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RESEARCH ARTICLE | *Obesity, Diabetes and Energy Homeostasis*

Cold acclimation triggers lipidomic and metabolic adjustments in the spotted wing drosophila *Drosophila suzukii* (Matsumara)

Thomas Enriquez and Hervé Colinet

Université Rennes 1, Centre National de la Recherche Scientifique, Rennes, France

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Enriquez T, Colinet H. Cold acclimation triggers lipidomic and metabolic adjustments in the spotted wing drosophila *Drosophila suzukii* (Matsumara). *Am J Physiol Regul Integr Comp Physiol* 316: R751–R763, 2019. First published April 3, 2019; doi:10.1152/ajpregu.00370.2018.—Chronic cold exposure is detrimental to chill susceptible insects that may accumulate chill injuries. To cope with deleterious effects of cold temperature, insects employ a variety of physiological strategies and metabolic adjustments, such as production of cryoprotectants, or remodeling of cellular membranes. Cold tolerance is a key element determining the fundamental niche of species. Because *Drosophila suzukii* is an invasive fruit pest, originating from East Asia, knowledge about its thermal biology is urgently needed. Physiological mechanisms underlying cold tolerance plasticity remain poorly understood in this species. Here, we explored metabolic and lipidomic modifications associated with the acquisition of cold tolerance in *D. suzukii* using Omics technologies (LC- and GC-MS/MS). In both cold-acclimated males and females, we observed physiological changes consistent with homeoviscous/homeophasic adaptation of membranes: reshuffling of phospholipid head groups and increasing unsaturation rate of fatty acids. Modification of fatty acids unsaturation were also observed in triacylglycerides, which would likely increase accessibility of lipid reserves. At the metabolic level, we observed clear-cut differentiation of metabolic profiles with cold-acclimated metabotypes showing accumulation of several potential cryoprotectants (sugars and amino acids). Metabolic pathway analyses indicated a remodeling of various processes, including purine metabolism and aminoacyl tRNA biosynthesis. These data provide a large-scale characterization of lipid rearrangements and metabolic pathway modifications in *D. suzukii* in response to cold acclimation and contribute to characterizing the strategies used by this species to modulate cold tolerance.

cold tolerance; homeoviscous adaptation; lipids; metabolic profiles; thermal plasticity

INTRODUCTION

Because of their viscous character, cellular membranes are thermosensitive structures (7). Extremely low temperatures can compromise their integrity by inducing the transition of membrane's phospholipid (PL) bilayer from a fluid, liquid-crystalline phase to a more rigid, lamellar-gel phase (53). These changes in membrane fluidity mechanically alter activity and function of membrane-bound enzyme (18, 41, 53), which may contribute to symptomatic loss of hydric and ionic homeostasis across membrane's bilayers at cold temperatures (59, 60, 69, 73). Consequences might include neuromuscular dysfunctions, chill coma, and, in the most extreme cases, death (44, 63, 72, 108). This is especially true for poikilothermic organisms, such as insects, as their body temperatures directly depend on their surrounding environment, and stressful thermal events, such as cold shock, can severely impair their physiological functioning (23).

Ectotherms can survive stressful low temperatures using physiological plasticity, such as cold acclimation (i.e., acquired cold tolerance subsequent to pre-exposure to cold temperatures). One of the most conserved physiological responses of ectotherms to low temperature is the preservation of membrane fluidity, referred to as homeoviscous adaptation of cell membranes (41, 92) or avoidance of membrane phase transition, referred to as homeophasic adaptation (42). These adjustments rely on remodeling the membrane's PL, for instance, by restructuring polar head groups, shortening fatty acid (FA) chains, or increasing FA unsaturation (53). Such modifications help maintain membrane's fluidity and functioning under cold temperature, by inducing modification of van der Waals forces within membrane PL bilayer (16, 64). Similar modifications occur in stored lipids, such as in triacylglycerides (TAG), in response to cold (57, 77, 102). Because TAG represents the major energy reserve in insects it plays an important role in overwintering and cold survival (36, 77, 91).

Cold acclimation is often correlated with recruitment of low molecular mass compounds (29, 56, 71, 103). These compatible osmolytes (traditionally sugars, polyols, and amino acids) are well known for their colligative effects at high concentrations (95, 109), but they may also play roles at low concentrations, for instance by stabilizing structures and integrity of proteins and membrane bilayers (5, 8, 20, 107). Cold acclimation may also induce large-scale changes in many enzyme activities, and therefore, metabolic pathways are putatively remodeled (71), such as pathways of central metabolism (12, 65, 66, 90).

The spotted wing drosophila, *Drosophila suzukii*, is an alien species in Europe (6) and North and South America (39, 62). Contrary to the common species *Drosophila melanogaster*, which lays its eggs in ripening fruits, *D. suzukii* females oviposit exclusively under the skin of mature fruits, which their larvae then consume. This fly is highly polyphagous and can exploit a large number of wild hosts (50), but it also feeds on a wide variety of cultivated fruit crops, including stone fruits, causing extensive loss of harvest and economic costs (32).

D. suzukii overwinters as adult in Northern America (99) and Europe (84). According to the definition of cold tolerance strategies (79), this fly is considered as chill susceptible.

Address for reprint requests and other correspondence: H. Colinet, Université de Rennes1, CNRS, ECOBIO-UMR 6553, 263 Avenue du Général Leclerc, 35042 Rennes, France (e-mail: herve.colinet@univ-rennes1.fr).

Indeed, it does not tolerate mild chilling (21, 26, 51, 88) and dies at temperatures well above its supercooling point (45). The overwintering success of *D. suzukii* likely relies on two main strategies: *1*) migration to favorable and buffered microhabitats, such as forests; indeed, underneath the forest mulch temperatures remain positive in winter (89, 111); and *2*) acquisition of cold tolerance via plastic responses (90). Several studies have shown that this fly is able to enhance its cold tolerance thanks to various cold acclimation types, such as rapid cold hardening (28, 101), adult gradual acclimation (45, 104), or developmental acclimation (90, 101, 105). Recently, cold-acclimated *D. suzukii* flies have been shown to survive temperatures as cold as −7.5°C (94). Using RNA sequencing, Shearer et al. (90) showed that a winter-acclimated phenotype of *D. suzukii* exhibits an upregulation of genes involved with ion transport, carbohydrate metabolism, and glycolysis. In a previously targeted time-series analysis (27), we investigated metabolic adjustments during cold acclimation in *D. suzukii* and showed that acclimation was associated with metabolic robustness during cold stress and recovery period. Despite recent progress in understanding the thermal biology of *D. suzukii*, physiological strategies underlying its cold tolerance plasticity remain poorly understood. Consequently, the aim of the present study was to investigate the impact of cold acclimation on physiological traits of importance regarding insect cold tolerance: composition of the membrane, lipid reserves, and metabolic responses. In this study, we combined developmental and adult cold acclimation to produce a cold-tolerant phenotype and then used lipidomic and metabolomic holistic approaches to explore physiological correlations associated with cold acclimation response in *D. suzukii*. We hypothesized that cold acclimation would increase cold tolerance in *D. suzukii* and predicted that cold acclimation will induce major physiological adjustments, such as PL changes consistent with homeoviscous or homeophasic adaptations (41), remodeling of TAG's carbon chains to increase their accessibility (87, 102), and metabolic pathway reorganizations to allow energy homeostasis and support cryoprotectants synthesis (e.g. sugars and amino acids) (11, 27, 56, 58, 71, 106).

MATERIALS AND METHODS

Flies Rearing and Acclimation Protocol

The *D. suzukii* line used in our experiments originated from wild flies collected from infested blueberries and raspberries in Thorigné Fouillard, France (48.0616N, −1.2387E) in September 2016. Flies were maintained under laboratory conditions for approximatively 20 generations before experiments. Flies were reared in glass bottles (100 ml) and supplied with a carrot-based food (in 1 liter of water: 15 g agar, 50 g sucrose, 50 g carrot powder, 30 g brewer yeast, 20 g cornmeal, 8 g kalmus, 8 ml Nipagin). Control flies were produced by placing bottles containing eggs in an incubator (model no. MIR-154-PE; Panasonic, Healthcare, Gunma, Japan) at 25°C, and a 12-h light/12-h dark cycle. After emergence, adults were maintained at 25°C for 7 days. To generate cold-acclimated flies, control adult flies (~50) were allowed to lay eggs in bottles for 24 h at 25°C. Then bottles with eggs were placed at 10°C (10-h light/14-h dark, same reference of incubator) to allow hatchling to develop into adults. After emergence, adults were kept at 10°C for 7 days. Consequently, flies were cold-acclimated during both development and at adult stage. Seven-day-old control or cold-acclimated flies were randomly taken from the rearing stock and used for subsequent experiments. All experiments were conducted on both females and males. Males were visually separated from females with an aspirator without CO_2 to avoid anesthesia stress (13).

Acute Stress

From each treatment group, flies were randomly taken and distributed in 10 replicates of 10 males or 10 females and subjected to −5°C for 100 min, using glass vials immersed in a glycol solution cooled by a cryostat (model Cryostat Lauda ECO RE 630; Lauda, Lauda-Königshofen, Germany). After cold exposure, flies were allowed to recover in 40-ml food vials maintained at 25°C (12-h light/13-h dark). Survival was assessed by counting the number of dead and living individuals in each vial at 4, 24, and 48 h after cold stress.

Critical Thermal Minimum

We used a glass knockdown column to estimate the critical thermal minimum (Ct_{min}) of *D. suzukii*. The column was a vertical, jacketed glass column (52 × 4.7 cm) containing several cleats to help flies not fall out the column while still awake. The column was linked to a cryostat (Cryostat Lauda ECO RE 630) to regulate internal temperature at 18°C. Temperature inside the column was continuously checked using a thermocouple K, placed at mid height in the center of the column, connected to a Tempscan C8600 scanning thermometer (Comark Instruments, Norwich, Norfolk, UK). Approximatively 60 flies of each treatments were introduced to the top of the column. Flies were allowed to equilibrate in the device for a few minutes, and then the temperature was decreased to −5°C at 0.5°C/min. At each fly passing out and falling out of the column the Ct_{min} (°C) was recorded.

Chill Coma Recovery Time

Chill coma recovery time (CCRT) is defined as the resurgence time of motor activity after a cold knockdown (22). To induce a chill coma, we subjected 40 control and 40 acclimated flies of both sexes to 0°C for 12 h, using food vials placed in a cooled incubator (model no. MIR-154-PE; Panasonic, Healthcare). Immediately after exposure to cold stress, flies were rapidly transferred into a 25°C regulated room, and we scattered them individually on a large plane surface. As each fly was able to stand up, the recovery time was recorded. This experiment ended after 120 min, and nonrecovered flies were then counted.

Omics Analyses

For each treatment and sex, five independent replicates (each consisting of a pool of ~40 seven-day-old flies) were snap-frozen in liquid N_2. Frozen samples were then sent to MetaSysX (Potsdam-Golm, Germany) where a coupled lipidomic and metabolomic nontargeted analysis was performed. Extraction of samples was performed according to MetaSysX standard procedure, a protocol modified from Giavalisco et al. (31).

LC-MS measurements, data processing, and annotation (hydrophilic and lipophilic analytes). The samples were measured with a Waters ACQUITY reversed phase ultraperformance liquid chromatography (Waters, Milford, MA) coupled to a Q Exactive mass spectrometer, which consists of an electrospray ionization source and an Orbitrap mass analyzer (Thermo Fisher Scientific, Bremen, Germany). C8 and C18 columns were used for the chromatographic separation of lipophilic and hydrophilic compounds, respectively. The mass spectra were acquired in full-scan MS positive and negative modes (mass range 100−1,500).

