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Larvae of *Drosophila melanogaster* exhibit transcriptional activation of immune response pathways and antimicrobial peptides during recovery from supercooling stress

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ABSTRACT

The biochemical and molecular mechanisms underlying insect cold acclimation prior to cold stress are relatively well explored, but the mechanisms linked to recovery and repair after cold stress have received much less attention. Here we focus on recovery from cold stress in the larvae of the vinegar fly (*Drosophila melanogaster*) that were exposed to two physiologically distinct cold stress situations: supercooling (S, survival > 95%) and freezing (F, survival < 10%), both at −5 °C. We analysed the metabolic and transcriptomic responses to cold stress via GC-MS/LC-MS and whole-genome microarrays, respectively. Both stresses (S and F) caused metabolic perturbations which were transient in supercooled larvae but deeper and irreversible in frozen larvae. Differential gene expression analysis revealed a clear disparity in responses to supercooling and freezing (less than 10% of DE genes overlapped between S and F larvae). Using GO term enrichment analysis and KEGG pathway mapping, we identified the stimulation of immune response pathways as a strong candidate mechanism for coping with supercooling. Supercooling caused complex transcriptional activation of innate immunity potential: from Lysozyme-mediated degradation of bacterial cell walls, recognition of pathogen signals, through phagocytosis and lysosomal degradation, Toll and Imd signaling, to upregulation of genes coding for different antimicrobial peptides. The transcriptomic response to freezing was instead dominated by degradation of macromolecules and death-related processes such as autophagy and apoptosis. Of the 45 upregulated DE genes overlapping in responses to supercooling and freezing, 26 were broadly ascribable to defense and repair functions.

1. Introduction

Insects are excellent animal models for studies on cold hardiness–the capacity to survive subzero body temperatures (Denlinger and Lee, 2010; Lee and Denlinger. 1991). Two broad categories of cold hardiness are commonly distinguished: insects may (i) supercool, i.e. maintain the body water in a liquid phase at subzero temperatures (the most common strategy) or (ii) survive freezing, i.e. survive formation of ice crystals inside the body (Salt, 1961; Sinclair et al., 2003; Zachariassen, 1985). This simple dichotomous classification, although long-debated in the field, will be sufficient for the purpose of the present study. In the scientific literature, considerable attention has been paid to analysis of physiological mechanisms underlying insect cold hardiness. Most studies have focused on physiological changes that are triggered by either seasonal acclimation and acclimatization (lab- and field-studies, respectively) or rapid cold hardening, i.e. *prior* to cold stress (see reviews; Lee, 2010; Overgaard and MacMillan, 2017; Teets and Denlinger, 2013; Toxopeus and Sinclair, 2018; Zachariassen, 1985). Such changes were mostly interpreted as preventing the occurrence of cold injury (i.e. increasing *resistance* to cold injury). For instance, accumulation of antifreeze proteins prevents freezing occurrence (Duman, 2015), accumulated small cryoprotective molecules stabilizes proteins and other

Abbreviations: DE, differential expression; F, freezing; FTR, fluctuating thermal regime; GO, gene ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; PRC, principal response curve; RDA, redundancy analysis; S, supercooling

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macromolecular structures (Timasheff, 2002), membrane restructuring can protect against unregulated phase transitions (Koštál, 2010), and altered activity of ion pumps and channels can prevent dissipation of membrane potentials upon cold exposure (Bayley et al., 2018). In addition to resistance mechanisms, cold hardiness also depends on an insect's ability to *tolerate* and/or repair the incurred injury (Koštál et al., 2019). For example, a rapid upregulation of various heat shock proteins (HSPs) is often observed after cold stress, and it is believed that this response helps in the re-folding or removal of proteins that were partially denatured during the cold stress (Rinehart et al., 2007; Štětina et al., 2018; Koštál and Tollarová-Borovanská, 2009; Zhao and Jones, 2012). The activation of tolerance mechanisms often requires energy allotments that may result in impaired fitness (e.g. decreased reproductive output) in cold stress survivors (Coulson and Bale, 1992). Hence, considering both resistance and tolerance mechanisms, the survival *and* the fitness of survivors dictate the final outcome of cold stress (Koštál et al., 2019). We argue that the contribution of tolerance mechanisms to insect cold hardiness is relatively understudied and we hope to ignite more interest in the analysis of physiological events *after* the cold stress, during the recovery period.

Here, we analyse the cold stress response of the vinegar fly (*Drosophila melanogaster*), and use untargeted metabolomics and transcriptomics approaches to obtain insight into the processes potentially involved in repair of cold injury. Specifically, we use the larval stage of *D. melanogaster*, for which detailed knowledge about cold hardiness was obtained in our earlier studies (Koštál et al., 2011, 2012; 2016a, 2016b; Rozsypal et al., 2018; Štětina et al., 2015). As a species of tropical origin, the vinegar fly is relatively susceptible to cold stress (Strachan et al., 2010). Nevertheless, it was shown that various simple laboratory acclimations, such as rapid cold hardening (a brief pre-treatment at sublethal low temperature prior to cold stress), long-term developmental cold acclimation (rearing the insects at a relatively low but developmentally-permissible temperature), and induction of quiescence (by exposing the larvae to temperatures just below the lower developmental threshold), dramatically increase the resistance of *D. melanogaster* to cold stress (Czajka and Lee, 1990; Jensen et al., 2007; Koštál et al., 2016a, 2016b; Rajamohan and Sinclair, 2008; Rako and Hoffmann, 2006). To explain this acclimation-linked increase in cold hardiness, several resistance mechanisms have been suggested for *D. melanogaster* (although most await direct functional validation): the accumulation of cryoprotective metabolites (such as proline and trehalose) (Koštál et al., 2011); modification of membrane phospholipid composition (Koštál et al., 2011; Overgaard et al., 2005, 2008); adjustments to the ionoregulatory system (MacMillan et al., 2015). Among potential tolerance mechanisms, the upregulation of HSP expression after cold stress received relatively high attention. In addition to several descriptive studies (Colinet et al., 2010a; Goto et al., 1998; Sinclair et al., 2007; Qin et al., 2005), functional validations were performed using genetically manipulated flies with altered expression of HSPs. However, the outcomes of validation studies were not straightforward; some suggested a weak role for HSPs in recovery after cold stress (Colinet et al., 2010b, 2010c; Štětina et al., 2015), while the others failed to detect a significant influence of HSPs on survival after cold stress (Nielsen et al., 2005; Udaka et al., 2013). The transcriptional activation of HSPs is a typical short-term response to cold stress. In the present study, by sampling insects 24 h after the cold stress we avoided the time window during which the transcriptional activation of HSPs is observed (Štětina et al., 2015). In this way, we hoped to detect other, long-term tolerance mechanisms that might be involved in repair of cold stress injury.

