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Divergent metabolomic profiles of cold-exposed mature and immature females of tropical *versus* temperate *Drosophila* species

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Abstract

Temperate species, contrary to their tropical counterparts, are exposed not only to thermally variable environments with low temperatures but also to long winters. Different selective pressures may have driven divergent physiological adaptations in closely related species with different biogeographic origins. To survive unfavourable winter conditions, *Drosophila* species in temperate areas generally undergo a period of reproductive dormancy, associated with a cold-induced cessation of oogenesis and metabolic reorganization. This work aims to compare cold tolerance and metabolic signatures of cold-exposed females exhibiting different reproductive maturity status (mature and immature females) of four *Drosophila* species from tropical vs. temperate origins. We expected that the capacity for delayed reproduction of immature females could result in the redirection of the energy-related metabolites to be utilized for surviving the cold season. To do so, we studied an array of 45 metabolites using quantitative target GC-MS profiling. Reproductively immature females of temperate species showed the lower CT_{min} and the faster chill coma recovery time (i.e. the most cold-tolerant group). Principal component analysis captured differences across species, but also between reproductive maturity states. Notably, temperate species exhibited

significantly higher levels of glucose, alanine, and gluconolactone than tropical ones. As proline and glycerol showed higher abundances in immature females of temperate species compared to the levels exhibited by the rest of the groups, we reasoned that glucose and alanine could serve as intermediates in the synthesis of these compatible solutes. All in all, our findings suggest that cold-exposed females of temperate species accumulate energy-related and protective metabolites (e.g. glycerol and proline) while delaying reproduction, and that these metabolites are relevant to cold tolerance even at modest concentrations.

Keywords

Fruit flies, cold adaptation, cold tolerance, ovarian maturity, metabolic responses, reproductive dormancy

1. Introduction

At high latitudes, ectotherms must exploit the warm season to sustain critical functions for growth, development and reproduction. Conversely, the maintenance of metabolic activity and homeostasis by means of seasonal cold-acclimation or cold adaptation are the major constraints they encounter during winter time (Williams *et al.*, 2014). Given that overwintering ectotherms are generally inactive and do not feed (Sinclair 2015), they must rely on a tight energy budget, and can hardly compensate for energetic deficiencies (Williams *et al.*, 2012). Thus, in response to changes in environmental stimuli (*e.g.*, length of the day and environmental temperatures), ectotherms switch in a coordinated way the metabolism of numerous biomolecules, such as polyols, amino acids, carbohydrates or phosphorylated metabolites, leading to changes in organism's resistance level to low temperatures. These changes can drive the accumulation of energy reserves (Hahn & Denlinger, 2011), and the synthesis of low-molecular weight compatible solutes (referred to as cryo/osmo-protectants) capable of stabilizing the membrane and protein structures (and their function) at low concentrations by preferential exclusion mechanisms, for instance (Gekko *et al.*, 1981; Yancey 2005). Additionally, at high concentrations, these compatible solutes can mitigate osmotic damages to cells due to colligative effects (Teets & Denlinger, 2013). For example, winter-acclimated insects often have increased levels of sugars (*e.g.*, trehalose or glucose) and polyols (*e.g.*, glycerol, sorbitol or inositol), with glycerol being the most

common compatible protective solute found in insects (Teets & Denlinger, 2013). At the onset of winter, glycogen stores are rapidly depleted and converted to sugar-alcohols or sugars (Storey & Storey, 1983; Shimada *et al.*, 1984). Proline is another protective molecule whose accumulation has been correlated with enhanced cold resistance and tolerance in a range of species including flies, beetles and crickets (Shimada & Riihimaa, 1990; Fields *et al.*, 1998; Ramlov, 1999, Toxopeus *et al.*, 2019). Accordingly, fly lines selected for cold-hardiness were characterized by altered amounts of metabolites involved in membrane lipid synthesis, energy balance, and proline metabolism (Misener *et al.*, 2001; Williams *et al.*, 2014).

Temperate species, contrary to their tropical counterparts, are exposed not only to thermally variable environments with lower temperatures, but also to longer winters. As a result, it is very likely that different selective pressures may have driven divergent metabolic adaptations among species living in regions with contrasting climates. For example, chill-tolerant *Drosophila* species have higher levels of sugars and free amino acids in their haemolymph, including protective solutes such as trehalose and proline (Olsson *et al.*, 2016), than their chill-susceptible counterparts thriving in warmer environments. The capacity for cryo/osmo-protectants accumulation may play important protective roles in cold tolerance possibly through their stabilizing properties (Gekko *et al.*, 1981; Yancey 2005; Kostal *et al.*, 2016) or by maintaining haemolymph osmolality despite low $[Na^+]$ and $[K^+]$ due to cold exposure (MacMillan *et al.*, 2015). Maintaining haemolymph water homeostasis during cold exposure can prevent chill-injuries that otherwise would lead to sublethal effects on performance and fitness or even death (MacMillan & Sinclair, 2011). Investigating to what extent such metabolic changes have evolved among different lineages and ecological scenarios can provide important support to the generality of the basis of cold adaptation.

The four species studied here belong to the *Drosophila repleta* group, which is a monophyletic group of Neotropical origin that mostly diversified in the American deserts thanks to the adoption of a cactophilic lifestyle (Wasserman, 1982). In this study, we focused on four members of the *buzzatii* complex (Hasson *et al.*, 2019), *Drosophila venezolana* as representative of the *martensis* cluster, while the other three, *Drosophila borborema*, *Drosophila buzzatii* and *Drosophila koepferae* are members of the *buzzatii* cluster. Recent phylogenomic studies of the *buzzatii* cluster, show that *D.*

buzzatii is the most basal lineage and, thus, the sister species of the clade formed by *D. koepferae* and *D. borborema* (Hurtado *et al.* 2019). Figure S1 shows the phylogenetic relationships of species under studied. While *D. venezolana* and *D. borborema* are inhabitants of tropical deserts where environmental temperatures are warm and mostly uniform throughout the year, *D. koepferae* and *D. buzzatii* can reach areas of higher latitudes or altitudes in subtropical and temperate regions, where cold seasons likely challenges survival and fertility (Mensch *et al.*, 2017). Climatic data from locations where the species were collected indicate that tropical and temperate species are exposed to very distinct conditions in nature (Figure S2). Specifically, winter temperatures markedly differ between biogeographic regions. For the temperate species, the minimum temperatures during the coldest months of the year fall below the lower thermal limit of ovarian development (*i.e.*, 10 °C), thus provoking reproductive arrest. Such a strategy is likely the most common, as immature overwintering females can maintain high reproductive output after prolonged low-temperature exposure (Mensch *et al.*, 2017). In contrast, for tropical species, mean temperatures are relatively warm (above 20 °C) and mostly uniform throughout the year.

