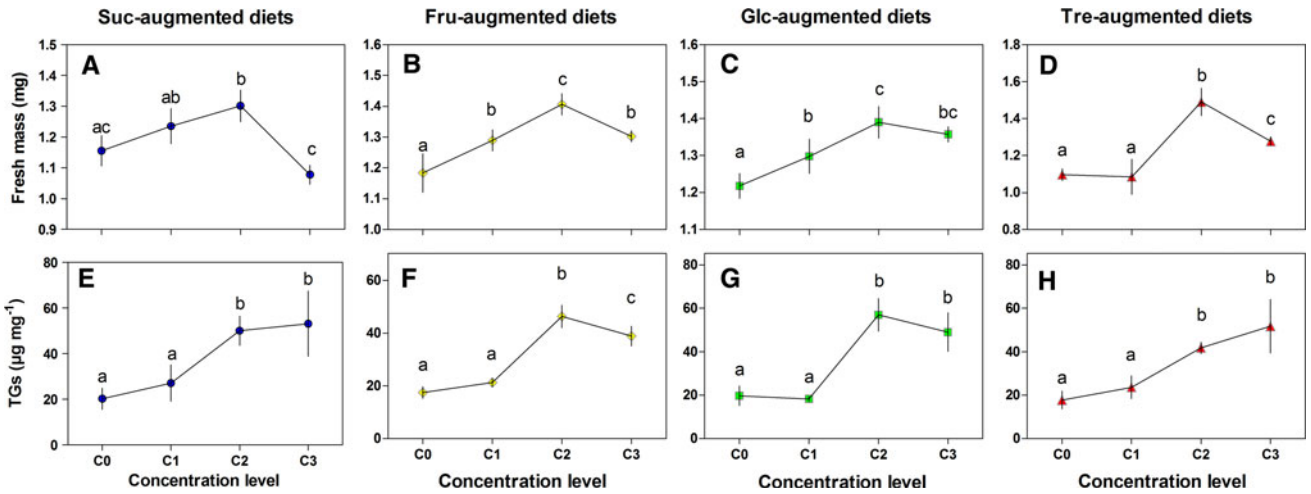


**Fig. 1** Fresh mass and triglycerides (TGs) content of *D. melanogaster* females reared on diets supplemented with sucrose (**a**, **e**), fructose (**b**, **f**), glucose (**c**, **g**) or trehalose (**d**, **h**) at four different concentration levels (from C0 to C3). See text and Table S1 for details on diets composition. Values are mean ± SE ($n = 5$). Different *letters* indicate significant difference ($P < 0.05$)

to the different dietary Suc doses (Monte-Carlo test, $P < 0.001$, Fig. 3). The first and second axes (LD1 and LD2) accounted for 23 and 31.6 % of the total inertia, respectively. LD1 was characterized by a clear-cut opposition between two groups: Suc-C0 and Suc-C1 opposed to Suc-C2 and Suc-C3 (Fig. 3). The VIP scores showed that metabolites contributing the most to LD1 were sorbitol, Suc, Fru, inositol, malate and xylitol that showed increasing concentrations with increasing Suc dose (Figs. 2, 3). To help put the above metabolic changes into context, we also performed MPEA to look for coordinated changes in metabolites that belong to the same pathway. MPEA comparisons among Suc-augmented groups revealed several enriched metabolic pathways; all were directly involved in carbohydrate metabolism (Table 2).

Concerning cold tolerance, we found that CCR significantly varied among the four nutritional groups (Mantel-Cox: $\chi^2 = 27.53$, $P < 0.001$; Fig. 4a). CCR was shorter in Suc-C0 and Suc-C2 groups than in Suc-C1 and Suc-C3 groups (Fig. 4a). Chronic and acute cold tolerances were also affected by dietary Suc ($\chi^2 = 21.64$, $P = 0.002$ and $\chi^2 = 156.3$, $P < 0.001$, respectively). Mortality was low in Suc-C0 and Suc-C1 groups, and then it increased with dietary Suc-enrichment (Fig. 4b, c).

### 3.2 Fructose-augmented diets

Flies reared on Fru-augmented diets successfully developed to adult stage, but development times varied among concentration groups. Flies reared on Fru-C0 and Fru-C1 took 10 days to emerge, while those on Fru-C2 and Fru-C3 took 11 and 13 days, respectively. The fresh mass of the flies varied with the Fru dose ($F = 25.18$, $P < 0.001$), with intermediate concentration (i.e. Fru-C2) producing the biggest flies (Fig. 1b). The TGs content also varied with Fru dose ($F = 97.10$, $P < 0.001$), with the flies reared on Fru-C2 and Fru-C3 being fatter than the flies reared on the other diets (Fig. 1f).

Low Fru concentrations were detected in Fru-C0 and Fru-C1 groups, while Fru concentration markedly increased in Fru-C2 and Fru-C2 groups (Fig. 2; Table S3). Several other metabolites had their concentrations affected by dietary Fru-enrichment (Fig. 2 and Table S3). The PLS-DA revealed a significant clustering effect according to dietary Fru dose (Monte-Carlo test, $P < 0.001$, Fig. 5). LD1 and LD2 accounted for 24.3 and 9.2 % of the total inertia respectively. LD1 was characterized by an opposition between two groups: Fru-C0 and Fru-C1 opposed to Fru-C2 and Fru-C3 (Fig. 5). The VIP scores showed that metabolites contributing the most to LD1 were malate, Fru, sorbitol, Tre, and inositol that showed increasing concentrations with increasing dietary Fru dose (Figs. 2, 5). MPEA comparisons among Fru-augmented groups revealed several enriched metabolic pathways; all were directly involved in carbohydrate metabolism (Table 2).