In the present study, we focus on recovery from cold stress in the cold-acclimated, quiescent larvae of *D. melanogaster* that were exposed to two physiologically distinct cold stress situations; supercooling (S) and freezing (F), both at −5 °C. Control larvae (C, not exposed to cold stress) served as a reference. The untargeted approach was used to describe the metabolic (via GC-MS/LC-MS analyses) and transcriptomic (via whole-genome microarrays) responses to cold stress linked to either successful recovery (> 95% survival to adult stage in supercooled larvae) or unsuccessful recovery (> 90% delayed mortality in frozen larvae). We asked two major questions: (i) How do the larvae survive after supercooling? Do they *resist* (avoid) the cold injuries (?), in which case we would not expect to see many differences between supercooled larvae and controls. Alternatively, do they *tolerate* (repair) cold injury (?), in which case we expected to obtain hints of what was damaged and/or what the repair mechanisms are. (ii) What characterizes the larvae exposed to freezing stress? Do they exhibit metabolic and transcriptomic responses that overlap with those observed in supercooled larvae (?), which would reveal general mechanisms that are induced irrespectively of the nature of the cold stress. Or do they show freezing-specific responses (?), which would mainly indicate the changes linked to severe freezing injury and mortality, typically occurring with a delay (> 1 day after stress).

2. Materials and methods

2.1. Insects and experimental design

All experiments were conducted in parallel with another study (Koštál et al., 2019) which focused on survival and fitness parameters of adults subjected to cold stress as larvae. The culture of vinegar flies, *Drosophila* (*Sophophora*) *melanogaster* of Oregon R strain (Lindsley and Grell, 1968) was maintained at a constant 18 °C and LD cycle of 12 h L/12 h D on agar-sugar-yeast-cornmeal diet in MIR 154 incubators (Panasonic Healthcare, Gunma, Japan). For experiments, the larvae were acclimated at a constant 15 °C and LD cycle until the age of 11 days (pre-wandering stage) and then moved to acclimation conditions under constant darkness and a fluctuating thermal regime (FTR) of 6 °C for 20 h/11 °C for 4 h, for three days. Under FTR conditions, larvae o *D. melanogaster* enter quiescence (i.e. developmental arrest induced directly by low temperature) and their cold hardiness increases (Koštál et al., 2016b). Note that control larvae (C) also experienced the FTR treatment in order to 'subtract' the effects of FTR on metabolic networks and gene expression patterns.

For a schematic depiction of the sampling plan and experimental design, see Fig. 1. The quiescent larvae were washed out of the diet and the first sample of larvae for metabolomics analysis (a pool of 10 larvae in each of 4 biological replicates) was taken prior to cold stress (at time

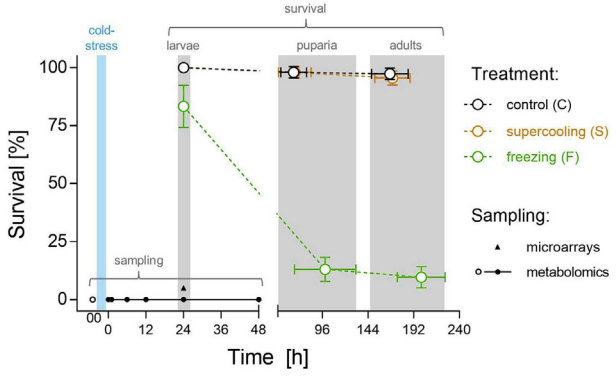


Fig. 1. Sampling plan and design of the experiment on metabolic and transcriptomic responses to cold stress in larvae of *Drosophila melanogaster* (S, supercooling; F, freezing; C, unstressed control). The first sample for metabolomics was taken prior to cold stress (time 00) and other samples were taken during recovery after cold stress (at 0, 1, 6, 12, 24, and 48 h). A single sample for microarray analysis was taken at 24 h of recovery after cold stress. The data on survival after the supercooling and freezing stresses were taken from a parallel study (Koštál et al., 2019). Each point represents a mean ± SD percent survivors and also a mean ± SD time of developmental transition (n = C, 300, C; S, 280, S; F, 900 larvae).

00). All remaining larvae were exposed to cold stress or served as controls. Control larvae were directly transferred into a new rearing vial with fresh larval diet and placed at constant 15 °C (i.e. no cold stress). Cold-stressed larvae were exposed to −5 °C as described elsewhere (Koštál et al., 2019):

- supercooling (S): 0 °C for 1 h, followed by ramping down to −5 °C over 30 min, maintaining at a constant −5 °C for 60 min, then ramping up to +5 °C over 40 min; or
- freezing (F): 0 °C for 1 h, followed by inoculative freezing stimulated by a small ice crystal, and ramping down to −2.5 °C over 180 min, then ramping down to −5 °C over 30 min, and finally ramping up to +5 °C over 40 min.

