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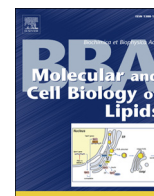
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# Uncovering the benefits of fluctuating thermal regimes on cold tolerance of drosophila flies by combined metabolomic and lipidomic approach

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## ABSTRACT

When exposed to constant low temperatures (CLTs), insects often suffer from cumulative physiological injuries that can severely compromise their fitness and survival. Yet, mortality can be considerably lowered when the cold stress period is interrupted by periodic warm interruption(s), referred to as fluctuating thermal regimes, FTRs. In this study, we have shown that FTRs strongly promoted cold tolerance of *Drosophila melanogaster* adults. We then assessed whether this marked phenotypic shift was associated with detectable physiological changes, such as synthesis of cryoprotectants and/or membrane remodeling. To test these hypotheses, we conducted two different time-series Omics analyzes in adult flies submitted to CLTs vs. FTRs: metabolomics (GC/MS) and lipidomics (LC/ESI/MS) targeting membrane phospholipids. We observed increasing levels in several polyhydric alcohols (arabitol, erythritol, sorbitol, mannitol, glycerol), sugars (fructose, mannose) and amino acids (serine, alanine, glutamine) in flies under CLT. Prolonged exposure to low temperature was also associated with a marked deviation of metabolic homeostasis and warm interruptions as short as 2 h were sufficient to periodically return the metabolic system to functionality. Lipidomics revealed an increased relative proportion of phosphatidylethanolamines and a shortening of fatty acyl chains in flies exposed to cold, likely to compensate for the ordering effect of low temperature on membranes. We found a remarkable correspondence in the time-course of changes between the metabolic and phospholipids networks, both suggesting a fast homeostatic regeneration during warm intervals under FTRs. In consequence, we suggest that periodic opportunities to restore system-wide homeostasis contribute to promote cold tolerance under FTRs.

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## 1. Introduction

Temperature affects virtually all aspects of ectotherms' life, including behavior, physiological processes, metabolic and signaling pathways and gene transcription [1]. For small ectotherms like insects, temperature can often reach stressing limits. Insects exposed to even relatively mild (sublethal) chilling for prolonged time may suffer from cumulative (so called indirect) physiological damages that can severely

compromise their fitness and survival. Studies of cold tolerance have generally been conducted under constant low temperatures (CLTs). However, the natural thermal environment is hardly ever stable, but rather fluctuates on scales ranging from hours to seasonal cycles. Not surprisingly, the responses of insects to constant temperatures significantly differ from those to fluctuating temperatures, and there is a growing body of literature describing the effects of thermal fluctuations on insect growth, life-history traits, stress tolerance and general physiology reviewed in [2].

Applying fluctuating temperatures during cold exposure can strongly affect insect's cold tolerance. Indeed, mortality is considerably lowered when a cold period is interrupted by brief warm interruption(s) (referred to as fluctuating thermal regime, FTR) [3–7]. Beneficial effects of FTRs have been reported when warm interruptions were applied on a daily basis [3–7] or just once within a prolonged cold stress [8,9]. Even short-term warm episodes (a few minutes to hours) are enough to mitigate cold-induced mortality [3,10–12]. The growing list of experimental evidence for the positive effect of FTRs suggests that mechanisms behind this process are highly conserved across insect taxa [2]. However, the benefits of FTRs hold true only for freeze-

**Abbreviations:** ANOVA, analysis of variance; CL, cardiolipin; CLT, constant low temperature; CO, constant control; FA, fatty acid; FTR, fluctuating thermal regime; GC/MS, gas chromatography-mass spectrometry; LC/ESI/MS, liquid chromatography combined with electrospray ionization mass spectrometry; phospholipids, PLs; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; LPC, lyso-PC; LPE, lyso-PE; LPG, lyso-PG; LPI, lyso-PI; MGDG, monogalactosyl diglyceride; PCA, principal component analysis; PC1, PC2 and PC3, first, second and third principal components; SFA, MUFA and PUFA, saturated, monounsaturated and polyunsaturated FA respectively; PUFA/MUFA, polyunsaturation ratio; UFA/SFA, unsaturation ratio; UI, unsaturation index.

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avoiding and chill-susceptible species. Indeed, periodic warming insects that survive in frozen state (freeze tolerant species) is detrimental because of repeated freeze-thaw cycles [2].

The physiological perturbations related to chilling are not yet fully characterized reviewed in [13]. Membranes are considered among the most thermally-sensitive macromolecular structures and are thus a primary target of chilling injuries. Chilling induces fluid to gel phase transition in cell membranes that can result in separation of phospholipids bilayer, change in permeability and sharp decline in the activity of membrane-bound enzymes [14–16]. These alterations can in turn severely compromise ion and water homeostasis across membranes [17–20], which causes neuromuscular alterations, loss of function, chill-coma, and ultimately chill injury and death [21–24]. Consequently, the remodeling of membrane lipids is a prime candidate mechanism underlying the positive effect of warm interruptions on insect cold tolerance under FTRs. Ectotherms typically compensate for temperature-induced changes in viscosity of membrane lipids by homeoviscous adaptation [14,25], which involves remodeling the chemical composition of phospholipids (PLs). When compensation is not possible or feasible, the membrane remodeling aims at preventing occurrence of unregulated membrane phase transitions and, this way, preserving the structural integrity of membranes exposed to thermal stresses [15]. Whether membrane composition is adaptable under the rapidly changing thermal conditions that characterize FTRs is not known. Another potential mechanism occurring during FTRs is the mobilization of cryoprotectants. These compatible solutes are known to protect against chilling injuries [23]. Cryoprotectants include polyhydric alcohols, sugars, and some free amino acids. A primary function of these molecules relates to their colligative effect at high concentrations, but they also play non-colligative protective roles at low concentrations by stabilizing macromolecules and membranes [26–29]. Therefore, we decided to directly test whether cryoprotectants can be mobilized during cold period and warm interruptions under FTRs.

Although the mechanisms underlying the benefits of FTRs are not yet fully known, a generally accepted assumption is that insects profit from periodic warming opportunities to physiologically recover from chilling injuries that accumulated during the preceding cold exposure [2,30]. However, direct evidence of such a physiological repair is still missing. Environmental stresses transiently disturb cellular homeostasis, and in response, cells activate a complex response to progressively restore homeostasis [31]. Time-series metabolomics can be useful to monitor metabolic homeostasis status (metabolic trajectories) during stress or recovery [32–34]. By analogy, analysis of lipidomic trajectories derived from large-scale temporal profiling of PLs can be used to track the dynamics of the homeostatic response in membranes during stress and recovery.

In this study, we applied CLTs and FTRs to adult *Drosophila melanogaster* flies and observed strong reduction of the cold-induced mortality under FTRs. Next, we hypothesized that this phenotypic change would be associated with detectable physiological signatures. Several hypotheses were tested: first, we expected adjustments of the concentrations of molecules with potential cryoprotective functions. Second, we expected membrane restructuring during either cold period or warm intervals in a direction that would support an increased cold tolerance. Finally, we assumed that FTRs will allow a fast homeostatic regeneration process. Specifically, we hypothesized that (i) metabolic and lipidomic trajectories will deviate markedly from the control state during cold periods and (ii) warm intervals will permit a return towards the initial homeostatic state.