Overwintering insects cope with environmental adversities (*e.g.*, cold, dryness, lack of food) by adopting a variety of dormant responses usually associated with reduced metabolism and increased stress tolerance. Many insects overwinter in diapause, a hormonally pre-programmed response to anticipated changes in conditions. Diapause is often induced by short-day photoperiods and sometimes additionally promoted by low temperatures (Kostal 2006). Another form of dormancy response is quiescence, which is a direct response driven by challenging conditions and which resumes as soon as favorable conditions return. Diapause and quiescence can occur at various developmental stages in different species and likely contributed to the establishment, maintenance, and spread of natural populations of temperate insects (Denlinger 2002; Kostal 2006).

In *Drosophila*, overwintering adults may enter a state of reproductive dormancy, usually associated with high lipid reserves for sustaining energy production, arrested ovarian development and increased cold tolerance (Vesala and Hoikkala, 2011; Mensch *et al.*, 2017; Lirakis *et al.*, 2018). Temperate *Drosophila* species, such as *D. suzukii*, are assumed to spend winter under a state of shallow reproductive dormancy (Toxopeus *et al.*, 2016), which is most likely a quiescence rather than a true diapause, since it consists

of a direct response to a limiting environmental factor (such as cold). When captured in winter, *Drosophila* flies have typically underdeveloped ovaries, reduced metabolism, and improved cold tolerance (e.g., Tatar *et al.*, 2001; Kubrak *et al.*, 2014; Shearer *et al.*, 2016; Enriquez *et al.*, 2019a). It is generally accepted that the expression of dormancy (*sensu lato*) facilitates the survival of fruit flies in geographic regions with cold climates such as in temperate areas (Fabian *et al.*, 2015). Yet, reproductive dormancy-like phenotypes have also been reported in tropical *Drosophila* species, likely as an ancestral stress response (Zonato *et al.*, 2017; Mensch *et al.*, 2017; Lavagnino *et al.*, 2020). Reproductive dormancy (*i.e.*, reversible arrested ovarian development) has been suggested to be a general stress response to cold temperature or starvation, and may affect the whole metabolism, as well as stress-resistance traits (Lirakis *et al.*, 2018). Consistently, we previously reported that females under simulated reproductive dormancy (referred here as “immature”) presented lower metabolic rate than females that had opportunity to mature their eggs upon emergence (referred here as “mature”) (Mensch *et al.*, 2017). Also, reproductively immature females showed higher cold tolerance than mature counterparts, and when flies returned to benign temperature, their fertility was also superior (Mensch *et al.*, 2017). These findings suggest a possible trade-off between fecundity and reproduction, as limited energy resources may allocate either to reproduction or stress-resistance traits (e.g., cold tolerance, starvation resistance, drought resistance). This Y model of resource allocation (Harshman and Zera, 2007; Kalra and Parkash, 2014) is expected if response traits are competing for a common pool of energy storage compounds (Zera and Harshman, 2001). The strategy of overwintering organisms would consist of using the energetic pools for survival, and invest in protective mechanisms that could drive recovery from the chill-coma, at the expense of delaying reproduction. In the present study, we performed a comparative metabolomic GC-MS-based analysis using four closely-related *Drosophila* species from different biogeographic origins in South America (two temperate *vs.* two tropical). For each of these species, we compared two experimental groups: mature *vs.* immature females, with the aim of studying their cold tolerance using two different metrics, chill coma recovery time (CCRT) and Critical Thermal minimum (CT_{min}). Both metrics are ecological relevant measurements of performance, since the effects of sub-lethal low temperatures on insect physiology are important determinants of insect activity, including foraging, mating, and predation avoidance (Hazell and Bale, 2011; Andersen *et al.*, 2015). We also searched for metabolic signatures correlated with cold adaptation

(*e.g.*, geographical origins: tropical *vs.* temperate), and with the reproductive maturity status (*i.e.*, mature *vs.* immature). Our hypotheses were that: 1) temperate species and immature females would have relatively lower CT_{min} and faster CCRT than their tropical and reproductively mature counterparts, 2) metabolic profiles would be context-specific, *i.e.* the set of protective compounds related to cold adaptation (temperate *vs.* tropical) would differ from the metabolites involved in reproductive maturity status (mature *vs.* immature).

2. Materials and Methods

2.1 Fly conditioning and thermal treatments

We used species from different biogeographic origins: *Drosophila buzzatii* and *D. koepferae*, as representative of temperate species, and *D. borborema* and *D. venezolana* for the tropical species. Details of fly stocks (collection and cultures under controlled laboratory conditions) are given in Mensch *et al.* (2017). All species were reared at low density (50 larvae per vial) at 25 °C under a 12h:12h light:dark (L:D) regime on an instant mashed potato medium hydrated with a water solution of the antifungal Nipagin (p-hydroxybenzoic acid methyl ester) (Mensch *et al.*, 2017). For each species, two experimental groups, differing by their timing of reproductive maturity, were compared: “immature” females (with only early egg chamber stages) *vs.* “mature” females (with vitellogenic mature eggs). To generate immature flies, a cohort of females, sexed within 6h of emergence, was kept at low temperature (10 and 12 °C for temperate and tropical species, respectively) and 10h:14h L:D photoperiod for 12 consecutive days after emergence. These low temperatures are the upper thermal limits that elicit the arrest of oogenesis in temperate and tropical species, respectively (Mensch *et al.*, 2017), and thus mimic the effect of a reproductive dormancy. For the mature flies, a cohort of newly emerged females was maintained at 25 °C for 5 days to allow full ovarian development (*i.e.*, yolk deposition). Afterwards, just as the immature females, these mature females were transferred to either 10 or 12 °C, for temperate and tropical species respectively, for the following 12 days under a 10h:14h L:D photoperiod. This experimental design allowed us to compare females exhibiting different states of their reproductive maturity (immature *vs.* mature) which were subsequently exposed to cold conditions for the same lapse of time (12 days).