CCR significantly varied among nutritional groups (Mantel-Cox: $\chi^2 = 88.78$, $P < 0.001$; Fig. 4d). CCR was shorter in Fru-C1 group, followed by Fru-C0 and Fru-C2 groups. CCR in Fru-C3 group was markedly longer with 36 % flies remaining in chill coma after 90 min of recovery (Fig. 4d). Chronic and acute cold tolerances were also affected by dietary Fru ($\chi^2 = 117.1$, $P < 0.001$ and $\chi^2 = 308.8$, $P < 0.001$, respectively). Mortality was low in Fru-C0 and Fru-C1 groups, and then it increased with dietary Fru-enrichment (Fig. 4e, f).

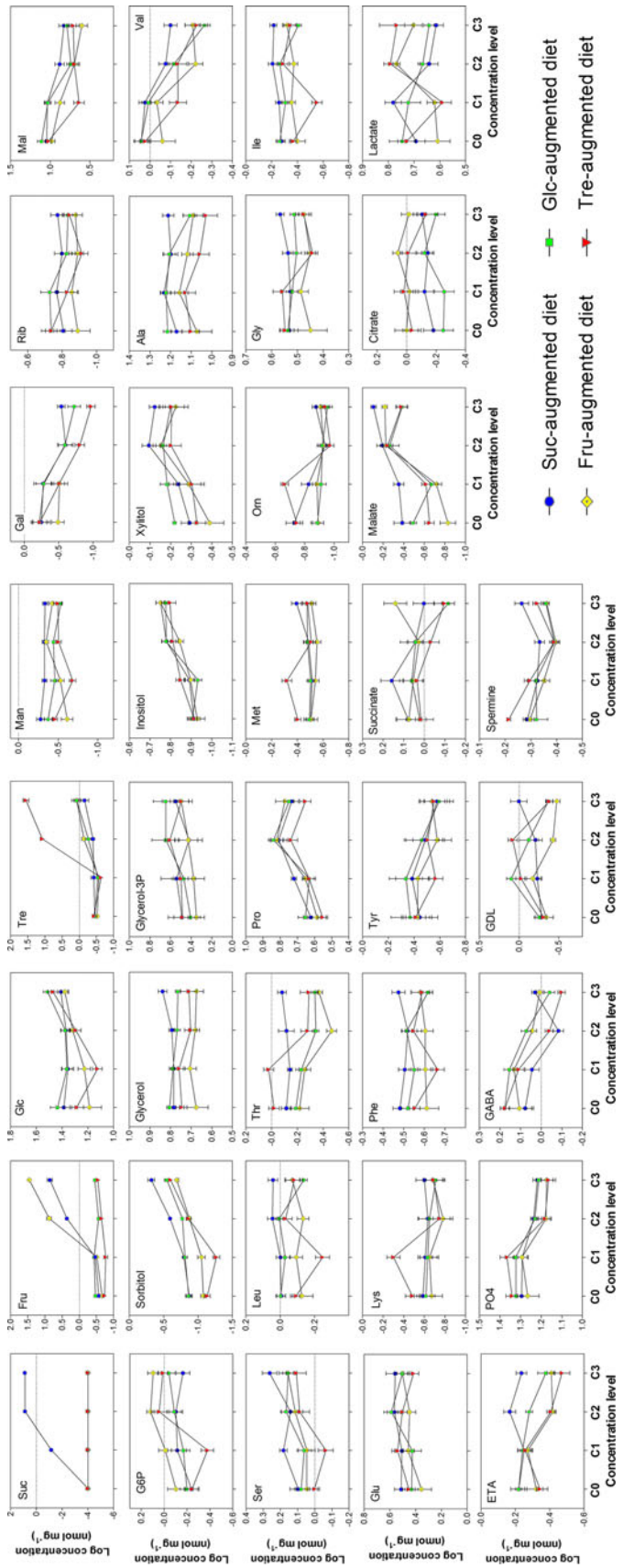


**Fig. 2** Changes of individual metabolite levels in relation to sugar dose (from C0 to C3) for each type of sugar supplemented in the diet (Suc or Fru or Glc or Tre). Values are mean ± SE ($n = 6$). Refer to Table 1 for compound abbreviations, Table S1 for details on diets composition and Table S3 for related statistics

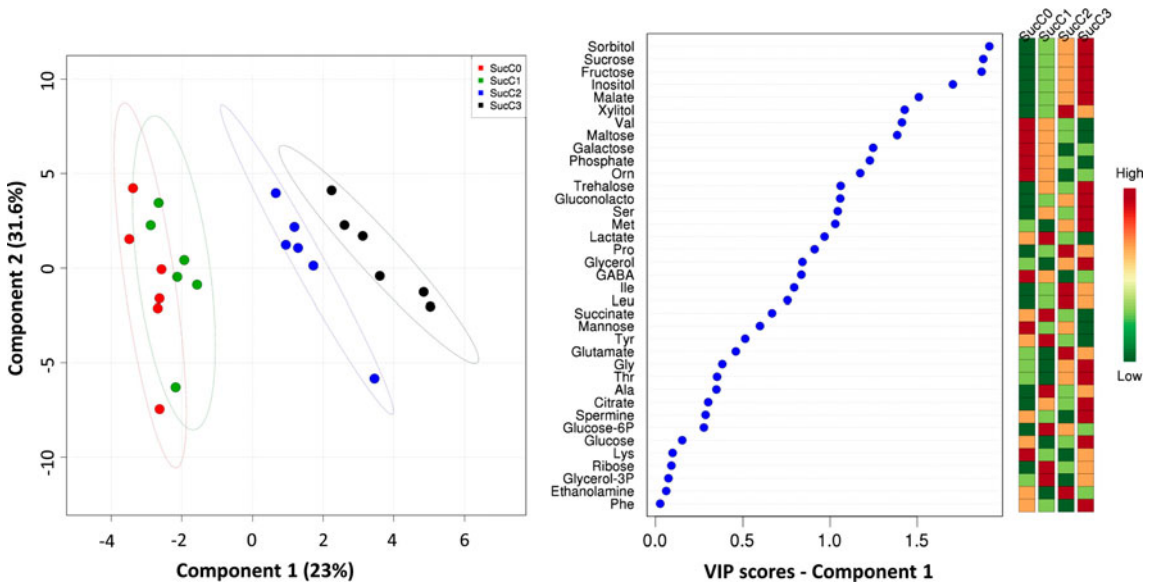


**Fig. 3** Projection of samples onto the first discriminant plane of the PLS-DA, showing a significant clustering effect according to the different dietary doses of sucrose (Suc-C0 and Suc-C1 opposed to Suc-C2 and Suc-C3). The variable importance plot shows the metabolites contributing the most to the first axis (based on VIP scores). Refer to Table 1 for compounds abbreviations