Extraction of the LC-MS data was accomplished with the software REFINER MS 10.5 (GeneData, https://www.genedata.com). Alignment and filtration of the LC-MS data were completed using in-house metaSysX software. After extraction of the peak list from the chromatograms, the data were processed, aligned, and filtered. Only features which were present in at least four out of the five replicates

of at least one of the sample groups were selected. At this stage in the process, an average retention time and an average mass-to-charge ratio values were given to the features. The alignments were performed for each platform independently (polar phase positive mode, polar phase negative mode, lipophilic phase positive mode, and lipophilic phase negative mode).

Data alignment was followed by the application of various filters to refine the data set, among them removal of isotopic peaks, removal of in-source fragments of the analytes (due to the ionization method), and removal of additional lower intense adducts of the same analyte to guarantee the quality of the data for further statistical analyses. The annotation of the sample content was accomplished by matching extracted data from chromatograms with metaSyX library of reference compounds and with metaSyX database of lipids in terms of accurate mass and retention time.

MS/MS lipid annotation. Chromatograms were recorded in the dd-MS2 Top-3 mode (data-dependent tandem mass spectrometry) with the following settings: full scan MS mode (mass range 100−1,500), NCE 25 (normalized collision energy). Acyl composition of di- and triacylglycerids was established from the $[M+H]^+$ precursor ion fragmentation with detection of $[Acyl+NH4]^+$ neutral losses in positive ion mode with further combinatorial calculation of the acyl composition. Acyl composition of phosphoglycerolipids was determined from the detection of $[Acyl\text{-}H]^-$ fragments of the corresponding precursors in the negative ion mode. Acyl composition of sphingolipids was established from the fragmentation pattern of $[M+H]^+$ precursor ions in the positive ionization mode.

GC-MS measurements data processing and annotation. The samples were measured on an Agilent Technologies gas chromatograph coupled to a Leco Pegasus HT mass spectrometer, which consists of an electron-impact ionization source and a time of fly mass analyzer (Leco, St. Joseph, MI). Column: 30 meter DB35; starting temperature: 85°C for 2 min; and gradient: 15°C per min up to 360°C. NetCDF files exported from the Leco Pegasus software were imported to "R" (R 3.4.3, R Core Team, 2016). The bioconductor package "TargetSearch" was used to transform retention time to retention index, to align the chromatograms, to extract peaks, and to annotate them by comparing the spectra and the retention index to the Fiehn Library and to a user created library. Annotation of peaks was manually confirmed in *Leco Pegasus*. Analytes were quantified using a unique mass.

Lipidomic and metabolomic data processing. Data from the five platforms were normalized to sample fresh weight and to the median intensity of 1,000 features per replicate group (including all the annotated features and highly abundant features with unknown name). Subsequently, the normalized data of annotated features from all five platforms were merged to the final data matrix.

Statistical Analyses

Cold tolerance. Analyses of acute cold survival and Ct_{min} were performed using R 3.4.3. We modeled acute cold survival by specifying a generalized linear mixed-effects model with a logistic link function for proportional outcomes (i.e., number of dead/alive per vial). The response variable was dependent on treatment type (cold acclimation vs. control), sex, time to survival measurement, time × treatment and time × sex interactions. As survival rate was repeatedly measured after 4, 24, and 48 h post-cold stress per vial, vial number was included in a generalized linear mixed-effects model as a random effect to account for repeated measurements. We analyzed the effects of variables with an ANOVA function using the "car" package (30). Ct_{min} values were compared using two-way ANOVA with treatment and sex as factors. CCRT data were analyzed using survival analyses with the software GraphPad Prism5. We made pairwise comparisons between each of the recovery curve using Gehan-Breslow-Wilcoxon tests. The α level of significance for survival analyses was adjusted with a Bonferroni correction ($\alpha = 0.008$).

Lipidomic profiling. Lipidomic data were divided into three data sets: PL, TAG, and other lipids that included ceramides, free lipids, and free FA. For PL and TAG data sets, between-class principal component analysis (PCA) (96) were run, using the "ade4" package in R (25). Monte Carlo tests were performed after each PCA to examine the significance of difference found among the classes (based on 1,000 simulations). Next, we independently calculated the following five indices for PL and TAG: *1*) ratio of unsaturation [UFA/SFA, i.e., cumulative percentage of all unsaturated FA (UFA) divided by the cumulative percentage of all saturated FA (SFA)]; *2*) ratio of polyunsaturation [PUFA/MUFA, i.e., cumulative percentage of polyunsaturated FA (PUFA) divided by the cumulative percentage of monounsaturated FA (MUFA)]; *3*) unsaturation index (UI):

$$\sum \frac{n * \left(\%\text{fatty acids with } n \text{ double bonds}\right)}{100};$$

4) cumulative percentage of all 16C FA divided by the cumulative percentage of all 18C FA (ratio 16C/18C); and *5*) cumulative percentage of short FA (≤16C) divided by the cumulative percentage of long FA (>16C) (ratio short/long). Lipids composition and the five calculated indices were analyzed in R, using two-way ANOVAs with sex, treatment (i.e., cold-acclimated vs. control) and their interaction as factors.

Metabolic Profiling

Metabolic compositions of flies were compared using between-class PCA in the "ade4" package in R (25). Monte Carlo tests were performed to examine the significance of the difference among the classes (based on 1,000 simulations). To identify the variables (i.e., metabolites) contributing the most to the PCA structure separation, the correlations to the principal components (PCs) were extracted and integrated into correlation circles.

In addition to multivariate analyses, we performed Student's *t*-tests on each identified metabolite to compare mean relative abundances (i.e., normalized area under the curve values, AUC) between cold-acclimated and control flies, independently for males and females. The list of all significantly affected metabolites ($P < 0.05$) (available in Supplemental Table S1) (https://figshare.com/articles/Supplementary_Tables_xls/7688354) was used to run Metabolite Set Enrichment Analysis (MSEA) in Metaboanalyst 4.0 (10), based on *D. melanogaster* reference metabolome. A hypergeometric test was used as over-representation analyses, and relative-betweenness centrality was used to analyze pathway topology. To confirm and ensure consistency of MSEA results, we also used another pipeline (Reactome: https://reactome.org/) to conduct metabolic pathways analysis.

RESULTS

Cold Tolerance

Figure 1*A* illustrates survival probability of control and cold-acclimated flies 4, 24, and 48 h after cold shock. Cold-acclimation promoted survival to −5°C in comparison with control conditions [$\chi^2 = 140.90$, degrees of freedom (df) = 1, $P < 0.001$, Fig. 1*A*], leading to 100% survival at 48 h after stress in cold-acclimated females. Globally, survival declines over time after cold stress ($\chi^2 = 41.67$, df = 1, $P < 0.001$, Fig. 1*A*), but this decline was more pronounced in control flies than in cold-acclimated flies (treatment × time interaction: $\chi^2 = 14.88$, df = 1, $P < 0.001$, Fig. 1*A*). There was no difference between male and female survival ($\chi^2 = 1.05$, df = 1, $P = 0.30$, Fig. 1*A*).

Figure 1*B* represent mean Ct_{min} values. Mean Ct_{min} was much lower in cold-acclimated flies than in control flies (−1.55 ± 0.17°C vs. 5.16 ± 0.05°C; $F_{(1,229)} = 966.14$, $P <$

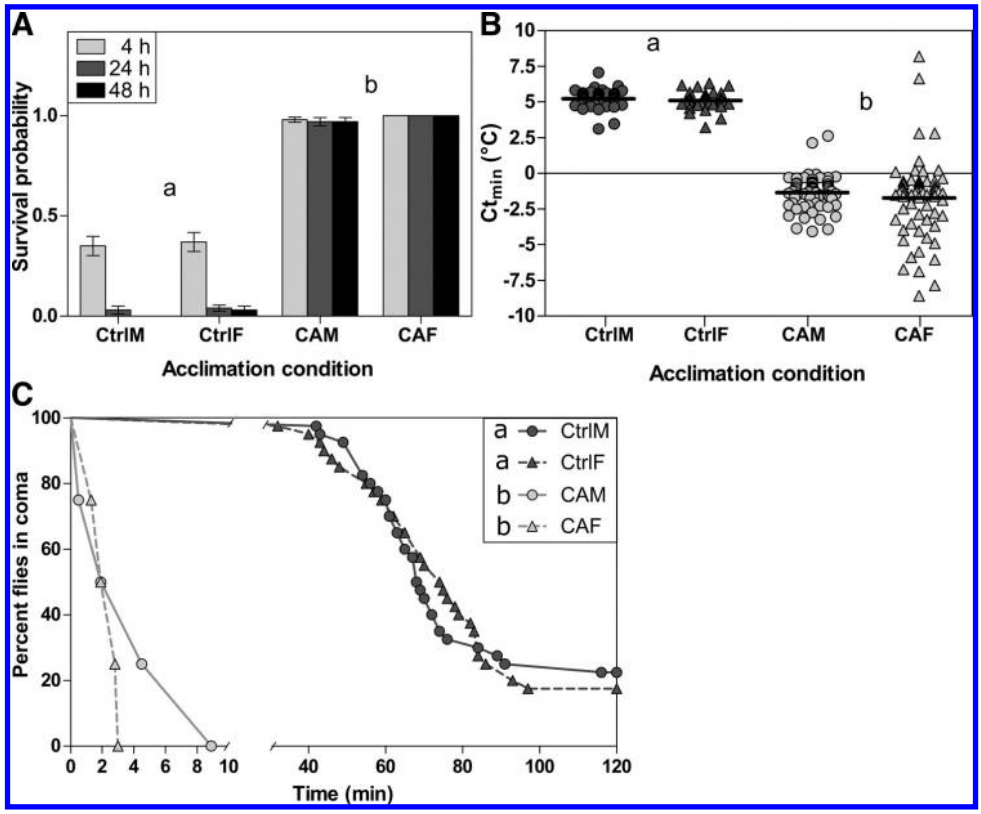

Fig. 1. Cold tolerance assays on control and cold-acclimated *Drosophila suzukii* males and females. *A*: acute cold stress survival after cold shock at −5°C for 100 min. Survival was measured 4, 24, and 48 h after cold shock (n = 10 replicates of 10 flies per sex and per condition). Bars represent survival probability means ± SE. Letters indicate differences between widespread effects of thermal treatments (GLMM Binomial, link Logit). *B*: critical thermal minimum (Ct_{min}). Temperature declined from 18°C to −5°C at −0.5°C/min and Ct_{min} have been recorded for each fly individually (n = 60 flies per sex and per condition). Each point represents an individual Ct_{min} and bars represent mean Ct_{min} in °C. Letters indicate differences between global effects of thermal treatments (two-way ANOVA). **C**: chill coma recovery time (CCRT). Flies were subjected to 0°C for 12 h, and then their individual CCRT was recorded at 25°C. Each point corresponds to the CCRT of one fly (n = 40 flies per sex and per condition). Letters indicate significant differences ($P < 0.008$, Gehan-Breslow-Wilcoxon Test). CtrlM, control males; CtrlF, control females; CAM, cold-acclimated males; CAF, cold-acclimated females.

0.001, Fig. 1*B*). Ct_{min} did not differ between males and females, nor did the interaction between treatment and sex [$F_{(1,229)} = 1.61$, $P = 0.20$; $F_{(1,229)} = 0.384$, $P = 0.53$, respectively].

CCRT curves are displayed in Fig. 1*C*. In control flies, chill coma recovery progressively started after 40 min; however, 20% of males and females were still in a coma after 120 min. Acclimated flies took only three and nine minutes to recover for females and males, respectively (Fig. 1*C*). As a result, there were significant differences between recovery curves for control and acclimated flies for both sexes (males: $\chi^2 = 64.00$, df = 1, $P < 0.008$; females: $\chi^2 = 64.00$, df = 1, $P < 0.008$). However, there were no differences in recovery times between males and females from both treatment groups (control group: $\chi^2 = 0.11$, df = 1, $P = 0.73$; cold-acclimated group: $\chi^2 = 0.18$, df = 1, $P = 0.66$).

