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**Dietary live yeast alters metabolic profiles, protein biosynthesis
and thermal stress tolerance of *Drosophila melanogaster***

Colinet Hervé* & Renault David

Université de Rennes 1, UMR CNRS 6553 Ecobio, 263 Avenue du Gal Leclerc, CS 74205,
35042 Rennes, France

*Address for correspondence:

Hervé Colinet, UMR CNRS 6553 Bât 14A, Université de Rennes1, 263 Avenue du Général
Leclerc CS 74205, 35042 Rennes Cedex, France.

Tel: +33 (0)2 23 23 36 67

Fax: +33 (0)2 23 23 50 26

Email: herve.colinet@univ-rennes1.fr

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Abstract

The impact of nutritional factors on insect's life-history traits such as reproduction and lifespan has been excessively examined; however, nutritional determinant of insect's thermal tolerance has not received a lot of attention. Dietary live yeast represents a prominent source of proteins and amino acids for laboratory-reared drosophilids. In this study, *Drosophila melanogaster* adults were fed on diets supplemented or not with live yeast. We hypothesized that manipulating nutritional conditions through live yeast supplementation would translate into altered physiology and stress tolerance. We verified how live yeast supplementation affected body mass characteristics, total lipids and proteins, metabolic profiles and cold tolerance (acute and chronic stress). Females fed with live yeast had increased body mass and contained more lipids and proteins. Using GC/MS profiling, we found distinct metabolic fingerprints according to nutritional conditions. Metabolite pathway enrichment analysis corroborated that live yeast supplementation was associated with amino acid and protein biosyntheses. The cold assays revealed that the presence of dietary live yeast greatly promoted cold tolerance. Hence, this study conclusively demonstrates a significant interaction between nutritional conditions and thermal tolerance.

1. Introduction

A number of studies have examined how nutrition affects various traits in insects, with special emphasis on the effects of dietary intake on reproduction and longevity (Le Bourg and Medioni, 1991; Chippindale et al., 1993; Leroi et al., 1994; Anagnostou et al., 2010). More specifically, the trade-off between reproduction and lifespan resulting from the qualitative and quantitative manipulations of diet has been studied extensively (Carey et al., 2008; Ellers et al., 2011; Moore and Attisano, 2011). *Drosophila melanogaster* is a very popular model used in the dietary restriction (DR) literature of gerontology because of its relatively short generation time and ease of handling for demographic analysis (Partridge et al., 2005). The composition of *Drosophila* sp. food recipes often varies among laboratories and medium types, but the standard ingredients usually include water, agar, sugar, killed yeast, and fungicides. In addition, the medium can be supplemented with live yeast. Often adding live yeast on the surface of the diet strongly stimulates females to lay eggs (Markow and O'Grady, 2006; Stocker and Gallant, 2008). Dietary yeast is a major source of nutrition for the adults and larvae of most saprophagous *Drosophila* (Diptera: Drosophilidae) (Begon, 1982). It provides essential nutrients for the developmental and reproductive processes such as amino acids, sterols, vitamins, and fatty acids (Davis, 1975; Anagnostou et al. 2010). As a result, the concentration of yeast in the artificial diet is the primary determinant of egg production in *D. melanogaster* (Sang and King, 1961; Skorupa et al., 2008), and the formation of yolk proteins can thus be strongly curtailed by depriving flies of nutritional proteins or essential amino acid present in yeasts (Sang and King 1961; Bownes et al., 1988; Chippindale et al., 1993). Hence, the incorporation of live yeast in mediums highly stimulates vitellogenesis because it provides essential nutrients that are not necessarily present in large amounts in the usual adult food (Sang and King, 1961; Simmons and Bradley, 1997).

Owing to the major importance of the nutritional status on physiological and biochemical processes of insects, any alteration of nutritional regime is likely to affect all aspects of their life, including not only reproduction but also stress tolerance (such as thermal tolerance) (Hallman and Denlinger, 1998; Chown and Nicolson, 2004; Nyamukondiwa and Terblanche, 2009; Andersen et al., 2010; Colinet and Boivin, 2011; Sisodia and Singh, 2012). Yet, a limited number of studies have considered the impact of nutritional resources on environmental stress tolerance, and more particularly on thermal tolerance (Andersen et al., 2010). In this particular respect, no clear-cut response has been observed as nutritional effects on thermal tolerance seem to be rather complex and involve many interacting factors.

Carbohydrate-rich diets tend to increase drosophilids' cold tolerance compared to protein-rich diets, and the opposite effect is observed on measures of heat resistance (Andersen et al., 2010; Sisodia and Singh, 2012). However, when supplemented at high levels, dietary sugars induce a severe nutritional imbalance and a pathological state in *D. melanogaster* (Wang and Clark, 1995; Skorupa et al., 2008; Musselman et al., 2011; Colinet et al., 2013a) and these high sugar doses negatively affect cold tolerance (Colinet et al., 2013a). Quantitative manipulation of food supply *via* dietary restriction (*i.e.* dilution.) has no detectable effect on cold tolerance (chill-coma recovery, CCR) of young flies and only marginally reduces cold tolerance later in adult life (Burger et al., 2007). Removing (or adding) live yeast from *D. melanogaster* food also impacts on thermal traits in a rather complex manner. Le Rohellec and Le Bourg (2009) found that removing live yeast only weakly decreased cold survival of females subjected to a 16 h coldshock (0 °C), but only when these were mated. In another study, absence of live yeast in food killed nearly 100% of flies (males and females) subjected to the same cold treatment, whereas access to live yeast resulted in medium to high survival rate depending on age (Le Bourg, 2010). Tolerance to heat (37 °C) was either unaffected (Le Bourg, 2010) or improved by removal of live yeast (but in young females only) (Le Rohellec and Le Bourg, 2009). From the above examples, it seems clear that nutritional status can be a significant component of thermal tolerance of insects, affecting both heat- and cold-related traits. It also appears that nutritional effects on thermal tolerance depend on several interacting factors including gender, mating status, and age. Although the physiological and biochemical basis of thermal responses is becoming clearer through metabolic and physiological studies (Overgaard et al., 2007; Doucet et al., 2009; Colinet et al., 2012a; Kostál et al., 2012; Storey and Storey, 2012; Teets and Denlinger 2013), there remains limited information on the physiology of nutrition-mediated variation in thermal tolerance. A way in which insects deal with nutrient variations is through altered physiology, namely by affecting developmental and metabolic processes (Markow et al., 1999). Therefore, it can be assumed that manipulating the source of essential nutrients found in live yeast, such as amino acids and proteins, could alter the physiology and also the general stress tolerance.

In the present study, we completed a comprehensive assessment of the impact of dietary live yeast supplementation on body mass characteristics, proteins, metabolic profiles and basal cold tolerance (to acute and chronic exposures) in *D. melanogaster* females. We hypothesized that the absence of the source of proteins (*i.e.* live yeast) from adult food would be associated with deep physiological alterations; therefore, we expected contrasted metabolic

profiles (*i.e.* metabotype) between yeast-deprived and yeast-fed females. Because live yeast is a rich source of proteins and amino acids, we hypothesized that pathways related to protein biosynthesis would be particularly targeted by dietary live yeast supplementation. In addition, we expected body mass parameters to be strongly curtailed by depriving females of live yeast. Finally, we hypothesized that the nutritional and the metabolic variations caused by manipulating dietary live yeast will translate into altered thermal stress tolerance.