Larval body fluids remained liquid throughout the S treatment, and were partially frozen in the F treatment (Koštál et al., 2012; Rozsypal et al., 2018). In the parallel study (Koštál et al., 2019), analogously treated larvae were maintained to score their survival to puparial and adult stages, and to follow fitness parameters in survivor adults. The survival data are reproduced in Fig. 1 for clarity.

The cold-stressed larvae were moved to new rearing tubes with fresh diet and placed at constant 15 °C and LD cycle. Samples for metabolomics were taken at 0 h (immediately after cold stress), 1 h, 6 h, 12 h, 24 h, and 48 h. Pools of 10 larvae (in each of 4 biological replicates) were washed out of the diet, quickly blotted on cellulose, transferred to 1.5 mL Eppendorf microvials (Thermo Fisher Scientific), weighed (fresh mass, FM), killed in liquid nitrogen, and stored at −80 °C until analysis. Samples for microarray analysis were taken at a single time, 24 h after cold stress. Pools of 10 larvae (in each of 3 biological replicates) were washed out of the diet, quickly blotted on cellulose, transferred to 1.5 mL microvials with 400 µL of RNA Extraction reagent (Amresco, Solon, OH, USA), cut to small pieces using fine scissors, and stored at −80 °C until analysis.

2.2. Metabolomics analysis

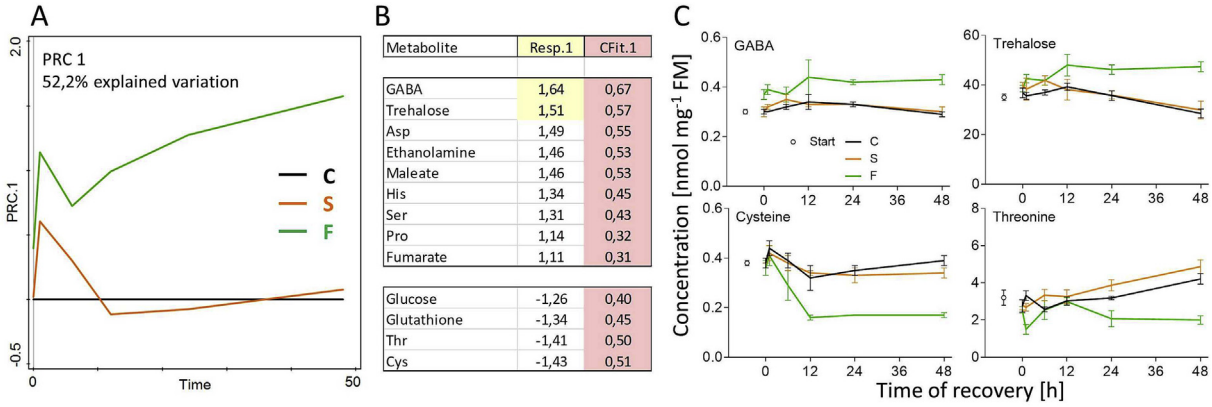
Larval metabolic composition was analysed using a set of chromatography methods coupled with mass spectrometry as described earlier (Štětina et al., 2018). Briefly, larvae were homogenized in a methanol:acetonitrile:water solution (volumetric ratio, 2:2:1) containing internal standards (*p*-fluoro-DL-phenylalanine, methyl α-D-glucopyranoside; Sigma-Aldrich, Saint Luis, MI, USA). Sugars and polyols were determined after *o*-methyloxime trimethylsilyl derivatization using a gas chromatograph (GC) with flame ionization detector GC-FID-2014 equipped with an AOC-20i autosampler (both from Shimadzu Corporation, Kyoto, Japan). Profiling of acidic metabolites was performed after treatment with ethyl chloroformate under pyridine catalysis and simultaneous extraction in chloroform (Hušek and Šimek, 2001). The analyses were conducted using a Trace 1300 GC combined with single quadrupole mass spectrometry (ISQ) (both from Thermo Fisher Scientific) and a liquid chromatograph Dionex Ultimate 3000 coupled with a high resolution mass spectrometer Q Exactive Plus (all from Thermo Fisher Scientific). All metabolites were identified against relevant standards and subjected to quantitative analysis using a standard calibration curve method. All standards were purchased from Sigma-Aldrich.

The results of metabolomics (summarized in the Supplementary Information, Dataset S1) were subjected to statistical analysis based on a constrained linear ordination method called redundancy analysis (RDA, Van den Wollenberg, 1977) using Canoco software, v5.04 (ter Braak and Šmilauer, 2012). RDA method computes ordination axes that summarize the effect of chosen predictors on the multivariate response data (here the metabolite concentrations). Concentrations of metabolites were first log-transformed (using a $ln(100*y + 1)$ formula) and then centered ($y'_i = y_i - \bar{y}$, where $\bar{y}$ is the mean of y_i values) and standardized ($y'_i = y_i/s_y$, where s_y is the standard deviation of y_i values). We used a specialized type of RDA called principal response curves (PRC), which focuses on time-dependent treatment effects on multivariate response data (Gaffney et al., 2018; Štětina et al., 2018; Van den Brink and ter Braak, 1999). Although the PRC analysis detects leading patterns in temporal metabolic profiles, its main difference from other similar tools, such as DBSCAN (Ester et al., 1996; Lehmann et al., 2018) is that the PRC analysis focuses on identification of *when* and *how* these profiles differ among different treatments (in our case C vs. S vs. F). Positions of treatment-time interaction terms on first and higher axes of the underlying partial RDA (with the main effect of time used as a covariate) are plotted against time, yielding one PRC curve for each experimental variant and axis. In addition, the temporal pattern of metabolic changes in the control variant was set as a reference value (0) and the temporal patterns of the cold-stressed treatments (S, F) therefore represent differences from the control.