### 3.3 Glucose-augmented diets

Flies reared on Glc-augmented diets successfully developed to adult stage in a synchronized fashion. All flies emerged after 10 days except for flies on Glc-C3 who emerged 1 day later (11 days). The fresh mass of flies varied with Glc dose ($F = 20.75$, $P < 0.001$), with intermediate concentration (i.e. Glc-C2) producing the biggest flies (Fig. 1c). The TGs content also varied with Glc dose ($F = 51.25$, $P < 0.001$), with the flies reared on Glc-C2 and Glc-C3 being fatter than flies reared on the other nutritional groups (Fig. 1g).

The Glc concentrations were generally very high in all nutritional groups and were affected by the sugar dose (Fig. 2 and Table S3). There was a trend towards an accumulation of Glc with Glc-enrichment, but we did not observe a striking response as with the other sugars (Fig. 2 and Fig. S1). Other metabolites had their concentrations affected by dietary Glc-enrichment (Fig. 2 and Table S3). The PLS-DA revealed a significant clustering effect according to Glc dose (Monte-Carlo test, $p < 0.001$, Fig. 6). LD1 and LD2 accounted for 32.9 and 24.8 % of the total inertia, respectively. LD1 was characterized by an opposition between two groups: Glc-C0 and Glc-C1 opposed to Glc-C2 and Glc-C3 (Fig. 6). The VIP scores showed that metabolites contributing the most to LD1 were Val, free phosphate ($PO_4$), GABA and maltose which had reduced levels with Glc enrichment, while sorbitol and inositol showed opposite response (Figs. 2, 6). MPEA comparisons among Glc-augmented nutritional groups revealed several enriched metabolic pathways, mainly involved in carbohydrate and amino acid metabolism (Table 2).

CCR significantly varied among nutritional groups (Mantel-Cox: $\chi^2 = 14.58$, $P = 0.002$; Fig. 4g). CCR was shorter in Glc-C0 and Glc-C1 groups than in Glc-C2 and Glc-C3 groups (Fig. 4g). Chronic and acute cold tolerances were also affected by dietary Glc ($\chi^2 = 98.59$, $P < 0.001$ and $\chi^2 = 273.7$, $P < 0.001$, respectively). Mortality was low in Glc-C0 and Glc-C1 groups, and then it increased with dietary Glc-enrichment (Fig. 4h, i).

### 3.4 Trehalose-augmented diets

Flies reared on Tre-augmented diets successfully developed to adult stage. There was a marked asynchrony in development times. Flies reared on Tre-C0 and Tre-C1 took 10 days to emerge, while those on Tre-C2 and Fru-C3 took respectively 11 and 14 days. The fresh mass of flies varied with Tre dose ($F = 45.41$, $P < 0.001$), with intermediate concentration (i.e. Tre-C2) producing biggest flies (Fig. 1d). The TGs content also varied with Tre dose ($F = 24.80$, $P < 0.001$), with flies on Tre-C2 and Tre-C3 being fatter than flies reared on the other nutritional groups (Fig. 1h).

Low Tre concentrations were detected in Tre-C0 and Tre-C1 groups, while Tre concentration markedly increased in Tre-C2 and Tre-C3 groups (Fig. 2; Table S3). Other metabolites had their concentrations affected by dietary Tre-enrichment (see Fig. 2). The PLS-DA revealed a significant clustering effect according to Tre dose (Monte-Carlo test, $P < 0.001$, Fig. 7). LD1 and LD2

