Transcriptomic and functional genetic evidence for distinct ecophysiological responses across complex life cycle stages

Running title: **Stage-specific ecophysiology**

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Summary Statement

RNAseq and gene knockout via transgenic RNAi lines suggest that physiological responses to low temperatures are largely distinct across life stages of the fly *Drosophila melanogaster*.

Abstract

Organisms with complex life cycles demonstrate a remarkable ability to change their phenotypes across development, presumably as an evolutionary adaptation to developmentally variable environments. Developmental variation in environmentally sensitive performance, and thermal sensitivity in particular, has been well documented in holometabolous insects. For example, thermal performance in adults and juvenile stages exhibit little genetic correlation (genetic decoupling) and can evolve independently, resulting in divergent thermal responses. Yet, we understand very little about how this genetic decoupling occurs. We tested the hypothesis that genetic decoupling of thermal physiology is driven by fundamental differences in physiology between life stages, despite a potentially conserved Cellular Stress Response. We used RNAseq to compare transcript expression in response to a cold stressor in *Drosophila melanogaster* larvae and adults and used RNAi (RNA interference) to test whether knocking down nine target genes differentially affected larval and adult cold tolerance. Transcriptomic responses of whole larvae and adults during and following exposure to -5°C were largely unique both in identity of responding transcripts and in temporal dynamics. Further, we found no relationship between stage-specificity and tissue-specificity of transcripts, suggesting that the differences are not simply driven by differences in tissue composition across development. In addition, RNAi of target genes resulted in largely stage-specific and sometimes sex-specific effects on cold tolerance. The combined evidence suggests that thermal physiology is largely stage-specific at the level of gene expression, and thus natural selection may be acting on different loci during the independent thermal adaptation of different life stages.

List of symbols and abbreviations

CSR – Cellular Stress Response

DAVID - The Database for Annotation, Visualization and Integrated Discovery

DE – Differentially Expressed

DGRP – Drosophila Genetic Reference Panel

61 dsRNA - Double-Stranded RNA
62 FDR – False Discovery Rate
63 FPKM – Fragments Per Kilobase of transcript per Million mapped reads
64 GO – Gene Ontology
65 HSP – Heat Shock Protein
66 KEGG – Kyoto Encyclopedia of Genes and Genomes
67 MDS – Multi-Dimensional Scaling
68 RNAi - RNA Interference
69 SNP – Single Nucleotide Polymorphism
70 TRiP – Transgenic RNAi Project
71

Introduction

Many organisms developing from juvenile life stages through adulthood are faced with changing environmental conditions that differ dramatically but predictably during development. These shifting conditions may include resource availability, predator/herbivore abundance, and abiotic factors such as temperature (Krebs and Loeschcke, 1995; Ragland and Kingsolver, 2008; Woods, 2013). To survive these environmental changes, organisms may also dramatically change their morphology, behavior, and physiology across development. For example, juvenile stages often specialize for feeding and growth, while adults primarily (and sometimes exclusively) disperse and mate (Kingsolver et al., 2011; McGraw and Antonovics, 1983; Moran, 1994; Schluter et al., 1991). These developmentally-variable environments and key fitness components (e.g., growth vs. reproduction) lead to shifting natural selection, which may favor different trait combinations in different life stages (Haldane, 1932; Moran, 1994). This is perhaps most apparent in organisms that metamorphose like amphibians and holometabolous insects. Their morphology has evolved independently in juvenile and adult stages that inhabit drastically different ecological niches. There are clear physiological differences across complex life cycle stages as well, in part because distinct developmental machinery underlies distinct morphologies and life history strategies across the life cycle (Arbeitman et al., 2002; Herrig et al., 2021; van Gestel et al., 2019). Such morphological and developmental decoupling supports the adaptive decoupling hypothesis, which posits that natural selection favors reduced genetic correlation across developmental stages to allow for stage-specific adaptation (Moran, 1994).

In addition to developmental differences in 'baseline' physiology, physiological responses to environmental perturbations may also vary across the life cycle. Many key studies have examined developmental variation in environmental responses by manipulating temperature, a nearly universal selective factor that often varies over the course of development (Bowler and Terblanche, 2008; Jensen et al., 2007; Klockmann et al., 2017). Most of these studies show that thermal responses (survival and various metrics of performance) have very low or absent genetic correlations between juvenile and adult stages of holometabolous insects (Dierks et al., 2012; Gilchrist et al., 1997; Loeschcke and Krebs, 1996; Tucić, 1979). Indeed, our recent studies show that the genetic correlation between juvenile and adult cold hardiness in the fly *Drosophila melanogaster* are not detectably higher than zero, with no evidence for pleiotropic effects of SNP

(single nucleotide polymorphism) variation on thermal performance across metamorphosis (Freda et al., 2017; Freda et al., 2019).

We reason that there are two hypotheses that could explain such extreme genetic decoupling of thermal physiology across development. The first, the 'developmentally distinct physiology' hypothesis, posits that environmental responses may indeed be very different across life stages, mirroring the differences in developmental regulation. In this scenario different genes would contribute to environmental responses across stages, with relatively low cross-stage pleiotropy.

Though the developmentally distinct physiology hypothesis is consistent with the observed lack of genetic correlations across life stages, it would be somewhat at odds with predictions based on the conserved cellular stress response. The Cellular Stress Response, or CSR, is an apparently conserved set of changes in cell physiology in response to a variety of environmental stressors (Kültz, 2005). For example, heat shock proteins and related chaperonins are up-regulated in response to multiple stressors, including temperatures that are relatively hot or cold compared to an organism's optimal environmental temperature (Colinet et al., 2010b; Philip and Lee, 2010; Yocum, 2001). If these heat shock responses and other elements of the CSR have a substantial role in whole-organism level environmental responses, then many elements of environmental physiological responses should be very similar across the life cycle. Some elements of environmental physiological responses are admittedly tissue specific. For example, ion homeostasis in the gut and central nervous system has a well-established role in the response to mild low temperatures in many insect species (MacMillan et al., 2015; Overgaard and MacMillan, 2017). However, such tissue-specific responses may also contribute similarly to environmental responses across life stages.