## 2. Material and methods

### 2.1. Fly culture

We conducted the experiments on a mass-bred *D. melanogaster* line derived from two wild populations collected in October 2010 in Brittany (France). Flies were maintained in laboratory in 200 mL bottles at 25 ±

1 °C (12 L:12D) on standard fly medium consisting of brewer yeast (80 g/L), sucrose (50 g/L), agar (15 g/L), and Nipagin® (8 mL/L). To generate flies for the experiments, groups of 15 mated females were allowed to lay eggs in 200 mL rearing bottles during a restricted period of 6 h under laboratory conditions. This controlled procedure allowed larvae to develop under uncrowded conditions. At emergence, adults were sexed visually (with an aspirator) without CO<sub>2</sub> to avoid stress due to anesthesia [35]. Individuals used in all experiments were synchronized at the age of 6-d-old to avoid effects on stress tolerance traits in maturing young adults (< 3-d-old) [36].

### 2.2. Cold-survival

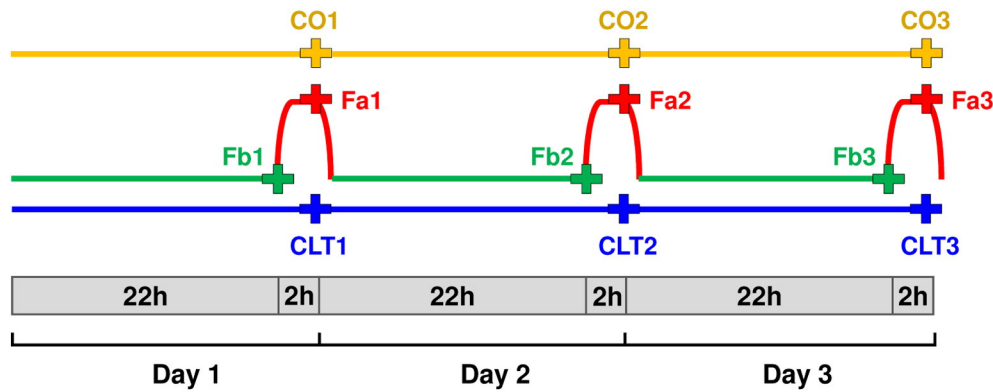
Cold survival of adult flies was tested either under constant low temperatures (CLTs) or fluctuating thermal regimes (FTRs). Four different temperatures were used: 2, 3, 4, and 5 °C. Preliminary experiments revealed that flies fall into chill-coma at all of these temperatures. For FTRs, the temperatures were the same except that the cold period was interrupted daily by a short episode at 20 °C for 2 h. These short warm intervals are known to promote cold survival in other species reviewed in [2]. For each experimental condition, several groups of 15 flies were placed into vials that contained only agar. Pure agar diet was used in order to avoid any confounding effect due to re-nutrition of flies during warming intervals (possible under FTR only). For each experimental condition, ten vials of 15 flies were placed inside programmed thermo-regulated incubators (Model SANYO MIR-153) set at the requested temperature (2, 3, 4, and 5 °C) and thermal regime (CLT or FTR) (i.e. 150 flies per experimental condition). Temperature was checked using automatic recorders (Hobo® data logger, model U12–012, Onset Computer Corporation, accuracy ± 0.35 °C). The experiment lasted for 10 consecutive days (240 h). Every 24 h, a vial with 15 flies was removed from the cold-incubator and transferred to the laboratory conditions to score the survival after 4 h and also after 24 h of recovery post stress. Survival was scored as the number of flies that could stand on legs. All these experiments were performed with males and females separately. The experiment was performed in 2013 and a complete replication was performed in 2014.

### 2.3. Experimental design for Omics profiling

Because the survival assays revealed that benefits of FTRs were particularly manifested in females exposed to 5 °C, we decided to use only females in this thermal condition for monitoring metabolic and lipidomic profiles in specific follow-up experiment (see Fig. 1). Only the first three days of exposure were targeted for Omics profiling to ensure the complete absence of dead individuals in samples. Females were exposed to cold treatments (CLT and FTR), and a third group of females was submitted to constant 20 °C as control (CO). Females were all virgin, synchronized at the age of 6-d-old and placed in incubators in vials with only agar. In CLT treatment, flies were assessed after 1, 2, and 3 days of cold stress at 5 °C (codes: CLT1 to 3) (see Fig. 1). The FTR treatment was divided into two sub-treatments: i) flies monitored at the end of the cold period, just before the onset of the warm interval (codes: Fb1 to 3 for FTR “before”) and ii) flies monitored after the recovery, at the end of the 2 h warm interval (codes: Fa1 to 3 for FTR “after”) (see Fig. 1). Finally, CO flies were monitored for 3 consecutive days at 20 °C (codes: CO1 to 3). For each time point, 14 replicates of 10 pooled females were used: 6 for metabolomics, 6 for lipidomics and 2 for checking the survival after 24 h recovery. Flies destined to biochemical analyzes were snap frozen in liquid N<sub>2</sub> before being stored at –80 °C for extractions.

### 2.4. Extractions and GC/MS-based metabolic profiling

Each sample was weighed (Mettler Toledo UMX2, accurate to 1 µg) before metabolite extractions. Sample preparation and derivatization



**Fig. 1.** Experimental design used for Omics profiling. Flies were exposed to constant low temperature at 5 °C (CLT, blue) or at constant standard temperature at 20 °C as control (CO, orange). Flies were also submitted to fluctuating temperature (5 °C/22 h + 20 °C/2 h) and were assessed just before the recovery at 20 °C (Fb, green) and also after 2 h of recovery at 20 °C (Fa, red). Samples were taken for 3 consecutive days.

were performed as previously described [33,37], with minor modifications. Briefly, after homogenization in 600  $\mu\text{L}$  of ice-cold methanol-chloroform solution (2:1, v:v) and phase separation with 400  $\mu\text{L}$  of ultrapure water, a 100  $\mu\text{L}$  aliquot of the upper phase, which contained polar metabolites, was *vacuum*-dried. The dry residue was resuspended in 30  $\mu\text{L}$  of 20  $\text{mg mL}^{-1}$  methoxyamine hydrochloride in pyridine before incubation under automatic orbital shaking at 40 °C for 60 min. Then, 30  $\mu\text{L}$  of BSTFA were added and the derivatization was conducted at 40 °C for 60 min under agitation. 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  $\text{min}^{-1}$ , from 170 to 280 °C at 7 °C  $\text{min}^{-1}$ , from 280 to 320 °C at 15 °C  $\text{min}^{-1}$ , 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 63 pure reference compounds at 1, 2, 5, 10, 20, 50, 100, 200, 500, 750, 1000, and 1500  $\mu\text{M}$  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. Quality controls at concentrations of 200  $\mu\text{M}$  were run every 15 samples. Arabinose (at 2 mM) was used as internal standard to account for potential loss during sample preparation. Calculated concentrations were adjusted according to internal standard.