**Table 2** Summary of the metabolic pathways that were significantly altered by dietary sugars

| Sugar | Pathway | Hits | Holm adjust *P* | FDR | Impact |
|---|---|---|---|---|---|
| Sucrose diet | Fructose and mannose metabolism | 3 | 0.000 | 0.000 | 0.197 |
| | Galactose metabolism | 9 | 0.002 | 0.001 | 0.457 |
| | Pyruvate metabolism | 2 | 0.010 | 0.004 | 0.000 |
| | Inositol phosphate metabolism | 1 | 0.027 | 0.006 | 0.204 |
| | Ascorbate and aldarate metabolism | 1 | 0.027 | 0.006 | 0.000 |
| | Starch and sucrose metabolism | 5 | 0.032 | 0.006 | 0.221 |
| Fructose diet | Fructose and mannose metabolism | 3 | 0.000 | 0.000 | 0.197 |
| | Galactose metabolism | 7 | 0.003 | 0.001 | 0.045 |
| | Pyruvate metabolism | 2 | 0.003 | 0.001 | 0.000 |
| | Glyoxylate and dicarboxylate metabolism | 2 | 0.013 | 0.003 | 0.333 |
| | Starch and sucrose metabolism | 4 | 0.014 | 0.003 | 0.197 |
| | Inositol phosphate metabolism | 1 | 0.031 | 0.005 | 0.204 |
| | Ascorbate and aldarate metabolism | 1 | 0.031 | 0.005 | 0.000 |
| Glucose diet | Starch and sucrose metabolism | 4 | 0.000 | 0.000 | 0.197 |
| | Pentose phosphate pathway | 4 | 0.000 | 0.000 | 0.070 |
| | Arginine and proline metabolism | 4 | 0.000 | 0.000 | 0.239 |
| | Amino sugar and nucleotide sugar metabolism | 3 | 0.000 | 0.000 | 0.157 |
| | Galactose metabolism | 8 | 0.000 | 0.000 | 0.415 |
| | Fructose and mannose metabolism | 3 | 0.001 | 0.000 | 0.197 |
| | Glutathione metabolism | 3 | 0.001 | 0.000 | 0.000 |
| | Beta-alanine metabolism | 1 | 0.002 | 0.000 | 0.000 |
| | Valine, leucine and isoleucine biosynthesis | 4 | 0.003 | 0.000 | 1.000 |
| | Pyruvate metabolism | 2 | 0.003 | 0.000 | 0.000 |
| | Butanoate metabolism | 2 | 0.012 | 0.001 | 0.182 |
| | Glycolysis or gluconeogenesis | 2 | 0.018 | 0.002 | 0.029 |
| | Glycine, serine and threonine metabolism | 3 | 0.036 | 0.004 | 0.571 |
| | Valine, leucine and isoleucine degradation | 3 | 0.041 | 0.004 | 0.000 |
| Trehalose diet | Galactose metabolism | 8 | 0.000 | 0.000 | 0.415 |
| | Fructose and mannose metabolism | 3 | 0.000 | 0.000 | 0.197 |
| | Starch and sucrose metabolism | 4 | 0.006 | 0.002 | 0.197 |
| | Pantothenate and CoA biosynthesis | 1 | 0.006 | 0.002 | 0.000 |
| | Inositol phosphate metabolism | 1 | 0.010 | 0.002 | 0.204 |
| | Ascorbate and aldarate metabolism | 1 | 0.010 | 0.002 | 0.000 |
| | Pentose phosphate pathway | 4 | 0.014 | 0.002 | 0.070 |
| | Valine, leucine and isoleucine biosynthesis | 4 | 0.023 | 0.003 | 1.000 |
| | Arginine and proline metabolism | 4 | 0.024 | 0.003 | 0.239 |
| | Valine, leucine and isoleucine degradation | 3 | 0.024 | 0.003 | 0.000 |

Comparisons are performed among the four concentration levels for each type of sugar. Only significantly enriched pathways with Holm-adjusted *P* value $< 0.05$ are included in the table

accounted for 32.7 and 26.9 % of the total inertia, respectively. LD1 was characterized by an opposition between two groups: Tre-C0 and Tre-C1 opposed to Tre-C2 and Tre-C3 (Fig. 7). The VIP scores showed that metabolites contributing the most to LD1 were sorbitol and Tre which had increased levels with Tre enrichment, while GABA and galactose showed opposite response (Figs. 2, 7). As for Glc, MPEA comparisons among Tre-augmented groups revealed several enriched metabolic pathways, mainly involved in carbohydrate and amino acid metabolism (Table 2).

CCR significantly varied among nutritional groups (Mantel-Cox: $\chi^2 = 40.56$, $P < 0.001$; Fig. 4j). CCR was shorter in Tre-C0 followed by Tre-C1 and Tre-C2 groups. CCR in Tre-C3 group was markedly longer, with 42 % flies remaining in chill coma after 90 min of recovery (Fig. 4j). Chronic and acute cold tolerances were also affected by dietary Tre ($\chi^2 = 107.5$, $P < 0.001$ and