Such conserved cellular and tissue-level responses would argue for a second, 'developmentally conserved physiology' hypothesis, positing that thermal physiology could be largely conserved across development, with only a few stage-specific processes harboring segregating genetic variation. This explanation is less obvious, but still consistent with the observed lack of genetic correlations for environmental physiology across life stages. In this scenario there may be many processes (e.g., the CSR) that universally affect thermal physiology across development, but

134 genetic loci that regulate these processes are highly conserved, and thus not genetically variable.
135 Genetic correlations only assess whether *variants* at loci affect two traits (e.g., juvenile and adult
136 performance), not whether a given locus itself affects the traits. Thus, these conserved loci would
137 not influence measures of genetic correlations. Rather, a subset of an environmental response
138 may be stage-specific and mediated by genetically variable loci. This scenario could also
139 generate low genetic correlations across life stages.
140
141 To test these two hypotheses, we examined physiological responses to cold across the life cycle
142 in *D. melanogaster*, using two approaches to compare larvae (juveniles) and adults separated by
143 a major metamorphic transition. First, we tested whether whole transcriptome responses to low
144 temperature exposure differ in identity of responding transcripts and/or their temporal patterns of
145 differential expression. Transcriptome sequencing provides a broad snapshot of organismal
146 physiology, and allowed us to assess the similarity of the environmental (temperature) response
147 across the two life cycle stages. Second, we tested whether knocking down a set of nine
148 candidate genes affected response to low temperature in larvae, adults, or both. We selected
149 these candidates based on a previous study that found evidence for knockout effects on cold
150 performance in adult *D. melanogaster* (Teets and Hahn, 2018). Though the sample of nine genes
151 is relatively small, it provides a first functional test for the presence of stage-specific (consistent
152 with the developmentally distinct physiology hypothesis) or cross-stage (consistent with the
153 developmentally conserved hypothesis) genetic effects on environmental physiology regardless
154 of genetic variability.
155
156 **Materials and Methods**

157 *Fly rearing*

158 We obtained all *D. melanogaster* (Meigen) lines (Table S1) from the Bloomington Drosophila
159 Stock Center (BDSC; Bloomington IN, USA) at Indiana University – specific lines used in this
160 study are described below. We reared flies at 25°C, 12:12 L:D in narrow vials on media
161 containing agar, cornmeal, molasses, yeast, and antimicrobial agents propionic acid and
162 Tegosept (Genesee Scientific, Morrisville NC, USA), as described previously (Freda et al., 2017;
163 Freda et al., 2019). We sorted parental flies from appropriate lines (details below) under light

164 CO_2 anesthesia and transferred them into fresh vials containing media sprinkled with dry, active
165 yeast to facilitate oviposition. We then transferred the parents each day for four consecutive days
166 into fresh vials to produce offspring for use in experiments. The vials from the first egg-laying
167 day were discarded to remove any residual effect of anesthesia on oviposition. We collected third
168 instar larvae and 5 d-old adults for use in both experiments described below. We extracted
169 experimental third instar feeding larvae from cultures 5 d post-oviposition using a 20% w/v
170 sucrose solution and following the protocol of Freda et al. (2017). Experimental adults were
171 collected and sorted into fresh vials under light CO_2 anesthesia 10 - 12 d post-oviposition (within
172 1 - 2 d of eclosion). These flies were held at 25°C, 12:12 L:D until 5 d-old to limit any carryover
173 effects of CO_2 exposure (Nilson et al., 2006).
174
175 *Experiment 1: Whole transcriptome response to low temperature*

176 To obtain a transcriptomic metric for how physiology changes during cold exposure and
177 subsequent recovery under benign conditions, we sampled whole-body transcriptomes of third
178 instar larvae and 5-day old adult *D. melanogaster* prior to, during, and after exposure to a cold
179 temperature (Fig. 1A).
180
181 We crossed five male and five virgin female flies from each of six Drosophila Genetic Reference
182 Panel (DGRP; Mackay et al. 2012; Huang et al. 2014) isogenic lines (Table S1) to produce
183 offspring for use in this experiment. We initially chose these six lines in order to compare three
184 lines exhibiting high cold tolerance in adults but not larvae, and three lines exhibiting high cold
185 tolerance in larvae but not adults (Table S1; Freda et al. 2017). However, initial analyses
186 revealed little evidence for transcriptomic variation tied to variance between these two classes of
187 fly lines, with apparent phenotypic effects highly influenced by outlier lines (Fig. S1). Thus, we
188 treated line (6 levels) as a fixed effect (random effects cannot be modeled using the methods that
189 we applied), providing replication across genetic backgrounds, and did not model phenotypic
190 effects in any of our subsequent analyses.
191