### 2.5. Extraction and LC/ESI/MS analysis of phospholipid composition

Total lipids were extracted twice in 400  $\mu\text{L}$  of chloroform:methanol solution (2:1, v:v) and evaporated to dryness. Total lipid extract was then separated into polar and non-polar classes by dissolution and liquid-liquid extraction between 2 mL acetonitrile in water (80:20) and 2 mL hexane. The lower aqueous acetonitrile phase containing polar lipids was used as a source of phospholipids. The samples were analyzed using high performance liquid chromatography (LC) combined with electrospray ionization mass spectrometry (ESI/MS) as described in previous studies [38,39]. Briefly, a LTQ-XL mass spectrometer (Thermo Fisher Scientific) equipped with ESI, Accela 600 pump HPLC system, and Accela AS autosampler (Thermo Fisher Scientific, San Jose, CA, USA) was used. A volume of 200  $\mu\text{L}$  was collected from the PL fractions, solvent was evaporated to dryness

and the residue was redissolved in 300  $\mu\text{L}$  of methanol. Aliquots of 5  $\mu\text{L}$  were injected into a Gemini C18 HPLC column (150  $\times$  2 mm ID, 3  $\mu\text{m}$  (Phenomenex, Torrance, CA, USA) thermostated at 35 °C. The mobile phase flow rate was 250  $\mu\text{L min}^{-1}$  with gradient elution of A:B:C (A = 10 mM ammonium acetate in methanol with ammonia (0.025%), B = 10 mM ammonium acetate in water, C = isopropanol - MeOH 8:2) - 0 min: 92:8:0, 7 min: 97:3:0, 12 min: 100:0:0, 19 min: 93:0:7, 20–23 min: 90:0:10, 24 min: 100:0:0 and for equilibration of column 26–45 min: 92:8:0. The ESI/MS was carried out either in the positive or the negative ion detection mode at potential + 3 kV or - 2.5 kV, with capillary temperature of 200 °C. Eluting ions were detected with full scan mode from 200 to 1000 Da with the collisionally induced MS2 fragmentations (collision energy 35%). Data were acquired and processed by means of XCalibur 2.1 software (Thermo Fisher Scientific). The responses of analyzed phospholipids were corrected by comparison to the signals of internal lipid standards that were obtained from Avanti Polar Lipids (Alabaster, AL, USA). As internal standards, we have used 17C FAs which are absent in our insect samples (PC<sub>17:0/17:0</sub> and PE<sub>17:0/17:0</sub> at 40  $\mu\text{g/ml}$  and PG<sub>17:0/17:0</sub> and PS<sub>17:0/17:0</sub> at 20  $\mu\text{g/ml}$ ). The corrected areas under individual analytical peaks were expressed in percentages assuming that the total area is 100%.

### 2.6. Statistics

Survival data were analyzed using a generalized linear model with logistic link function for binary outcome. The survival data was dependent on stress duration (1 to 10 days), sex, temperature (2, 3, 4, 5 °C) and thermal regime (CLT vs. FTR) as well as the second-order interactions. The two replicated experiments (2013 and 2014) were analyzed separately. Variations in the level of all individual metabolites or PLs were analyzed using analysis of variance (ANOVA) with thermal treatment (CLT, Fb, FA and CO) and stress duration (1, 2, 3 days) as crossed factors. To compare the temporal metabolic and lipidomic profiles among the different conditions, a between-class Principal Component Analysis (PCA) was used to identify the main patterns and clustering. A Monte Carlo test was then performed to examine the significance of the difference among the classes (based on 1000 simulations). To identify the variables (i.e. metabolites or PLs) contributing the most to the structure separation, the correlations to the principal components were extracted and ranked. Data were scaled and mean-centered prior to the PCAs. PLs composition and calculated indices were analyzed within each sampling duration using one-way ANOVA followed by Tukey's multiple comparison tests. Analyses were performed using the statistical software 'R 3.0.3'.

### 3. Results

#### 3.1. Cold survival under constant and fluctuating regimes

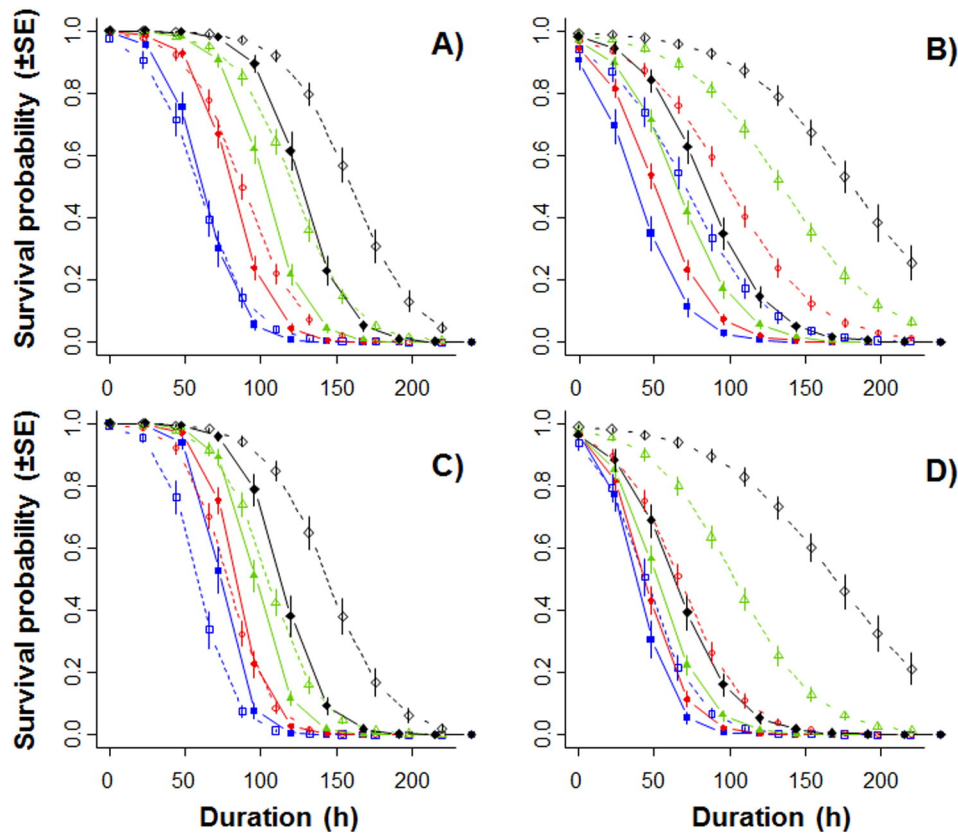
Mortality scored 4 h after exposing the flies to CLT or FTR is shown in Fig. 2. As expected, mortality was higher at lower temperatures and increased when stress duration increased in both sexes. However, the survival was clearly superior under FTR compared to CLT, particularly in females and at the highest tested temperature (5 °C), and this was observed in both experiment replicates. The statistical analyzes are summarized in Table 1 for both replications separately. All the main effects were significant, as well as most of the second order interactions. Nearly identical survival patterns were observed when mortality was scored after 24 h of recovery (presented in Supplemental Fig. S1).

As we found that benefits of FTRs were particularly manifested in females exposed to 5 °C, this condition was used for temporal Omics profiling over the three first days (Fig. 1). No mortality was observed in any of these treatments, except after 3 days at 20 °C where some females died (5%) likely because of starvation effects which negatively interfered at this temperature. Therefore, we decided to exclude this specific condition (3 days at 20 °C) from all further analyzes.