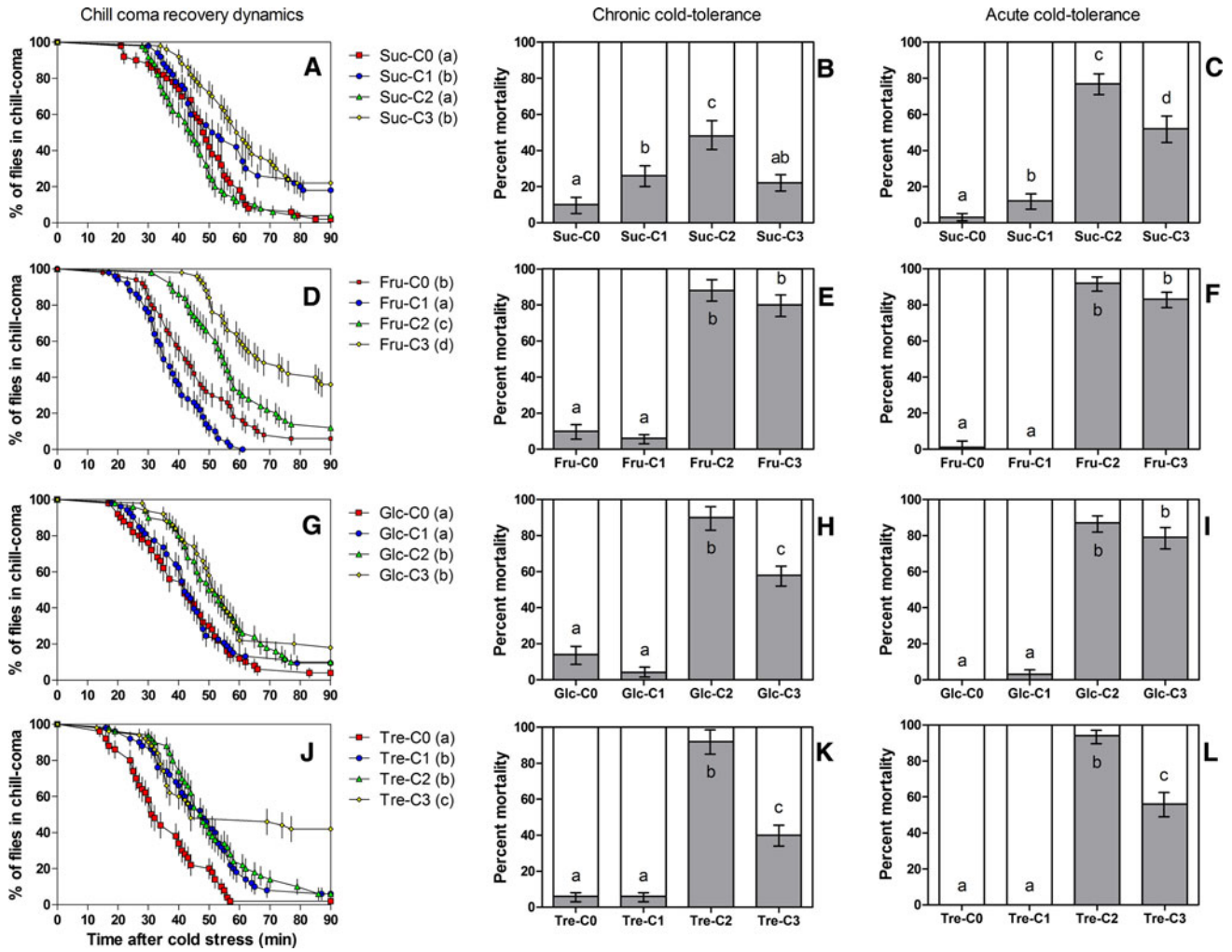


**Fig. 4** Composite panel summarizing all cold tolerance assays. Temporal recovery curves of adults exposed to chronic cold stress (16 h at 0 °C) in relation to sugar dose (from C0 to C3) are shown in panels **a**, **d**, **g**, and **j** for diets enriched with Suc, Fru, Glc and Tre, respectively. Each *dot* represents the mean proportion of recovering flies in relation to time after cold stress (±SE, $n = 50$). Different *letters* in the legends indicate significant difference among the recovery curves. Mortality rates, assessed 24 h after the chronic cold stress, are shown in panels **b**, **e**, **h**, and **k** for each targeted sugar and concentration level. Mortality rates assessed 24 h after an acute cold stress (−3.5 °C for 2 h) are shown in panels **c**, **f**, **I**, and **l** for each targeted sugar and concentration level ($n = 100$). Different *letters* indicate significant difference ($P < 0.05$)

$\chi^2 = 270.8$, $P < 0.001$, respectively). Mortality was low in Tre-C0 and Tre-C1 groups, and then it increased with dietary Tre-enrichment (Fig. 4k, l).

## 4 Discussion

### 4.1 Sugar incorporation and fat body stores

In the present study, we tested whether increased body sugars via dietary manipulation would affect thermal tolerance of *D. melanogaster*. We found that rearing of larvae and then adults on sugar-enriched diets resulted in sugars being incorporated. At high concentrations (C2 and C3), striking increases of sugar compounds were found in adult whole-body extracts, except for Glc which only moderately increased. The fact the Glc was only moderately accumulated suggests an effective homeostatic control of this highly-abundant compound (Haselton and Fridell 2010). Musselman et al. (2011) reported that larvae reared on high-sugar diets accumulated high levels of Glc and Tre. By contrast, Koštál et al. (2012) reported that feeding of larvae on trehalose-augmented diet only moderately increased Tre body concentration. Theses incongruities may arise from different concentrations and genotypes being used in these two studies. Indeed, change in metabolic traits, such as hemolymph sugar level, is known to be mainly driven by genetic-by-diet interaction (Reed et al. 2010). There might be differences in the incorporation of sugars according to the stage being exposed; indeed, larvae

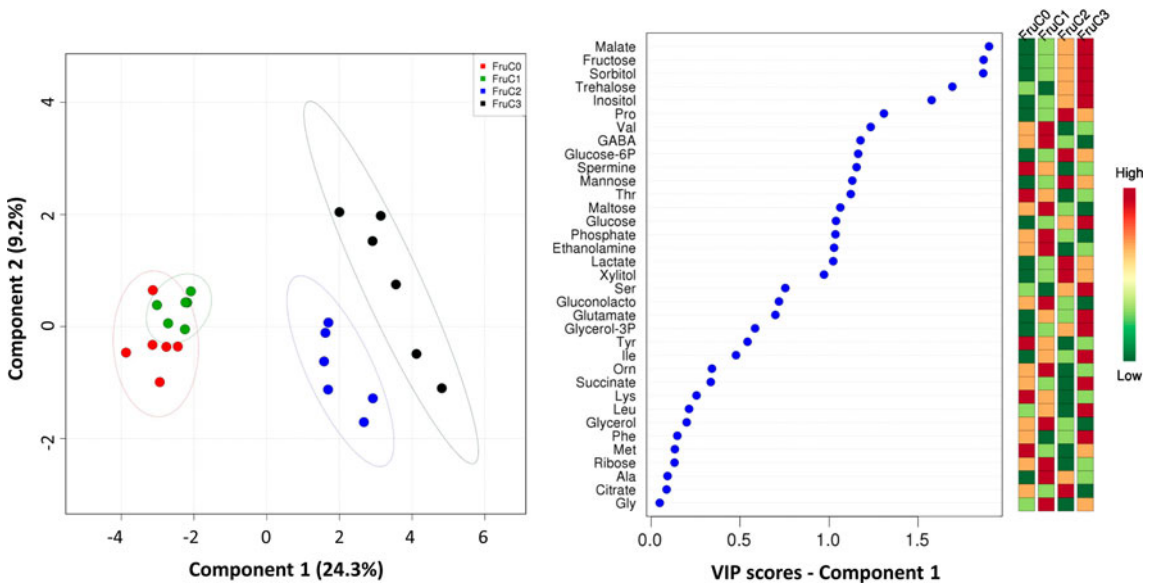


**Fig. 5** Projection of samples onto the first discriminant plane of the PLS-DA, showing a significant clustering effect according to the different dietary doses of fructose (Fru-C0 and Fru-C1 opposed to Fru-C2 and Fru-C3). The variable importance plot shows the metabolites contributing the most to the first axis (based on VIP scores). Refer to Table 1 for compounds abbreviations