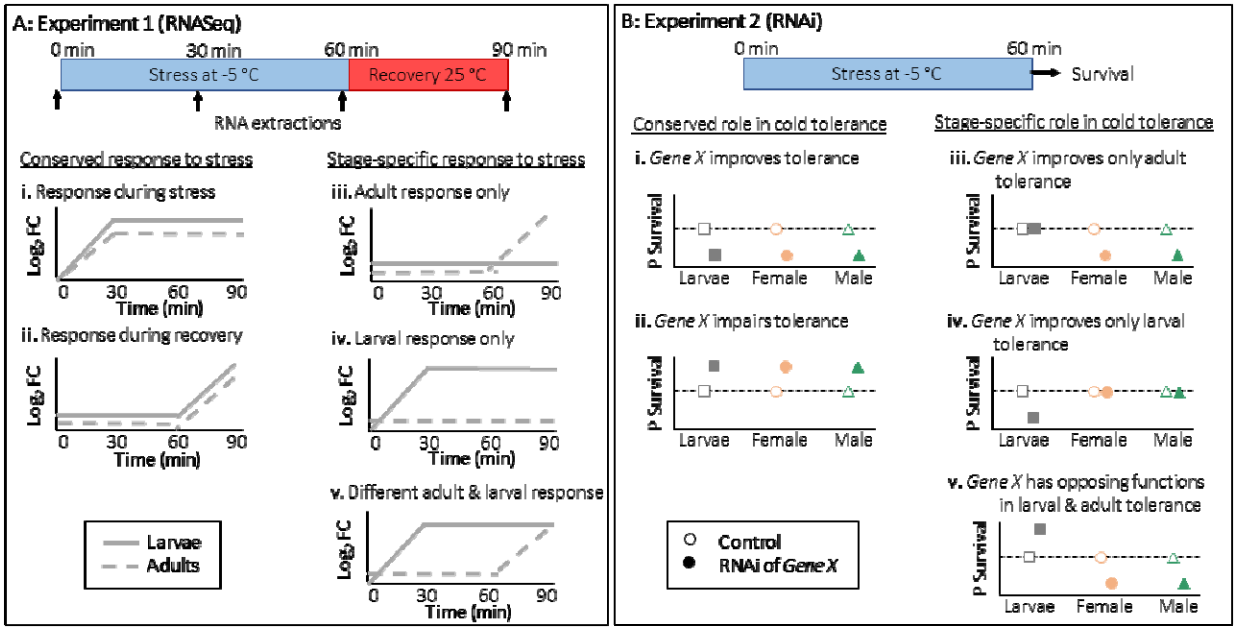

Figure 1. Summary of methods and example predictions for Experiments 1 and 2. Time courses at the top of each column show sampling time points during the **(A)** RNAseq experiment or the treatment prior to the measure of survival in the **(B)** RNAi line experiments. Panel **A** illustrates predicted patterns of differentially-expressed genes in *Drosophila melanogaster* during and after cold stress, with examples of (i, ii) larvae and adults exhibiting a conserved transcriptional response to cold and (iii, iv, v) larvae and adults exhibiting different transcriptional responses to cold. Predictions (log2 fold change, FC) do not differentiate between up- and down-regulated transcripts. Panel **B** illustrates predicted effects of target gene (*Gene X*) RNA interference (RNAi) on the proportion of surviving *D. melanogaster* after cold stress compared to control flies (no RNAi), with examples of the RNAi having a similar effect on cold tolerance of larvae, female adults, and male adults (i, ii) and the RNAi having life stage-specific effects on cold tolerance (iii, iv, v). We predict that knocking out a gene with a positive effect (improves cold tolerance) decreases survival of RNAi lines, whereas knocking out a gene with a negative effect (impairs tolerance) increases survival of RNAi lines.

Each experimental replicate consisted of 10 offspring (10 larvae or 5 male + 5 female adults). To minimize stochastic, environmental effect, each replicate group of 10 offspring was homogenized together to create pools for RNA sequencing. The vial flug (Genesee Scientific, Catalog # 49-102) for each replicate was moistened with water to inhibit desiccation during and after cold exposure. We took an initial sample at 25°C prior to cold exposure (time zero, t0), then exposed all remaining replicates to -5°C by immediately immersing fly vials in a temperature-controlled recirculating bath (ECO RE 2025, Lauda Corporation, Lauda-Königshofen, Germany). We confirmed that vials rapidly reached test temperatures and that larval food did not freeze during treatments (Freda et al., 2017) We then took samples at 30 and 60 minutes during

the cold exposure (t30 and t60, respectively). At 60 minutes all remaining vials were removed from the bath and placed back at 25°C, and one final sample was taken 30 minutes after this transfer (30 minutes of recovery, or 90 minutes total, t90). All samples were immediately snap-frozen in liquid N_2, ground into Tri-reagent (Zymo Research, Irvine CA, USA), then frozen at -80°C until RNA purification. The overall experimental design included 6 lines by 2 stages by 4 time points by 3 replicates, yielding 144 total samples.

RNA extraction, library preparation, sequencing, and initial informatics

To extract RNA from DGRP lines for RNASeq, we homogenized each sample (pool of 10 individuals) with a plastic micropestle in Tri-reagent (Zymo) and used the Zymo Direct-zol total RNA extraction kit according to manufacturer's instructions. We prepared our cDNA libraries using a RNA-tag sequencing approach, as described previously (Lohman et al., 2016). Resulting libraries were sequenced on 5 lanes as 100 bp single-end reads on an Illumina HiSeq 2500 at Kansas University's Genome Sequencing Core Laboratory, resulting in an average of 6 million reads per sample. The library size for each sample is available in Table S2. We used STAR (Dobin et al., 2013) to map reads to the *D. melanogaster* reference genome (version 6.06) obtained from FlyBase (Gramates et al., 2017), with >95% total mapped reads across all samples. Read counts per gene and per isoform were generated using RSEM (Li and Dewey, 2011). After filtering out all gene models not covered by at least one read in 50% of samples, we retained 13,242 genes. Following normalization of read counts across libraries using the weighted trimmed mean of M-values (TMM) method (Robinson and Oshlack, 2010), we examined variation among libraries using a Multi-Dimensional Scaling (MDS) plot generated using the 500 genes with the highest root-mean-square $\log_2$-fold change among samples (Robinson et al., 2010). We removed 10 samples that were very clear outliers on the MDS plot (Fig. S2) and exhibited low read counts (less than 200,000 reads) compared to the median read count of 4,808,878 before outliers were removed (Table S2). After removing outliers, all stage × time × line combinations were represented by at least two replicates (Table S3).