Omics Analyses

Raw data from GC- and LC-MS/MS are available in the Supplementary Data set 1 (https://figshare.com/articles/Supplementary_data_set_1_-_GCMS-LCMS_Data_xls/7688351).

Lipidomic Profiles

Coupled GC- and LC-MS/MS analyses resulted in 313 annotated lipid features. After discarding features containing NA values, we retained 109 PL, 109 TAG, and 51 other features (diacylglycerols, ceramides, free FA, and free lipids). In subsequent analyses, we focused only on PL and TAG data sets. Figure 2, *A* and *B*, presents the results of the PCAs showing the ordination of classes within the first plane for PL and TAG, separately. In both cases, the PCA revealed four clearly distinct, nonoverlapping lipidotypes. Differences between control and cold-acclimated flies were mainly explained by the first axis of the PCAs (PC1 = 67.5% of total inertia for PL and 60.58% for TAG). Sexes differentiated along the second axis with 21.59% of total inertia for PL and 31.53% for TAG (PC1 + PC2 = 89.39% for PL and 92.11% for TAG). Correlation values with PC1, PC2, and PC3 for each lipidomic feature are available in Supplementary Table S2 for PL and Table S3 for TAG (https://figshare.com/articles/Supplementary_Tables_xls/7688354). Differences between lipidotypes were confirmed with Monte Carlo randomizations (observed $P < 0.001$ for both PL and TAG).

PL abbreviations of the various families are available in Supplemental Table S4 (https://figshare.com/articles/Supplementary_Tables_xls/7688354). Relative proportions of the PL families relative to thermal treatments and sexes are shown in Fig. 3, *A*-*G*. Relative proportions were calculated as the sum of AUC of each PL divided by the sum of AUC of all lipid features. We also calculated the ratio of PE/PC (phosphatidylethanolamine/phosphatidylcholine), which is displayed in Fig. 3*H*. Cold-acclimated flies were characterized by a higher relative proportion of lysoPC, lysoPE, and PG (Fig. 3 *A, B*, and *E*, $F_{(1,16)} = 6.91$, $P < 0.05$; $F_{(1,16)} = 7.78$, $P < 0.05$; $F_{(1,16)} = 24.99$, $P < 0.001$), whereas PC were more abundant in control flies (Fig. 3*C*, $F_{(1,16)} = 7.13$, $P < 0.05$). PS were relatively more abundant in cold-acclimated flies, but in females only (Fig. 3*G*, treatment × sex interaction: $F_{(1,16)} = 5.12$, $P < 0.05$). A similar difference was observed for PE in cold-acclimated females, while males followed the opposite trend (Fig. 3*D*, treatment × sex interaction: $F_{(1,16)} = 14.29$, $P < 0.01$). Consequently, this

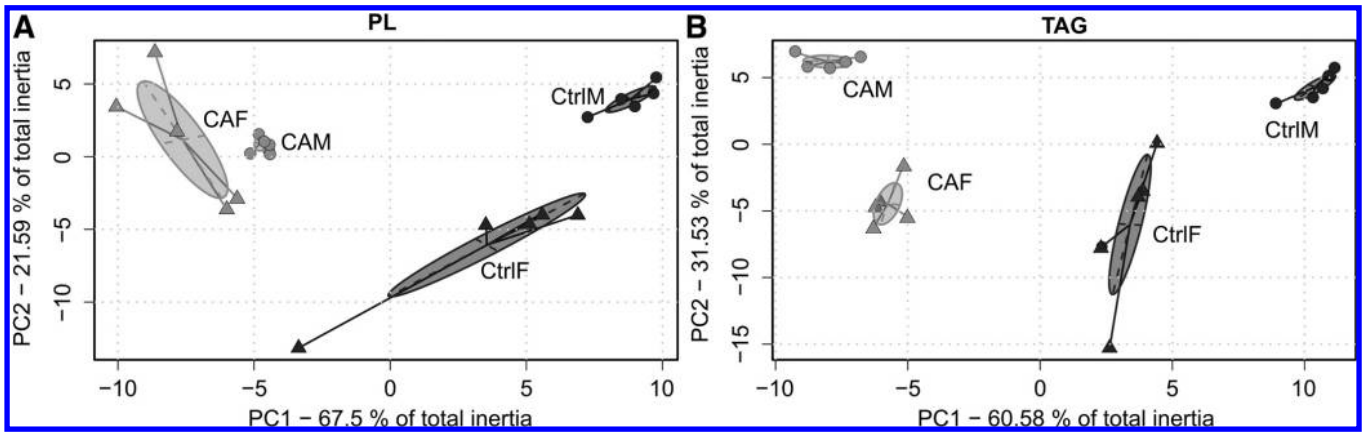

Fig. 2. Principal component analyses (PC1 vs. PC2) on *A*: the 109 phospholipids (PL) and *B*: the 109 triacylglycerides (TAG) identified in control and acclimated flies. Differences between lipidotypes were confirmed with Montecarlo randomizations (observed $P < 0.001$ for both PL and TAG). CtrlM, control males; CtrlF, control females; CAM, cold-acclimated males; CAF, cold-acclimated females.

pattern reverberated on a PE/PC ratio, which was higher in cold-acclimated females than in control females but lower in cold-acclimated males than in control males (Fig. 3*H*, treatment × sex interaction: $F_{(1,16)} = 8.18$, $P < 0.05$). Although we also compared the relative proportions of TAG between cold-acclimated and treated flies, we did not observe any differences (control females: 0.84 ± 0.005%; control males: 0.82 ± 0.002%; acclimated females: 0.82 ± 0.006%; acclimated males: 0.85 ± 0.002%; effect of thermal treatment: $F_{(1,16)} = 1.10$, $P = 0.31$; effect of sex: $F_{(1,16)} = 3.02$, $P = 0.10$).

FA Compositions of Carbon Chains

The metrics used to characterize FA compositions of PL and TAG are shown in Fig. 4. The list of abbreviations for these ratios is available in Supplemental Table S4 (https://figshare.com/articles/Supplementary_Tables_xls/7688354). Cold-acclimated flies showed a higher UFA/SFA ratio than control flies in both PL and TAG (Fig. 4*A*), $F_{(1,16)} = 143.20$, $P < 0.001$; $F_{(1,16)} = 2448.91$, $P < 0.001$, respectively). Likewise, cold-acclimated flies had a higher PUFA/MUFA ratio than controls in PL and TAG (Fig. 4*B*,

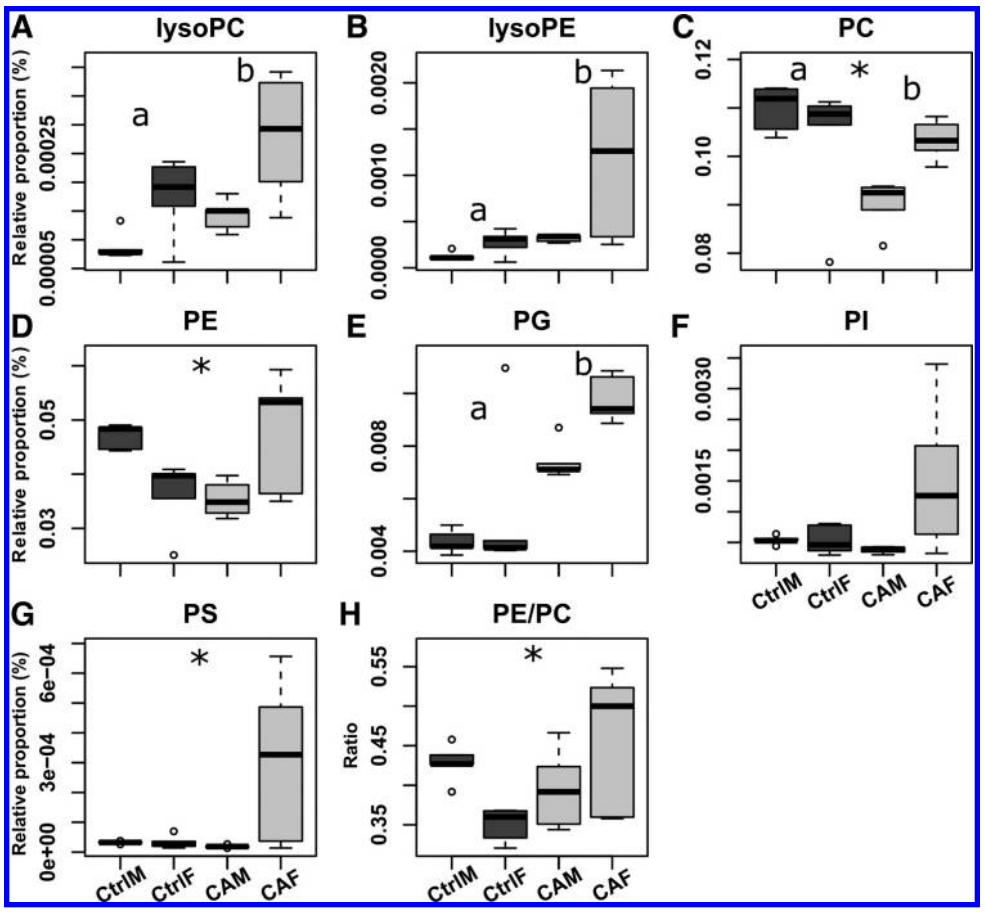

Fig. 3. *A*–*G*: relative proportions of the various families of phospholipids following the different thermal treatments. *H*: ratio of the relative proportion of PE on PC. [a,b]Differences between global effects of thermal treatments; *interactive effect between sexes and thermal treatments (two-way ANOVA); open circles, outliers. CtrlM, control males; CtrlF, control females; CAM, cold-acclimated males; CAF, cold-acclimated females; lysoPC, lyso phosphatidylcholine; lysoPE, lyso phosphatidylethanolamine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine.