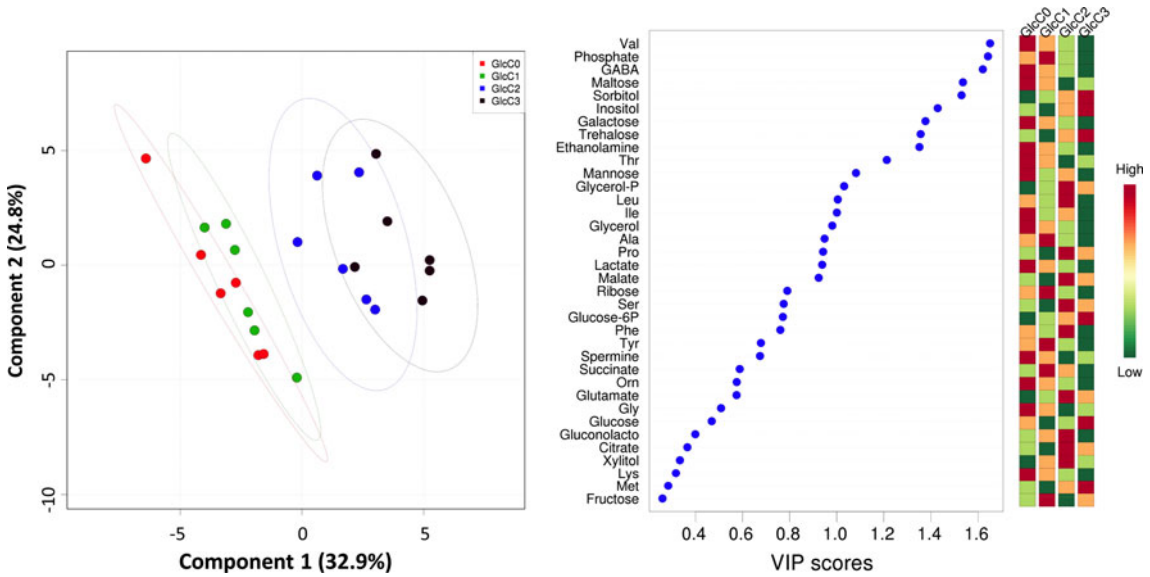


**Fig. 6** Projection of samples onto the first discriminant plane of the PLS-DA, showing a significant clustering effect according to the different dietary doses of glucose (Glc-C0 and Glc-C1 opposed to Glc-C2 and Glc-C3). The variable importance plot shows the metabolites contributing the most to the first axis (based on VIP scores). Refer to Table 1 for compounds abbreviations

possess metabolic specializations that are not present in adults (Haselton and Fridell 2010). In the present study, larvae and then adults were reared on sugar-enriched diets, and we found a significant increase of body sugars and TGs content. Flies reared on concentrations C2 and C3 were clearly fatter than those reared on the other nutritional groups. Increased body fat is a typical response to high-sugar feeding (Wang and Clark 1995; Matzkin et al. 2011). It has been shown in flies reared on sugar-enriched diets, at concentrations similar to those used in the present study (C2 and C3), that resources are strongly diverted towards fat storage resulting in severe obesity (Skorupa et al. 2008; Musselman et al. 2011). The elevation in TGs level is an important marker of obesity in flies and is associated with disruptions of lipid and sugar homeostasis, mitochondrial function, and many other biochemical processes (Birse

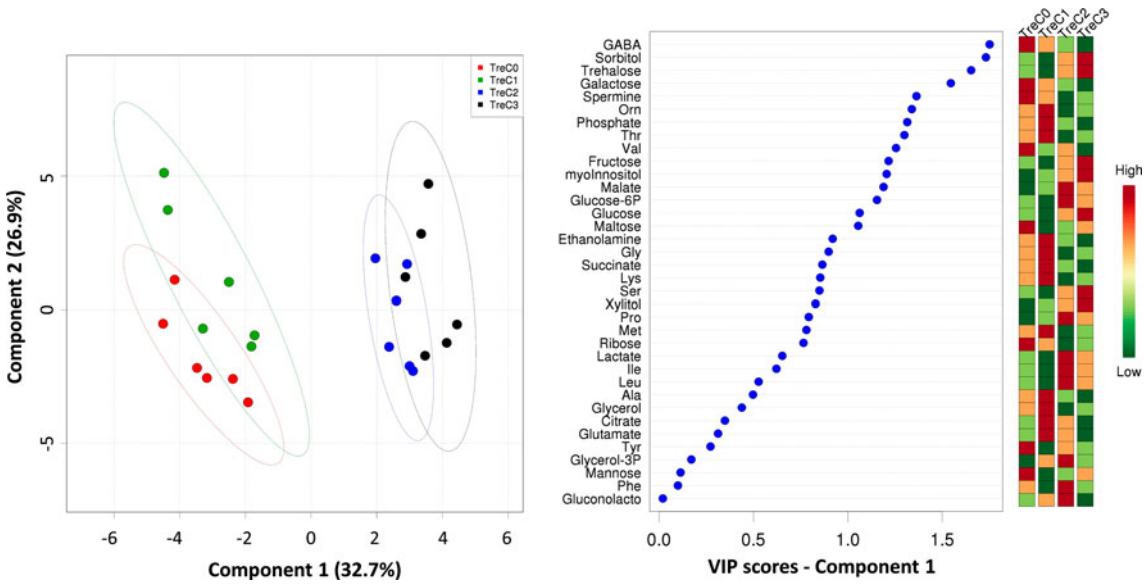


**Fig. 7** Projection of samples onto the first discriminant plane of the PLS-DA, showing a significant clustering effect according to the different dietary doses of trehalose (Tre-C0 and Tre-C1 opposed to Tre-C2 and Tre-C3). The variable importance plot shows the metabolites contributing the most to the first axis (based on VIP scores). Refer to Table 1 for compounds abbreviations

et al. 2010). Therefore, in the light of the TGs data, it seems that flies reared on C2 and C3 diets were characterized by such a syndrome.