245 Statistical modelling of temperature- and stage-specific transcription

246 Our main goal in Experiment 1 was to quantify whether and how the transcriptional response to
247 low temperatures varied between larval and adult life history stages. We expected that many
248 transcripts would be differentially expressed (DE) between life stages because they have very
249 distinct tissue compositions (Arbeitman et al., 2002). Thus, though we estimated gross life stage
250 differences and other contrasts, the parameter of primary interest was a stage × time interaction,
251 indicating stage-specific thermal response during and/or after low temperature exposure (see Fig.
252 1A for example predicted gene expression trajectories). Below, we detail nested, ad hoc model
253 selection to best estimate that parameter and characterize thermal response trajectories for
254 transcripts with stage-specific expression patterns. The code for this analysis is also publicly
255 available (see Data Availability section). We recognize that transcripts/effects removed from
256 these models may also be of interest, but they were not the focus of this study.
257
258 We started with a full generalized linear model with binomial error fitted using the *edgeR*
259 package (Robinson et al., 2010) in R (R Core Team, 2021) to predict the mean read count for
260 each transcript, then removed effects and transcripts to estimate stage × time (interaction) effects
261 that did not depend on DGRP line. The full model included regression coefficients modelling the
262 effects of *stage, time, line*, and all two-way interactions and the three-way interaction of these
263 variables. Statistical inferences from this model identified 130 transcripts with a significant
264 (FDR < 0.05) three-way interaction term. We then removed all transcripts with significant three-
265 way interactions, then fit a reduced model omitting the three-way interaction, which identified 19
266 transcripts with a significant two-way interaction between *time* and *line*. We removed these
267 transcripts, then fit our final, reduced model including all main effects plus the *stage* × *time* and
268 *stage* × *line* two-way interactions.
269
270 The transcripts of primary interest in our final, reduced model were those that either 1) had a
271 significant main effect of *time* but no *stage* × *time* interaction, or 2) had a significant *stage* ×
272 *time* interaction. The former are transcripts that respond to low temperature in similar ways in
273 both life history stages, while the latter are transcripts that exhibit distinct responses to cold in
274 larvae vs. adult flies. We used linear contrasts to estimate the trajectories of differential
275 expression over time for all transcripts in both of these categories by estimating the $\log_2$ fold

change between each time point relative to time zero (t0). This model also allowed us to identify transcripts that had a significant main effect of *stage* or a *stage* × *line* interaction, but no *stage* × *time* interaction. These were not of primary interest, but allowed us to estimate how much of the transcriptome was differentially expressed between life history stages but not responsive to cold. Finally, we used the DAVID functional annotation tool (Huang et al., 2009a; Huang et al., 2009b) to identify functional categories enriched in the set of transcripts illustrating stage-specific responses to cold temperatures.

Tests for the influence of tissue-specific gene expression

Transcriptomics from whole bodies are coarse measurements that ignore tissue-specificity of gene expression, and in this case differential expression in response to changing temperatures. However, they provide a comprehensive snapshot of whole-organism physiological responses. We could not directly assess how differences in tissue composition across stages might contribute to different transcriptomic responses without tissue-specific RNA libraries. Rather, we tested whether genes that exhibit high levels of tissue-specific expression in *D. melanogaster* were overrepresented in sets of transcripts that we identified as differentially expressed between life stages, or exhibiting stage-by-time interactions.

We quantified tissue specificity of *D. melanogaster* transcripts using data from FlyAtlas2 (Leader et al., 2018) as described in (Cridland et al., 2020). We calculated τ for each transcript, a value ranging from 0 to 1, with higher numbers associated with greater tissue-specificity (Yanai et al., 2005). As in (Cridland et al., 2020), if fragments per kilobase of transcript per million mapped reads (FPKM) for whole bodies was less than 2, we set it equal to 2 to avoid inflated estimates for genes with very low expression. We then calculated a normalized expression value for each tissue as the FPKM for that tissue divided by the FPKM for the whole body of the sex/life stage from which the tissue was derived. Finally, we calculated the tissue specificity index, τ, as follows:

$$\tau = \frac{\sum_{i=1}^{N}(1-x_i)}{N-1} \quad (1)$$

Where x_i is the normalized expression value for the i^{th} tissue divided by the maximum normalized expression value across tissues and N is the number of tissues. We then calculated

the median τ for a given set of transcripts, e.g., the set exhibiting significant stage-by-time interactions in the above generalized linear models. We compared that point estimate against the median τ for 10,000 random samples with the same sample size as the tested set of transcripts to generate a permutation-based p-value.

Experiment 2: RNAi to test stage-specific functional effects

In order to functionally test whether genes can have stage-specific effects on cold tolerance, we compared the effect of knocking down target gene expression on survival of third instar larvae and 5 day-old adult females and males following a cold stress (Fig 1B). Gene knockdown was achieved using TRiP (Transgenic RNAi Project) lines (Table S1) as described in Teets and Hahn (2018). Briefly, five virgin females from each TRiP line carrying dsRNA under the control of a UAS promoter were crossed to five males of a driver line carrying the GAL4 gene under the control of an actin promoter to produce F_1 offspring for experiments. The GAL4 driver promotes expression of dsRNA in all tissues to knock down expression of the target gene in the F_1 generation. We measured survival of groups of 20 larvae or 20 adults (10 adult females and 10 adult males) kept in single fly vials after a 60 min exposure to -5°C, with at least three replicates vials of each stage per line (Fig 1B). The cold treatment was chosen because 40 - 60% of control flies (no RNAi) survived this temperature, allowing us to detect effects of RNAi that either increased or decreased survival relative to the control.

We exposed flies to a -5°C cold stress by immersing vials of larvae and adults in a temperature-controlled Arctic A40 recirculating bath (ThermoFisher, Denver CO, USA) containing 50% (v/v) propylene glycol in water. The fly vials for larvae contained fresh medium, and larvae were allowed to burrow into the food prior to cold treatment via holes poked in the medium; the fly vials for adults were empty (Freda et al., 2017). We verified the temperature in vials using a 36-AWG type-T copper-constantan thermocouple (Omega Engineering, Norwalk CT, USA) interfaced with Picolog v6 software (Pico Technology, Cambridge, UK) via a Pico Technology TC-08 unit. After a 60 min exposure to -5°C, we returned groups of larvae or adult flies to 25°C, 12:12 L:D to recover. Larvae recovered from cold exposure in the same vials and were monitored for adult eclosion over the next 10 d. We classified larval survivors as those that completed development and eclosed as adults (Freda et al., 2017). Adults recovered in small

petri dishes containing an approximately 1 cm^3 piece of fly food medium. We classified adult survivors as those that were motile (could walk/fly independently) 24 h post-cold stress (Jakobs et al., 2015).