2.2 Critical Thermal minimum (CT_{min})

At the end of cold exposures (12 days at 10 or 12 °C), for each experimental group, approximately 20 flies were placed on a temperature plate (Pelt-plate; Sable System International (SSI), Las Vegas, NV, USA) covered by a cylindrical acrylic container (5 cm diameter and 1 cm high), which avoid flies to fly and escape. The walls of the containers were covered with fluon to prevent flies from climbing and to ensure that flies were standing on the plate, minimizing any possible bias resulting from a thermal gradient in the air. The temperature plate was connected to a temperature controller (Pelt-5; SSI), where a descending temperature ramp was set up at a rate of -0.25 °C min^{-1} for 50 min. Before starting the ramp, insects were kept for 15 min at 10 °C. CT_{min} was defined as loss of coordinated muscle function (Terblanche *et al.*, 2006). It was determined when insect did not respond to a mechanical perturbation, *i.e.*, soft touch, and the temperature at which this occurred was noted.

2.3 Chill coma recovery time (CCRT)

At the end of cold exposure, approx. 50 females of each experimental condition (immature *vs.* mature) and of each species were transferred without anaesthesia to vials set in boxes containing melting ice. After 12h at 0 °C, females were allowed to recover at 25 °C. Recovery from chill coma was individually measured as the time (in minutes) elapsed until flies could stand up on their legs (David *et al.*, 1998).

2.4 GC-MS metabolic profiling

At the end of cold exposure, for each experimental condition and species, ten replicates, each consisting of 20 females, were snap-frozen in liquid nitrogen for each of the eight treatment combinations (4 species x 2 phenotypes). The samples were stored at -80 °C until being processed. Before metabolic profiling, females were placed on a glass plate on ice to isolate the heads that were then rapidly returned to -80 °C . To prevent thawing of the samples, this protocol was done for one replicate (one pool of 20 female heads) at a time. We decided to use only the heads to assess circulating metabolites in haemolymph. The rationale was to avoid confounding effects of metabolites derived from eggs in the case of mature females. Fresh mass of each sample was measured to the nearest 0.01 mg using an analytical balance (Mettler AJ100, Toledo, OH, USA). Samples were sent on dry ice to the EcoChim platform at University of Rennes (Rennes, France) for GC-MS analysis. Sample preparation and derivatization were performed as

previously described in Colinet et al. (2016), with the following minor modifications. Briefly, after homogenization in 750 μL of ice-cold methanol-chloroform solution (2:1, v:v) and phase separation with 500 μL of ultrapure water, a 100 μL aliquot of the upper phase was vacuum-dried. The dry residue was resuspended in 30 μL of 20 mg mL^{-1} methoxyamine hydrochloride in pyridine before incubation under automatic orbital shaking at 40 $^{\circ}\text{C}$ for 60 min. Then, a volume of 30 μL of BSTFA was added, and the derivatization was conducted at 40 $^{\circ}\text{C}$ for 60 min under agitation. A CTC CombiPal autosampler (PAL System, CTC Analytics AG, Zwingen, Switzerland) 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 $^{\circ}\text{C}$ at 5 $^{\circ}\text{C min}^{-1}$, from 170 to 280 $^{\circ}\text{C}$ at 7 $^{\circ}\text{C min}^{-1}$, from 280 to 320 $^{\circ}\text{C}$ at 15 $^{\circ}\text{C min}^{-1}$, and then remained at 320 $^{\circ}\text{C}$ 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 μM concentrations were run concurrently. Chromatograms were deconvoluted using XCalibur 2.0.7, and metabolite levels were quantified using the quadratic calibration curve for each reference compound and concentration. Quality controls at concentrations of 200 μM were run every 20 samples. In this study, a total of 45 metabolites was detected and quantified.

2.5 Statistical analyses

To assess the effects of reproductive maturity status and biogeographical origin on flies' thermal tolerance, we used separate linear models for each cold tolerance metric (*i.e.*, CT_{min} and CCRT) employing the package “glmmTBM”, version 1.0.2.1, and the “glmmTBM” function (Brooks *et al.*, 2017). Reproductive maturity status and origin were considered as fixed and crossed factors. DHARMA package was used to test normality and heteroscedasticity (Hartig, 2020). We then used the package “emmeans” for *post hoc* multiple mean comparisons across groups (Lenght, 2021). For CCRT, data were log-transformed for normalization. Changes in the concentrations of each metabolite were analyzed by two-way ANOVAs using the function 'Anova' from the 'car' package (Fox and Weisberg, 2019), followed by *post-hoc* Tukey tests. Again,

reproductive maturity status and origin were considered as fixed and crossed factors. Metabolite concentrations were also analyzed using a principal component analysis (PCA) of the factextra R Package (Kassambara and Mundt, 2020). Data were scaled and mean-centered prior to the PCAs. To identify the metabolites contributing the most to PCA structure separation, the correlations to the principal components (PCs) were extracted and ranked. Then, the list of all significantly affected metabolites was used to run a Pathway Enrichment Analysis in Metaboanalyst 4.0 (Chong *et al.*, 2018), based on *D. melanogaster* reference metabolome.

3. Results

3.1 CT_{min}

CT_{min} was affected by reproductive maturity status ($p < 0.001$), biogeographic origin ($p < 0.001$) and their interaction ($p < 0.001$; see Table 1). According to *post-hoc* comparisons, reproductively immature females of temperate species had the lowest CT_{min} value with 1.02 ± 1.4 °C (mean \pm SD) (Figure 1). The rest of the experimental groups showed higher CT_{min} values, ranging from 3.7 to 4.5 °C (Figure 1).