#### 3.2. Metabolic profiling

Among the 63 metabolites included in our spectral library, 43 were detected in the samples. The list of detected metabolites with their abbreviations is available in Table S1A and raw data for individual metabolites are provided in Table S1B. Among these 43 compounds, we found free amino acids (13), gamma amino acid (1), sugars (8), polyols (8), carboxylic acids (5), acidic sugar (glyceric acid), polyamines (3), alpha hydroxyl acid (1) and other molecules (3). Trehalose, glucose,

proline and glutamine were the most abundant metabolites detected in whole-body extracts. Changes of all individual metabolite levels in relation to treatments and durations are illustrated in the panel Supplemental Fig. S2 and the corresponding univariate statistical outputs are provided in Table S2A. Changes of metabolite according to treatments were characterized using between-classes PCA and the ordination of classes within the first plane is presented in Fig. 3A. PC1 (53.8%) and PC2 (20.9%) cumulated 74.7% of total inertia (Fig. 3B). PC3 accounted for only 8% of total inertia and mainly represented within-treatment inertia. The Monte-Carlo randomizations confirmed the significance of the differences among classes ( $P < 0.001$ ). The PC1 and PC2 scores (i.e. projection of centroids) of each treatment group are shown in Fig. 3C and D. These analyzes revealed a clear opposition between all treatments exposed to cold (CLT1 to 3 and Fb1) that were negatively correlated to PC1 and the other treatments that were positively correlated to PC1. From Fig. 3C, it became clear that metabolic profiles departed significantly from their initial state (i.e. CO) in all CLT treatments, showing a strong homeostatic deviation, before coming back to control state during the fluctuations (especially after 2 h recovery). PC2 explained the time-course of the metabolic response from day1 (positive association) to day3 (negative association), day2 being intermediate. The correlation of each metabolite to PC1 and PC2 are shown in Supplemental Fig. S3 A & S3B. Among the metabolites contributing the most to PC1, we found almost all the detected polyols (arabitol, erythritol, sorbitol, mannitol, glycerol), some amino acids (Ser, Ala, Glu) and sugars (Man, Fru) that were negatively correlated to PC1 (i.e. more abundant in CLT flies) (see Supplemental Figs. S2 and S3 A). Some of these compounds showed rather large magnitude of fold-change accumulation compared to CO flies (e.g. erythritol: 22 fold, Man: 12 fold, Fru: 12 fold, sorbitol: 5 fold). Succinate, Tyr, inositol and Pro were among the few metabolites that were positively correlated to PC1, and thus more abundant in CO



**Fig. 2.** Probability of mortality ( $\pm$  SE) as function of cold exposure duration of *D. melanogaster* adults exposed to 2, 3, 4, and 5 °C (blue, red, green, and black respectively) under either constant (straight line) or fluctuating temperature (dotted line), in males (A, C) and females (B, D). Under the fluctuating thermal regime, the cold exposure was interrupted daily by a 2 h break at 20 °C. The experiment lasted for 10 consecutive days (240 h) and every 24 h survival scored in each condition after the flies had recovered at 20 °C for 4 h. The experiment was replicated twice, in 2013 (A, B) and 2014 (C, D). Probability lines and estimates SE were obtained from fitted generalized linear model with binomial logit link function.

**Table 1**  
Results from generalized linear model performed on survival data with logistic link function for binary outcome. The survival was dependent on stress duration (1 to 10 days), sex, temperature (2, 3, 4, 5 °C) and thermal regime (CLT vs. FTR). The two replicated experiments (2013 and 2014) were analyzed separately.

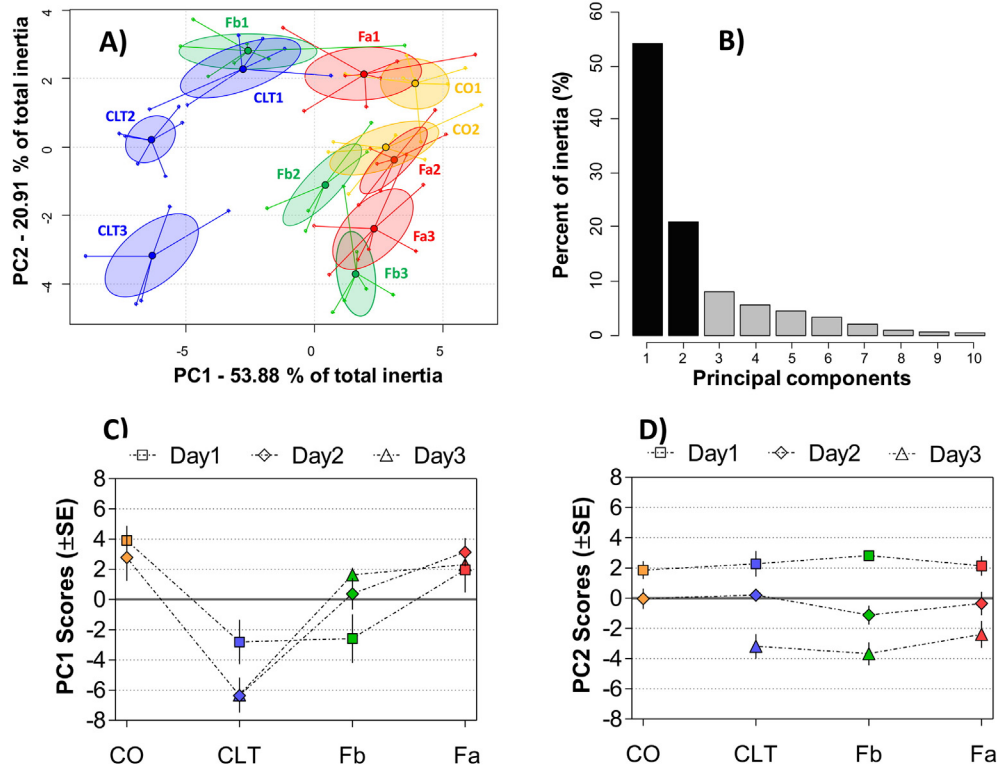
Source (2013 experiment)	Chi <sup>2</sup>	Df	P	Source (2014 experiment)	Chi <sup>2</sup>	Df	P
sex	11.81	1	0.001	sex	60.18	1	<0.001
temperature	389.29	1	<0.001	temperature	338.28	1	<0.001
regime	162.26	1	<0.001	regime	85.67	1	<0.001
duration	1955.97	1	<0.001	duration	2081.56	1	<0.001
sex*regime	34.57	1	<0.001	sex*regime	44.7	1	<0.001
regime*duration	24.1	1	<0.001	regime*duration	22.77	1	<0.001
sex*duration	29.24	1	<0.001	sex*duration	26.19	1	<0.001
sex*temperature	17.04	1	<0.001	sex*temperature	1.04	1	0.308
temperature*regime	0.77	1	0.380	temperature*regime	11.54	1	<0.001
temperature* duration	7.4	1	0.007	temperature* duration	40.13	1	<0.001

and/or Fa flies (Supplemental Fig. S3 A). Concerning PC2, amino acids (Thr, Lys, Orn) and polyamines (putrescine and spermidine) were the most positively correlated metabolites (i.e. temporal increase over the 3 days of experiment), while other amino acids (Ile, Leu, Val) and sugars (Tre and Glc) were negatively correlated to PC2 (i.e. temporal decrease over time) (Supplemental Figs. S2 and S3B).