Fly lines

Experiment 2 included 11 TRiP lines (Table S1) whose cold tolerance in adult females has previously been characterized: two control (non-RNAi) lines and nine lines that each knocked down expression of a target gene (Teets and Hahn, 2018). We reasoned that these genes previously had observable effects on adult cold responses, and thus would provide an appropriate test for whether those responses carry over to other life history stages. Two control lines were required because the dsRNA insertion site (and therefore the genetic background) differed among RNAi lines: four lines (+ one control) had an attP2 insertion site, while five lines (+ one control) had an attP40 insertion site (Table S1). Originally, we also planned on knocking out genes identified as having universal or stage-specific responses to cold in Experiment 1, but these experiments were truncated by lab closures during the coronavirus pandemic of 2020.

Statistical analysis

For each of the nine target genes in Experiment 2 (Table S1), we compared the survival post-cold stress of RNAi and control flies with the same genetic background (attP2 or attP40 insertion sites). We used the *nlme* function in the *lme4* package in R (Bates et al., 2014) to fit generalized linear mixed models with binomial error and a logit link function. We modelled survival as a function of the fixed effects of line (control/RNAi), stage (larvae/adult female/adult male), and their interaction and random (subject level) effects of vial. Example predictions for the effect of RNAi on survival for genes with stage-specific function are in Fig. 1B.

Results

Differential gene expression in response to cold is largely stage-specific

A large number of transcripts were significantly (FDR < 0.05) differentially expressed between larval and adult life stages regardless of time sampled during cold treatment (n=10,966, Fig. 2). A smaller, but still sizeable number of transcripts changed in abundance over time. However,

only 21 transcripts changed in a similar pattern in both life stages (significant main effect of time, no *stage* × *time* interaction), while the bulk of the temperature-sensitive transcripts changed over time in a stage-specific manner (n=880 with significant stage × time interaction).

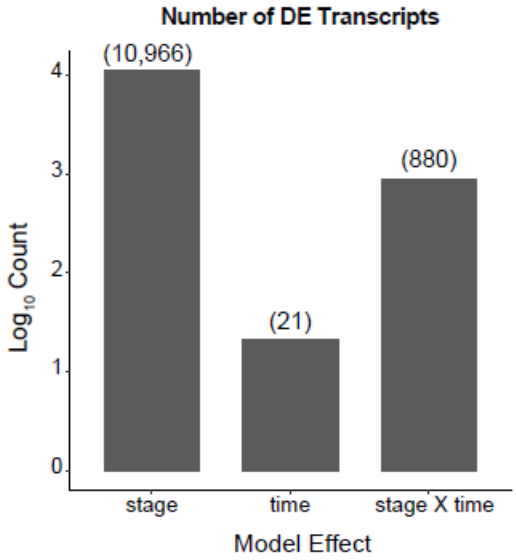

Figure 2. Number of transcripts demonstrating significant (FDR < 0.05) effects of *stage* (larva or adult), *time* (t0, t30, t60, t90), or a *stage* × *time* interaction during and after cold stress in *D. melanogaster*. The y-axis is $\log_{10}$ scaled; actual counts are reported above each bar.

Patterns of change over time were also distinct between life stages. Using linear contrasts, we identified many more transcripts that were significantly DE across at least one time point in larvae (n=763) compared to adults (n=121). Subdividing these into transcripts up-regulated or down-regulated over time on average revealed that most cold-sensitive transcripts in larvae were up- or down-regulated during the cold exposure and remained at similar levels during recovery (Fig. 3A). In contrast, far fewer transcripts were cold-sensitive in adults, and these were mainly up-regulated only during recovery, as has been observed following both cold and heat exposure in other studies of *D. melanogaster* adults (Colinet et al., 2010a; Sinclair et al., 2007; Sørensen et al., 2005; Fig. 3B). Transcripts that changed significantly over time in larvae did not change over time in adults (Fig. 3A; Adult trajectories remain flat). Transcripts significantly up-regulated over time in adults did tend to be up-regulated in larvae as well (Fig. 3B). However, those larval expression trajectories did not demonstrate the same, pronounced up-regulation during recovery observed in adults. The small number of transcripts (n = 21) with significant main effects of *time*

but no *stage* × *time* interaction were up-regulated over time in various patterns during cold exposure and recovery (Fig. S3).