2.3. Microarray analysis

Differential expression of *D. melanogaster* genes in response to cold stress was assayed using the FL003 *D. melanogaster* microarrays designed by the Flychip facility in Cambridge (http://www.flychip.org.uk/). Each microarray contains 18,240 spots, composed of transcript-specific oligonucleotides and controls developed by the International *Drosophila* Array Consortium (INDAC). A complete list of the 14,183 assayed transcripts represented on each microarray is shown in the Supplementary Information, Dataset S2. The methods were described in detail earlier (Koštál et al., 2017). Briefly, total RNA was isolated from larvae using Ribozol and treated with DNase I (Invitrogen, Carlsbad, CA, USA). First-strand cDNA was synthesized using Superscript III reverse transcriptase (Invitrogen). Second-strand DNA was synthesized using DNA Polymerase I and *E. coli* Ligase (both from Invitrogen) and the resulting DNA was purified with the Wizard SV Gel and PCR Clean-up-System (Promega, Madison, WI, USA). Next, one µg of double-stranded DNA was taken for Klenow labeling using Cyanine-5 dCTP (Cy5, Perkin Elmer, Waltham, MA, USA) and BioPrime DNA Labeling System (Invitrogen). Unincorporated dye was removed using the Illustra AutoSeq G-50 Dye Terminator Removal Kit (GE Healthcare). The Cy5-labeled DNA sample was blocked with sonicated salmon sperm DNA (Invitrogen), and used for hybridization on the microarrays. An automatic Hybridization Station HS4800 Pro (Tecan, Mannedorf, Switzerland) was used to perform all hybridization steps. All microarrays were scanned using the ScanArray Gx Microarray Scanner (PerkinElmer) at a resolution of 5 µm and the fluorescence was quantified using ScanArray Express v4.0.0.0004 software (Perkin Elmer).

The microarray signals passed multiple quality checks using R-based transcriptomics data preprocessor software RobiNA (Lohse et al., 2012). Using the same software, the log2 fluorescence values, normalized using quantile normalization (Supplementary Information, Dataset S2), were obtained from ScanArray data files. To minimize the risk of having transcripts with low fluorescence values showing high fold changes, no background subtraction was done. The log2 fluorescence values were first subjected to RDA statistical methods using Canoco software, v5.04 (ter Braak and Šmilauer, 2012) in order to assess clustering of transcriptional profiles according to treatment (C, S, F). Next, pairwise comparisons (S vs. C and F vs. C) were conducted using the limma R package (Ritchie et al., 2015). Genes with absolute fold changes > 1.5 fold and Benjamini and Hochberg adjusted P-value (FDRs, false discovery rates) < 0.1 were considered significantly differentially expressed (DE). Then, Gene Ontology (GO) and KEGG Pathway Enrichment Analysis were conducted on the DE genes using the R package ClusterProfiler (Yu et al., 2012). GO term categories with a minimum count of five significant DE genes and adjusted P-value < 0.1 were considered significantly enriched. KEGG pathways with adjusted P-value < 0.05 were considered significantly enriched. The Pathview R package (Luo and Brouwer, 2013) was then used to visualize the DE genes together with their fold change expression on KEGG pathway



Metabolite	Resp.1	CFit.1
GABA	1,64	0,67
Trehalose	1,51	0,57
Asp	1,49	0,55
Ethanolamine	1,46	0,53
Maleate	1,46	0,53
His	1,34	0,45
Ser	1,31	0,43
Pro	1,14	0,32
Fumarate	1,11	0,31
Glucose	-1,26	0,40
Glutathione	-1,34	0,45
Thr	-1,41	0,50
Cys	-1,43	0,51

Fig. 2. Summary of metabolic PRC 1-type response to cold stress in *Drosophila melanogaster* larvae (see section 2.2. for explanation of PRC analysis). (**A**) The metabolic changes in control (C) larvae were set as the reference value (0) and the temporal patterns (curves) of responses to supercooling (S) and freezing (F) represent a difference from the control. (**B**) Numeric results of PRC analysis for the most important metabolites– drivers of the PRC 1-type response. The parameter 'Resp.' describes the magnitude of response of a given metabolite in the statistical model; the parameter 'Cfit.' describes the proportion of explained variance by the statistical model (see Table S1 in Supplementary Information for complete results). (**C**) Examples of real temporal profiles in concentrations of four select metabolites most representative of the PRC 1-type response.

maps.

3. Results

3.1. Metabolome profiling

The results of metabolomics analysis of 35 different compounds are given in Dataset S1. Fig. S1 exhibits temporal profiles for three prominent amino acids: Asn, Gln, and Pro, which were present in relatively high concentrations in larvae, but did not rank very highly in the PRC analysis. The temporal profiles of Asn were practically equal in all three treatments. The profiles of Gln and Pro differed between treatments: the profile for frozen larvae deviated from the profiles for control and supercooled larvae, which were closely similar. The same major theme, a similarity of C and S profiles vs. deviation of the F profile, emerged from the comparison of the temporal trends in total (sum) concentrations of all targeted metabolites (Fig. S2), and also from the PRC analysis (presented in detail in Table S1).

The first set of PRC curves, explaining 52.2% of fitted variation in the metabolomics dataset, is shown in Fig. 2. The PRC 1 set clusters the metabolites exhibiting two clearly different temporal profiles: one similar for control and supercooled larvae vs. one for frozen larvae. In supercooled larvae there was an initial perturbation (until 12 h after cold stress), but the concentrations later matched the values observed in controls. In frozen larvae, these same metabolites showed a clear trend toward increasing deviation from controls over time (Fig. 2A). The list of important drivers of PRC 1-type response is shown in Fig. 2B (for more detail, see Table S1). Examples of temporal courses in the four most important metabolites according to PRC 1 set are given in Fig. 2C.