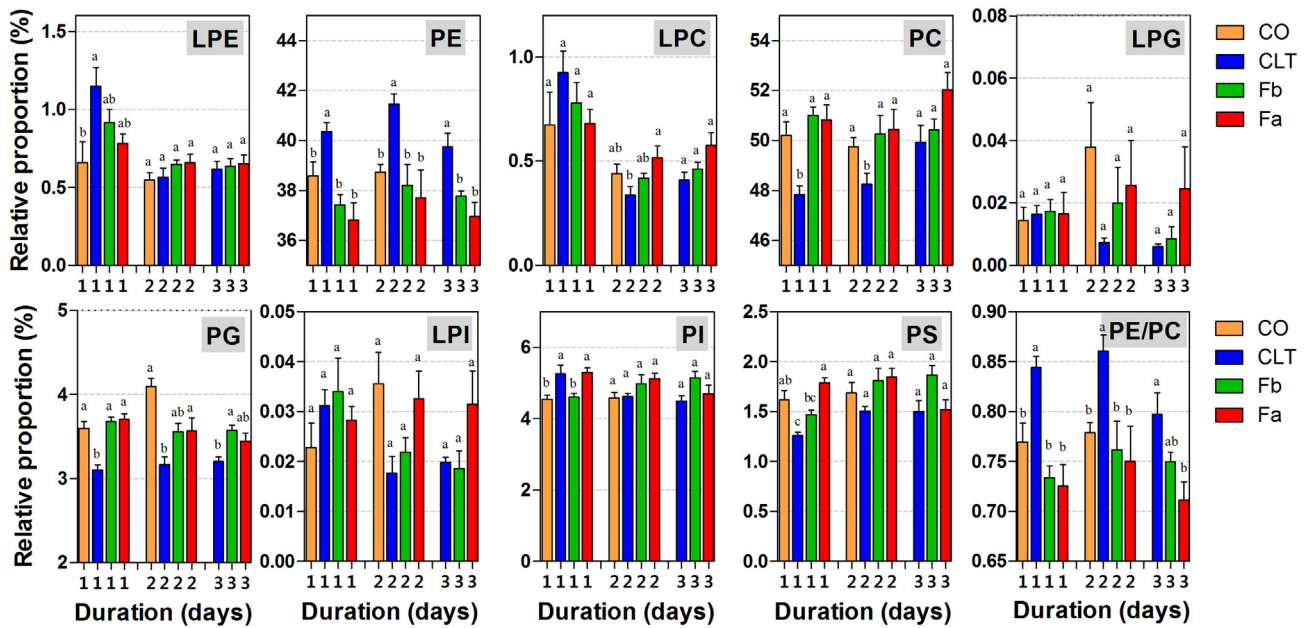
### 3.3. Phospholipids profiling and patterns

The LC/ESI/MS analysis detected 101 different PL molecular species belonging to different classes: phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylglycerol (PG), phosphatidylinositol (PI), phosphatidylserine (PS), lyso-PE (LPE), lyso-PC (LPC), lyso-PG (LPG), lyso-PI (LPI), and monogalactosyl diglyceride (MGDG). Mean relative proportions of all phospholipids detected within all experimental conditions are summarized in Supplemental Table S3 (analytical

data). Relative proportions of each PL class and fatty acyl, as well as calculated indices are provided in Supplemental Table S4 (summarized data). The dominant PLs classes in our whole-body extract samples were PCs (47–52%) and PEs (37–41%), followed by minor PIs (4–5%), PGs (3–4%), PSs (1–2%) and Lyso-PLS (1%). This pattern follows the general trend in insects [40] and fits with *Drosophila* lipidome [41]. Most of the PL molecular species exhibited statistically significant changes according to thermal treatment (48%) and/or duration of exposure (37%) (Supplemental Table S2B). The compounds PC<sub>16:1/16:1</sub> and PC<sub>18:1/16:1</sub> were the most abundant PLs; their combined relative proportion contributed up to 20% to the total PLs. The mean changes in the relative proportions of different PL classes according to treatment and durations are summarized in Fig. 4 and in Supplemental Table S4. Some changes in relative proportions among treatments were statistically significant in some PL classes (ANOVA,  $P < 0.05$ ) (Fig. 4). The most striking changes were: (i) a consistent increase in PEs in all the



**Fig. 3.** Metabolic profiling based on GC/MS analyses of *D. melanogaster* whole-body extracts from individuals sampled for three consecutive days at constant (CO and CLT) or fluctuating temperature (Fb or Fa) (see Fig. 1 for design and codes). Changes of individual metabolite levels in relation to the thermal treatment are shown in Supplemental Fig. S2. (A) Between-class PCA showing PC1 against PC2 (cumulating 74.7% of inertia). Lines link individuals to their respective centroids ( $n = 6$ ). (B) Percent of inertia for each principal component. Mean scores ( $\pm$  SE) (i.e. projection of centroids) on PC1 (C) and PC2 (D) according to treatments are shown for the three sampling days. These multivariate analyzes show that PC1 explains an opposition between the treatments continuously exposed to cold (CLTs and Fb1) and the other treatments. PC2 explains a temporal pattern from day 1 to day 3. Note that the condition CO3 was discarded (see Section 3.1 for details). Correlations of the different metabolite concentrations to PC1 and PC2 are illustrated in Supplemental Fig. S3.



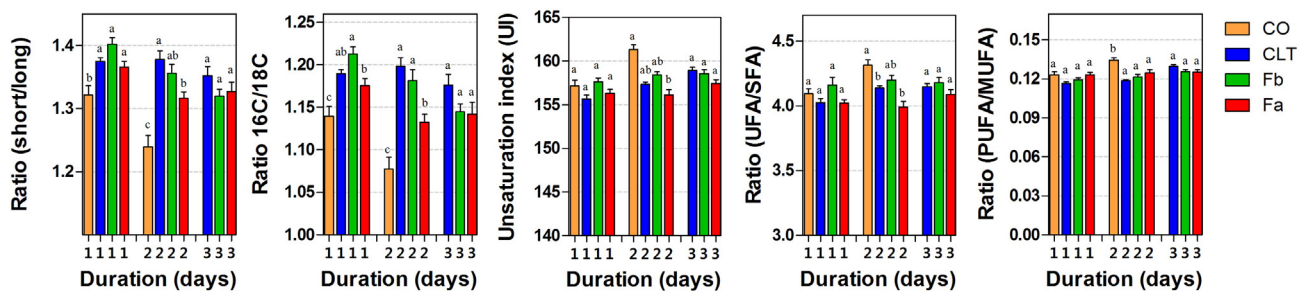
**Fig. 4.** Relative proportion (%) of PL classes resulting from LC/ESI/MS analytical data (Table S3). PLs molecular species composition was assessed in *D. melanogaster* adults sampled for three consecutive days either under constant temperature (cold at 5 °C: CLT or control 20 °C: CO) or under fluctuating thermal regime (before 2 h recover: Fb or after 2 h recovery: Fa). Lyso-Phosphatidylethanolamine (LPE); Phosphatidylethanolamine (PE); Lyso-Phosphatidylcholine (LPC); Phosphatidylcholine (PC); Lyso-Phosphatidylglycerol (LPG); Phosphatidylglycerol (PG); Lyso-Phosphatidylinositol (LPI); Phosphatidylinositol (PI); Phosphatidylserine (PS). Each bar is a mean ( $\pm$  SE) from six biological replications. For each PL class, the scale of y-axis may be different. Note that the condition CO3 was discarded (see Section 3.1 for details). Data were analyzed within each sampling duration using one-way ANOVA followed by Tukey's multiple comparison tests (means flanked by different letters are significantly different).

CLT groups, and (ii) a corresponding decrease in PGs and PCs in the same treatments. It resulted that PE/PC ratio was strongly increased in individuals continuously exposed to cold (CLTs). The relative proportion LPEs also increased at cold (CLTs), but this was only manifested on the first day (Fig. 4).