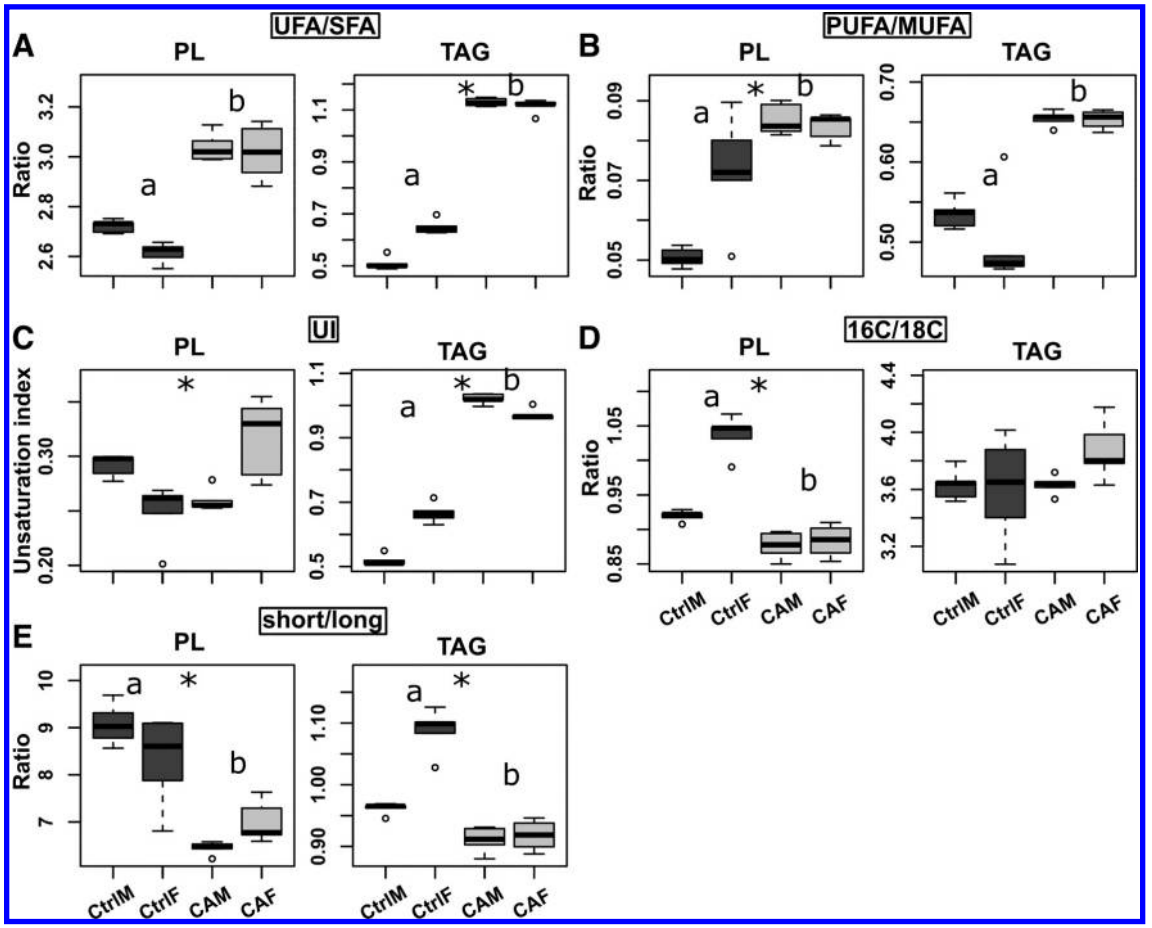

Fig. 4. *A–E*: ratios and unsaturation index calculated on fatty acid chains of phospholipids (PL) and triacylglycerides (TAG). Refer to MATERIALS AND METHODS for the calculation of the different ratios and indexes. [a,b]Differences between global effect of thermal treatments; *interactive effect between sexes and thermal treatments (two-way ANOVA); open circles, outliers; CtrlM, control males; CtrlF, control females; CAM, cold-acclimated males; CAF, cold-acclimated females; TAG, triacylglycerides; SFA, saturated fatty acids; UFA, unsaturated fatty acids; MUFA, mono-unsaturated fatty acids; PUFA, poly-unsaturated fatty acids; UI, unsaturation index; 16C or 18C, chains with 16 or 18 carbon atoms; short: FA carbons chains ≤ 16C; long: carbon chains > 16C.

$F_{(1,16)} = 89.51$, $P < 0.001$; $F_{(1,16)} = 43.48$, $P < 0.001$, respectively). UI was relatively higher in cold-acclimated flies than in control flies for TAG (Fig. 4*C*), $F_{(1,16)} = 1742.17$, $P < 0.001$), whereas for PL, it was higher only in cold-acclimated females than control females, which resulted in a significant treatment × sex interaction effect (Fig. 4*C*), $F_{(1,16)} = 21.63$, $P < 0.001$). Carbon chains lengths ratios were globally lower in cold-acclimated flies than in control flies, except for TAG, which showed no variation in 16C/18C ratio (16C/18C: Fig. 4*D*, PL: $F_{(1,16)} = 104.35$, $P < 0.001$; TAG: $F_{(1,16)} = 1.80$, $P = 0.20$; Short/long: PL: Fig. 4*E*, $F_{(1,16)} = = 97.95$, $P < 0.001$; TAG: $F_{(1,16)} = 56.86$, $P < 0.001$).

Metabolic Profiling

Coupled GC-MS/LC-MS analyses produced 312 metabolic features, among which 239 features were identified. From these 239 metabolites, 53 were discarded because they contained missing values. The relative concentrations of the 186 remaining metabolites, relative to treatments are available in Supplemental Fig. S1 (https://figshare.com/articles/Figure_S1_pdf/7688357).

Abbreviations and chemical classes to which these metabolites belong are displayed in Supplemental Table S5 (https://figshare.com/articles/Supplementary_Tables_xls/7688354). Metabolites were attributed to chemical classes based on the Human Metabolome Data Base (http://www.hmdb.ca/).

The PCA of metabolomic samples, relative to thermal treatments and sex is shown in Fig. 5*A*. The ordination of classes within the first plane of the PCA showed four distinct nonoverlapping metabotypes (i.e., the metabolic compositions resulting from each phenotype). Differences between cold-acclimated and control flies were mainly explained by PC1, which accounted for 58.64% of total inertia. Sexes were opposed along PC2 which accounted for 30.26% of total inertia (PC1 + PC2 = 88.9% of total inertia). Differences between metabotypes were confirmed with Monte Carlo randomizations (observed $P < 0.001$). Free amino acids, carbohydrates, and analogs of purine and pyrimidine were the main metabolite classes of the data set. To increase visibility, the projections of these variables into correlation circles are shown separately in Fig. 4, *B–D*. These three correlation circles resulted from the same PCA. In addition, the list of all correlation values associated with PC1 and PC2 for the 186 metabolites are available in Supplementary Table S5 https://figshare.com/articles/Supplementary_Tables_xls/7688354). The levels of several amino acids were

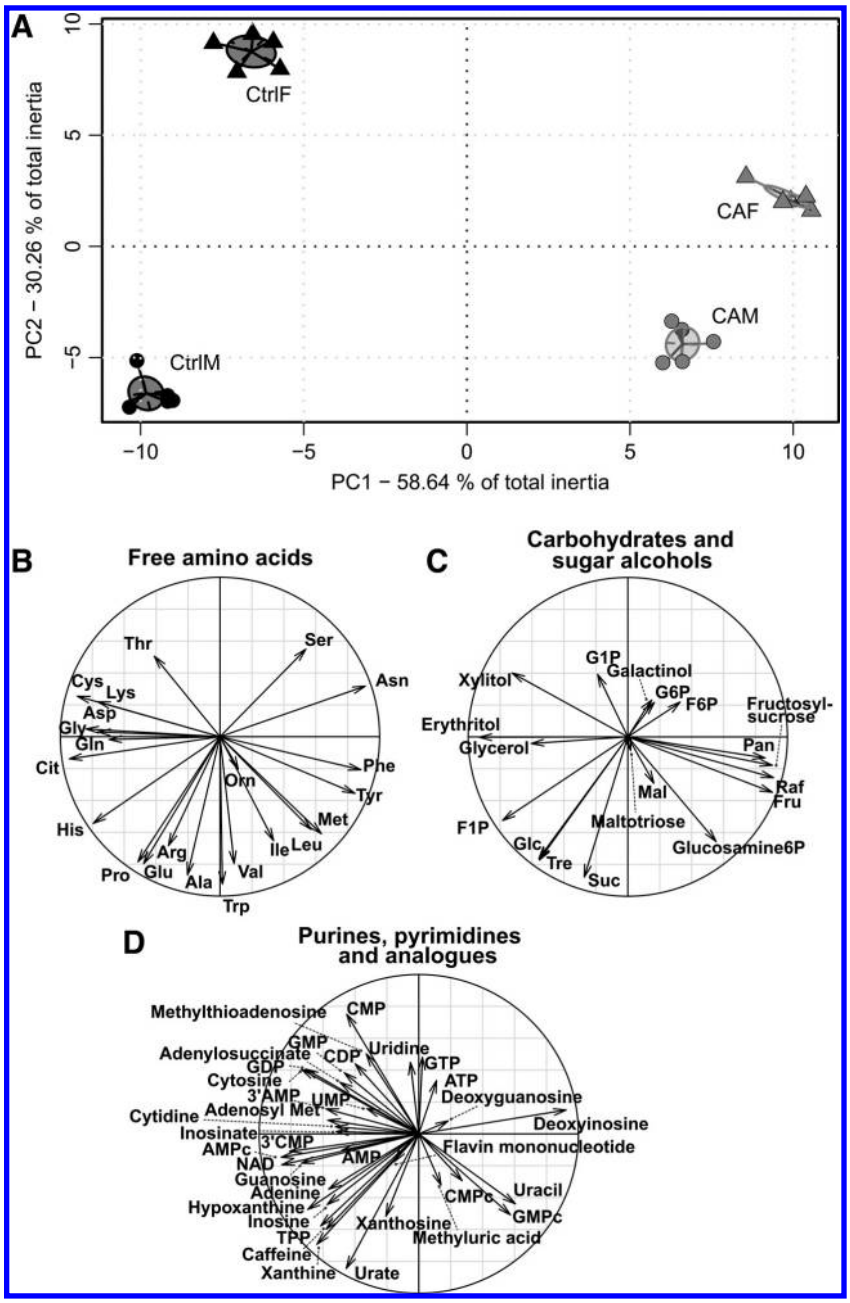

Fig. 5. *A*: principal component analysis (PC1 vs. PC2) on the 186 metabolites identified in our control and cold-acclimated flies. Differences between metabotypes were confirmed with Montecarlo randomizations (observed $P < 0.001$). Correlation circles from the principal component analysis on *B*, free amino acids; *C*, carbohydrates and sugar alcohols; and *D*, purines, pyrimidines, and analogs. CtrlM, control males; CtrlF, control females; CAM, cold-acclimated males; CAF, cold-acclimated females.

positively correlated with PC1 (i.e., relatively more abundant in cold-acclimated flies) (Ser, Asn, Phe, Tyr, Met, Leu, etc.) (Fig. 4*B*). The relative concentrations of several sugars were also positively correlated with PC1, such as fructosylsucrose, Pan, Raf, and Fru, whereas control flies were linked with several polyols (xylitol, erythritol, glycerol; Fig. 4*C*). Finally, the relative concentrations of the majority of purines and pyrimidines were negatively correlated with PC1 (i.e., relatively more abundant in controls), and only a few metabolites of this class, such as deoxyinosine, uracil, and GMPC, were positively correlated with PC1 (Fig. 4*D*).

Metabolite Set Enrichment Analysis

Univariate analyses showed that the relative level of 177 metabolites differed significantly between control and cold-acclimated flies in males, whereas 112 metabolites differed significantly in females (Supplemental Table S1, https://figshare.com/articles/Supplementary_Tables_xls/7688354). To test for enrichment of biologically meaningful pathways, MSEA was performed in males and females separately, using the lists of differentially expressed compounds. MSEA identified eight significantly impacted pathways in males and three in females. These pathways, as well as the metabolites involved in them, are displayed in Table 1. Two pathways were shared between males and females: aminoacyl-tRNA biosynthesis, and purine metabolism. These patterns were confirmed with the Reactome pipeline; outcomes of these analyses are available in Supplementary Tables S6 and S7 for males and females, respectively (https://figshare.com/articles/Supplementary_Tables_xls/7688354).