The other sets of PRC curves had much less explanatory power than PRC 1. The PRC 2 response (15.6% of explained variation) is depicted in Fig. 3 and the PRC 3 response (10.4% of explained variation) is depicted in Fig. S3. In supercooled larvae, the metabolites driving the PRC 2 response showed initial perturbations but finally their concentrations matched the values observed in controls. In frozen larvae, the PRC response drivers exhibited a transient peak at 12 h of recovery (for instance: alanine, lactate, and succinate in Fig. 3C), and/or a gradual decline far below the values of controls (for instance: glucose in Fig. 3C).

3.2. Gene transcript profiling

The results of microarray analyses of responses of 14,183 mRNA transcripts to cold stress (taken at 24 h after cold stress) are summarized in the Supplementary Information, Dataset S2. The transcriptomic responses significantly differed among treatments. The RDA found distinct clustering according to treatments (Fig. 4A), and suggested that the global transcriptomic responses to two different stresses, supercooling and freezing, share relatively little in common. The partial similarity between the two cold stress responses was detectable as a shift of both the S-cluster and F-cluster from the control cluster (C) along axis 2. Axis 2, however, explained only 16.9% of variation and, moreover, there were high within-treatment variations along the axis. The prevailing dissimilarity of responses to supercooling and freezing is suggested by their clear separation and shifts to opposite directions along axis 1 (37.1% explained variance).

The analysis of differential expression (for detailed summary see Supplementary Information, Tables S2A–D) identified 282 DE genes (227 upregulated; 55 downregulated) in response to supercooling, and 476 DE genes (256 upregulated; 220 downregulated) in response to freezing (both compared to control). Only 45 upregulated genes (10.3% of total) overlapped between responses to supercooling and freezing. The overlap was even smaller (2 genes; 0.7%) for downregulated genes (Fig. 4B, see Supplementary Information, Table S3 for a complete list of overlapping upregulated genes). These results confirmed the prevailing disparity of responses to supercooling and freezing.

The GO term enrichment analysis helped to identify biological processes in which the responses to supercooling and freezing either differ or overlap. Firstly, the GO terms related to chitin metabolism, cuticle development, and also the term 'response to toxic substance' were enriched among upregulated genes after both supercooling and freezing. The other terms that were enriched among upregulated genes differed between two stresses. Thus, the defense and immune responses were upregulated after supercooling, while a number of different pathways, dominated by amino acid catabolism, were upregulated after freezing (Fig. S4). Secondly, the terms linked to actin polymerization were enriched among downregulated genes after both supercooling and freezing. The processes related to muscle and cuticle development, cell division, phosphorylation, and phosphate metabolism were specifically downregulated after supercooling. A number of processes, dominated by response to oxidative stress and defense and immune responses, were specifically downregulated after freezing (Fig. S5).

The mapping of DE genes on KEGG pathways helped to further dissect the commonalities and differences in responses to supercooling and freezing. Because we set the cut-off criteria for significant response in a non-conservative (less stringent) way, the KEGG mapping sometimes identified pathways based on just one or two DE loci in a whole pathway. In the Supplementary Information, Tables S4A–D (where all

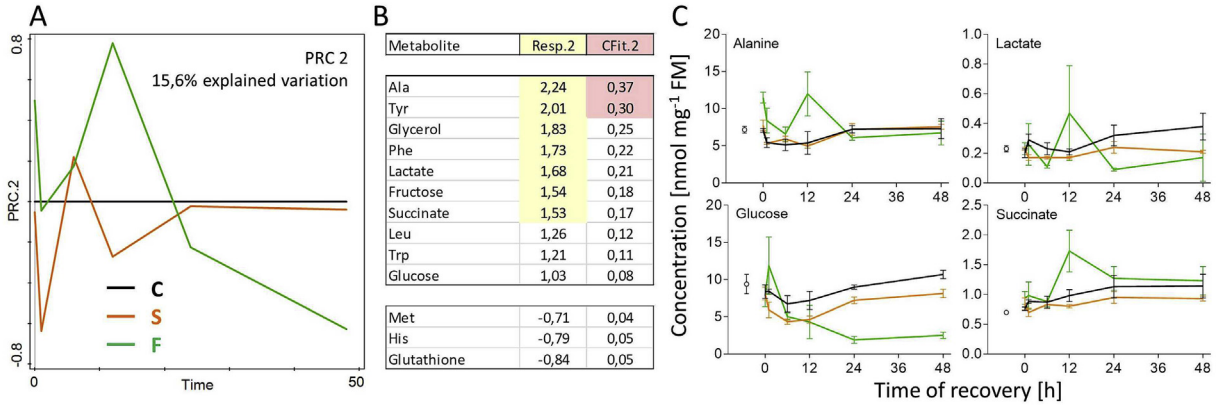


Fig. 3. Summary of metabolic PRC 2-type response to cold stress in *Drosophila melanogaster* larvae (see section 2.2. for explanation of PRC analysis). (**A**) The metabolic changes in control (C) larvae were set as the reference value (0) and the temporal patterns (curves) of responses to supercooling (S) and freezing (F) represent a difference from the control. (**B**) Numeric results of PRC analysis for the most important metabolites– drivers of the PRC 2-type response. The parameter 'Resp.' describes the magnitude of response of a given metabolite in the statistical model, the parameter 'Cfit.' describes the proportion of explained variance by the statistical model (see Table S1 in Supplementary Information for complete results). (**C**) Examples of real temporal profiles in concentrations of four selected metabolites most representative of the PRC 2-type response.