2. Materials and methods

2.1. Fly culture and diets

We conducted the experiments on a mass-bred *D. melanogaster* line derived from the mix of two wild populations collected in October 2010 and September 2011 at Plancoët (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 deactivated brewer yeast (80 g/L) (MP Biochemicals, Illkirch, France), sucrose (50 g/L), agar (15 g/L), kalmus (9 g/L) and Nipagin® (8 mL/L) as described previously (Colinet et al., 2013a). To generate flies for the experiments, groups of 15 mated females were allowed to lay eggs during a restricted period of 6 h in bottles (200 mL) containing 25 mL of standard fly medium. This controlled procedure allowed larvae to develop under uncrowded conditions at 25 ± 1 °C (16L:8D). At emergence, adult flies were allowed to age for six days on different diets and controls. The diets were changed every day for six consecutive days. Two different experiments were used to assess the effect of adult dietary live yeast supplementation (see Fig. 1 for experimental design).

- **Experiment 1** (conducted in 2012): minimal control diet *versus* live yeast-supplemented diet. Sugar and agar [SA] *versus* sugar, agar, live yeast [SAY(+)].
- **Experiment 2** (conducted in 2013): standard control diet *versus* live yeast-supplemented diet. Sugar, agar, killed yeast [SAY(-)] *versus* sugar, agar, killed yeast and live yeast [SAY(±)].

In the first experiment, emerging flies did not have any nutrient supply except from sugar. It is thus conceivable that these flies could suffer from malnutrition. Therefore, a second

experiment was designed with a standard diet as control that contains protein supply [SAY(-)] rather than a minimal diet [SA], in order to assess the effect of dietary live yeast supplementation without any putative malnutrition. In both experiments, the amounts of sugar, agar and killed yeast when supplied were 50 g/L, 15 g/L and 80 g/L respectively. When supplemented, the live yeast was provided with *ad libitum* paste placed on the surface of the food [*i.e.* for SAY(+) and SAY(\pm)]. We used synchronized six day-old adults for all assays to avoid the uncontrolled variation of stress tolerance during the first days of age (Colinet et al., 2013b). Adults were sexed visually (with an aspirator) without CO₂ to avoid any confusing metabolic effects due to anaesthesia (Colinet and Renault, 2012), and only females were kept. Six day-old females from each nutritional group were either directly used for the cold assays or snap-frozen in liquid nitrogen and stored at -80 °C for the other assays.

2.2. Body mass and protein levels

We assessed total protein content using the Bradford procedure (Bradford, 1976). Twelve biological replicates, each consisting of a pool of three females, were used for each experimental condition. Each sample was *vacuum*-dried (GENEVAC, model DNA-23050-B00) set at 30 °C for 24 h and then weighed (dry mass, Mettler Toledo UMX2, accurate to 1 µg) before proteins were extracted in a phosphate buffer (100 mM KH₂PO₄, 1 mM DTT and 1 mM EDTA, pH 7.4, Foray et al., 2012) and homogenized using bead-beating at 25 Hz for 1.5 min. The concentration of total proteins was then measured in the whole body extracts using Bio-Rad Protein Assay (catalog number 500-0006) following manufacturer's instructions.

For each nutritional treatment, 15 females were subjected to individual fresh mass (FM) measurements (Mettler Toledo UMX2, accurate to 1 µg). Then, individual females were dried at 60 °C for two days, and reweighed to measure dry mass (DM). Water mass (WM) was determined by subtracting DM from FM. Water content (WC) represents the ratio WM/FM. Lean dry mass (LDM) was measured by extracting total lipids in a chloroform/methanol solution (Folch reagent 2:1, v:v) for one week under continuous agitation. The samples were then dried at 60 °C to eliminate residues of the extracting solution before measurement of LDM. Body lipid mass (LM), corresponding to DM-LDM, was calculated (see Colinet et al., 2006). Folch reagent may extract a small fraction of other

compounds than lipids, but measurements obtained with this method are still considered as a good index of lipid content for comparative studies ([Williams et al., 2011](#)).

2.3. Cold tolerance assays

Different metrics were used to assess cold tolerance. First, recovery time following a non-lethal chronic cold stress was measured (*i.e.* chill-coma recovery, CCR). Fifty females were exposed to 0 °C for various durations: 8, 10 and 12 h for the flies of the experiment 1 [*i.e.* SA *vs.* SAY(+)], and 10 and 12 h for the flies of the experiment 2 [*i.e.* SAY(-) *vs.* SAY(±)]. Cold-exposed flies were then allowed to recover at 25 ± 1 °C (16L:8D) and recovery times were individually recorded; flies were considered recovered when they stood up. A cold incubator (Model MIR-153, SANYO Electric Co. Ltd, Japan) was used for the assays. 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 after 24 h (*i.e.* latent damage assessment).

Second, tolerance to acute cold stress was measured. A total of 100 females (5 replicates, 20 females per replicate) were placed in 42 mL glass vials immersed in a glycol solution cooled to -3.5 °C for different durations: 90, 120 and 135 min for the flies of the experiment 1 [*i.e.* SA *vs.* SAY(+)], and 90 and 120 min for the flies of the experiment 2 [*i.e.* SAY(-) *vs.* SAY(±)]. After the acute cold stress, the flies were returned to 25 °C on their respective diet, and the mortality was scored after 24 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.

2.4. Metabolic fingerprinting

The metabolic effect of dietary live yeast supplementation was assessed by comparing the metabolotypes of SA *vs.* SAY(+) (experiment 1) and SAY(-) *vs.* SAY(±) (*i.e.* experiment 2). To ensure that the differences observed were not only related to presence/absence of live yeast in the gut content, we included an additional treatment where flies were starved before sampling. In this **experiment 3** (conducted in 2013), the same flies as in the experiment 2 were starved for 8 h on agar before their metabolic profiles were compared. Hence, we compared the

following conditions: sugar, agar, killed yeast, plus 8h starvation (**St-SAY-**) *versus* sugar, agar, killed yeast and live yeast, plus 8h starvation (**St-SAY±**) (see Fig. 1).

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 1 µg) before metabolite extractions. Sample preparation and derivatization were performed as previously described (Colinet et al., 2012b), with minor modifications. Briefly, after homogenisation in methanol-chloroform solution (2:1, v:v) and phase separation with 400 µL of ultrapure water, an 120 µL aliquot of the upper phase, which contained polar metabolites, was *vacuum*-dried. The dry residue was resuspended in 30 µL of 20 mg mL⁻¹ methoxyamine hydrochloride in pyridine before incubation under automatic orbital shaking at 40 °C for 60 min. Then, 30 µL of MSTFA were added 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). 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 at 320 °C for 4 min. 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 63 pure reference compounds included in our custom spectral database. Calibration curves for 60 pure reference compounds at 5, 10, 20, 50, 100, 200, 500, 750, 1000, 1500 and 2000 µM concentrations were run concurrently. Chromatograms were deconvoluted using XCalibur 2.0.7, and metabolite levels were quantified using the quadratic calibration curve for each reference compound and concentration. Arabinose was used as the internal standard (see Colinet et al., 2012b). Among the 63 metabolites included in our spectral library, 37, 34 and 34 compounds were detected in the samples from experiment 1, 2 and 3 respectively (see Table 1 for compounds' list and abbreviations).