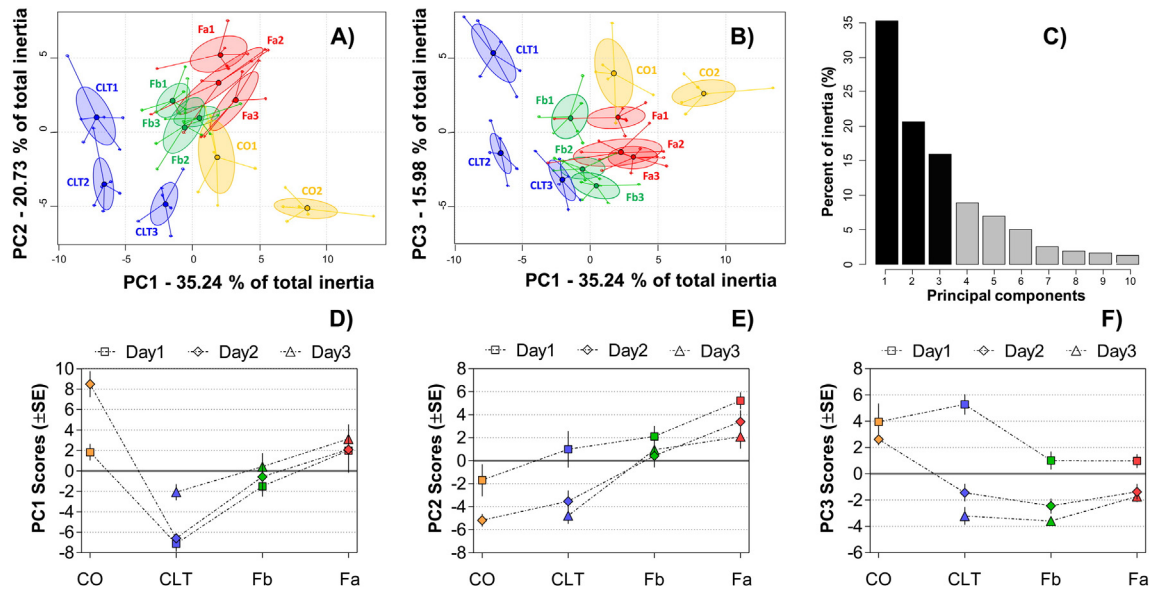
Indices of PLs remodeling were calculated from LC/ESI/MS analytical data (Supplemental Table S3) and are summarized in Fig. 5 and in Supplemental Table S4. The most notable changes concerned the length of FA chains. Because 16C and 18C FAs prevail in membrane lipids of most insects, average chain length is often estimated based on the ratio of 16C/18C. The ratios short/long and the 16C/18C both indicated that FA chains tended to be shorter in treatments exposed to low temperature (CLT and Fb) compared to control at 20 °C (CO) (ANOVA,  $P < 0.05$ ) (Fig. 5). After 2 h of recovery at 20 °C (i.e. treatments Fa), the FA chains tended to be of intermediate length between cold and control, suggesting a fast return towards the initial condition during the 2 h recovery at 20 °C. Concerning the indices of FAs desaturation (unsaturation index, UFA/SFA, PUFA/MUFA, Supplemental Table S4),

we did not detect any consistent change according to treatments, except a small variation on the second day due to CO flies only (Fig. 5).

Global changes of lipidotype according to treatments clearly separated the groups into different clusters corresponding to the different treatments (Fig. 6A & B). PC1 and PC2 accounted for 35.2 and 20.7% inertia. PC3 was also considered here as it still accounted for 15.9% of total inertia. Together, these three components cumulated 71.8% of the total inertia (Fig. 6C). The Monte-Carlo randomizations confirmed the significance of the differences among classes ( $P < 0.001$ ). The projection of the scores on PC1, PC2 and PC3 are illustrated in Fig. 6D-F. Together, these multivariate analyzes revealed patterns that are strikingly consistent with those observed with the metabolic profiling. Explicitly, constant cold treatments (CLTs) were all negatively associated with PC1, while treatments exposed to 20 °C, either constantly (CO1 and CO2) or transiently (Fa1 to 3) were positively associated with PC1. This means that lipidomic profiles strongly deviated from their initial state in CLT flies before tending towards control state after 2 h recovery (Fig. 6A,D). PC2 was positively associated with a cluster that comprised



**Fig. 5.** Indices of PLs remodeling calculated from LC/ESI/MS analytical data (Table S3). PLs molecular species composition was assessed in *D. melanogaster* adults sampled for three consecutive days either under constant temperature (cold at 5 °C: CLT or control 20 °C: CO) or under fluctuating thermal regime (before 2 h recover: Fb or after 2 h recovery: Fa). The ratio short/long is the cumulative percent of short fatty acids ( $\leq 16C$ ) divided by the cumulative percent of long fatty acids ( $> 16C$ ). The ratio 16C/18C is cumulative percent of all 16C fatty acids divided by the cumulative percent of all 18C fatty acids. The unsaturation index (UI) is the sum of the percent unsaturated FAs multiplied by their number of double bonds. The unsaturation ratio (UFA/SFA) is the cumulative percent of all unsaturated fatty acids (UFA) divided by the cumulative percent of all saturated fatty acids (SFA) and the polyunsaturation ratio (PUFA/MUFA) is the cumulative percent of poly-unsaturated fatty acids (PUFA) divided by the cumulative percent of mono-unsaturated fatty acids (MUFA). Note that the condition CO3 was discarded (see Section 3.1 for details). Each bar is a mean ( $\pm$  SE) of six biological replications. Data were analyzed within each sampling duration using one-way ANOVA followed by Tukey's multiple comparison tests (means flanked by different letters are significantly different).



**Fig. 6.** Phospholipids profiling based on LC/ESI/MS analyzes of PLs in the polar fraction of lipids extracted from whole bodies of *D. melanogaster*. Individuals were sampled for three consecutive days at constant (CO and CLT) or fluctuating temperature (Fb or Fa) (refer to Fig. 1 for experimental design and codes). The between-class PCA shows PC1 against PC2 (A) and PC1 against PC3 (B). Percent of inertia for each principal component is shown in (C). PC1, PC2 and PC3 cumulate 71.95% inertia. Mean scores ( $\pm$ SE) (i.e. projection of centroids) on PC1 (D), PC2 (E) and PC3 (F) according to treatments are show for the three sampling days. These multivariate analyzes show that PC1 explains a clear-cut opposition between cold treatments (CLT and Fb) and all treatments at 20 °C (Fa or CO). PC2 is associated with a cluster of the three Fa treatments opposed to the other treatments. PC3 shows a temporal pattern where all treatments from day 1 (and CO2) are opposed to the other treatments from day2 and 3. Note that the condition CO3 was discarded (see Section 3.1 for details). See Supplemental Fig. S4(A,B,C) for correlations of PLs to the three PCA axes.

all fluctuating treatments (mostly Fa treatments) (Fig. 6A,E), and PC3 explained a temporal pattern where all treatments from day1 (as well as CO2) were opposed to the other treatments from day2 and 3 (Fig. 6B,F).