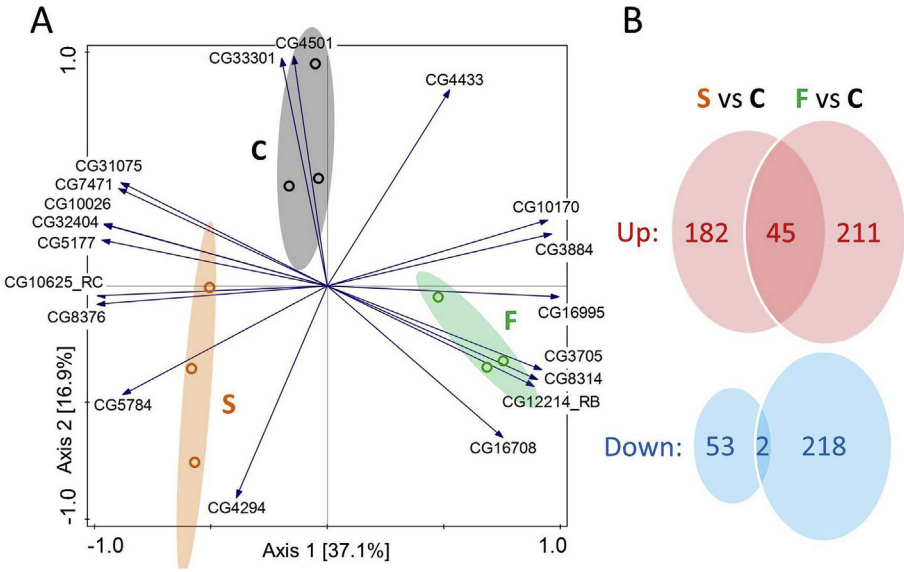


Fig. 4. Summary of the transcriptomic response in *Drosophila melanogaster* larvae to cold stress (see Tables S2, S3, and S4 in Supplementary Information for details). (**A**) The RDA analysis shows distinct clustering of control (C), supercooling (S), and freezing (F) treatments and also the loading vectors for selected mRNA transcripts [19 best fitting transcripts (fitting to statistical model > 97.5%) are shown; the transcript variant is explicitly mentioned only for variants RB, RC; all other transcript variants are RA). (**B**) Venn diagrams showing results of differential gene expression (DE) analysis. The numbers of significant DE genes (up- and downregulated) in pairwise comparisons S vs. C and F vs. C are shown.

results of KEGG analyses are summarized) we provide KEGG maps only for the most significant pathways. Firstly, the pathways 'Lysosome' (Fig. S6), and 'Toll and Imd signaling' (Fig. S7) were identified as most significantly upregulated in response to supercooling, while a great number of amino acid and other metabolites' pathways (mostly supported by a single locus) were found upregulated in response to freezing (Figs. S8–S10). Secondly, no downregulated pathway was identified in response to supercooling, while two pathways most downregulated in response to freezing were 'Lysosome' (Fig. S11) and 'Sphingolipid metabolism' (Fig. S12); we also added a KEGG map for 'Toll and Imd signaling' (Fig. S13). Although the Toll and Imd pathway signaling was not significantly downregulated in statistical terms (adjusted P-value = 0.11), its downregulation trend after freezing stress contrasted with the upregulation trend after supercooling stress.

Based on analysis of DE genes, GO enrichment, and KEGG mapping, we designed a schematic overview of the most important defense processes and effector molecules that were found differently regulated after supercooling and freezing (Fig. 5): namely (i) degradation of bacterial walls via Lysozymes (LysS, FBgn0004430; LysC, FBgn0004426); (ii) phagocytosis followed by lysosomal degradation of microbes via different acidic hydrolases such as cathepsins (Bace, FBgn0032049; CG33128, FBgn0053128), glycosidases (LAMAN: LManIII, FBgn0032066; HEX A/B: Hexo1, FBgn0041630), and lipase (LIPA: Lip3, FBgn0023495); and (iii) Toll and Imd signaling cascades that mediate response to microbial immune challenge and lead to the production of antimicrobial proteins (AMPs) such as Drosomycins (Drsl1, FBgn0052274; Drsl4, FBgn0052282), Attacins (AttA, FBgn0012042; AttB, FBgn0042581), Diptericins (DptA, FBgn0004240; DptB, FBgn0034407), Cecropins (CecA1, CG1365; CecA2, FBgn0000277), and Immune induced molecules (IM1, FBgn0034329; IM23, FBgn0034364).

4. Discussion

The larvae of *D. melanogaster* showed markedly different metabolic and transcriptomic responses to supercooling and freezing, both at −5 °C. The two cold stresses differed both in duration (the total exposure time at subzero temperature was 110 min and 230 min for S and F treatments, respectively), and in whether or not ice crystals formed inside the body. Freezing, in contrast to supercooling, is associated with osmotic dehydration of cells linked to a whole complex of deleterious consequences (for review, see Muldrew et al., 2004). Rather than directly comparing the responses to two different stresses, we will discuss responses to two stresses more or less independently focusing on specific questions: (i) what, if anything, the larvae do in order to survive

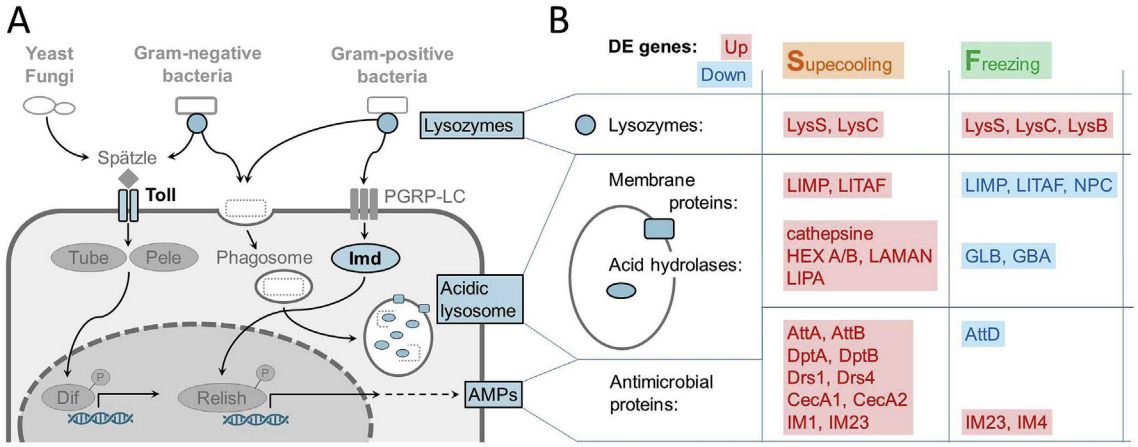


Fig. 5. Schematic overview of (**A**) Toll and Imd signaling cascades that mediate the response to microbial immune challenges and lead to stimulation of defense response effector molecules such as Lysozymes, components of acidic lysosomes, and antimicrobial peptides. These processes and effectors were all upregulated after supercooling according to DE analysis, GO term enrichment analysis, and KEGG pathway mapping analysis (for more details see Figs. S7 and S13). (**B**) A list of effector molecules of the defense response that were transcriptionally either upregulated (pink rectangles) or downregulated (blue rectangles) in response to supercooling and freezing (see sections 3.2 and 4.2. for explanations). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