2.5. Statistics

Since allometric relationship may exist between the body mass parameters and size, we first determined if the variables were linearly related to LDM (with least-squares regressions)

(Packard and Boardman, 1999). Analysis of covariance (ANCOVA) was then used if linear relationships was established, using LDM as co-variable, whereas analysis of variance (ANOVA1) was used with nutritional treatment as factor when the allometric relationship was not found. The same approach was used for analysing the protein content but with DM as co-variable. Comprehensive details on regression statistics and individual plots are shown in [supplementary file S1](#). For cold tolerance, Chi-square contingency tests were used to compare mortality rates between nutritional groups (with Yates' correction to prevent overestimation of statistical significance). For CCR, the data were used to generate temporal recovery curves which were compared with Mantel-Cox (Log rank) test. This non parametric method tests the null hypothesis that there is no difference between the populations in the probability of an event at any time point (i.e. a curve comparison test). Analyses were performed using Prism v. 5.01 (GraphPad Software, Inc., San Diego, CA, USA, 2007) or the statistical software 'R 2.13.0' (R Development Core Team, 2008). For metabolic data, a principal component analysis (PCA) was performed on the whole dataset to detect the compounds contributing the most to the separation between the nutritional groups. The inertia calculated in the PCA represents the part of the total variance that is due to the difference between modalities. Scaled data (i.e. mean-centered and divided by \sqrt{SD}) were used in the multivariate analyses to prevent the effects of the metabolite concentration means and ranges of variability on the correlations with the principal components (PCs). This analysis was performed using the 'ade4' library in the statistical software 'R 2.13.0'. In addition, to look for evidence of enriched metabolic pathways in response to dietary live yeast supplementation, metabolite pathway enrichment analysis (MPEA) was conducted using MetPA online package, with *D. melanogaster* specific library (Xia and Wishart, 2010), as previously described (Colinet et al., 2013a).

3. Results

3.1. Body mass and protein levels

The [Figure 2](#) summarizes the variations in mass parameters according to nutritional treatments. The DM corresponds to the sum of LM and LDM, and FM corresponds to the sum of LM, LDM and WM ([Fig. 2](#)). Females fed with live yeast [SAY(+) and SAY(\pm)] were heavier in terms of FM and DM than their counterparts fed without live yeast. Since FM and

DM were linearly related to LDM ($P < 0.05$; see [supplementary file S1](#)), we used ANCOVA to assess the effect of nutritional treatment with LDM as co-variable. The effect of the treatment remained significant even when the allometric effect of size was removed (FM: $F = 30.69$, $df = 3$, $P < 0.001$; DM: $F = 8.27$, $df = 3$, $P < 0.001$; $n = 15$). Multiple comparisons revealed that $SA < SAY(-) < SAY(+) = SAY(\pm)$ for FM, and $SA < SAY(-) = SAY(+) = SAY(\pm)$ for DM. The WM was also correlated to LDM ($P < 0.05$; see [supplementary file S1](#)). The ANCOVA revealed a significant effect of the treatment ($F = 29.94$, $df = 3$, $P < 0.001$; $n = 15$). Multiple comparisons revealed that $SA < SAY(-) < SAY(+) = SAY(\pm)$ for WM. Contrary to WM, the WC was unrelated to LDM ($P > 0.05$; see [supplementary file S1](#)) and the ANOVA did not detect variation according to the diet treatment ($F = 0.197$, $df = 3$, $P = 1.61$; $n = 15$). The LM was unrelated to LDM ($P > 0.05$; see [supplementary file S1](#)), and ANOVA detected a significant effect of nutritional treatment, with a lower total lipid content for the treatment SA ($F = 10.4$, $df = 3$, $P < 0.001$; $n = 15$). The LDM varied according to nutritional treatment ($F = 76.14$, $df = 3$, $P < 0.001$; $n = 15$), with the following rank order: $SA < SAY(-) < SAY(+) < SAY(\pm)$. Finally, the total protein content was not related to DM ($P > 0.05$; see [supplementary file S1](#)), and ANOVA revealed a significant effect of nutritional treatment ($F = 155.3$, $df = 3$, $P < 0.001$; $n = 12$), with higher protein contents in females fed with live yeast [*i.e.* $SA < SAY(-) < SAY(+) < SAY(\pm)$].

3.2. Cold tolerance

Concerning cold tolerance, we found that CCR significantly varied between the two nutritional groups of the experiment 1, with females fed on SAY(+) diet recovering faster than females fed on SA diet ([Fig. 3](#)). This difference manifested for all the durations of cold stress that were tested in the experiment 1 (8h: $Chi^2 = 19.17$, $df = 1$, $P < 0.001$; 10 h: $Chi^2 = 16.29$, $df = 1$, $P < 0.001$; 12 h: $Chi^2 = 14.65$, $df = 1$, $P < 0.001$; $n = 50$). Survival after chronic cold stress was also affected by nutritional regime. For all the durations of cold stress at 0 °C (8, 10 and 12 h), the post-stress mortality was significantly lower when females fed on SAY(+) diet compared to SA diet (8 h: $Chi^2 = 21.23$, $df = 1$, $P < 0.001$; 10 h: $Chi^2 = 19.10$, $df = 1$, $P < 0.001$; 12 h: $Chi^2 = 21.23$, $df = 1$, $P < 0.001$; $n = 50$) ([Fig. 3](#)). Finally, the acute cold tolerance also varied with the nutritional regimes of the experiment 1 ([Fig. 3](#)). For all the durations of cold stress at -3.5 °C (90, 120 and 135 h), the post-stress mortality was significantly lower when females fed on SAY(+) diet compared to SA diet (90 min: Chi^2

=39.61, $df = 1$, $P < 0.001$; 120 min: $Chi^2 = 35.57$, $df = 1$, $P < 0.001$; 135 min: $Chi^2 = 17.56$, $df = 1$, $P < 0.001$; $n = 100$).

The cold tolerance of the flies from the experiment 2 was also affected by the nutritional treatments; however, this was not manifested on CCR. Females feeding on SAY(-) and SAY(\pm) recovered from chronic cold stress with similar temporal dynamics (10 h: $Chi^2 = 2.55$, $df = 1$, $P = 0.10$; 12 h: $Chi^2 = 1.01$, $df = 1$, $P = 0.31$; $n = 50$) (Fig. 4). On the other hand, the survival after the chronic cold stress was affected by the nutritional regimes. For both durations of chronic cold stress (10 and 12 h), the post-stress mortality was significantly lower when females fed on SAY(\pm) diet compared to SAY(-) diet (10 h: $Chi^2 = 5.02$, $df = 1$, $P = 0.025$; 12 h: $Chi^2 = 16.94$, $df = 1$, $P < 0.001$; $n = 50$) (Fig. 4). Finally, the acute cold tolerance also varied with the nutritional regimes of the experiment 2. For both durations of acute cold stress at -3.5 °C (90 and 120 h), the post-stress mortality was significantly lower when females fed on SAY(\pm) diet compared to SAY(-) diet (90 min: $Chi^2 = 69.01$, $df = 1$, $P < 0.001$; 120 min: $Chi^2 = 66.66$, $df = 1$, $P < 0.001$; $n = 100$) (Fig. 4).