The variables (PLs) contributing the most to the structure separation along PC1, PC2 and PC3 were ranked according to their correlation value and are presented in Supplemental Fig. S4 A,B,C. From these, we found that short PEs (e.g. PE<sub>16:1/14:0</sub>, PE<sub>14:0/18:1</sub>, PE<sub>16:1/16:0</sub>) and LPEs (e.g. LPE<sub>16:0</sub>, LPE<sub>14:0</sub>, LPE<sub>18:0</sub>) were strongly negatively correlated to PC1 (i.e. relatively more abundant in CLTs), while PGs (e.g. PG<sub>18:2/18:1</sub>, PG<sub>18:3/18:1</sub>, PG<sub>16:0/18:2</sub>) and PCs (PC<sub>14:0/12:0</sub>, PC<sub>18:2/18:1</sub>, PC<sub>18:0/18:1</sub>) were strongly positively correlated to PC1 (i.e. relatively more abundant in COs and FAs) (Supplemental Fig. S4 A). The PLs that were the most positively correlated to PC2 included some PIs (e.g. PI<sub>18:0/18:1</sub>, PI<sub>16:0/16:1</sub>, PI<sub>18:1/18:1</sub>) and PCs (e.g. PC<sub>16:0/16:1</sub>, PC<sub>16:0/14:0</sub>, PC<sub>16:1/12:0</sub>). These were relatively more abundant in Fa flies, so after 2 h recovery (Supplemental Fig. S4B). Finally, the PLs that were the most positively correlated to PC3 (i.e. more abundant at the start of experiment on day1) comprised exclusively Lysp-PLs (e.g. LPG<sub>16:1</sub>, LPC<sub>14:0</sub>, LPC<sub>16:1</sub>, LPE<sub>18:1</sub>), while those that were negatively correlated (i.e. less abundant at the start of experiment) comprised various PLs such as PS<sub>18/2:18:2</sub>, PC<sub>16/0:16:0</sub>, and PE<sub>14/1:12:0</sub> (Supplemental Fig. S4C).

#### 4. Discussion

In accordance with the literature on other insects, we confirmed that applying FTRs to *D. melanogaster* adults significantly reduces cold-induced mortality. Our findings provide additional support for the general occurrence of FTR-linked improvement of cold tolerance across insect taxa [2]. The promoting effect of FTR was particularly manifested in females and at the least stressful temperatures (4 and 5 °C). It is conceivable that at the lower temperatures (2 and 3 °C), physiological damages had reached levels that cannot be fully counterbalanced within 2 h of daily recovery. We further assessed whether such a shift in cold tolerance phenotype is associated with detectable physiological changes. Repairing mechanisms under FTRs are under investigation but are

still rather hypothetical. However, it appears clear that benefits of FTRs are somewhat related the re-establishment of overall ion homeostasis and electrochemical potentials across specific membranes during warm spells [30]. That is because chilling may induce separation of phospholipids bilayer and alter the permeability, and may also reduce the activity of membrane-bound enzymes, including the primary ion-pumping systems such as Na<sup>+</sup>/K<sup>+</sup>-ATPase [14,15,24]. As a result, the ion homeostasis is disturbed at cold and the electrochemical membrane potentials partially or completely dissipate which leads to rapid development of chill injury and mortality [17–20,24]. The positive effect of warm periods under FTR is likely realized via supporting the active re-establishment of ion homeostasis by allowing the functionality of ATP-generating pathways and ion pumping ATPases, as indicated in *Pyrhocoris apterus* and *Alphitobius diaperinus* [30]. Theoretically, membrane phospholipid composition could be adaptively remodeled during warm periods in a direction that would counterbalance the negative effects of cold on membrane functions as mentioned above. Moreover, the cryoprotectants could mitigate the alterations in biological membranes by stabilizing their structures [26–29]. Consequently, we expected some changes in membrane phospholipid composition and/or in the concentrations of compounds with cryoprotective functions (polyols, sugars, amino acids) under FTRs. We did observe increasing levels in several polyhydric alcohols (arabitol, erythritol, sorbitol, mannitol, glycerol), sugars (Fru, Man) and amino acids (Ser, Ala, Glu). However, these changes were manifested in CLT treatments not in FTR treatments. Prolonged chilling may be required to trigger these accumulations and periodic warmings may offset this response. Glycerol and many other polyols (e.g. ribitol, sorbitol and *myo*-inositol) have often been associated with cold-tolerance e.g. [23,27,42,43]. Similarly, prolonged exposure to moderately low temperature (4 °C) elevated levels of several polyols (glycerol, sorbitol) and amino acids (Ala, Glu) in *Sarcophaga crassipalpis* [44]. In beetle *A. diaperinus* exposed to cycling regimes (12 h cold/12 h warm), changes in the concentration of cryoprotectants (glycerol and glucose) were observed only after the end of cold period and turned back to control value after the warm period [45]. The only metabolites that showed higher level at the end of the warm recovery were succinate, Tyr and Pro. Relative increased in Pro could be of interest as



this free amino acid is a particularly potent cryoprotectant [46,47]. However, we consider it premature to speculate that Pro has causally contributed to cold tolerance of FTR flies as the concentrations and the magnitude of the fold-change accumulations remained low (1.5 fold). Because metabolite levels result from flux of many metabolic pathways and processes, it is difficult to depict whether the observed changes represent a beneficial (protective) or detrimental (dysregulation) signal; it may be a combination of both depending on the metabolite considered. In spite of this, metabolic profiling can be useful to monitor temporal changes of overall homeostasis under specific treatments [32–34]. There is evidence for strong deviation of metabolic profiles when chill-susceptible insects are exposed to chilling [33,34,48,49]. It is not yet clear whether this marked deviation reflects only a degenerative syndrome, resulting for instance from uncoupling among various metabolic pathways at cold [44], or whether it partially entails a compensatory protective response, such as synthesis cryoprotectants. However, when insects are either adapted (genetically) or acclimated (plastically) to low temperature, they clearly possess the ability to hamper this cold-induced homeostatic deviation [33,34,49], suggesting that robustness in metabolic networks are key element of cold tolerance. No study so far has investigated the temporal maintenance/deviation of metabolic networks over the course of FTRs. We observed that metabolic trajectories deviated markedly from the control state during cold exposure, suggesting that flies under CLT were in a physiological state distinctively divergent and manifestly unfavorable compared to counterparts. Most importantly, we confirmed that warm intervals allowed a fast homeostatic regeneration towards initial state. Recovery as short as 2 h was sufficient to periodically return the metabolic system to functionality (as suggested by longer survival under FTR). We argue that periodic opportunities to restore metabolic networks contributes to cold tolerance of flies under FTRs.

Number of previous studies have investigated PLs composition of *D. melanogaster* in response to thermal acclimation [50–52] or thermal adaptation [53–56]; however, so far no study has analyzed temporal remodeling of PLs during rapid temperature shifts under FTRs. In addition, these earlier studies have generally analyzed the composition of FAs and/or head groups separately and in limited number of molecular species (often <20 species). Here we provide structural information of a large set of PLs (101 different molecular species), including the information on PLs head groups and FA chains. This large dataset coupled with time-series measurements allowed us to assess whether a rapid restructuring occur under FTRs in a direction that would support theory of homeoviscous adaptation [14,25] and/or protection of membrane integrity at low temperatures [15]. Concerning head groups, we repeatedly found a relative increase in PEs in all the CLT treatments, and a corresponding decrease in PGs and PCs. The biosynthesis of PEs includes several pathways in which free ethanolamine (ETA) and the amino acid Ser are key precursors [57]. Interestingly, Ser was among the most influential metabolite associated with PC1 (Supplemental Fig. S3 A). Both Ser and ETA were more abundant in CLT flies (Supplemental Fig. S2) which may relate to the PE accumulation in CLT flies. The relative proportion of PEs (ratio PE/PC) was significantly higher in CLT flies than in the other groups. Ectotherms can compensate for temperature-induced changes in the viscosity of membrane lipids by increasing the proportion of PE head groups at the expense of PC head groups [14,15]. The ethanolamine moiety occupies a smaller area than the choline. PEs have a conical conformation, while PCs are more cylindrical. It results that PEs pack much less efficiently into the lipid bilayer [58]. The increased relative proportion of PEs at cold can be an attempt to compensate for the ordering effects of low temperatures and prevent unregulated transition of membrane bilayer into the non-functional gel phase. In insects, relative increase in PEs occurs during gradual cold acclimation [59,60], but also over shorter periods, as observed in response to rapid cold hardening (RCH) [61] but [54]. The relative proportion LPEs also increased at cold (i.e. CLTs), but this was only observed on the first day. Lyso-PLs were found in membranes in relatively low quantities