### 4.2 Effects of sugar enrichment on cold tolerance

Selection for cold tolerance has been linked to increased TGs level (Chen and Walker 1994). In addition, accumulation of hemolymph sugars has been associated with cold tolerance acquisition in drosophilids (Kimura 1982; Overgaard et al. 2007; Koštál et al. 2011a, 2012; Colinet et al. 2012a, Vesala et al. 2012). We thus speculated that increased body sugars would promote cold tolerance. Contrary to our expectation, cold tolerance was negatively affected by exogenous sugars. This was particularly manifested in phenotypes that were reared on high sugar concentrations (C2 and C3). These flies suffered from high post-cold stress mortality in nearly all assays, compared to concentrations C0 and C1. In some cases, the experimental treatment C3 was slightly less detrimental than C2. These variations of thermotolerance between C2 and C3 are not clear, but they might denote a sort of pessimal level at C2. Chill coma recovery, an ecologically relevant measure of thermal adaptation (Terblanche et al. 2011), was also affected by the concentration of dietary sugars. Chill coma recovery patterns varied among the different types of sugars, in such way that a gradual dose–response was not always observed. In spite of this, we also found that high sugar doses (C2 and/or C3) were always detrimental compared to controls (C0). In the light of the presumed association between body sugars and cold tolerance, these results may at first seem surprising. Highly concentrated sugar diets may pose an osmotic challenge, which may negatively interact with cold tolerance. However, flies are able to withstand large variance in food osmolarity by regulating water flux via the cuticle and spiracles (Pierce et al. 1999), and hence this is not likely to be the driving force in our data. The decreased cold tolerance in sugar-enriched phenotypes probably occurred because flies were 'constrained' to accumulate exogenous sugars. By contrast, endogenous sugar accumulation, occurring via thermal acclimation for instance, likely involves an intended and regulated process rather than a constrained one. It has been suggested that stress tolerance can be promoted via exogenous increase of cryoprotectants, but only if the organism is set in 'accumulation mode' (Koštál et al. 2011b, 2012). In our experiment, flies were reared under standard conditions that do not induce diapause (25 ± 1 °C and 16L:8D). Even if *D. melanogaster* only shows a weak diapause response (Saunders et al. 1989), it cannot be excluded that high levels of sugars could exert a protective role on cold tolerance but only in the diapause phenotype. Indeed, diapause often represents a prerequisite for full expression of cold-tolerance processes (Vesala et al. 2012).

### 4.3 Side-effects of high dietary sugars

It is not known why feeding on sugar-enriched diets negatively affected thermal traits but an obvious first hypothesis is that high dietary sugars created a metabolic imbalance and a nutritional stress, which might have affected cold tolerance. This 'metabolic imbalance hypothesis' is supported by

metabolic fingerprinting which clearly showed that a wealth of metabolites had their concentrations altered by high sugar doses, demonstrating a loss of metabolic homeostasis (Malmendal et al. 2006). Similarly to the patterns of cold tolerance and TGs accumulation, we found a clear segregation among the metabotypes that formed two contrasted clusters (i.e. C0 and C1 opposed to C2 and C3). MPEA also revealed that feeding on sugar-enriched diets was associated with several enriched metabolic pathways related mostly to carbohydrate and amino acid metabolism. These observations further support the hypothesis that rearing on high dietary sugars induces system-wide metabolic remodeling likely associated with carbohydrate metabolism imbalance.

*Drosophila* larvae consume a wide range of necrotic fruits. This species is thus supposed to be adapted to diets rich in sugars (Sang 1956; Matzkin et al. 2011). In spite of this, it was shown that high levels of dietary sugar induce a severe nutritional imbalance and a pathological state in *D. melanogaster* (Skorupa et al. 2008; Musselman et al. 2011). We found that high sugar concentrations (especially C3) caused a marked developmental delay together with a fresh mass reduction, which denote a detrimental (pathological) effect. Our data are in accordance with those of Musselman et al. (2011) who reported that high-sucrose feeding (Suc 1 M) resulted in a dramatic delay (3–5 days) in developmental time when compared with flies reared on control food (Suc 0.15 M). As observed here, these authors reported that other sugars (Glc and Fru) produced a developmental delay (Musselman et al. 2011). Reduction of body mass and developmental delay are typical responses to hyperglycemia and reflect severe nutritional imbalance (Reed et al. 2010; Matzkin et al. 2011, Musselman et al. 2011).

Several adverse effects related to sugar metabolism disorders are referred to as carbohydrate toxicity (Lushchak et al. 2011). Particularly Glc and Fru can glycate proteins to form toxic advanced glycation end-products (AGEs), such as glyoxal or methylglyoxal (Brownlee 2005; Negre-Salvayre et al. 2009). Transcriptional responses of high-sugar-fed *Drosophila* indicate metabolic toxicity mechanisms (Musselman et al. 2011). A futile cycle of lipid hydrolysis and re-esterification seems to occur with dietary sugar excess. The bioactive lipids generated by such process are supposed to be a source of cellular damages (Musselman et al. 2011). In addition to producing potentially toxic products, excessive dietary sugars can negatively affect antioxidant defense of *D. melanogaster* (Lushchak et al. 2011; Rzezniczak et al. 2011). This might be important, as production of reactive oxygen species (ROS) is known to be related to chilling injury in insects (Rojas and Leopold 1996; Grubor-Lajsic et al. 1997; Jing et al. 2005; Lalouette et al. 2011). Rzezniczak et al. (2011) found that tolerance to paraquat was reduced when flies were fed on sugar-rich diet because rearing on such diet generates a pro-oxidant effect. Moreover, Musselman et al. (2011) reported several pathways affected by high dietary Suc, including oxidative stress. Therefore, the higher cold susceptibility of sugar-enriched phenotypes might be related to (i) the presence of toxic products and/or (ii) the altered antioxidant defense. Finally, Suc overconsumption leads to obesity, insulin resistance and insulin-defective growth phenotypes in *D. melanogaster* (Skorupa et al. 2008, Musselman et al. 2011). Insulin signaling pathway is known to regulate resistance to stress (Broughton et al. 2005; Partridge et al. 2011) and seems directly implicated in the response to cold (Teets et al. 2012). Therefore, alteration of this pathway might also account for the reduced cold tolerance of sugar-enriched phenotypes.