Table 1. *Outcomes from pathway analysis using Metaboanalyst 4.0*

Pathway	Total	Hits	*P*	Impact	Decreased Metabolites	Accumulated metabolites
			Males			
Histidine metabolism	7	4	<0.01	1	His; histamine; imidazoleacetic acid; methylhistidine	
Alanine, aspartate and glutamate metabolism	23	7	<0.01	0.635	Asp; Gln; Glu; fumarate	Arginiosuccinicate; Asn; glucosamine 6-phosphate
Ascorbate and aldarate metabolism	6	3	<0.05	0.5	Myo-inositol; D-glucuronic acid; D-saccharic acid	
Cysteine and methionine metabolism	25	6	<0.05	0.496	L-Cysteine; *S*-adenosyl methionine; 5′-methylthioadenosine	L-methionine;L-serine; L-Cystathionine
Arginine and proline metabolism	37	10	<0.01	0.294	Gln; Cit; Asp; Pro; Glu; *S*-adenosylmethionine; hydroxyproline; fumarate	argininosuccinicate; urea
Purine metabolism	64	14	<0.01	0.147	GDP; xanthine; Gln; AMPc; AMP; deoxyadenosine monophosphate; hypoxanthine; inosine; urate; guanosine; GMPc; adenine; allantoate	Deoxynosine; deoxyguanosine
Aminoacyl-tRNA biosynthesis	67	15	<0.01	0.137	His; Gln; Cys; Gly; Lys; Pro; Glu; Asp	Ser; Met; Asn; Phe; Ile; Leu; Tyr
Starch and sucrose metabolism	17	6	<0.01	0.129	Suc; Tre; Glc; D-glucuronic acid	Fru, Mal
Taurine and hypotaurine metabolism	6	3	<0.05	0.5	Cys; taurine; hypotaurine	
			Females			
Purine metabolism	64	11	<0.05	0.167	GDP; xanthine; AMPc; AMP; GMP; urate; guanosine; allantoate; adenylsuccinic acid	Deoxynosine; deoxyguanosine
Aminoacyl-tRNA biosynthesis	67	14	<0.01	0.137	His; Cys; Gly; Asp; Lys; Pro; Glu	Asn; Phe; Ser; Met; Leu; Trp; Tyr

Analysis was performed on pooled down- and upregulated metabolites. Total refers to the number of metabolites included in the respective pathways, and hits refers to the number of metabolites from the input list Supplemental Table S1 (see https://figshare.com/articles/Supplementary_Tables_xls/7688354) identified in the pathway. The *P* value resulted from metabolite set enrichment analysis (MSEA). Impact corresponds to the pathway impact value calculated from pathway topology analysis. List of metabolites identified in each pathway are separated depending on their fold change in cold-acclimated flies in comparison with controls (decreased or increased). Males and females have been analyzed separately.

DISCUSSION

Cold Acclimation Increases Cold Tolerance of D. suzukii

As hypothesized, combined developmental and adult cold acclimation greatly promoted cold tolerance in both males and females *D. suzukii*, and this was consistently observed with all tested metrics (i.e., survival, CCRT, and Ct_{min}. See Fig. 1). The basal cold survival of control flies measured after exposure to −5°C for 100 min was similar to data reported by Jakobs et al. (45), and Everman et al. (28), (i.e., 20% survival after 1 h at −7°C and 10% after 1 h at −6°C, respectively), and cold acclimation greatly promoted acute cold stress survival, as previously reported (105). Shorter CCRT after adult acclimation has also been reported by Jakobs et al. (45). Finally, as observed here, Toxopeus et al. (101), also observed a clear decrease in Ct_{min} after combined developmental and adult acclimation.

Several studies have reported that cold tolerance is highly plastic in *D. suzukii* (37), especially when flies are cold-acclimated during both developmental and adult stages (90, 101, 105), which could in part explain the fly's success at overwintering. Furthermore, previous work showed that developmental cold acclimation of *D. suzukii* under laboratory conditions resulted in relatively bigger, darker and more cold-tolerant flies, similar to individuals captured during winter in invaded areas (90, 101, 105). Recently, it was shown that laboratory maintenance does not alter ecological (stress tolerance) and physiological patterns of *Drosophila* species (68). Yet, caution should be exercised in the extrapolation of these data to field reality because results were obtained from a laboratory-adapted line exposed to controlled thermal environment. In natural environment, insects may be exposed to multiple and repeated stresses that may affect patterns of cold tolerance (52).

Cold Acclimation-Driven Phospholipidic Readjustments

The mechanisms underlying cold tolerance acquisition in *D. suzukii* remain poorly understood and our goal here was to explore lipidomic readjustments driven by cold acclimation. Exposure to cold acclimation often induces marked alterations in lipid composition and physical properties (fluidity) of membranes, a response conserved among taxa (18, 33, 40, 41, 57, 83). We identified >100 different PL. The PL composition of membranes was mainly dominated by PE and PC in *D. suzukii* (see Supplemental Table S2 (https://figshare.com/articles/Supplementary_Tables_xls/7688354), similarly to the membrane composition of *D. melanogaster* (9, 15, 38, 46, 56). Cold acclimation induced significant changes in PL composition that resulted in a distinct differentiation among the lipidotypes (Fig. 2) and reshuffled the PL headgroups (Fig. 3). The major changes induced by acclimation were observed in both males and females, although responses were sometimes sex specific, which might explain why lipidotypes of males and females did not overlap. LysoPE and lysoPC were relatively more abundant in cold-acclimated males and females. Even if LysoPL represent only a minor proportion of membrane's PL, they seem to play important roles in membrane response to cold temperatures in *D. melanogaster* (15). LysoPL have an in-

verted conical shape, which disrupts the tight packaging of PL membranes, thus increasing their fluidity (53). Their precise functions relative to cold tolerance, however, still need to be clearly defined. Moreover, PG accumulated in response to cold acclimation in both sexes, whereas PS accumulated only in females. PG are common PL of cell membranes and play important roles in response to environmental variations and in membrane fluidity (67). PC declined after cold acclimation in both sexes, while PE and the PE/PC ratio declined in cold-acclimated males and increased in cold-acclimated females. Changes in the PE/PC ratio is a common response to cold temperatures in insects (33, 100). In males and females *Drosophila melanogaster*, cold acclimation, or fluctuating cold temperatures leads to an augmentation of the PE/PC ratio (15, 16, 83), but not rapid cold hardening (70). PC arbore a cylindrical form, which provides membranes a highly organized, compact and rigid structure (53). Consequently, a decline in PC at the expense of other PL is supposed to increase membrane fluidity (40, 53).

Lipid restructuring was also visible at the scale of FA chains of PL: we observed relative increases in the proportions of FA unsaturation and polyunsaturation after cold acclimation (Fig. 4, *A* and *B*). As mentioned earlier, desaturation of FA is a common response to cold in poikilotherms (53) and insects (3, 49, 57). In *Drosophila* species, several studies reported no evidence of marked desaturation of FA in response to low temperatures (15, 16, 70, 76, 83); however, other studies have reported increases in unsaturation ratios in response to cold selection (34) or temperature shifts (17). Desaturation of FA is linked to fatty-acyl-CoA desaturase activity (19, 49). These enzymes insert cis double bonds into carbon chains of FA, resulting in a bend of ~30°. Therefore, UFA occupy more space in the PL bilayer than do SFA, resulting in a reduction in the tight packing of PL, which, in turn, increases membrane fluidity (53).

Fatty acids of PL were also characterized by an increase of their carbon chain lengths after cold acclimation (a decline in the 16C/18C ratio and short/long ratio, (Fig. 4, *D* and *E*). Usually in insects, cold hardiness is linked to FA shortening (1, 2, 74), but there are several cases where the opposite pattern has been observed (3, 4, 54, 85). In *Drosophila* species, the pattern varies, with some studies reporting an increase (15, 76, 78, 82), some a decrease (83), and some no change (81) in 16C/18C ratios in response to various low temperature treatments. These incongruities may be due to the variety of cold treatments applied and analyzed, ranging from short-term plastic responses to cold to long-term cold adaption. Koštál (53) suggested that desaturation of FA and shortening of carbon chain length could occur alternatively in organisms to obtain a similar result: an increase in membrane fluidity.

The overall PL adjustments resulting from gradual cold acclimation in *D. suzukii*, (PL headgroup modifications and desaturation of FA) are typical responses fitting with homeoviscous adaptation, thus confirming our hypothesis. These modifications may contribute to the maintenance of membrane fluidity and functions under cold temperatures and partly explain the enhanced cold tolerance of cold-acclimated flies.

In this work, we used a cold acclimation protocol similar to that of Toxopeus et al. (101). These authors showed that female flies exposed to this cold acclimation had arrested ovarian development. Therefore, differences observed here between cold-acclimated and control flies could also result, at least in part, from differences in reproductive status of flies (35). However, the fact that cold acclimation patterns were found in both males and females suggests that differential oogenesis was not a major confounding effect. Both lipidomic and metabolomics profiles change with age and aging (14, 35, 43, 86). Since physiological age of acclimated flies could slightly differ from that of control, this may partly account for differences between our treatment groups.

Changes in Stored Lipids

Cold acclimation did not induce changes in the relative amount of TAG found in *D. suzukii* males or females, thus confirming previous results (101). However, we noticed an increase of the proportions of UFA and PUFA in TAG from cold-acclimated flies (Fig. 4, *A* and *B*). TAG desaturation is commonly observed before or on experiencing a decline in body temperature among poikilotherms (41), including insects (3, 36, 57, 102) such as drosophilids (76, 77). TAG represents the major energy source in insect tissues (36, 91). Low temperatures increase the viscosity of TAG, which tends to hamper their accessibility for basal metabolism. As for desaturation of FA in membrane PL, desaturation of TAG decreases their melting point, thus increasing fluidity and accessibility at cold temperatures (57, 87, 102).

Changes in Metabolic Composition

Through alteration of many metabolic pathways (Table 1), cold acclimation resulted in metabotypes that were clearly distinct from those of nonacclimated counterparts in both males and females (Fig. 5*A*). Only two altered pathways were shared between cold-acclimated males and females: purine metabolism and aminoacyl-tRNA biosynthesis. The majority of purines and pyrimidines were correlated with nonacclimated controls (Fig. 5*D*). Changes in purine metabolites and purine metabolism in insects in response to low temperatures have been reported in previous studies (71, 106). In the psyllid *Diaphorina citri* for example, it was evidenced that after 5 days of exposure at 35, 20, or 5°C the nucleotides and sugar-nucleotides were all correlated with warm temperatures, reflecting the pattern found in our present study. Purine and pyrimidine nucleotides have essential functions: building blocks of DNA and RNA, energy carriers, and cell signaling molecules, and they play a central part in metabolism (47). Because of these multiple roles, it would be premature to speculate about the precise involvement of these molecules in cold acclimation. However, we can assume that low temperature during acclimation may affect intracellular nucleotide pools, which could consequently affect the kinetics of metabolism.

Purines are also directly involved in transcription and translation (47). Therefore, the alteration of purine metabolism, as suggested by our data, could be linked to the alteration of the aminoacyl-tRNA biosynthesis pathway (Table 1). RNA sequencing has revealed that aminoacyl-tRNA biosynthesis was the most altered pathway after cold-acclimation in *D. melanogaster* (71). Similarly, in *D. suzukii*, RNA sequencing demonstrated that cold acclimation resulted in the downregulation of several functions and processes involved in DNA translation (90). All these modifications could be linked with a global

decline of the translational machinery due to low temperatures (98).

We hypothesized that cold acclimation would be correlated with mobilization of cryoprotectant molecules, specifically amino acids (e.g., Pro, Arg) and sugars (e.g., Glc or Tre). According to PCA, it appeared that metabolic profiles of cold-acclimated flies were correlated with relative increases in many amino acids (Ser, Asn, Phe, Tyr, Met, Leu, etc., Fig. 5*B*). This was reflected in MSEA, which detected the involvement of pathways related to several amino acids in cold-acclimated males. Amino acids are known to act as cryoprotectants in insects, especially Pro (56, 58, 61), Arg (55), and Ala (65, 75). However, in this study, changes in Ala and Pro levels were correlated with differences between sexes but not with thermal treatments (Fig. 5*B*), although their metabolic pathways were significantly altered in males (Arg and Pro metabolism; Ala, Asp, and Glu metabolism, see Table 1). Other amino acids (such as Val, Leu, Ser, Thr, Ile, Asn, His, or Glu) are known to accumulate in response to cold in *D. melanogaster* (11, 56). In a previous targeted study (27), we observed a similar mobilization of amino acids after cold acclimation in *D. suzukii*. Except from Pro and Arg that have strong and well-defined cryoprotective roles in promoting cold/freeze tolerance in *Drosophila*, many other amino acids can also show mild positive effects at some concentrations, such as Val, Ile, Leu, and Asn (55). Our previous results (27) showed that amino acids concentrations in acclimated flies did not exceed the nmol/mg. At these concentrations it is unlikely that amino acids contribute to cold tolerance through colligative effects, but they could still act at low concentrations by protecting and stabilizing structures of macromolecules (107).