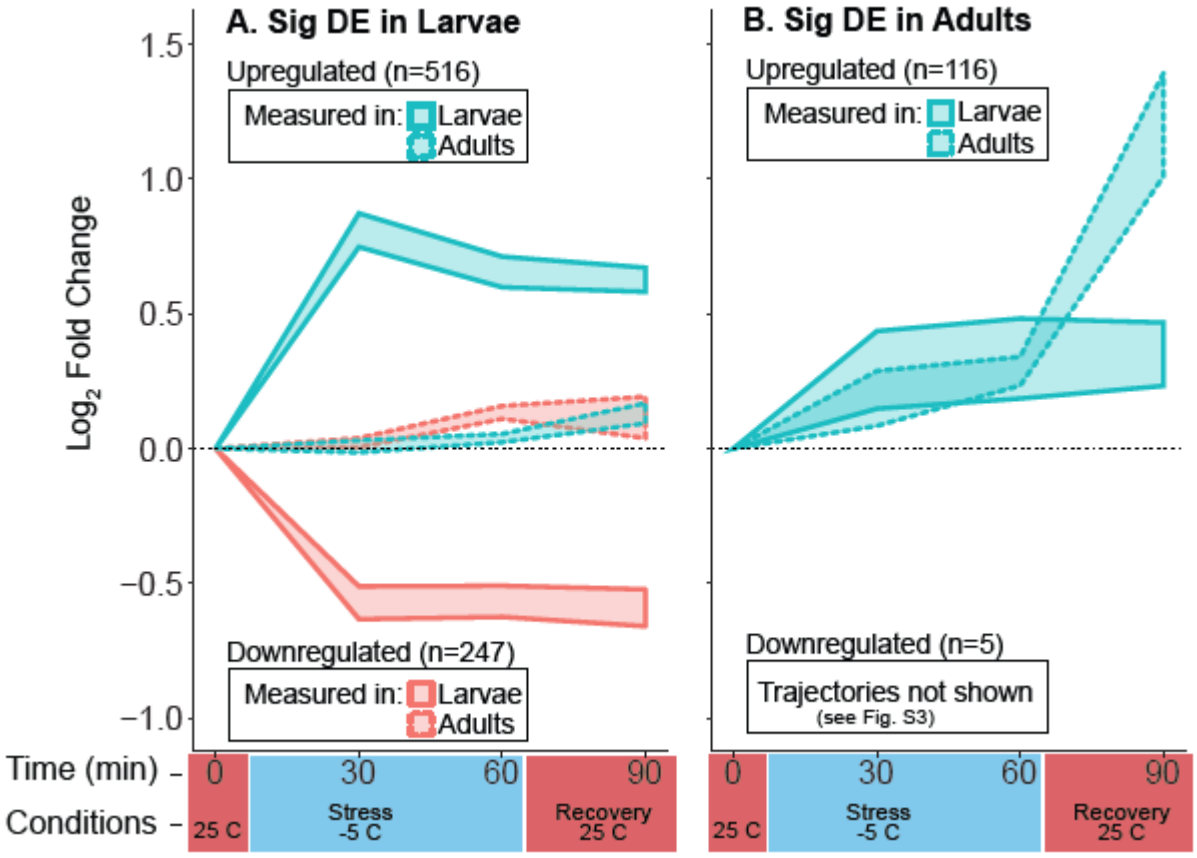

Figure 3. Trajectories of differential expression (DE) during and after cold exposure for the group of transcripts with significant (FDR < 0.05) DE across at least two time points in *D. melanogaster* **(A)** larvae or **(B)** adults. For comparison, the same transcripts measured in larvae (solid borders) and adults (dashed borders) are plotted. Blue indicates transcripts significantly up-regulated on average in (A) larvae or (B) adults, while pink indicates transcripts down-regulated on average in those respective life stages. Y-axis values are $\log_2$ fold changes at each time point relative to the first (t0) time point. Shaded regions are 95% confidence intervals (c.i.) for each group of transcripts designated in the legends. For example, the blue shaded region with a solid boundary in (A) represents 516 transcripts significantly up-regulated (on average) in larvae, while the blue shaded region with a dashed boundary represents those same transcripts measured in adults. Darker red and blue on the x-axis denote the temperature at which individuals were sampled over the time course.

Closer examination of genes in several functional categories identify candidate mechanisms underlying the cold response that are also stage-specific. Functional enrichment analysis of the

763 and 121 gene significantly differentially expressed over time in larvae and adults, respectively, and with significant *stage* × *time* interactions identified many overrepresented (FDR < 0.05) functional categories (including Uniprot keyword searches – UPK; Gene Ontology groups – GO; Interpro protein domains – INTERPRO; and Kyoto Encyclopedia of Genes and Genomes pathways – KEGG; Table S4). Below we focus on members of select categories enriched in either larvae (GO Autophagy, INTERPRO Basic leucine zipper, KEGG Fatty Acid metabolism), adults (UPK Stress response), or both (GO Response to bacterium).

Transcripts participating in autophagy, often involved in clearance of cellular damage and nutrient recycling during energy stress (Kroemer et al., 2010), were mainly up-regulated during and after cold exposure in larvae (Fig. 4A). Transcripts with basic leucine zipper domains, largely transcription factors playing roles in developmental regulation, exhibited similar patterns in larvae (Fig. 4B). Transcripts participating in fatty acid metabolism, potentially influencing lipid metabolism or temperature-induced changes in membrane fluidity (Clark and Worland, 2008; Koštál, 2010) were mainly down-regulated during and after cold exposure in larvae (Fig. 4C). All of the transcripts in these three functional categories demonstrated little change during and after cold exposure in adult flies. In contrast, transcripts associated with stress response, mainly chaperonins, exhibited the most pronounced changes only during recovery in adults (Fig. 4D). Though some of these transcripts also changed over time in larvae, many were down-regulated, including multiple copies of the well-known, temperature-inducible stress response gene Hsp70.