(around 1%) which is consistent with earlier reports [39]. Lyso-PLs have the shape of an inverted cone which disrupts tight packing into membranes and decreases the order, and hence increases the fluidity. Increased relative proportion of LPEs at cold is thus consistent with the physical properties of the prevailing thermal conditions. The specific role of minor membrane phospholipid classes in shaping thermal responses are still poorly known in insects and only start to be unraveled [39].

We also noted that the increase in relative proportion of PEs at cold occurred at the expense of some PGs. Phosphatidylglycerols are mostly cylindrical and bilayer-forming PLs [62]. The concurrent decrease of tubular-conformed PGs and increase in levels of conical-conformed PEs may also contribute to counteract the ordering effect at cold. Interestingly, members of PGs are considered as high melting point PLs and are associated with chilling susceptibility in plants [63]. Phosphatidylglycerols-derived lipids are mitochondrial cardiolipin (CL) precursors; therefore, PGs have a crucial role in cell physiology, including in stress response because CL synthesis is highly regulated and modulated under stress situations [62]. The level of PGs parallels the rate of oxygen consumption and oxidative metabolism [41]. Therefore, decreased relative level of PGs at cold could also result from reduced aerobic metabolism, which is consistent with the reduced level of succinate at cold (Supplemental Figs. S2, S3 A).

Some molecular species of PLs were specifically more abundant in recovering Fa flies (highly correlated to PC2 in Supplemental Fig. S4B). The synthesis of PLs takes place by the condensation of CDP-diacylglycerol with inositol [63,64]. PI synthase activity is tightly regulated by inositol level [63]. Interestingly, all polyhydric alcohols detected in our metabolic profiles were at higher level in CLT flies, with the exception of inositol which was more abundant in Fa flies (see Supplemental Figs. S2, S3 A) and may have served as precursor of PIs synthesis. Laboratory acclimation experiments have shown that PIs are much less dependent on environmental temperature than the other PLs, which suggests that PIs have different roles from those of PEs and PCs in membrane function and temperature adaptation [65]. Indeed, PIs and phosphorylated derivatives (phosphoinositides) play a central role in cell signaling and membrane trafficking in eukaryotes [63]. At this stage it is premature to suggest that these functions are implicated in the recovery mechanisms occurring during warm intervals but it may be an area to explore, especially since members of phosphoinositide signaling pathway are well-known to mediate stress responses in plants and yeasts [64,66].

We did not detect any consistent changes related to FA desaturation according to treatments. This is consistent with earlier reports that failed to detect any correlation between FA unsaturation and cold exposure in drosophilids [50,51,53,54,59]. On the contrary, the ratios 16C/18C indicated that FA chains tended to be shorter in flies sampled from the cold (i.e. CLT and Fb) compared to flies exposed to continuous 20 °C (CO). After 2 h of recovery under FTR, the FA chains length tended to be intermediate, suggesting a partial remodeling towards the initial condition during the warm intervals. Owing to the greater area of hydrophobic interactions, the PLs containing long-chain FAs have a higher melting temperature than short-chain FAs [67]. The modulation of FA chain length may be a strategy for mitigating membrane's ordering and increased viscosity at cold. FA shortening is commonly found in insects exposed to cold [16]. In some species, FA desaturation did not markedly vary with cold treatment but the 16C/18C ratio increases [68,69]. In fact, FA shortening and FA unsaturation can be alternatively used to achieve a similar function [16]. Previous reports have shown that lipid composition is adjustable within very short periods, as little as a few hours for instance in insects exposed to RCH [50,61] or in intertidal molluscs that deal with ample daily thermal variations [70,71]. Our global lipidomic profiles show that rapid changes in PLs occur under FTRs. The lipidotypes, which integrate all detected PLs, significantly differed according to treatments: they strongly deviated from their initial state in all CLT treatments. Also, the global lipid composition was biased towards the lower temperature of the cycle (5 °C) before the

recovery and towards the higher temperature of the cycle (20 °C) by the end of recurrent recovery, which means that a fast homeostatic regeneration occurs during warm intervals. The variations in membrane lipid composition reported in this study, being manifest within only a 2 h period, are among the fastest recognized adjustments to environmental change.

It is remarkable that lipidomic trajectories completely mirrored metabolic trajectories. This implies that warm intervals involve a system-wide homeostatic regeneration process, of which membrane remodeling is likely just a facet. Relatively rapid changes (e.g. diurnal fluctuations) in lipid composition have also been reported in plants and fishes and these data suggest that alterations in lipid composition and membrane viscosity do not occur passively in response to changes in external temperature [72–74]. Membrane viscosity is not just a direct reflection of external temperature but is subject to active homeostatic regulation, even when normal mechanisms are impaired [75]. The patterns of change in membrane lipid composition noted here are in accordance with thermal compensation of membrane function. Rapid adjustments in the lipid composition under FTRs may reset the optimal functioning membranes and that of associated proteins (e.g. ion pumping system). We argue that this likely participates in mitigating the accumulation chilling damages. Cell membrane is a primary site of chilling injury [76] and persisting exposure to low temperature can result in leakage of ions and other solutes across cell membranes exposed to cold [17–20]. The complemented restoration of metabolic and PLs homeostasis under FTRs must definitively contribute to offset these deleterious cumulative effects at continuous low temperature.

#### 4.1. Conclusion

In this study, we have shown that FTRs promoted cold tolerance of *D. melanogaster* flies. We could not correlate this phenotypic change with accumulation of cryoprotectants under FTRs. However, we found that prolonged exposure to low temperature was associated with a marked deviation of metabolic homeostasis and that recovery as short as 2 h was sufficient to periodically return the metabolic system to functionality. Lipidomics revealed that relative proportion of PEs increased and FAs chains tended to be shorter in flies at cold, likely to compensate for the ordering effect of low temperature. A striking observation was remarkable correspondence in the time-course of changes between metabolic network and PLs profiles, both suggesting a fast homeostatic regeneration during warm intervals. Therefore, we conclude that this rapid restoration process likely contributes to offset the accumulation of chilling injuries. Our multi-Omics approach revealed a set of metabolites and PLs putatively linked to recovery process under FTRs. These represent good candidates for further targeted studies. Finally, the adjustments of certain classes of PLs over temperature shifts were connected with corresponding changes in the levels of some metabolites known as biosynthetic precursors. Using combinations of techniques such as stable isotope tracer studies will permit to causatively associate these changes. Pharmacological inhibition of membrane restructuring [77] could also be useful approach to depict the precise role of membrane properties on cold survival under constant and fluctuating thermal regimes.

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#### Transparency document

The Transparency document associated with this article can be found, in online version.

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