3.3. Metabolic fingerprinting

The metabolic profiles of flies from experiment 1 showed that a number of metabolites had their concentrations affected by dietary live yeast, which resulted in contrasted metabotypes between the two nutritional groups (Fig. 5). A clear-cut separation was observed along the first principal component (PC1) of the PCA, which accounted for 43.9% of the total inertia (Fig. 5). GDL, Fru, Glc, Tre and sorbitol were the molecules the most positively correlated to PC1 (*i.e.* accumulated in SA flies), whereas on the opposite side, the amino acids Val, Ile, Leu, Thr, Gly, Phe and Glu were the molecules the most negatively correlated to PC1 (*i.e.* accumulated in SAY(+) flies) (Fig. 5). The other principal components accounted for 28.4% (PC2) and 11.3% (PC3) of the total inertia and mainly represented within-treatment variations. MPEA based on the metabolites that were positively correlated to PC1 revealed three enriched metabolic pathways (Holm adjust $P < 0.05$), and all were directly involved in carbohydrate metabolism. MPEA based on all the metabolites that were negatively correlated to PC1 revealed three enriched metabolic pathways; all were directly involved in amino acids and protein biosynthesis (see Dataset S1 for detailed concentrations and fold changes).

Similar results were obtained with the flies from experiment 2. A clear-cut separation was observed along the PC1 of the PCA, which accounted for 47.1% of the total inertia (Fig. 6). Fru, Tre, xylitol, Glc and GDL were the most positively correlated to PC1 (*i.e.* accumulated in SAY(-) flies), whereas on the opposite side, Glu, inositol, Leu, Phe and Val were the most negatively correlated metabolites to PC1 (*i.e.* accumulated in SAY(\pm) flies) (Fig. 6). The other principal components accounted for 31.6% (PC2) and 6.4% (PC3) of the total inertia and mainly represented within-treatment variations. MPEA also revealed that that carbohydrate metabolism was enriched in the SAY(-) flies, while amino acids and protein biosynthesis were enriched in the SAY(\pm) flies (see Dataset S2 for detailed concentrations and fold changes).

Finally, the addition of a starvation period to empty the gut content of the flies before assessing the flies (*i.e.* experiment 3) resulted in a similar metabolic response. Again, a clear-cut separation was observed along the PC1, which accounted for 57.3% of the total inertia (Fig. 7). Xylitol, Man, Ala, Fru, Tre and Glc were the most positively correlated metabolites to PC1 (*i.e.* accumulated in St-SAY(-) flies), whereas Glu, Thr, Ile, Phe, inositol and Leu were the most negatively correlated to PC1 (*i.e.* accumulated in SAY(\pm) flies) (Fig. 7). The other principal components accounted for 21.2% (PC2) and 7.15% (PC3) of the total inertia. MPEA also revealed that that carbohydrate metabolism was enriched in the St-SAY(-) flies, while amino acids and protein biosynthesis were enriched in the St-SAY(\pm) flies (see Dataset S3 for detailed concentrations and fold changes).

4. Discussion

Dietary yeast is a major source of nutrition for the adults and larvae of most saprophagous *Drosophila* sp. (Diptera: Drosophilidae) (Begon, 1982), and as a consequence, yeast is typically incorporated into artificial diets (Markow and O'Grady, 2006; Stocker and Gallant, 2008). Dietary yeast provides essential nutrients such as amino acids, sterols, vitamins, and fatty acids (Davis, 1975; Anagnostou et al., 2010). We assumed that removing or adding live yeast from adult food at eclosion would be associated with physiological remodelling that would subsequently affect fitness-related traits such as body size and stress tolerance. In the present study, we completed a comprehensive assessment of the impact of

365 dietary live yeast supplementation on body mass characteristics, stored proteins, metabolic
366 profiles and basal cold tolerance (to acute and chronic exposures) in *D. melanogaster* females.

367 We expected body mass parameters to be affected by dietary live yeast
368 supplementation. Indeed, the body mass of the flies is known to reflect protein level in food,
369 with high levels of dietary yeast leading to heavier flies (Skorupa et al., 2008). We have
370 conclusively shown that body mass parameters (FM, DM, WM, LM and LDM) increased
371 when females were fed with live yeast, which is consistent with previous studies (Simmons
372 and Bradley, 1997; Le Rohellec and Le Bourg, 2009). For all the considered mass parameters,
373 the SA flies had significantly smaller values than the SAY(-) flies which shows that SA flies
374 disproportionally suffered from the complete lack of dietary protein and suggests a
375 malnutrition in this group. Concerning the fat (*i.e.* LM), we found that the SA flies had lower
376 stored fat than the live yeast-fed flies [*i.e.* SAY(+), SAY(\pm)], but this reduction was not
377 observed in SAY(-) flies. Hence, the reduction of fat was not related to the suppression of live
378 yeast *per se*, but to the complete suppression of proteins supply from the diet (*i.e.* SA). It was
379 previously reported that the increase in body mass with dietary live yeast is almost
380 exclusively due to increased ovary size (Simmons and Bradley, 1997), and ovaries comprise
381 approximately 15% of the body lipids of insects (Lease and Wolf, 2011). The lower LM of
382 the flies on SA diet is not surprising as these flies had small ovaries and hardly produced eggs
383 (data not shown). The LDM was different among all treatments and a corresponding pattern
384 was observed for the protein content. This suggests that feeding on a diet that contains killed
385 yeast [SAY(-)] provides proteins to the flies, but feeding on a diet that also contains live yeast
386 provides additional amounts of proteins. Storage of proteins is largely independent of dietary
387 carbohydrates but is almost exclusively determined by the presence and concentration of yeast
388 in the medium (Skorupa et al., 2008). Our data corroborate this idea.

389 A way in which insects deal with nutrient variations is through altered physiology,
390 namely by affecting developmental and metabolic processes (Markow et al., 1999). Therefore,
391 we assumed that manipulating the adult food (*via* live yeast supplementation) would be
392 associated with physiological changes that would translate into contrasted metabolic profiles
393 between nutritional groups. We have conclusively shown that a number of metabolites had
394 their concentrations affected by the nutritional treatments, which resulted in contrasted
395 metabotypes between live yeast-supplemented flies [SAY(+) and SAY(\pm)] and the control
396 flies. Whatever the control used [SA or SAY(-) or St-SAY(-)], a similar response was
397 repeatedly observed : sugars (Fru, Glc, and Tre) exhibited elevated amounts in the control