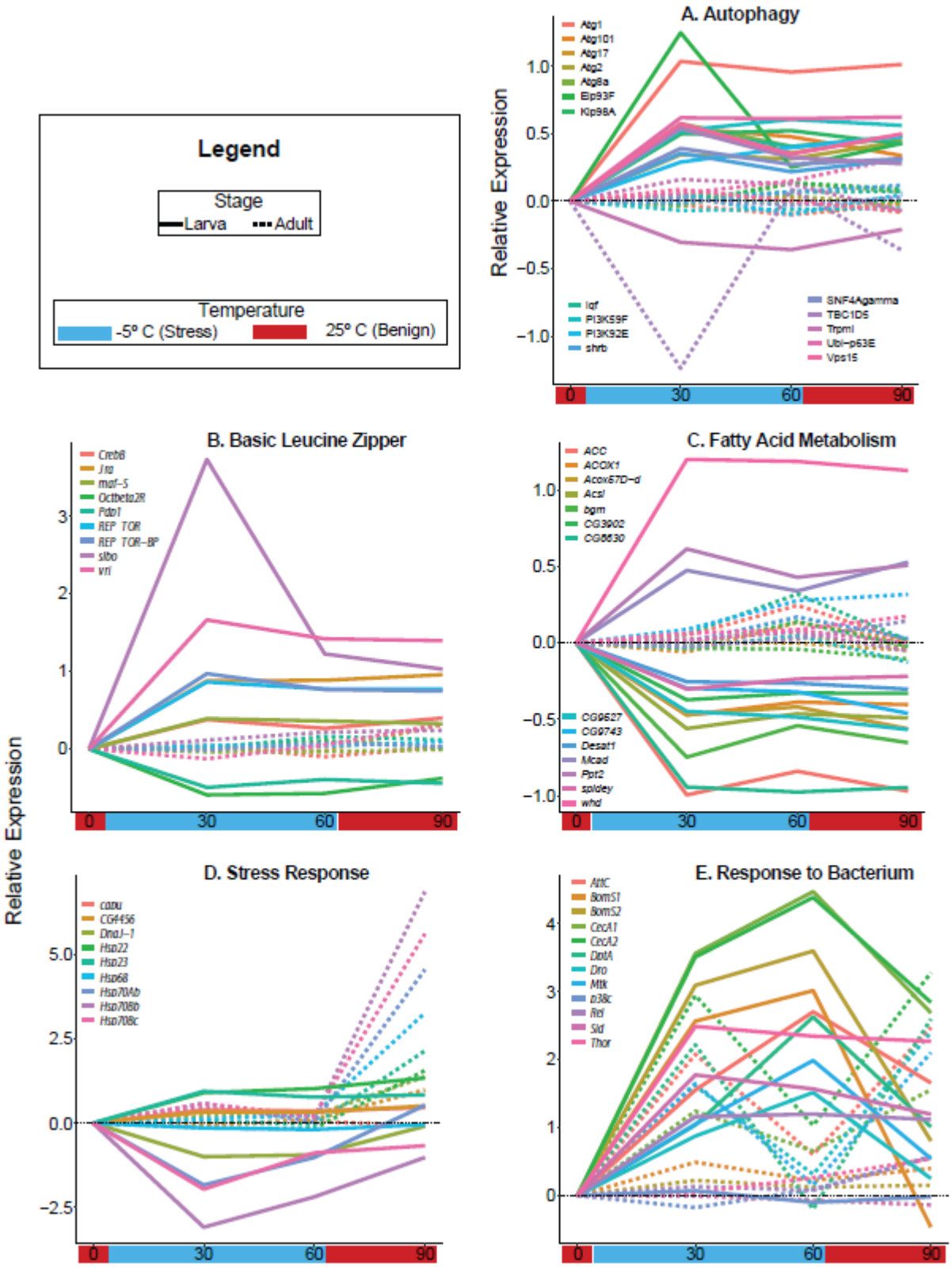

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Figure 4 (previous page). Trajectories of differential expression during (t30, t60) and after (t90) cold exposure for transcripts in select functional categories enriched in the set of all transcripts significantly differentially expressed across at least two time points in *D. melanogaster* larvae or adults. Y-axis values are $\log_2$ fold changes at each time point relative to the first (t0).

Like transcripts in the stress response category, some transcripts associated with the immune response (within the Response to Bacterium GO group) responded to cold in larvae and adults, though again demonstrating stage-specific patterns (Fig. 4E). The immune response has previously been implicated in responses to thermal extremes in insects (Ferguson et al., 2018; Salehipour-shirazi et al., 2017; Sinclair et al., 2013). All but one transcript in the category were substantially up-regulated during cold exposure in larvae, but tended to decrease in relative abundance during recovery. In adults, transcripts for *Attacin-C* (*AttC*), two Cecropins (*CecA1*, and *CecA2*), *Diptericin A* (*DptA*), and *Metchnikowin* (*Mtk*) were up-regulated at 30 minutes during cold exposure, down-regulated by 60 minutes, then up-regulated again during recovery. One additional Cecropin (*CecC*) was up-regulated over time in a similar pattern for larvae versus adults (main effect of *time* but no *stage* × *time* interaction; Fig. S3).

Differential expression is unrelated to tissue specificity

We found no evidence that transcripts differentially expressed between whole-body extracts from different stages tended to be more tissue specific. Rather, we found a slight tendency for that set of transcripts to exhibit less tissue specificity than chance expectations. The median τ for the set of 10,931 transcripts with stage or stage-by-line effects (FDR < 0.05) was 0.88, and we did not observe a value this small in 10,000 random samples of 10,931 transcripts (median τ of random samples = 0.90; $p < 0.0001$).

Similarly, we found no evidence that transcripts with *stage* × *time* interactions (different responses to the temperature treatments across stages) tended to be more tissue specific. The median τ for the set of 849 transcripts with *stage* × *time* effects (FDR < 0.05) was 0.82, and we did not observe a value this small in 10,000 random samples of 849 transcripts (median τ of random samples = 0.90; $p < 0.0001$).

Some knockdowns had stage-specific effects, but none had consistent cross-stage effects

We observed that the effect of RNAi of target genes on cold tolerance could be stage-specific, although this effect was not universal and was complicated by sex. Three genes of the nine genes tested in this study exhibited clear stage-specific effects of RNAi on cold hardiness (Fig. 5, Table S5). Knockdown of *CG10505* or *Clk* decreased adult, but not larval, survival relative to control flies after a cold stress, suggesting these two genes are important for adult cold tolerance only. Conversely, knockdown of *Ir85a* decreased larval, but not adult, survival, suggesting this gene is important for larval cold tolerance only. RNAi of three other genes had both stage- and sex-specific effects on cold hardiness (Fig. 5, Table S5). *klu* knockdown only increased female adult survival, but had no effect on larvae or male adults. *CG32533* or *mthl15* knockdown had opposite effects on larvae (low survival) and adults (high survival) of one sex only – either female (*CG32533*) or male (*mthl15*). The expression of these two genes therefore seems important for larval cold tolerance but detrimental to either female or male adult cold tolerance. We observed no significant effect of RNAi on cold hardiness for the remaining three genes (*NtR*, *pigs*, *psh*), although *pigs* and *psh* trended toward stage-specific effects (Fig. 5, Table S5).