after supercooling (S vs C comparison); (ii) what characterizes the survivor (but mostly the destined-to-die) larvae exposed to freezing (F vs C comparison).

4.1. Response to supercooling indicates tolerance of injury and active repair processes

Considering high survival after supercooling (> 95% survival to adult stage) and relatively weak sublethal effects (a reduction of reproductive capacity of survivor females to 75.7% of controls), a question emerged as to whether the temperature of −5 °C was stressful at all. In other words, we were not able to decide conclusively in our parallel study (Koštál et al., 2019) whether the quiescent larvae *resisted* the supercooling injury (no injury occurred), or *tolerated* the incurred injury and/or actively repaired it. Detailed analysis of metabolic and transcriptomic changes indicated that the response to supercooling in quiescent larvae of *D. melanogaster* differed from controls. The supercooled larvae had to cope with cold-induced perturbation of metabolic homeostasis and recruited specific transcriptomic responses, likely at an energetic cost. These results suggest that the supercooling treatment is likely stressful, and the response to supercooling is, at least partially, of tolerance type.

Metabolomics analysis revealed that supercooling was associated with a transient initial perturbation of metabolism. During the first 12 h of recovery after supercooling stress, various metabolites showed rapid fluctuations in concentration that were not seen in controls. Nevertheless, the metabolic profiles of supercooled larvae returned to 'normality', and were strikingly similar to profiles taken in control larvae screened 24 h or 48 h after cold stress. These results indicate that supercooling was not simply resisted but rather perceived as a sort of stress resulting in misbalance of the metabolic network. As the effect on the larval metabolic network was only transient, the results further suggest that the feedback-loop mechanisms were sufficiently robust to return the challenged organism back to metabolic homeostasis within 24 h. In accordance with our observations, various metabolic perturbations caused by cold stress were observed earlier in adults of *D. melanogaster* (Colinet et al., 2012; Overgaard et al., 2007), and it was shown that metabolic networks are more robust (less responsive to cold shock) in the lineages of *D. melanogaster* selected in laboratory for increased cold hardiness (Williams et al., 2014).

Microarray analysis further corroborated the distinction between supercooled and control larvae. Of the 282 transcripts differentially expressed in response to supercooling (2.0% of the total 14,183 mRNA transcripts analysed), 227 were up- and 55 were downregulated. The downregulation response was relatively weak and the GO term enrichment analysis suggested, perhaps not surprisingly, that various developmental processes (e.g. muscle cell development, cuticle development, cell division, developmental maturation, cellular component biogenesis, and organelle assembly) were downregulated. In addition, the GO terms related to actin filament organization were enriched among the downregulated genes (in response to both supercooling and freezing). Cytoskeletal stabilization and rearrangement has emerged as a recurring theme linked to both insect cold acclimation (Kim et al., 2006; Des Marteaux et al., 2017, 2018a,b), and cold injury repair (Kayukawa and Ishikawa, 2009; Teets et al., 2012), particularly with regards to defense of polymerized actin. KEGG mapping failed to identify any enriched pathway downregulated in response to supercooling. In contrast, the upregulation response to supercooling exhibited the single strongest unifying theme: immune defense, which was reflected in both GO term enrichment and KEGG mapping analyses (Fig. 5).

4.2. Response to supercooling is dominated by transcriptional upregulation of immune response pathways and antimicrobial peptides

The GO term enrichment and KEGG mapping analyses revealed the 'Defense response to Gram-positive bacterium' (GO:0050830), 'Toll and Imd signaling pathway' (KEGG pathway dme04624), and 'Lysosome' (KEGG pathway dme04142) as the most significantly upregulated processes in response to supercooling in larvae of *D. melanogaster* (Fig. 5A). For instance, the genes encoding Spheroide (FBgn0030774) and Spätzle processing enzyme (SPE, FBgn0039102) were upregulated in response to supercooling. Spheroide and SPE are serine proteases, and SPE integrates responses to various infections as well as cellular damage (Buchon et al., 2014). SPE is activated by proteases released from virulent fungi and bacteria (Gottar et al., 2006), by signals released from necrotic cells (Ming et al., 2014), and also by microbial cell wall components (Gobert et al., 2003). Activated SPE cleaves Spätzle proteins, leading to Toll pathway activation in the fat body and haemocytes (Buchon et al., 2009). Activated Toll pathway, in turn, leads to the systemic production of antimicrobial proteins (AMPs), with antifungal peptide Drosomycin as a principal target (Valanne et al., 2011). Accordingly, KEGG mapping and DE analysis identified significant upregulation in a whole set of genes coding for effector molecules of

immune response such as Lysozymes, various antimicrobial proteins, and different acidic hydrolases (Fig. 5B), which may help to digest macromolecules after phagocytosis of microbial cells and formation of acidic lysosomes (Gao et al., 2017). Such complex transcriptional response suggests that immune response pathways are activated upon supercooling stress.