whereas amino acid amounts (Val, Ile, Leu, Thr, Gly, Phe and Glu) were more abundant in the live yeast-supplemented groups. The fact that the relative abundance of sugars was higher in SA metabotype is not surprising, as these flies were fed on a minimal diet with no access to any source of proteins from adult eclosion. For the flies fed on SAY(-) and St-SAY(-) diets, the increased levels of sugars likely translates that these diets were proportionally richer in sugar than the corresponding live yeast-supplemented diets. We also found that GDL, sorbitol and xylitol contributed to the control metabotypes. GDL is a metabolite (a lactone) resulting from the degradation of Glc through the pentose phosphate pathway (Garrett and Grisham, 1999). Polyols such as sorbitol are derived from hexose monophosphates and can be produced from both Glc and Fru (Storey, 1983; Wolfe et al., 1998). The higher relative abundance of these sugar-related compounds is thus congruent with the nutritional regime of the flies. Moreover, MPEA revealed several enriched metabolic pathways associated with the control metabotypes [SA or SAY(-) or St-SAY(-)], and all of them were directly involved in the carbohydrate metabolism. This further confirmed the relative higher impact of sugars in shaping the metabotype of these nutritional groups. Concerning the live yeast-fed flies, we found a higher relative abundance of amino acids (*e.g.* Val, Ile, Leu, Thr, Gly, Phe and Glu) associated with these nutritional groups. This response was observed whatever the treatment used [SAY(+) or SAY(\pm), or St-SAY(\pm)]. This is congruent with the nutritional regime of these flies. Live yeast is known to provide essential nutrients such as proteins and amino acids (Davis 1975; Anagnostou et al. 2010). This most likely explains why MPEA revealed several enriched metabolic pathways related to amino acids and protein biosynthesis in these nutritional groups. This biological interpretation also coincides with the larger body protein content detected in these nutritional groups. The differences observed in the metabolic profiles between the live yeast-supplemented and the control groups may also be partly due to different food intake and thus incorporation of nutrients. Indeed, food intake increases with concentration of dietary yeast in *D. melanogaster* (Min and Tara, 2006). The fact that metabolic patterns were consistent among experiments suggest that (i) live yeast promotes amino acids biosynthesis even when the flies are already fed with killed yeast, and (ii) that differences observed were not related to presence/absence of live yeast in the gut content.

Many insect species feed on yeasts and the effects of this nutritional resource on the growth, fecundity and survival has been demonstrated in a wide range of species (*e.g.* Starmer and Fogleman, 1986; Ganter 2006; Anagnostou et al. 2010). In spite of this, there is limited information on nutrition-mediated variations in stress tolerance in insects, and more

particularly regarding thermal tolerance (Andersen et al., 2010). Here, we report convincing evidence that supplementing adult flies with sources of dietary proteins and amino acids (live yeast) promoted their subsequent cold tolerance. This positive effect of live yeast was repeatedly found in almost all of the metrics used to assess their cold tolerance (acute and chronic tolerance), and for all the stress intensities applied in the first experiment. In the second experiment, CCR was not affected by live yeast supplementation, but all the other assays (post-stress survival) supported a positive effect of live yeast on cold tolerance. Previous works reported an effect of dietary yeast on *Drosophila* cold tolerance, but the effects ranged from weak to very intense, and were thus difficult to interpret. For instance, Le Rohellec and Le Bourg (2009) found that removing live yeast weakly decreased cold survival of females subjected to a 16h cold-shock (0 °C), but only when these were mated. In another study, the absence of live yeast in food killed nearly all flies (males and females) subjected to the same cold treatment, whereas access to dietary yeast resulted in medium to high survival rates, depending on the age of the specimens (Le Bourg, 2010). These incongruities likely arise from the fact that nutrition-related variation in thermal tolerance involves interacting factors such as age, mating and gender. It remains unclear why in our study the CCR was affected by the nutritional treatment in the experiment 1 but not in the experiment 2. Longer temporal recovery dynamics of the SA flies (experiment 1) could result from the lack of essential nutrients necessary for an optimal functioning of the whole-system physiology, or from an excessive amount of consumed sugars (as sugar was the sole source of food in this specific group). With regard to sugars, it appears that carbohydrate-enriched diets tend to increase *Drosophila* cold tolerance (Andersen et al., 2010; Sisoda and Singh, 2012). However, when provided at too high levels, dietary sugars induce a severe nutritional imbalance and a pathological state in *D. melanogaster* (Wang and Clark, 1995; Skorupa et al., 2008; Musselman et al., 2011; Colinet et al., 2013a), which in turn negatively affects cold tolerance including CCR (Colinet et al., 2013a). In spite of this, our data and earlier observations (Le Rohellec and Le Bourg, 2009; Le Bourg, 2010) all converge towards the same conclusion that cold tolerance of the females of *D. melanogaster* is generally promoted by dietary live yeast. Females fed with live yeast had increased body mass and contained more lipids and proteins, and MPEA corroborated that live yeast supplementation was associated with amino acids and protein biosyntheses. Interestingly, it was previously found in *D. melanogaster* that the level of glycogen, triglycerides, and total proteins was higher in cold-selected than in control lines (Chen and Walker, 1994). The same authors also noted that these levels quickly decreased 24 h after a cold stress and

suggested that higher storage of energy reserves entails increased cold tolerance of cold-selected lines. Thus, the higher energy reserves of the live yeast-supplemented flies may explain why cold survival (assessed after 24 h) was higher in this nutritional group.

Stressful conditions are known to critically increase energy expenditure because the repairing mechanisms require excess of energy (Parsons, 1991). We suggest that in nutrient-unbalanced conditions (*e.g.* SA), individuals might disproportionately suffer from stressful conditions because the metabolically available energy is already constrained. The ability to synthesize essential stress-related proteins, due to dietary depletion of amino acids and protein building blocks could be an alternate explanation for the reduced cold tolerance. Hence, dietary balance is likely to be a key point of environmental stress physiology. Stress tolerance is probably compromised under conditions of excessive nutritional imbalance, as for life-history traits (Skorupa et al., 2008). In the natural environment, larvae may occasionally face nutritional stress and this might further affect the stress tolerance of the adults (carry-over effect), however, this question has not been examined. This study conclusively demonstrates an interaction between dietary live yeast and thermal stress tolerance of *D. melanogaster* females. Whether dietary live yeast positively affects the tolerance to other stressors remains to be further examined. Moreover, the mechanistic link between thermal tolerance and dietary live yeast remains an open question. Understanding the link between thermal stress tolerance and nutrient quality represents an important step in physiological ecology that may further add to our understanding of thermal biology of ectotherms.

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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
Fumarate

Other metabolites

Lactate
Ethanolamine (ETA)
Free phosphate (PO₄)
Gamma-aminobutyric acid (GABA)
Glucono delta-lactone (GDL)
Spermine

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Figure 1: Schematic diagram of the experimental design used to investigate the effect of dietary live yeast supplementation on mass parameters, cold tolerance and GC-MS metabolic profiles of *D. melanogaster*. In all experiments, the flies developed from egg to adult on a standard diet [SAY(-)]. Emerging females were then fed on different diets for 6 days: SA vs. SAY(+) for experiment 1 and SAY(-) vs. SAY(±) for experiment 2. In the experiment 3, females were fed on the same experimental conditions as in experiment 2, but they were starved for 8 h before sampling, St-SAY(-) vs. St-SAY(±). Symbols S, A and Y for sugar, agar and yeast, respectively. Sign (+), (-) and (±) for live yeast only, killed yeast only, and both live and killed yeast, respectively.