We also observed changes in the relative level of several carbohydrates linked to cold-acclimated flies (e.g., Pan, Raf, Fru, fructosylsucrose, Fig. 5*C*). In addition, the starch and sucrose metabolism pathway was significantly altered in cold-acclimated males (Table 1). Cold tolerance is associated with increased carbohydrate concentrations in various insect species (24, 110) and in *D. melanogaster* (11, 56, 80). Furthermore, in *D. suzukii* and *D. melanogaster*, transcriptomic data suggest that carbohydrates and starch and sucrose metabolism pathways were altered by cold acclimation (71, 90). Tre is one of the carbohydrates that is most commonly correlated with cold tolerance (48, 56, 95); but in the present study, Tre was not linked with cold-acclimated flies. In a previous work, however, we showed that several carbohydrates accumulate in *D. suzukii* after cold acclimation, including Tre (27).

The protocol used in the present study was somewhat specific, as it combined juvenile and adult cold acclimation. Physiological patterns may vary by using different acclimation procedures (stage, temperature, or photoperiod for example). In addition, it is conceivable that metabolic remodeling observed after acclimation might result from nonadaptive changes that are nonessential for cold tolerance acquisition. Indeed, it is possible that some of these changes resulted for moderate low temperature during cold acclimation, as cold exposure is known to trigger metabolic alteration (11, 80, 97, 106). However, we previously showed that after a similar cold acclimation procedure, *D. suzukii* flies were able to maintain metabolic homeostasis after a cold stress, contrary to non-cold acclimated individuals (27).

Perspectives and Significance

This study represents the first large-scale analysis of cold acclimation response in the invasive pest *D. suzukii*, based on combined lipidomic and metabolic approaches. We showed that acquiring cold tolerance thanks to cold acclimation generated a phenotype that was clearly distinct from control phenotypes at both the lipidomic and metabolic levels. This suggests a system-wide reprogramming in response to cold acclimation across a variety of metabolic pathways and lipidic changes. We showed that acquired cold tolerance in *D. suzukii* correlated with homeoviscous adaptations of membrane PL, increased fluidity of stored lipids, and deep metabolic adjustments involving compatible solutes, such as sugars and various amino acids. The acclimated phenotype resulting from our experiments was relatively similar to those observed in wild flies captured in autumn and winter. These winter flies probably resulted from larvae and pupae that developed in fruits in autumn (50). Hence, they may acclimate during both development and as adult (28, 84, 90, 93, 101, 105). Therefore, it is likely that wild *D. suzukii* can use physiological adjustments similar to the ones we described here to enhance their cold tolerance and successfully overwinter in invaded areas. Our data provides novel information on *D. suzukii* cold physiology and contributes to a better understanding of its thermal biology. Future studies should analyze the physiological strategies used by *D. suzukii* in nature during autumn and winter to assess if they use strategies similar to what we identified in laboratory cold-acclimated flies.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

T.E. and H.C. conceived and designed research; T.E. performed experiments; T.E. analyzed data; T.E. and H.C. interpreted results of experiments; T.E. prepared figures; T.E. drafted manuscript; T.E. and H.C. edited and revised manuscript; T.E. and H.C. approved final version of manuscript.

REFERENCES

1. **Bahrndorff S, Petersen SO, Loeschcke V, Overgaard J, Holmstrup M.** Differences in cold and drought tolerance of high arctic and sub-arctic populations of *Megaphorura arctica* Tullberg 1876 (Onychiuridae: Collembola). *Cryobiology* 55: 315–323, 2007. doi:10.1016/j.cryobiol.2007.09.001.
2. **Bashan M, Akbas H, Yurdakoc K.** Phospholipid and triacylglycerol fatty acid composition of major life stages of sunn pest, *Eurygaster integriceps* (Heteroptera: Scutelleridae). *Comp Biochem Physiol B Biochem Mol Biol* 132: 375–380, 2002. doi:10.1016/S1096-4959(02)00045-3.
3. **Bashan M, Cakmak O.** Changes in composition of phospholipid and triacylglycerol fatty acids prepared from prediapausing and diapausing individuals of *Dolycoris baccarum* and *Piezodorus lituratus* (Heteroptera: Pentatomidae). *Ann Entomol Soc Am* 98: 575–579, 2005.

4. **Bennett VA, Pruitt NL, Lee RE Jr.** Seasonal changes in fatty acid composition associated with cold-hardening in third instar larvae of *Eurosta solidaginis*. *J Comp Physiol B* 167: 249–255, 1997. doi:10.1007/s003600050071.
5. **Cacela C, Hincha DK.** Low amounts of sucrose are sufficient to depress the phase transition temperature of dry phosphatidylcholine, but not for lyoprotection of liposomes. *Biophys J* 90: 2831–2842, 2006. doi:10.1529/biophysj.105.074427.
6. **Calabria G, Máca J, Bächli G, Serra L, Pascual M.** First records of the potential pest species *Drosophila suzukii* (Diptera: Drosophilidae) in Europe. *J Appl Entomol* 136: 139–147, 2012. doi:10.1111/j.1439-0418.2010.01583.x.
7. **Cao-Hoang L, Dumont F, Marechal PA, Gervais P.** Inactivation of *Escherichia coli* and *Lactobacillus plantarum* in relation to membrane permeabilization due to rapid chilling followed by cold storage. *Arch Microbiol* 192: 299–305, 2010. doi:10.1007/s00203-010-0555-y.
8. **Carpenter JF, Crowe JH.** The mechanism of cryoprotection of proteins by solutes. *Cryobiology* 25: 244–255, 1988. doi:10.1016/0011-2240(88)90032-6.
9. **Carvalho M, Sampaio JL, Palm W, Brankatschk M, Eaton S, Shevchenko A.** Effects of diet and development on the *Drosophila* lipidome. *Mol Syst Biol* 8: 600, 2012. doi:10.1038/msb.2012.29.
10. **Chong J, Soufan O, Li C, Caraus I, Li S, Bourque G, Wishart DS, Xia J.** MetaboAnalyst 4.0: towards more transparent and integrative metabolomics analysis. *Nucleic Acids Res* 46: W486–W494, 2018. doi:10.1093/nar/gky310.
11. **Colinet H, Larvor V, Laparie M, Renault D.** Exploring the plastic response to cold acclimation through metabolomics: Metabolomics of cold acclimation response. *Funct Ecol* 26: 711–722, 2012. doi:10.1111/j.1365-2435.2012.01985.x.
12. **Colinet H, Nguyen TTA, Cloutier C, Michaud D, Hance T.** Proteomic profiling of a parasitic wasp exposed to constant and fluctuating cold exposure. *Insect Biochem Mol Biol* 37: 1177–1188, 2007. doi:10.1016/j.ibmb.2007.07.004.
13. **Colinet H, Renault D.** Metabolic effects of CO^2 anaesthesia in *Drosophila melanogaster*. *Biol Lett* 8: 1050–1054, 2012. doi:10.1098/rsbl.2012.0601.
14. **Colinet H, Renault D.** Similar post-stress metabolic trajectories in young and old flies. *Exp Gerontol* 102: 43–50, 2018. doi:10.1016/j.exger.2017.08.021.
15. **Colinet H, Renault D, Javal M, Berková P, Šimek P, Koštál V.** Uncovering the benefits of fluctuating thermal regimes on cold tolerance of drosophila flies by combined metabolomic and lipidomic approach. *Biochim Biophys Acta* 1861: 1736–1745, 2016. doi:10.1016/j.bbalip.2016.08.008.
16. **Cooper BS, Hammad LA, Fisher NP, Karty JA, Montooth KL.** In a variable thermal environment selection favors greater plasticity of cell membranes in *Drosophila melanogaster*. *Evolution* 66: 1976–1984, 2012. doi:10.1111/j.1558-5646.2011.01566.x.
17. **Cooper BS, Hammad LA, Montooth KL.** Thermal adaptation of cellular membranes in natural populations of *Drosophila melanogaster*. *Funct Ecol* 28: 886–894, 2014. doi:10.1111/1365-2435.12264.
18. **Cossins AR.** *Temperature Adaptation of Biological Membranes*. Portland, OR: Portland Press, 1994.
19. **Cossins AR, Murray PA, Gracey AY, Logue J, Polley S, Caddick M, Brooks S, Postle T, Maclean N.** *The role of desaturases in cold-induced lipid restructuring*. Portland Press Limited, 2002.
20. **Crowe JH, Crowe LM, Carpenter JF, Rudolph AS, Wistrom CA, Spargo BJ, Anchordoguy TJ.** Interactions of sugars with membranes. *Biochim Biophys Acta* 947: 367–384, 1988. doi:10.1016/0304-4157(88)90015-9.
21. **Dalton DT, Walton VM, Shearer PW, Walsh DB, Caprile J, Isaacs R.** Laboratory survival of *Drosophila suzukii* under simulated winter conditions of the Pacific Northwest and seasonal field trapping in five primary regions of small and stone fruit production in the United States. *Pest Manag Sci* 67: 1368–1374, 2011. doi:10.1002/ps.2280.
22. **David RJ, Gibert P, Pla E, Petavy G, Karan D, Moreteau B.** Cold stress tolerance in *Drosophila*: analysis of chill coma recovery in *D. melanogaster*. *J Therm Biol* 23: 291–299, 1998. doi:10.1016/S0306-4565(98)00020-5.
23. **Denlinger DL, Lee RE Jr (Eds.).** *Low Temperature Biology of Insects*. New York: Cambridge University Press, 2010.
24. **Ditrich T, Koštál V.** Comparative analysis of overwintering physiology in nine species of semi-aquatic bugs (Heteroptera: Gerromorpha). *Physiol Entomol* 36: 261–270, 2011. doi:10.1111/j.1365-3032.2011.00794.x.
25. **Dray S, Dufour A-B.** The ade4 package: implementing the duality diagram for ecologists. *J Stat Softw* 22: 1–20, 2007. doi:10.18637/jss.v022.i04.
26. **Enriquez T, Colinet H.** Basal tolerance to heat and cold exposure of the spotted wing drosophila, *Drosophila suzukii*. *PeerJ* 5: e3112, 2017. doi:10.7717/peerj.3112.
27. **Enriquez T, Renault D, Charrier M, Colinet H.** Cold acclimation favors metabolic stability in *Drosophila suzukii*. *Front Physiol* 9: 1506, 2018. doi:10.3389/fphys.2018.01506.
28. **Everman ER, Freda PJ, Brown M, Schieferecke AJ, Ragland GJ, Morgan TJ.** Ovary development and cold tolerance of the invasive pest *Drosophila suzukii* (Matsumura) in the central plains of Kansas, United States. *Environ Entomol* 47: 1013–1023, 2018. doi:10.1093/ee/nvy074.
29. **Foray V, Desouhant E, Voituron Y, Larvor V, Renault D, Colinet H, Gibert P.** Does cold tolerance plasticity correlate with the thermal environment and metabolic profiles of a parasitoid wasp? *Comp Biochem Physiol A Mol Integr Physiol* 164: 77–83, 2013. doi:10.1016/j.cbpa.2012.10.018.
30. **Fox J, Weisberg S.** *An R Companion to Applied Regression* (2nd ed.). Thousand Oaks, CA: Sage, 2011.
31. **Giavalisco P, Köhl K, Hummel J, Seiwert B, Willmitzer L.** 13C isotope-labeled metabolomes allowing for improved compound annotation and relative quantification in liquid chromatography-mass spectrometry-based metabolomic research. *Anal Chem* 81: 6546–6551, 2009. doi:10.1021/ac900979e.
32. **Goodhue RE, Bolda M, Farnsworth D, Williams JC, Zalom FG.** Spotted wing drosophila infestation of California strawberries and raspberries: economic analysis of potential revenue losses and control costs. *Pest Manag Sci* 67: 1396–1402, 2011. doi:10.1002/ps.2259.
33. **Goto SG, Katagiri C.** Effects of acclimation temperature on membrane phospholipids in the flesh fly *Sarcophaga similis*. *Entomol Sci* 14: 224–229, 2011. doi:10.1111/j.1479-8298.2010.00439.x.
34. **Goto SG, Udaka H, Ueda C, Katagiri C.** Fatty acids of membrane phospholipids in *Drosophila melanogaster* lines showing rapid and slow recovery from chill coma. *Biochem Biophys Res Commun* 391: 1251–1254, 2010. doi:10.1016/j.bbrc.2009.12.053.
35. **Guan XL, Cestra G, Shui G, Kuhrs A, Schittenhelm RB, Hafen E, van der Goot FG, Robinett CC, Gatti M, Gonzalez-Gaitan M, Wenk MR.** Biochemical membrane lipidomics during *Drosophila* development. *Dev Cell* 24: 98–111, 2013. doi:10.1016/j.devcel.2012.11.012.
36. **Hahn DA, Denlinger DL.** Energetics of insect diapause. *Annu Rev Entomol* 56: 103–121, 2011. doi:10.1146/annurev-ento-112408-085436.
37. **Hamby KA, Bellamy DE, Chiu JC, Lee JC, Walton VM, Wiman NG, York RM, Biondi A.** Biotic and abiotic factors impacting development, behavior, phenology, and reproductive biology of *Drosophila suzukii*. *J Pest Sci* 89: 605–619, 2016. doi:10.1007/s10340-016-0756-5.
38. **Hammad LA, Cooper BS, Fisher NP, Montooth KL, Karty JA.** Profiling and quantification of *Drosophila melanogaster* lipids using liquid chromatography/mass spectrometry. *Rapid Commun Mass Spectrom* 25: 2959–2968, 2011. doi:10.1002/rcm.5187.
39. **Hauser M.** A historic account of the invasion of *Drosophila suzukii* (Matsumura) (Diptera: Drosophilidae) in the continental United States, with remarks on their identification. *Pest Manag Sci* 67: 1352–1357, 2011. doi:10.1002/ps.2265.
40. **Hazel JR.** Cold Adaptation in Ectotherms: Regulation of Membrane Function and Cellular Metabolism. In: *Animal Adaptation to Cold*, edited by Wang LCH. Berlin, Heidelberg: Springer Berlin Heidelberg, 1989, p. 1–50.
41. **Hazel JR.** Thermal adaptation in biological membranes: is homeoviscous adaptation the explanation? *Annu Rev Physiol* 57: 19–42, 1995. doi:10.1146/annurev.ph.57.030195.000315.
42. **Hazel JR, Williams EE.** The role of alterations in membrane lipid composition in enabling physiological adaptation of organisms to their physical environment. *Prog Lipid Res* 29: 167–227, 1990. doi:10.1016/0163-7827(90)90002-3.
43. **Hoffman JM, Soltow QA, Li S, Sidik A, Jones DP, Promislow DEL.** Effects of age, sex, and genotype on high-sensitivity metabolomic profiles in the fruit fly, *Drosophila melanogaster*. *Aging Cell* 13: 596–604, 2014. doi:10.1111/acel.12215.
44. **Hosler JS, Burns JE, Esch HE.** Flight muscle resting potential and species-specific differences in chill-coma. *J Insect Physiol* 46: 621–627, 2000. doi:10.1016/S0022-1910(99)00148-1.