3.2 *Chill coma recovery time*

CCRT was affected by reproductive maturity status ($p < 0.001$), biogeographic origin ($p < 0.001$) and their interaction ($p < 0.01$; Table 1). According to *post-hoc* comparisons, reproductively immature flies recovered faster than mature flies in both origins (Figure 1). For temperate species, either reproductively immature or mature females had a mean CCRT of less than four minutes; tropical species recovery took much longer (Figure 1). The fastest and slowest chill coma recovery times were observed for immature females of temperate species (2.2 ± 1.3 °C min), and in mature females of tropical origin (90.6 ± 48.7 °C min), respectively. After 12h at 0 °C, fly mortality was negligible, except for *D. venezolana* which exhibited a drop in survival of 38% for immature, and 58% for mature females.

3.3 *Metabolite concentrations*

3.3.1 *Univariate analysis*

Concentrations of all individual metabolites are available in the Supplementary Figures S3-9. Univariate analyses showed that 29 out of 45 metabolites significantly changed between biogeographic origins ($p < 0.05$) (temperate vs. tropical), and 28 out of 45 between immature vs. mature females (Supplementary Table S1). 15 out of 45 metabolites exhibited a significant origin-by-reproductive maturity status interaction ($p < 0.05$, see Supplementary Table S1). Metabolites were grouped according to the major metabolic classes they belong and corresponding figures are displayed in supplementary material (e.g., sugars, TCA intermediates, amino acids, see Figures S3-S9).

3.3.2 Multivariate Analysis

We investigated the changes in the concentrations of the 45 metabolites that were present in detectable amounts in all samples (listed in Supplementary Table S2) using PCA analysis, which clusters all treatments, and searches for statistically significant differences among them (Monte Carlo test, $P < 0.001$). The PCA resulting from the analysis of all metabolites is shown in Figures 2 and 3. Most experimental groups were differentiated within the first three planes of the PCA, which cumulated 63% of inertia. Differences among species (particularly among *D. buzzatii*, *D. koepferae* and *D. venezolana*) were partly explained along PC1, which accounted for 34.1% of total inertia. Also, tropical species (*D. borborema* and *D. venezolana*) and temperate species (*D. buzzatii* and *D. koepferae*) were clearly opposed along PC2, which accounted for 17.3% of total inertia (Figure 2). Finally, PC3 (11.3% of total inertia) captured differences related to the reproductive maturity. Reproductively immature and mature females had their centroids on the negative and positive values of PC3 in all species, respectively (Figure 3). For *D. borborema*, despite centroids were opposed along PC3, the ellipses partly overlapped between reproductively immature and mature; yet, these conditions were more clearly discriminated along the PC1 axis (Figure 2). Correlation values of all the metabolites to PC1, PC2 and PC3 are shown in Figures S10, S11 and S12, respectively. Figure 4 also shows the changes in concentration of the individual metabolites that were among the most correlated (negatively or positively) to these three PCs. The levels of several organic acids were positively correlated to PC1 (i.e., relatively more abundant in mature and immature *D. venezolana* or in mature *D. borborema* and *D. buzzatii*) (e.g., citric acid, malic acid) (Figure 4 and S10). Conversely, concentrations of several phosphorylated compounds (glycerol 3-P, fructose 6-P,

glucose 6-P) and polyols (glycerol, sorbitol, inositol) were negatively correlated to PC1. Glycerol was the only polyol that showed high (on average of 9.7 nmol/mg) and variable concentrations across origins and reproductive conditions (Figure 4). We also found low levels of sorbitol (0.1 nmol/mg) and inositol (0.2 nmol/mg) varying across origins and reproductive conditions (see Figure S7 and Table S1). Concerning the levels of TCA intermediates, low levels of malic and citric acids contrasted with high levels of succinic acid in species of both biogeographic origins and both maturity classes (Figure 4). Notably, several sugars were negatively correlated to PC2 (*i.e.* more abundant in temperate species) and other sugars positively correlated to PC2 (*i.e.* more abundant in tropical species). In effect, glucose and gluconolactone showed significantly higher abundances in temperate species compared to tropical ones (Figure 4). The opposed pattern was found for trehalose, with lower concentrations in temperate species compared to tropical ones (Figure 4). Also, we found high concentrations of alanine (on average of 34.7 nmol/mg) that significantly differed between tropical and temperate species. GABA and some free amino acids (leucine, lysine, valine and proline) were negatively correlated to PC3 (*i.e.*, relatively more abundant in immature flies) (Figure 4 and S12). While the concentration of GABA was relatively higher in immature than mature females of both origins, proline level (on average of 14.8 nmol/mg) was only higher in immature females of temperate species (Figure 4).

3.5 Pathway analysis

To identify biologically meaningful pathways linked to metabolites that changed between temperate and tropical species, a Metabolite Set Enrichment Analysis was performed. The analysis identified 12 significantly enriched pathways. These pathways are displayed in Figure S13. Additionally, we identified 12 pathways associated with reproductive maturity (Figure S14). Five pathways were shared between both factors, *i.e.* species and reproductive maturity: aminoacyl-tRNA biosynthesis, glycerophospholipid metabolism, glycerolipid metabolism, pentose and glucuronate interconversion and valine, leucine and isoleucine biosynthesis.

4. Discussion

The present study aimed to investigate how adaptation to different climates (temperate vs. tropical) has driven differences in cold tolerance and search for metabolic signatures correlated with cold adaptation. Also, we compared cold-exposed females that have

reached reproductive maturity or not (mature *vs.* immature). We expected that 1) temperate species and immature females would show lower cold tolerance than their tropical and reproductively mature counterparts, 2) metabolic profiles would be context-specific, *i.e.* the set of compounds related to cold adaptation (temperate *vs.* tropical) would differ from the metabolites involved in reproductive maturity status (mature *vs.* immature). Though our study does not provide information on how fluxes occur through metabolic pathways, metabolomics can be a useful tool to infer the relative importance of specific biochemical processes underlying cold tolerance (Enriquez *et al.*, 2018; Enriquez and Colinet, 2019a).