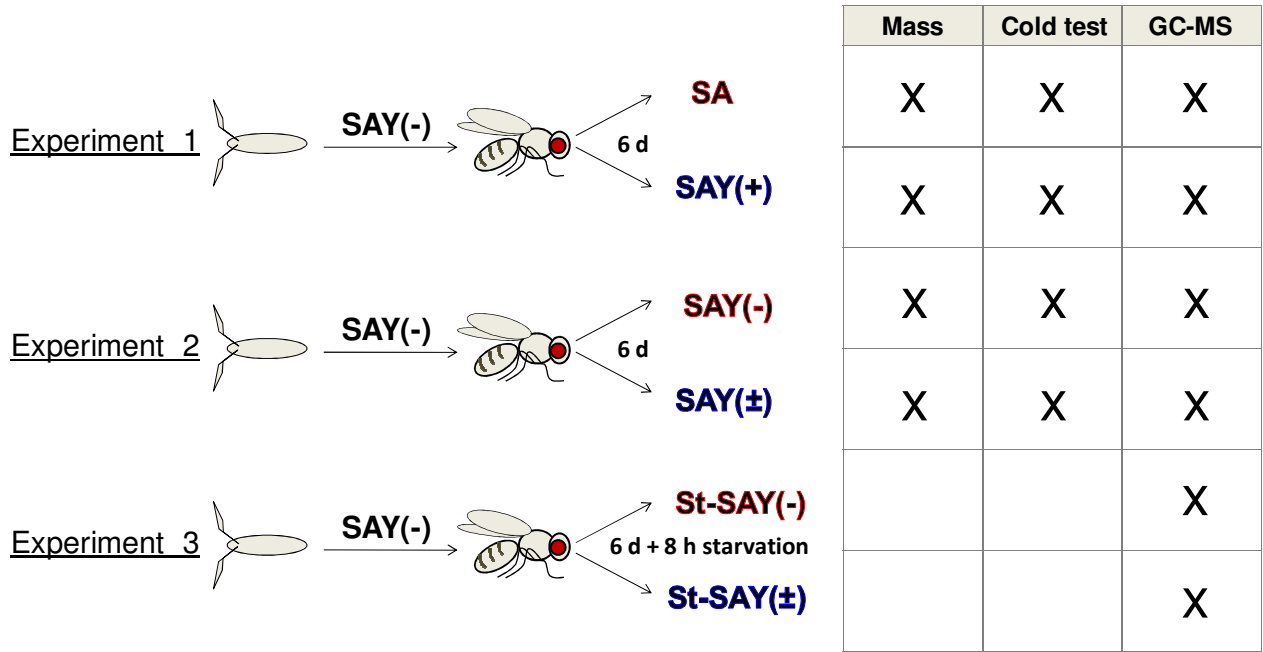


Figure 2: (A) Body mass parameters showing changes in lipid mass (LM), lean dry mass (LDM), and water mass (WM) according to nutritional treatment [SA, SAY(+), SAY(-), SAY(\pm)] ($n = 15$). (B) Total protein content of female *D. melanogaster* ($n = 12$).

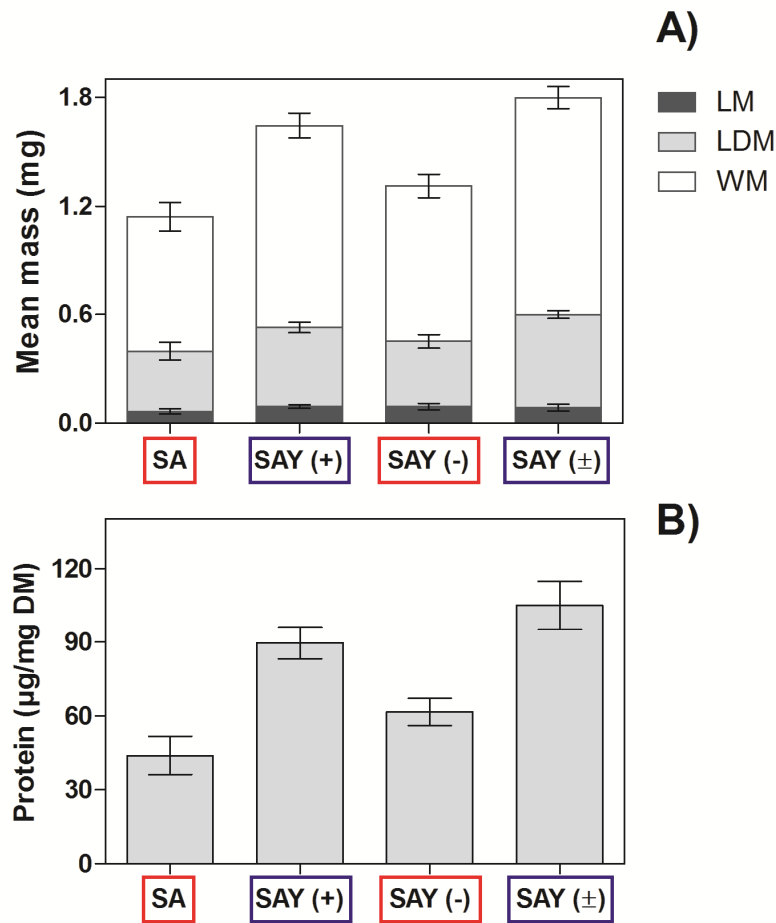


Figure 3: Composite panel summarizing all the cold tolerance assays of the experiment 1. Temporal recovery curves of live yeast-fed females [blue line, SAY(+)] and yeast-deprived females [red line, SA] exposed to chronic cold stress (0 °C) for various durations: 8, 10 and 12 h in figures A, D, and G, respectively. Each line represents the mean proportion (\pm 95% confidence interval) of recovering flies in relation to time after cold stress ($n = 50$). Mortality rates, assessed 24 h after the chronic cold stresses, are shown in figures B, E and H for each nutritional treatment [SA vs. SAY(+)] ($n = 50$). Mortality rates assessed 24 h after an acute cold stress (-3.5 °C) for various durations: 90, 120, 135 min are shown in figures C, F and I, respectively ($n = 100$). The black part of the bars represents the percent mortality and grey part is percent survival.