It has been reported earlier that cold stress may induce transcriptional immune response in adults of *D. melanogaster* (Salehipour-Shirazi et al., 2017; Sinclair et al., 2013; Zhang et al., 2011). The relationship between potential and realized immune response is not, however, straightforward. The estimates of immunity based on gene expression (potential) need not match the realized ability to defend against pathogens (Fedorka et al., 2007). Indeed, some authors reported that cold stress increases resistance to fungal infection (Le Bourg et al., 2009), while others showed that realized immunity actually decreases after cold shock in *D. melanogaster* (Salehipour-Shirazi et al., 2017). Analysis of the cold-related immunity in different insects revealed a high level of idiosyncrasy (Ferguson and Sinclair, 2017). It has been shown that low temperatures can both suppress and activate immune responses in ectothermic animals depending on species, overwintering habitat, level of cold stress, type of pathogen, and also depending on which particular metric of immune response is measured (Ferguson et al., 2018). Therefore, our results on complex transcriptional activation of immune response pathways after supercooling in larvae of *D. melanogaster* will require rigorous functional validation at the level of realized immunity.

At this stage of research we are unable to conclusively decide on neither the triggering signals nor the adaptive meaning of the observed transcriptional activation. The transcriptional response could be triggered by microbial signals, perhaps coming from Lysozyme-mediated degradation of bacterial walls of gut microbiota (Douglas, 2015; Engel and Moran, 2013; Moghadam et al., 2018). Alternatively, the response may be triggered by signals released from cold-damaged cells (Ming et al., 2014), or non-specifically by low temperature via broader activation of multiple defense responses, for instance to the occurrence of partially denatured/misfolded proteins (Ananthan et al., 1986; Kaunisto et al., 2016; Štětina et al., 2015; Wallin et al., 2002). The response could be adaptive (helping to cope with increased microbial attack after cold stress), or prophylactic (preparing the organism for the case of increased microbial attack), or even maladaptive (consuming energy when no increase in microbial attack occurs). One way to assess adaptiveness of the immune response for survival after cold stress would be to compare the cold stress responses between wild and mutant or RNAi lineages of *D. melanogaster* deficient in Toll, Imd and other relevant pathways. An alternative way would be to expose wild-type animals to different infection loads and observe how the survival and fitness of survivors are influenced after cold stress.

4.3. Response to freezing includes downregulation of immune response pathways

The exposure to freezing at −5 °C was clearly stressful to larvae of *D. melanogaster* as only approximately one in ten treated larvae survived to adulthood. Nevertheless, most larvae were still alive 24 h after the stress and exhibited delayed mortality (Koštál et al., 2019). In addition, the reproductive capacity of freezing-stress-survivor females was reduced to 47.9% relative to controls (Koštál et al., 2019). Hence, the described freezing-induced metabolic and transcriptomic patterns will likely combine the responses linked to upcoming mortality (unsuccessful repair) and to survival (tolerance and partial repair).

Metabolomics analysis showed that the perturbation of metabolic networks was deeper in frozen larvae relative to those that were supercooled and, importantly, this perturbation was irreversible in most frozen larvae. The PRC curve analyses suggested that the initial phase of recovery (until 6 h after stress) was associated with similar disturbance trends in supercooled and frozen larvae. Afterwards, however, the frozen larvae deviated increasingly from controls and supercooled larvae as recovery time progressed. Most frozen larvae died during the interval of 24–96 h after the stress and their mortality was preceded by a peak (at 12 h of recovery) of anaerobic end-products such as alanine, lactate, and succinate. At the same time (12 h), peaks of some amino acids (Gln, Phe, Pro, Ser, Thr, Trp, Tyr, Val) were observed in frozen but not supercooled larvae. This result corresponds well with the results of GO term enrichment analysis (returning 'Amino-acid catabolic process' as the most significantly upregulated process) and KEGG mapping (returning many different amino acid metabolisms among the upregulated pathways). We interpret these results as signifying gradual failure of life functions, including respiration and aerobic energy production (6–12 h after the stress), prevailing degradation of macromolecules (12–24 h after stress), and death (24–96 h after stress).

Some elements of transcriptomic responses were shared between supercooled and frozen larvae. Although only 45 DE genes (less than 10% of all DE genes) were shared, the list contained 11 genes to which different defense functions are broadly ascribable, including those coding for Lysozymes LysS, LysC, and Immune induced molecule IM23. Another 15 genes on the list are broadly related to degradation of macromolecules (proteases, glucosidases, lipases), which can also be regarded as a part of the defense or injury-repair response. In contrast to supercooled larvae, the frozen larvae showed a *down*regulation of Toll and Imd signaling pathways. Thus, it seems that both supercooling and freezing triggered some defense mechanisms, but the response to freezing was less complex or not complete, perhaps interrupted and overwhelmed by death-related processes (see downregulation of elements in several death-related pathways in frozen larvae: 'Autophagy', 'Apoptosis', and 'Sphigholipid metabolism'). Due to the nontrivial relationship between transcriptional activation (potential) and realized immune response (Ferguson et al., 2018; Ferguson and Sinclair, 2017), we cannot decide (based on our current results) whether relatively weak transcriptional activation of the immune response was a consequence or a cause of freezing injury and developing mortality in frozen larvae.

Collectively, the stimulation of immune response pathways appears as a strong candidate tolerance mechanism for coping with supercooling stress in larvae of *D. melanogaster*. Here we described complex transcriptional activation of innate immunity: from Lysozyme-mediated degradation of bacterial cell walls, recognition of pathogens' signals, through phagocytosis and lysosomal degradation, Toll and Imd signaling pathways, to upregulation of genes coding for effector molecules – antimicrobial peptides. It will require further effort, however, to learn whether this elevation of immune potential is reflected in an adaptive increase of realized immunity.

Conflicts of interest

The authors have declared that no competing interest exists.

Data accession

Microarray raw data were deposited in the ArrayExpress database at EMBL-EBI (www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-7401.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ibmb.2019.01.006.

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