45. **Jakobs R, Gariepy TD, Sinclair BJ.** Adult plasticity of cold tolerance in a continental-temperate population of *Drosophila suzukii*. *J Insect Physiol* 79: 1–9, 2015. doi:10.1016/j.jinsphys.2015.05.003.
46. **Jones HE, Harwood JL, Bowen ID, Griffiths G.** Lipid composition of subcellular membranes from larvae and prepupae of *Drosophila melanogaster*. *Lipids* 27: 984–987, 1992. doi:10.1007/BF02535576.
47. **Kamatani N, Jinnah HA, Hennekam RC, van Kuilenburg ABP.** Purine and pyrimidine metabolism. In: *Emery and Rimoin's Principles and Practice of Medical Genetics*, edited by Rimoin D, Pyeritz R, Korf B. New York: Elsevier, 2014, p. 1–38.
48. **Kandror O, DeLeon A, Goldberg AL.** Trehalose synthesis is induced upon exposure of *Escherichia coli* to cold and is essential for viability at low temperatures. *Proc Natl Acad Sci USA* 99: 9727–9732, 2002. doi:10.1073/pnas.142314099.
49. **Kayukawa T, Chen B, Hoshizaki S, Ishikawa Y.** Upregulation of a desaturase is associated with the enhancement of cold hardiness in the onion maggot, *Delia antiqua*. *Insect Biochem Mol Biol* 37: 1160–1167, 2007. doi:10.1016/j.ibmb.2007.07.007.
50. **Kenis M, Tonina L, Eschen R, van der Sluis B, Sancassani M, Mori N, Haye T, Helsen H.** Non-crop plants used as hosts by *Drosophila suzukii* in Europe. *J Pest Sci (2004)* 89: 735–748, 2016. doi:10.1007/s10340-016-0755-6.
51. **Kimura MT.** Cold and heat tolerance of drosophilid flies with reference to their latitudinal distributions. *Oecologia* 140: 442–449, 2004. doi:10.1007/s00442-004-1605-4.
52. **Knapp M, Vernon P, Renault D.** Studies on chill coma recovery in the ladybird, *Harmonia axyridis*: Ontogenetic profile, effect of repeated cold exposures, and capacity to predict winter survival. *J Therm Biol* 74: 275–280, 2018. doi:10.1016/j.jtherbio.2018.04.013.
53. **Koštál V.** Cell structural modifications in insects at low temperature. In: *Low Temperature Biology of Insects*, edited by Denlinger DL and Lee RE Jr. New York: Cambridge University Press, 2010, p. 116–140.
54. **Koštál V, Berková P, Šimek P.** Remodelling of membrane phospholipids during transition to diapause and cold-acclimation in the larvae of *Chymomyza costata* (Drosophilidae). *Comp Biochem Physiol B Biochem Mol Biol* 135: 407–419, 2003. doi:10.1016/S1096-4959(03)00117-9.
55. **Koštál V, Korbelová J, Poupardin R, Moos M, Šimek P.** Arginine and proline applied as food additives stimulate high freeze tolerance in larvae of *Drosophila melanogaster*. *J Exp Biol* 219: 2358–2367, 2016. doi:10.1242/jeb.142158.
56. **Koštál V, Korbelová J, Rozsypal J, Zahradníčková H, Cimlová J, Tomčala A, Šimek P.** Long-term cold acclimation extends survival time at 0°C and modifies the metabolomic profiles of the larvae of the fruit fly *Drosophila melanogaster*. *PLoS One* 6: e25025, 2011. doi:10.1371/journal.pone.0025025.
57. **Koštál V, Simek P.** Changes in fatty acid composition of phospholipids and triacylglycerols after cold-acclimation of an aestivating insect prepupa. *J Comp Physiol B* 168: 453–460, 1998. doi:10.1007/s003600050165.
58. **Koštál V, Šimek P, Zahradníčková H, Cimlová J, Štětina T.** Conversion of the chill susceptible fruit fly larva (*Drosophila melanogaster*) to a freeze tolerant organism. *Proc Natl Acad Sci USA* 109: 3270–3274, 2012. doi:10.1073/pnas.1119986109.
59. **Koštál V, Vambera J, Bastl J.** On the nature of pre-freeze mortality in insects: water balance, ion homeostasis and energy charge in the adults of *Pyrrhocoris apterus*. *J Exp Biol* 207: 1509–1521, 2004. doi:10.1242/jeb.00923.
60. **Koštál V, Yanagimoto M, Bastl J.** Chilling-injury and disturbance of ion homeostasis in the coxal muscle of the tropical cockroach (*Nauphoeta cinerea*). *Comp Biochem Physiol B Biochem Mol Biol* 143: 171–179, 2006. doi:10.1016/j.cbpb.2005.11.005.
61. **Koštál V, Zahradnícková H, Šimek P.** Hyperprolinemic larvae of the drosophilid fly, *Chymomyza costata*, survive cryopreservation in liquid nitrogen. *Proc Natl Acad Sci USA* 108: 13,041–13,046, 2011. doi:10.1073/pnas.1107060108.
62. **Lavagnino NJ, Díaz BM, Cichón LI, De La Vega G, Garrido SA, Lago JD, Fanara JJ.** New records of the invasive pest *Drosophila suzukii* (Matsumura) (Diptera: Drosophilidae) in the South American continent. *Rev Soc Entomol Argent* 77: 27–31, 2018. doi:10.25085/rsea.770105.
63. **Lee RE Jr.** A primer on insect cold-tolerance. In: *Low Temperature Biology of Insects* edited by Denlinger DL and Lee RE Jr. New York: Cambridge University Press, 2010, p. 3–34.
64. **Lee RE Jr, Damodaran K, Yi S-X, Lorigan GA.** Rapid cold-hardening increases membrane fluidity and cold tolerance of insect cells. *Cryobiology* 52: 459–463, 2006. doi:10.1016/j.cryobiol.2006.03.003.
65. **Li Y, Zhang L, Chen H, Koštál V, Simek P, Moos M, Denlinger DL.** Shifts in metabolomic profiles of the parasitoid *Nasonia vitripennis* associated with elevated cold tolerance induced by the parasitoid's diapause, host diapause and host diet augmented with proline. *Insect Biochem Mol Biol* 63: 34–46, 2015. doi:10.1016/j.ibmb.2015.05.012.
66. **Lu Y-X, Zhang Q, Xu W-H.** Global metabolomic analyses of the hemolymph and brain during the initiation, maintenance, and termination of pupal diapause in the cotton bollworm, *Helicoverpa armigera*. *PLoS One* 9: e99948, 2014. doi:10.1371/journal.pone.0099948.
67. **Luévano-Martínez LA, Kowaltowski AJ.** Phosphatidylglycerol-derived phospholipids have a universal, domain-crossing role in stress responses. *Arch Biochem Biophys* 585: 90–97, 2015. doi:10.1016/j.abb.2015.09.015.
68. **Maclean HJ, Kristensen TN, Sørensen JG, Overgaard J.** Laboratory maintenance does not alter ecological and physiological patterns among species: a *Drosophila* case study. *J Evol Biol* 31: 530–542, 2018. doi:10.1111/jeb.13241.
69. **MacMillan HA, Andersen JL, Davies SA, Overgaard J.** The capacity to maintain ion and water homeostasis underlies interspecific variation in *Drosophila* cold tolerance. *Sci Rep* 5: 18,607, 2015. doi:10.1038/srep18607.
70. **MacMillan HA, Guglielmo CG, Sinclair BJ.** Membrane remodeling and glucose in *Drosophila melanogaster*: a test of rapid cold-hardening and chilling tolerance hypotheses. *J Insect Physiol* 55: 243–249, 2009. doi:10.1016/j.jinsphys.2008.11.015.
71. **MacMillan HA, Knee JM, Dennis AB, Udaka H, Marshall KE, Merritt TJS, Sinclair BJ.** Cold acclimation wholly reorganizes the *Drosophila melanogaster* transcriptome and metabolome. *Sci Rep* 6: 28999, 2016. doi:10.1038/srep28999.
72. **MacMillan HA, Sinclair BJ.** Mechanisms underlying insect chill-coma. *J Insect Physiol* 57: 12–20, 2011. doi:10.1016/j.jinsphys.2010.10.004.
73. **MacMillan HA, Williams CM, Staples JF, Sinclair BJ.** Reestablishment of ion homeostasis during chill-coma recovery in the cricket *Gryllus pennsylvanicus*. *Proc Natl Acad Sci USA* 109: 20750–20755, 2012. doi:10.1073/pnas.1212788109.
74. **Michaud MR, Denlinger DL.** Oleic acid is elevated in cell membranes during rapid cold-hardening and pupal diapause in the flesh fly, *Sarcophaga crassipalpis*. *J Insect Physiol* 52: 1073–1082, 2006. doi:10.1016/j.jinsphys.2006.07.005.
75. **Michaud MR, Denlinger DL.** Shifts in the carbohydrate, polyol, and amino acid pools during rapid cold-hardening and diapause-associated cold-hardening in flesh flies (*Sarcophaga crassipalpis*): a metabolomic comparison. *J Comp Physiol B* 177: 753–763, 2007. doi:10.1007/s00360-007-0172-5.
76. **Ohtsu T, Katagiri C, Kimura MT.** Biochemical aspects of climatic adaptations in *Drosophila curviceps, D. immigrans*, and *D. albomicans* (Diptera: Drosophilidae). *Environ Entomol* 28: 968–972, 1999. doi:10.1093/ee/28.6.968.
77. **Ohtsu T, Katagiri C, Kimura MT, Hori SH.** Cold adaptations in *Drosophila*. Qualitative changes of triacylglycerols with relation to overwintering. *J Biol Chem* 268: 1830–1834, 1993.
78. **Ohtsu T, Kimura MT, Katagiri C.** How *Drosophila* species acquire cold tolerance: qualitative changes of phospholipids. *Eur J Biochem* 252: 608–611, 1998. doi:10.1046/j.1432-1327.1998.2520608.x.
79. **Overgaard J, MacMillan HA.** The integrative physiology of insect chill tolerance. *Annu Rev Physiol* 79: 187–208, 2017. doi:10.1146/annurev-physiol-022516-034142.
80. **Overgaard J, Malmendal A, Sørensen JG, Bundy JG, Loeschcke V, Nielsen NC, Holmstrup M.** Metabolomic profiling of rapid cold hardening and cold shock in *Drosophila melanogaster*. *J Insect Physiol* 53: 1218–1232, 2007. doi:10.1016/j.jinsphys.2007.06.012.
81. **Overgaard J, Sørensen JG, Petersen SO, Loeschcke V, Holmstrup M.** Changes in membrane lipid composition following rapid cold hardening in *Drosophila melanogaster*. *J Insect Physiol* 51: 1173–1182, 2005. doi:10.1016/j.jinsphys.2005.06.007.
82. **Overgaard J, Sørensen JG, Petersen SO, Loeschcke V, Holmstrup M.** Reorganization of membrane lipids during fast and slow cold hardening in *Drosophila melanogaster*. *Physiol Entomol* 31: 328–335, 2006. doi:10.1111/j.1365-3032.2006.00522.x.
83. **Overgaard J, Tomčala A, Sørensen JG, Holmstrup M, Krogh PH, Šimek P, Kostál V.** Effects of acclimation temperature on thermal