### 4.4 Metabolic correlates

Except from sugars, a number of metabolites had their concentrations affected by dietary sugar- enrichment. These compounds might be viewed as potential fingerprint of the metabolic alterations in sugar-enriched phenotypes. The metabolites contributing the most to structure the separation among the different metabotypes included polyols, mainly sorbitol, which concentration increased in parallel with sugar enrichment. This response was found whatever the type of sugar tested. Sorbitol is derived from hexose monophosphates and can be produced from both Glc and Fru (Storey 1983; Wolfe et al. 1998). A common feature of hyperglycemia is the activation of the polyol pathway (Lorenzi 2007). Cells normally use Glc for energy, but excessive Glc can enter the polyol pathway, where it is reduced to sorbitol by aldose reductase (Lee and Chung 1999). Depending upon the severity of hyperglycemia, up to 30 % of Glc can be diverted to the polyol pathway (Singh et al. 2009) and sorbitol level is tightly correlated to Glc level (Aida et al. 1990). This pathway has several disadvantages as it produces both ROS and AGEs (Lee and Chung 1999; Lorenzi 2007; Singh et al. 2009). The increasing sorbitol level with sugar-enrichment may thus denote a flux of metabolized Glc through the polyol pathway. This might explain why genes encoding polyol pathways are modulated by high-sugar feeding in *Drosophila* (Musselman et al. 2011). It is worth mentioning that endogenous accumulation of sorbitol generally confers cold tolerance in overwintering insects (e.g. Storey et al. 1981; Walters et al. 2009). However, cold tolerant insects accumulate sorbitol at levels 1,000× greater than that observed in the present study (Storey et al. 1981; Walters et al. 2009). Thus, despite the significant variation of sorbitol with dietary sugars, it is very unlikely that the concentrations reached could have promoted cold tolerance.

Malate is another potential biomarker of metabolic alterations in sugar-enriched phenotypes, particularly in

phenotypes reared on Suc- and Fru-diets. When malate concentration increases, malate dehydrogenase activity (MDH) is strongly activated (Mullinax et al. 1982). This enzyme plays a central role in the biosynthesis of lipids by catalyzing the NADP-dependent oxidative decarboxylation of malate to pyruvate plus carbon dioxide, and generating NADPH (Farkaš and Knopp 1998). MDH activity is known to be affected by dietary sugar in *D. melanogaster*, being significantly increased at high concentrations of disaccharide (Suc) (Geer et al. 1978; Farkaš and Knopp 1998). Therefore, higher malate levels in sugar-enriched phenotypes might be related to biosynthesis of lipids, which is consistent with the TG accumulation observed in the flies reared at C2 and C3.

Finally, among the most influential metabolites, we also found that GABA level was reduced in concert with increasing sugars dose, particularly in phenotypes reared on Glc- and Tre-diets. Pancreatic $\beta$-cells of mammals contain high amounts of GABA. High concentrations of Glc decrease both the content and release of islet GABA, which results in stimulation of insulin secretion (Pizarro-Delgado et al. 2010). Similarly, GABA is involved in inhibitory regulation of insulin producing cells in *Drosophila* (Enell et al. 2010). This metabolite is therefore directly involved in regulation of insulin signaling pathway and therefore also in stress response (Enell et al. 2010). The reduction of GABA level with increasing sugars dose is thus congruent with these functions under high sugar dose.

## 5 Concluding remarks

Overall, our data demonstrate that dietary supplementation of sugars resulted in these compounds being incorporated and stored as fat. Contrary to our expectation, cold tolerance of *D. melanogaster* was negatively affected by high levels of exogenous sugars. This most likely resulted from a metabolic disturbance and a nutritional stress that in turn negatively affected cold tolerance. The developmental delay and fresh mass reduction confirm that adult flies suffered from nutritional stress and were likely in a pathological state. Several metabolites are potential fingerprints of metabolic alterations of the sugar-enriched phenotypes (e.g. sorbitol, malate and GABA). To our knowledge, this study is among the first that demonstrates a negative interaction between nutrition and thermal stress. Further experiments would be necessary to decipher the underpinnings of such interaction. Presence of toxic products (AGE or ROS) and altered antioxidant defense are potential research directions to explore. A comprehensive knowledge of the factors affecting thermal traits is necessary for understanding how temperature determines mortality, and hence population dynamics and biogeography. In this context, it would be intriguing to study the connection between nutritional conditions and thermal traits under natural conditions. As a fruit generalist, *D. melanogaster* is expected to feed on sugar-rich diets (Matzkin et al. 2011). Yet larvae can successfully grow without any sugar (Sang 1956), as found in C0 diets. Variations of sugar levels in fruits might be an important source of variability in cold tolerance under field conditions and feeding on sugar-rich fruits could be deleterious. Yet, due to the seasonal pattern of food/fruit availability, this issue might be avoided, as feeding period on sugar-rich mature fruits is likely restricted to late summer, while temperature drop will occur during winter starvation period. From a practical point of view, it is worth mentioning that many laboratories use different diets and techniques for rearing *Drosophila* flies. Concentrations of sugars in standard diets typically range between 50 and 150 g/L (5–15 % w/v) (Lewis 1960; Brent and Oster 1974; Backhaus et al. 1984; Toivonen et al. 2007). According to our data, this could affect thermal tolerance, so that it might become difficult to compare the results obtained from various studies. For this reason a careful attention should be given to diet composition.

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