4.1 Cold tolerance differences between temperate and tropical species.

4.1.1 The putative role of osmoprotectants

Flies were cold-exposed to a relatively low temperature (10 °C), similar to the mean temperature of the coldest month found in the regions inhabited by the temperate *Drosophila* species used in this study, but differing from the one encountered by their tropical relatives (Figure S2). In temperate regions, the minimum temperature of the coldest month reaches values around zero degrees, and flies can fall into chill-coma under these conditions. As expected, immature females of temperate species had the lowest mean values of CT_{min} , while the rest of the experimental groups showed intermediate mean values of CT_{min} . Though both metrics of cold tolerance (*i.e.*, CCRT and CT_{min}) detected differences across species, CCRT was more sensitive in distinguishing temperate *vs.* tropical species. In effect, we found that temperate species recovered significantly faster from chill-coma than tropical ones, with immature females of temperate species exhibiting the shortest recovery times. The reason for this difference could be associated with the distinct capacity of temperate and tropical species to maintain haemolymph ion balance during acute cold-stress (MacMillan *et al.*, 2015). Indeed, it has been shown in tropical *Drosophila* species that haemolymph ion balance collapse triggers a loss in muscle membrane potential and delayed recovery time, whereas in temperate species, the stability of ion balance results in fast recovery from coma by the accumulation of compatible protective solutes (Olsson *et al.*, 2016). The analysis of the metabolic profiles could distinguish tropical *vs.* temperate species (along PC2), and the metabolites associated with this clear-cut opposition included glucose, alanine, and gluconolactone, which were all more abundant in temperate

species. We reasoned that glucose and alanine could potentially serve as intermediates in pathways involved in the synthesis of protective solutes. In fact, modest amounts of many cryoprotectants can also act as osmoprotectants (MacMillan *et al.*, 2015). For example, *D. melanogaster* selected for fast CCRT synthesized amino acids (*e.g.*, glutamic acid, alanine, serine and proline) and glucose at a faster rate than lines selected for slow CCRT (Williams *et al.*, 2016), suggesting that the rapid synthesis of cryoprotectants/osmoprotectants can represent a metabolic signature of cold adaptation. Interestingly, glycerol and proline were relatively more abundant in immature females of temperate species (*i.e.*, the most cold-tolerant ones) compared to the levels exhibited by the tropical species or mature females of temperate origin. Even if there were significant variations, the concentrations observed (on average of 14.8 nmol/mg for proline, and 9.7 nmol/mg for glycerol) remained too low to speculate about any colligative effect. Yet, it is established that these osmolytes can protect and stabilize the native macromolecular structures of proteins and biological membranes by various mechanisms when they are at low concentrations (Yancey 2005; Overgaard *et al.*, 2007). We also noted a low level of glyceric acid in temperate *Drosophila* species, which could be due to its conversion into glycerol *via* oxidation. Also, gluconolactone, an oxidized derivative of glucose, exhibited a higher concentration in temperate species. As gluconolactone results from the degradation of glucose through the pentose phosphate pathway (Garrett and Grisham, 1999), our results collectively suggest that this pathway was boosted in temperate cold-exposed females, as indicated in the enrichment analysis (Figure S13). A similar finding has been reported earlier from diapausing mites (Khodayari *et al.*, 2013). In short, our findings suggest that cold tolerance is associated with an increased abundance of candidate protective solutes like glycerol and proline. However, whether the ionic balance is actively maintained through osmoprotection by these metabolites or only represents metabolite variations in the absence of a causal relationship needs to be tested in future studies.

4.1.2 The role of energy metabolism

An interesting feature captured by CT_{min} and CCRT was that immature females had higher cold tolerance than mature ones, a pattern that was particularly clear in temperate species. Several lines of evidence support the fact that the immature stage is likely the most common overwintering stage in the temperate species *D. buzzatii* and *D. koepferae*. For example, after a prolonged period of cold exposure the fertility of

overwintered immature females is superior in comparison with mature ones (Mensch *et al.*, 2017) and also compared to mated females (Kreiman and Mensch, personal communication). In addition, immature females of temperate species are characterized by an increased lifespan, remarkably reaching three months under winter-like conditions, and thus matching the duration of the winter season (Kreiman and Mensch, personal communication). In this context, our results are in agreement with the Y-model of resource allocation, which states that limited energy resources are allocated either to survival or reproduction (Harshman and Zera, 2007; Kalra and Parkash, 2014). As a consequence, the accumulation of energy reserves for overwintering, but also subsequent post-overwintering activities, is expected to be a metabolic feature that promotes winter survival. We suggest that the capacity for delayed reproduction of immature females could result in the use of some energy metabolites (*e.g.*, glucose, alanine) for the synthesis of osmoprotectants (see 4.1.1) and/or for surviving long cold winters.

In females of temperate origin, we found increased levels of several glycolysis-related metabolites: glucose, gluconolactone, lactic acid and alanine were more abundant, while glyceric acid was less abundant in temperate cold-exposed flies. The higher level of alanine and lactic acid may suggest partial entry into anaerobic metabolism at cold (Feala *et al.*, 2007), but it can also be the result of the aerobic glycolysis that can occur in both larvae and adults (Tennesen *et al.*, 2014; and Krejčová *et al.*, 2019). Another interesting feature was the variation among the concentration of TCA intermediates: in all cases, low levels of malic and citric acids contrasted with high levels of succinic acid. This pattern may indicate a partial stalling of certain steps within the TCA cycle at cold, which would be consistent with the metabolic activity reduction observed in immature females of temperate origin (Mensch *et al.*, 2017). Such energy conservation should be particularly important for surviving cold seasons like the ones temperate species are exposed to. Thus, it would be useful in future studies to quantify the activity of the glycolytic and TCA pathways in flies not exposed to low temperature to distinguish whether the metabolic effects observed here also occur in flies independently of the exposure to low temperature.