tolerance and membrane phospholipid composition in the fruit fly *Drosophila melanogaster*. *J Insect Physiol* 54: 619–629, 2008. doi:10.1016/j.jinsphys.2007.12.011.

84. **Panel ADC, Zeeman L, van der Sluis BJ, van Elk P, Pannebakker BA, Wertheim B, Helsen HHM.** Overwintered *Drosophila suzukii* are the main source for infestations of the first fruit crops of the season. *Insects* 9: 1–18, 2018. doi:10.3390/insects9040145.
85. **Pruitt NL, Lu C.** Seasonal changes in phospholipid class and class-specific fatty acid composition associated with the onset of freeze tolerance in third-instar larvae of *Eurosta solidaginis*. *Physiol Biochem Zool* 81: 226–234, 2008. doi:10.1086/524394.
86. **Pujol-Lereis LM, Fagali NS, Rabossi A, Catalá Á, Quesada-Allué LA.** Chill-coma recovery time, age and sex determine lipid profiles in *Ceratitis capitata* tissues. *J Insect Physiol* 87: 53–62, 2016. doi:10.1016/j.jinsphys.2016.02.002.
87. **Rozsypal J, Koštál V, Berková P, Zahradníčková H, Šimek P.** Seasonal changes in the composition of storage and membrane lipids in overwintering larvae of the codling moth, *Cydia pomonella*. *J Therm Biol* 45: 124–133, 2014. doi:10.1016/j.jtherbio.2014.08.011.
88. **Ryan GD, Emiljanowicz L, Wilkinson F, Kornya M, Newman JA.** Thermal tolerances of the spotted-wing Drosophila *Drosophila suzukii* (Diptera: Drosophilidae). *J Econ Entomol* 109: 746–752, 2016. doi:10.1093/jee/tow006.
89. **Santoiemma G, Trivellato F, Caloi V, Mori N, Marini L.** Habitat preference of *Drosophila suzukii* across heterogeneous landscapes. *J Pest Sci* 92: 485–494, 2019. doi:10.1007/s10340-018-1052-3
90. **Shearer PW, West JD, Walton VM, Brown PH, Svetec N, Chiu JC.** Seasonal cues induce phenotypic plasticity of *Drosophila suzukii* to enhance winter survival. *BMC Ecol* 16: 1–18, 2016. doi:10.1186/s12898-016-0070-3.
91. **Sinclair BJ, Marshall KE.** The many roles of fats in overwintering insects. *J Exp Biol* 221, *Suppl* 1: jeb161836, 2018. doi:10.1242/jeb.161836.
92. **Sinensky M.** Homeoviscous adaptation--a homeostatic process that regulates the viscosity of membrane lipids in *Escherichia coli*. *Proc Natl Acad Sci USA* 71: 522–525, 1974. doi:10.1073/pnas.71.2.522.
93. **Stephens AR, Asplen MK, Hutchison WD, Venette RC.** Cold hardiness of winter-acclimated *Drosophila suzukii* (Diptera: Drosophilidae) adults. *Environ Entomol* 44: 1619–1626, 2015. doi:10.1093/ee/nvv134.
94. **Stockton DG, Wallingford AK, Loeb GM.** Phenotypic plasticity promotes overwintering survival in a globally invasive crop pest, *Drosophila suzukii*. *Insects* 9: 1–16, 2018. doi:10.3390/insects9030105.
95. **Storey KB, Storey JM.** Biochemistry of cryoprotectants. In: *Insects at Low Temperature*. Boston: Springer, 1991, p. 64–93.
96. **Dolédec S, Chessel D.** Recent developments in linear ordination methods in environmental sciences. *Adv Ecol* 1: 133–155, 1991.
97. **Teets NM, Peyton JT, Ragland GJ, Colinet H, Renault D, Hahn DA, Denlinger DL.** Combined transcriptomic and metabolomic approach uncovers molecular mechanisms of cold tolerance in a temperate flesh fly. *Physiol Genomics* 44: 764–777, 2012. doi:10.1152/physiolgenomics.00042.2012.
98. **Thieringer HA, Jones PG, Inouye M.** Cold shock and adaptation. *BioEssays* 20: 49–57, 1998. doi:10.1002/(SICI)1521-1878(199801)20:1<49:AID-BIES8>3.0.CO;2-N.
99. **Thistlewood HMA, Gill P, Beers EH, Shearer PW, Walsh DB, Rozema BM, Acheampong S, Castagnoli S, Yee WL, Smytheman P, Whitener AB.** Spatial analysis of seasonal dynamics and overwintering of *Drosophila suzukii* (Diptera: Drosophilidae) in the Okanagan-Columbia basin, 2010-2014. *Environ Entomol* 47: 221–232, 2018. doi:10.1093/ee/nvx178.
100. **Tomcala A, Tollarová M, Overgaard J, Simek P, Kostál V.** Seasonal acquisition of chill tolerance and restructuring of membrane glycerophospholipids in an overwintering insect: triggering by low temperature, desiccation and diapause progression. *J Exp Biol* 209: 4102–4114, 2006. doi:10.1242/jeb.02484.
101. **Toxopeus J, Jakobs R, Ferguson LV, Gariepy TD, Sinclair BJ.** Reproductive arrest and stress resistance in winter-acclimated *Drosophila suzukii*. *J Insect Physiol* 89: 37–51, 2016. doi:10.1016/j.jinsphys.2016.03.006.
102. **van Dooremalen C, Ellers J.** A moderate change in temperature induces changes in fatty acid composition of storage and membrane lipids in a soil arthropod. *J Insect Physiol* 56: 178–184, 2010. doi:10.1016/j.jinsphys.2009.10.002.
103. **Vesala L, Salminen TS, Koštál V, Zahradníčková H, Hoikkala A.** Myo-inositol as a main metabolite in overwintering flies: seasonal metabolomic profiles and cold stress tolerance in a northern drosophilid fly. *J Exp Biol* 215: 2891–2897, 2012. doi:10.1242/jeb.069948.
104. **Wallingford AK, Lee JC, Loeb GM.** The influence of temperature and photoperiod on the reproductive diapause and cold tolerance of spotted-wing drosophila, *Drosophila suzukii*. *Entomol Exp Appl* 159: 327–337, 2016. doi:10.1111/eea.12443.
105. **Wallingford AK, Loeb GM.** Developmental acclimation of *Drosophila suzukii* (Diptera: Drosophilidae) and its effect on diapause and winter stress tolerance. *Environ Entomol* 45: 1081–1089, 2016. doi:10.1093/ee/nvw088.
106. **Williams CM, Watanabe M, Guarracino MR, Ferraro MB, Edison AS, Morgan TJ, Boroujerdi AFB, Hahn DA.** Cold adaptation shapes the robustness of metabolic networks in *Drosophila melanogaster*. *Evolution* 68: 3505–3523, 2014. doi:10.1111/evo.12541.
107. **Yancey PH.** Organic osmolytes as compatible, metabolic and counteracting cytoprotectants in high osmolarity and other stresses. *J Exp Biol* 208: 2819–2830, 2005. doi:10.1242/jeb.01730.
108. **Yocum GD, Žďárek J, Joplin KH, Lee RE Jr, Smith DC, Manter KD, Denlinger DL.** Alteration of the eclosion rhythm and eclosion behavior in the flesh fly, *Sarcophaga crassipalpis*, by low and high temperature stress. *J Insect Physiol* 40: 13–21, 1994. doi:10.1016/0022-1910(94)90107-4.
109. **Zachariassen KE.** Physiology of cold tolerance in insects. *Physiol Rev* 65: 799–832, 1985. doi:10.1152/physrev.1985.65.4.799.
110. **Zeng J-P, Ge F, Su J-W, Wang Y.** The effect of temperature on the diapause and cold hardiness of *Dendrolimus tabulaeformis* (Lepidoptera: Lasiocampidae). *Eur J Entomol* 105: 599–606, 2008. doi:10.14411/eje.2008.080.
111. **Zerulla FN, Schmidt S, Streitberger M, Zebitz CP, Zelger R.** On the overwintering ability of *Drosophila suzukii* in South Tyrol. *J Berry Res* 5: 41–48, 2015. doi:10.3233/JBR-150089.