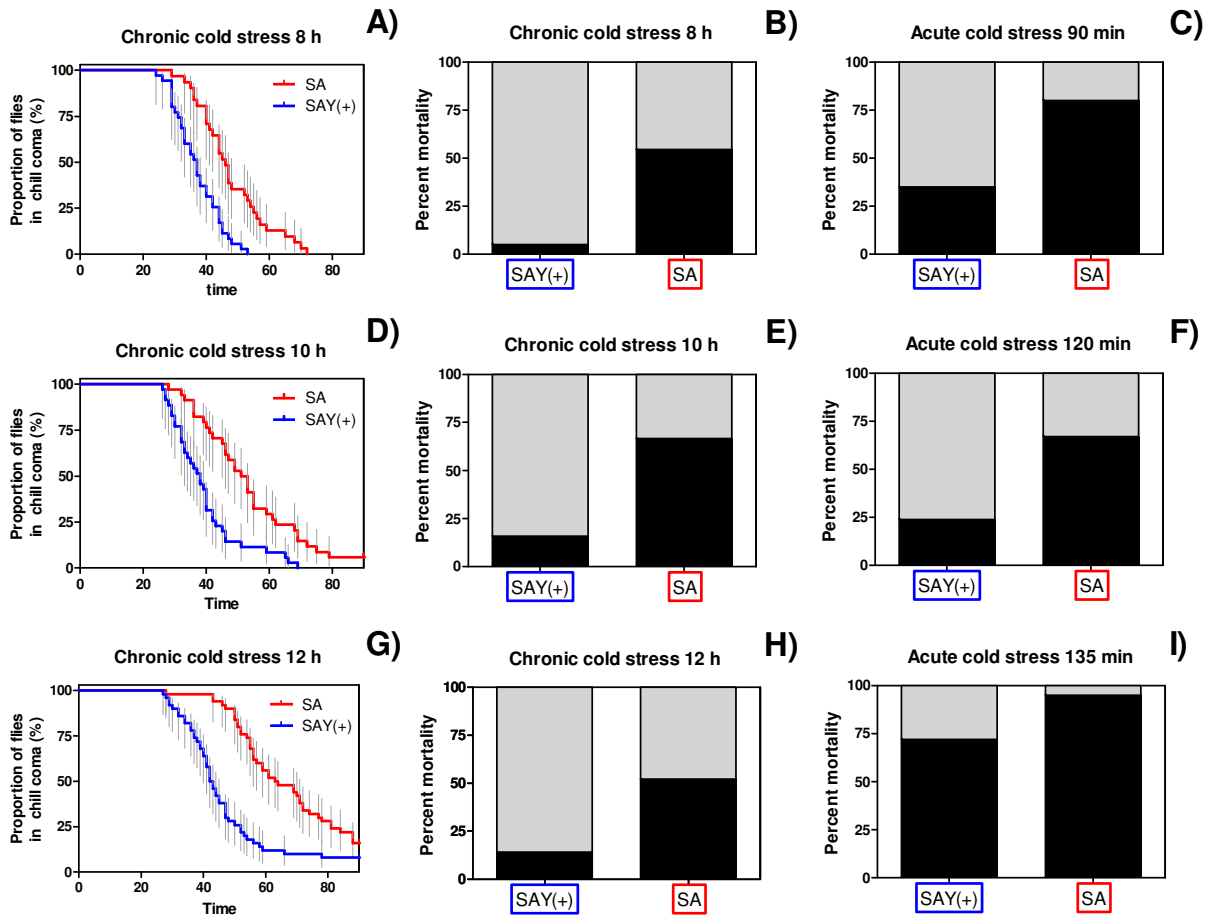


Figure 4: Composite panel summarizing the cold tolerance assays of the experiment 2. Temporal recovery curves of live yeast-fed females [blue line, SAY(\pm)] and live yeast-deprived females [red line, SAY(-)] exposed to chronic cold stress (0 °C) for various durations: 10 and 12 h in figures A and D, respectively. Each line represents the mean proportion (\pm 95% confidence interval) of recovering flies in relation to time after cold stress ($n = 50$). Mortality rates, assessed 24 h after the chronic cold stresses, are shown in figures B, and E for each nutritional treatment [SAY(-) vs. SAY(\pm)] ($n = 50$). Mortality rates assessed 24 h after an acute cold stress (-3.5 °C) for various durations: 90 and 120 min are shown in figures C and F, respectively ($n = 100$). The black part of the bars represents the percent mortality and the grey part is percent survival.

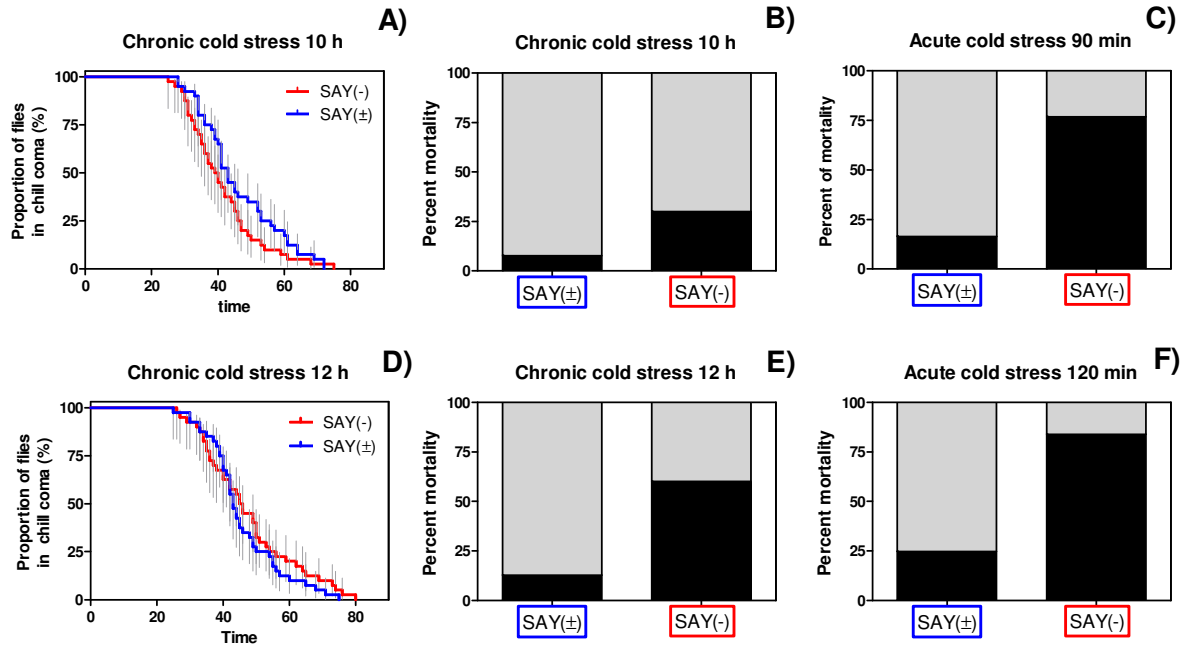


Figure 5: (A) Multivariate analysis (PCA) based on the GC/MS metabolomic data of the experiment 1 illustrating the plotting of PC1 against PC2. The unit "d" (top right of the plot) represents the side-length of a square in the grid. A clear separation was observed between live yeast-fed [blue ellipse, SAY(+)] and yeast-deprived metabolotypes [red ellipse, SA]. Lines link replicates to their respective centroids ($n = 6$). (B) Correlation values of the different metabolite concentrations to the principal components PC1 in the principal component analysis. Correlations are ranked on Y-axis according to their values. Blue bars for negative correlations (*i.e.* accumulated in SAY(+) flies) and red bars for positive correlations (*i.e.* accumulated in SA flies). See [Dataset S1](#) for detailed concentrations and fold changes.