4.2 Reproductively immature females display higher cold tolerance: possible role of GABA

Entering into coma (*i.e.*, at CT_{min}) is associated to spreading depolarization, a general silencing of the central nervous system (CNS), followed shortly by a loss of muscle function (Andersen and Overgaard, 2019). Thus, neuromuscular dysfunction (*i.e.*, loss of muscle membrane potential) could be buffered in immature females by a neuroprotective mechanism. One intriguing result was the increased levels of gamma-aminobutyric acid (GABA) in immature flies compared to mature ones. GABA was the most highly correlated metabolite to PC3, associated to reproductively immature females. GABA is synthesized by decarboxylation of glutamic acid, which was also among the most correlated to PC3 and relatively more abundant in immature flies. For many years, it has been known that GABA accumulates in plants upon various abiotic stress, including exposure to saline and cold environments (Kinnersley and Turano, 2000; Mazzucotelli *et al.*, 2006), but its role in insects' cold tolerance remains uncharted. GABA is a major inhibitory neurotransmitter in insects and is widely distributed in the central nervous system (CNS) (Wilson and Laurent, 2005). Interestingly, several genes coding for neurotransmitter GABA receptors or transporters were up-regulated in *D. sukuzii* or *Gryllus pennsylvanicus* after cold acclimation (Des marteaux *et al.*, 2017; Enriquez and Colinet, 2019b). Several lines of evidence suggest that paralysis during cold exposure results from direct inhibitory effects of low temperature on neuromuscular function (*e.g.*, Des marteaux *et al.*, 2017; Andersen *et al.*, 2018). Thus, we speculate that increased concentrations of GABA may buffer depolarization within CNS, resulting in a neuroprotective mechanism that lowers CT_{min} of immature females. It is worth mentioning that in the present study, all metabolites were extracted only from the heads of the flies, a fact that is especially relevant here, as GABA modulation plays a significant role in the CNS.

We cannot rule out the possibility, that differences in cold tolerance between mature and immature females might be partially attributed to a slightly age-related difference. Indeed, several studies support an age-dependent decline in thermal tolerance even at a young age (Colinet *et al.*, 2013, 2016; Xue and Ma, 2020). While the reproductively immature females were 12 days-old flies, the mature ones were 17 days old, since mature females were maintained at 25 °C for 5 days post-emergence to allow full ovarian maturation before being cold-exposed for 12 days as the immature flies. It is worth noticing that females of both experimental groups were virgins, so mating status

cannot explain differences, as observed in other studies (Javal *et al.*, 2016; Xue and Ma, 2020).

4.3 Evolution of cold tolerance in cactophilic flies

Our findings provide evidence that under winter-like conditions, temperate species showed higher levels of glucose, alanine and gluconolactone than tropical ones. Such energetic metabolites are probably utilized in surviving long cold seasons or in the synthesis of osmoprotectants, such as proline and glycerol. Our results also suggest that the modulation of glycolysis- and TCA-related metabolites may have contributed to the climate adaptation of flies and, thus, the expansion of *D. buzzatii* and *D. koepferae* towards greater latitudes and pre-Andean highlands. In this context, we may ask whether cold-hardy species acquired mechanisms of cold tolerance independently (*i.e.*, adaptive convergence) or, if mechanisms of cold tolerance were acquired in their common ancestor, and then lost in the lineage leading to *D. borborema*. Further experiments including more species of both the *martensis* and the *buzzatii* clusters, and other cold treatments evaluating metabolites during and after acute cold exposure are necessary to differentiate between these hypotheses.

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Data Accessibility

The raw data can be accessed through the following link:

doi:10.6084/m9.figshare.14607876.v1

Conflict of interest

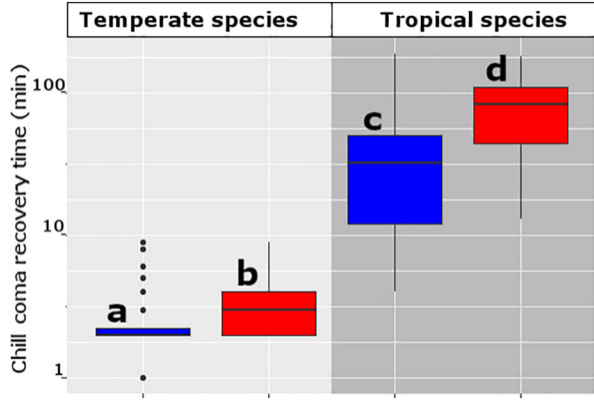
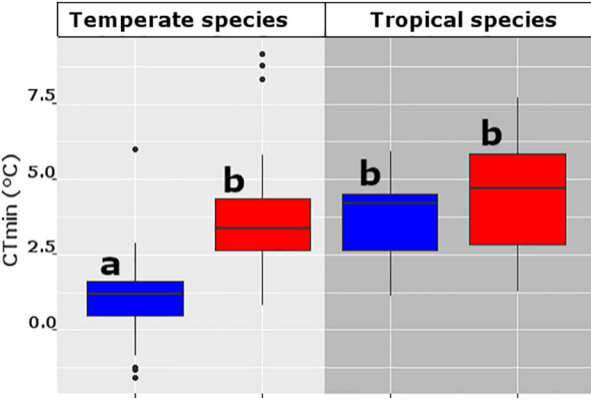
We declare no conflict of interest.

Figure 1. Cold tolerance of tropical vs. temperate species exhibiting different reproductive maturity status (mature in red vs. immature in blue). All groups were scored for cold tolerance after spending 12 days at mild cold conditions (see Material and methods). **Left panel:** CT_{min} of the different experimental groups. **Right panel:** Chill coma recovery time (CCRT) of the different experimental groups after spending 12h at 0 °C. Different letters indicate significant differences in Tukey tests ($P < 0.05$).

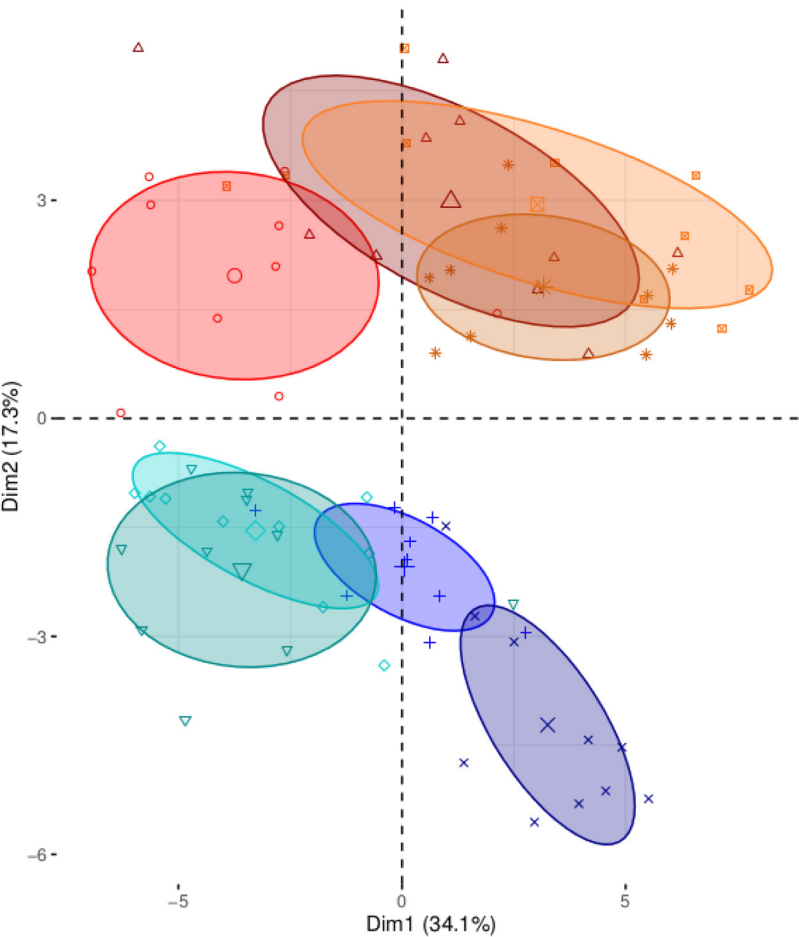
Figure 2. Principal component analyses (PCA) displaying the two first components (PC1 vs. PC2). The analysis was based on 45 quantified metabolites detected across all treatments combinations (*i.e.*, 4 species x 2 reproductive maturity status). Temperate species: *D. koe* for *D. koepferae* [cyan], *D. buz* for *D. buzzatii* [blue]; tropical species: *D. bor* for *D. borborema* [red] and *D. ven* for *D. venezolana* [orange]. Within species: letter I for immature and letter M for mature flies.

Figure 3. Principal component analyses (PCA) displaying the second and third components (PC2 vs. PC3). The analysis was based on 45 quantified metabolites detected across all treatments combinations (*i.e.*, 4 species x 2 reproductive maturity status). Temperate species: *D. koe* for *D. koepferae* [cyan], *D. buz* for *D. buzzatii* [blue]; tropical species: *D. bor* for *D. borborema* [red] and *D. ven* for *D. venezolana* [orange]. Within species: letter I for immature and letter M for mature flies.

Figure 4. Concentration changes of the individual metabolites among the most correlated to the three first PCs of the PCA. **(A-B)** Metabolites positively correlated to PC1; **(C-E)** Metabolites negatively correlated to PC1; **(F-H)** Metabolites negatively correlated to PC2; **(I)** Metabolite positively correlated to PC2; **(J-L)** Metabolites negatively correlated to PC3. Blue boxes depict immature females and red boxes mature ones.