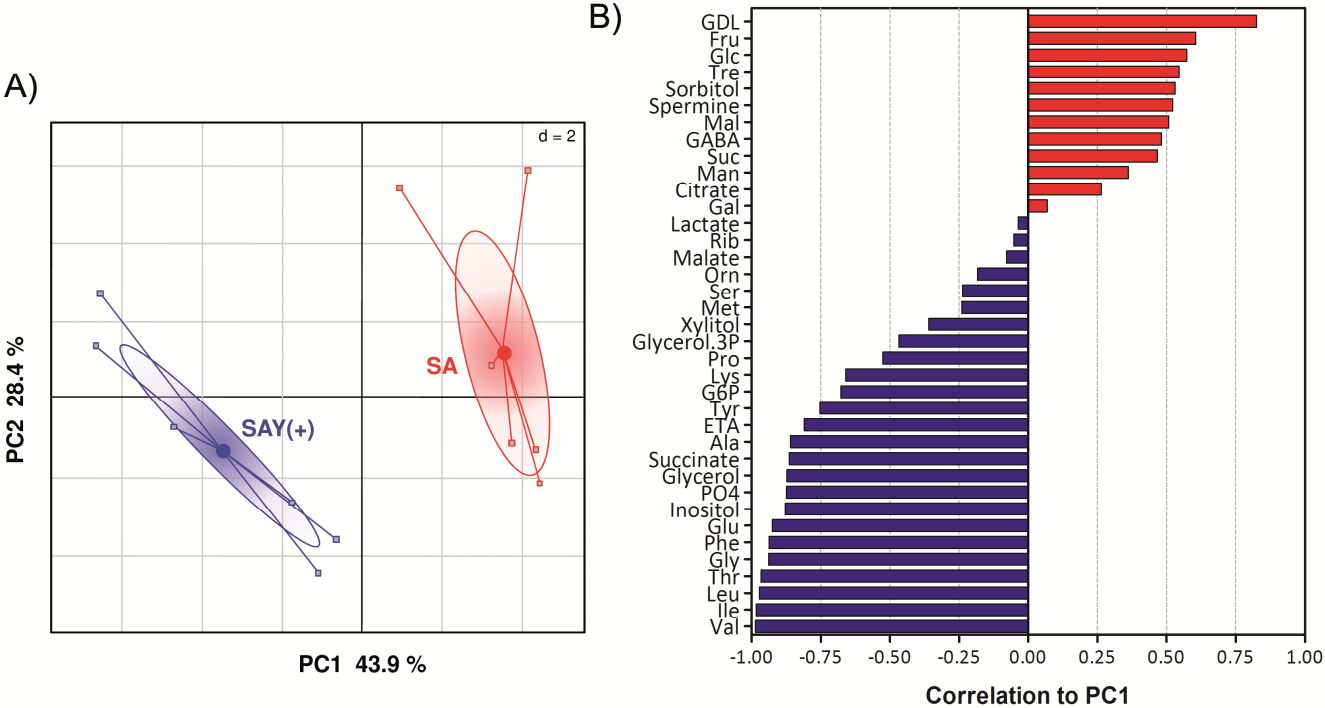


Figure 6: (A) Multivariate analysis (PCA) based on the GC/MS metabolomic data of the experiment 2 illustrating the plotting of PC1 against PC2. The unit "d" (top right of the plot) represents the side-length of a square in the grid. A clear separation was observed between live yeast-fed [blue ellipse, SAY(\pm)] and live yeast-deprived metabolotypes [red ellipse, SAY(-)]. Lines link replicates to their respective centroids ($n = 6$). (B) Correlation values of the different metabolite concentrations to the principal components PC1 in the principal component analysis. Correlations are ranked on Y-axis according to their values. Blue bars for negative correlations (*i.e.* accumulated in SAY(\pm) flies) and red bars for positive correlations (*i.e.* accumulated in SAY(-) flies). See [Dataset S2](#) for detailed concentrations and fold changes.

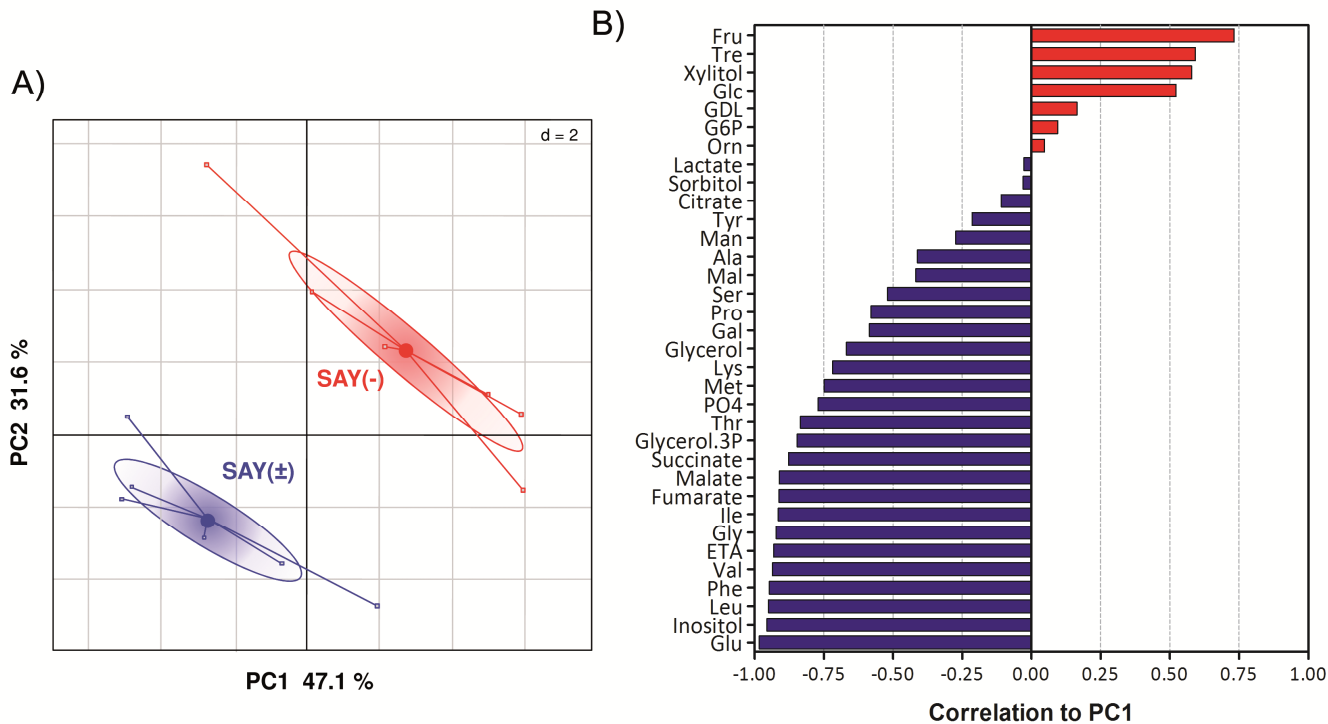


Figure 7: (A) Multivariate analysis (PCA) based on the GC/MS metabolomic data of the experiment 3 illustrating the plotting of PC1 against PC2. The unit "d" (top right of the plot) represents the side-length of a square in the grid. A clear separation was observed between live yeast-fed [blue ellipse, St-SAY(\pm)] and live yeast-deprived metabolotypes [red ellipse, St-SAY(-)]. Lines link replicates to their respective centroids ($n = 6$). (B) Correlation values of the different metabolite concentrations to the principal components PC1 in the principal component analysis. Correlations are ranked on Y-axis according to their values. Blue bars for negative correlations (*i.e.* accumulated in St-SAY(\pm) flies) and red bars for positive correlations (*i.e.* accumulated in St-SAY(-) flies). See [Dataset S3](#) for detailed concentrations and fold changes.