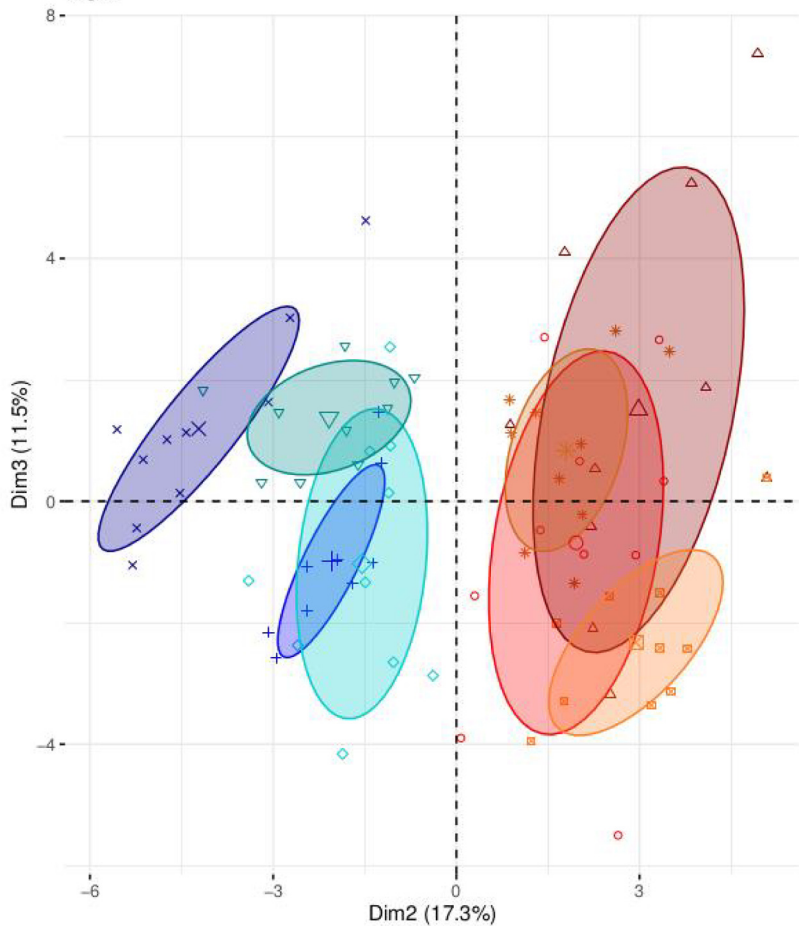
PCA



Groups

Tropical speciesTemperate species

PCA



Groups

Tropical speciesTemperate species

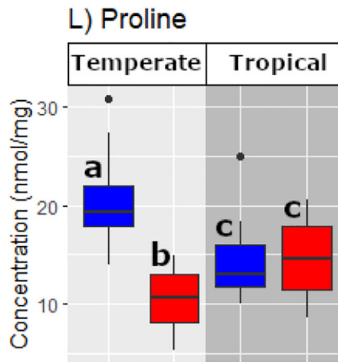
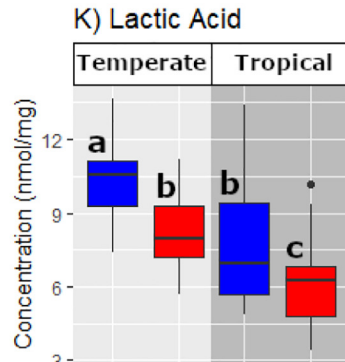
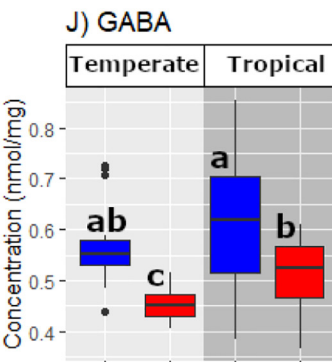
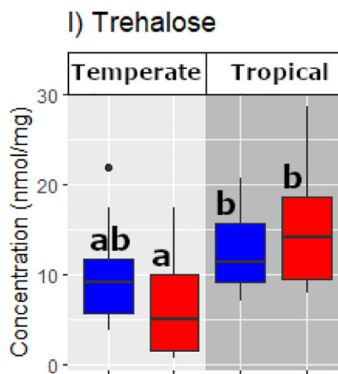
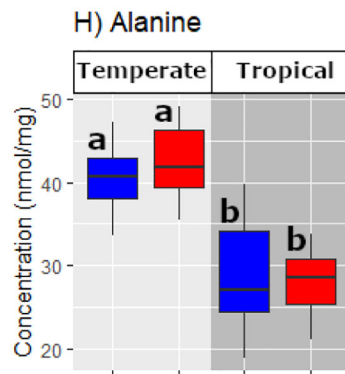
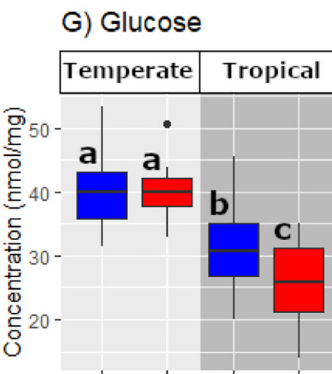
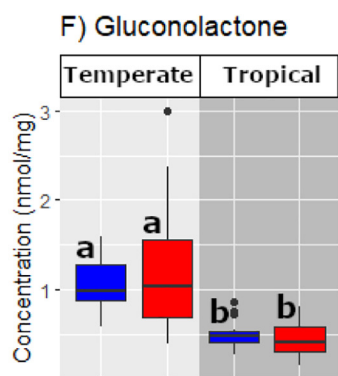
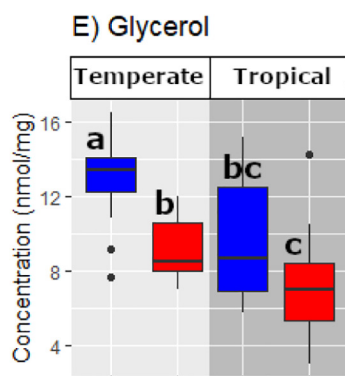
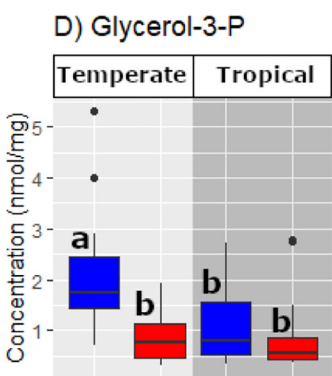
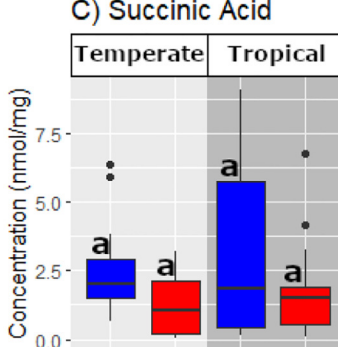
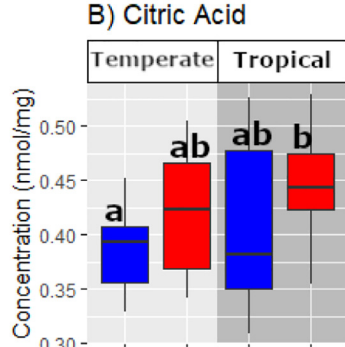
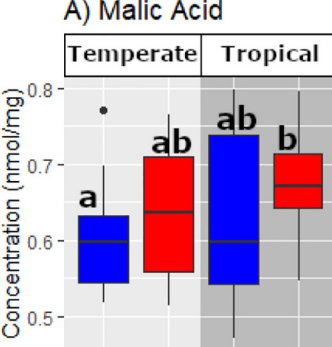


Table 1. Analysis of variance of Chill coma recovery time (CCRT) and CT_{min}

CT_{min}

Variables	df	p-value
Reproductive maturity status	1	5.34*10 ⁻¹⁵
Origin	1	2.2*10 ⁻¹⁶
Interaction	1	0.0001

CCRT

Variables	df	p-value
Reproductive maturity status	1	2.2*10 ⁻¹⁶
Origin	1	2.2*10 ⁻¹⁶
Interaction	1	0.0023

Supplementary data

Supplementary material

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Graphical abstract

Highlights

- Reproductive maturity status (immature vs. mature) affects cold tolerance
- Immature females of temperate origin display the highest cold tolerance
- Glucose show the highest levels across all metabolites in temperate species
- Immature females of temperate species accumulate proline and glycerol
- Metabolic cold adaptations are revealed by comparing immature and mature females