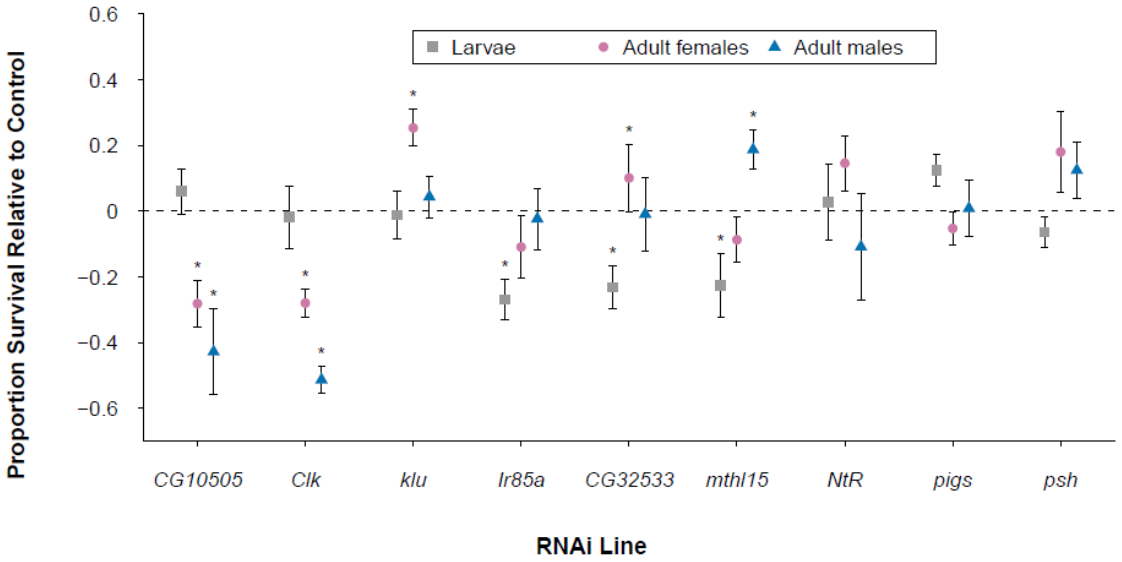

Figure 5. Proportion survival of *D. melanogaster* larvae and adults from lines with RNAi of target gene relative to control lines (no RNAi) following 1 h cold shock at -5°C. Each point represents the mean proportion survival of the RNAi line minus the mean proportion survival of the appropriate control line. Error bars indicate s.e.m., calculated from the proportion survival of three or more replicates of 20 (larvae) or 10 (adult female or male) RNAi individuals. Asterisks indicate a significant effect of RNAi on proportion survival for larvae, adult females, or adult males, based on logistic regressions (Table S4).

Discussion

Cold tolerance physiology is largely distinct across metamorphosis

Our results generally support the 'developmentally distinct physiology' hypothesis, showing that both the expression and function of genes pertinent to cold hardiness differ dramatically across development in *D. melanogaster*. Transcriptional responses to cold in larvae and adults differed in timing (during vs. after cold stress), magnitude (many more DE transcripts in larvae), and constituent genes. In addition, of the nine genes whose expression we knocked down via RNAi, most of them (six) affected adult and larval cold hardiness differently. Though differences in tissue composition across life stages probably have some influence on transcriptional responses to cold, they do not appear to account for the majority of whole-organism transcriptional differences in the thermal response across stages. Other studies have demonstrated transcriptional differences across stages in a complex life cycle (Arbeitman et al., 2002; Chevalier et al., 2006; Sanil et al., 2014; Strode et al., 2006), but this is the first study to our knowledge that demonstrates distinct transcriptome-wide environmental responses across life stages, with additional support from functional genetics experiments.

Although classic CSR genes (e.g. heat shock proteins; HSPs) were not similarly regulated in response to cold in both adults and larvae, we had minor support for the 'developmentally conserved physiology' hypothesis based on transcription of immune response genes. Immunity-related genes have been identified as cold-responsive in a number of other studies of adult drosophilid flies (MacMillan et al., 2016; Sinclair et al., 2013; Vermeulen et al., 2013). However, to our knowledge this is the first study to find similar results in adults and larvae, both of which upregulated antimicrobial genes. The function of immunity genes in cold-mediated responses remains unknown, though Vermeulen et al. (2013) suggest that some constituent genes may play a role in repair of cellular damage through their known effects on wound healing. The consistency with which these genes are observed in cold responses across species (Cheng et al., 2017; Salehipour-shirazi et al., 2017; Su et al., 2019; Sun et al., 2019), and here across stages, suggests that they play a specific role in cold physiology, and are not just a general stress response *a la* the CSR.

Though some changes in transcription in response to environmental stress undoubtedly have important, adaptive benefits (Chen et al., 2018; Feder, 1999; Feder and Hofmann, 1999; Feder and Krebs, 1998), differences in baseline (unperturbed) physiology may be equally important. In particular, organisms may have higher fitness when exposed to stress because they are physiologically better prepared prior to stress exposure (Hercus et al., 2003; Krebs and Loeschcke, 1994). To be sure, we have shown that many transcripts differ in expression between stages in benign (baseline) conditions, but this largely reflects the massive developmental differences between the stages. These data do not allow us to identify which of these differences might contribute to differences in expression during and after stress, or to whole organism performance in response to stress, for that matter. However, to the extent that baseline transcriptomes heavily influence transcriptomic responses to a stressor, this still implies that (baseline) physiology affecting cold performance is distinct between life stages.

Cold hardiness is associated with a muted transcriptional response to cold

Differences between life stages in transcriptomic responses to cold stress likely reflect differences in cold stress resistance between stages. Though not definitively established, a relatively clear pattern is emerging from transcriptomic studies: species or populations that are the most stress resistant are also the least transcriptomically-responsive to environmental stressors. Or, more generally, species or populations that more frequently encounter a given environment tend to have more muted transcriptomic responses to that environment. This is true for Trinidadian guppies responding to predator cues (Ghalambor et al., 2015), fruit-feeding flies responding to different host fruits (Ragland et al., 2015), and marine invertebrates (Lockwood et al., 2010; Schoville et al., 2012), rice plants (Zhang et al., 2012), and other drosophilid flies (Königer and Grath, 2018; Parker et al., 2015) responding to thermal stressors. Adult *D. melanogaster* survive cold stressors better than larvae (Freda et al., 2017; Jensen et al., 2007), and we observed relatively few cold-sensitive transcripts in adults in this study. Moreover, the identity of transcripts involved in the larval transcriptomic response suggest more severe cold-induced damage in larvae compared to adults. Larvae differentially expressed autophagy genes during cold stress, suggesting that larvae need to mitigate cellular damage (i.e. degrade damaged cellular components; Kroemer et al., 2010) or to redistribute macromolecules and energy needed

546 for cell differentiation or growth (Neufeld, 2012; Wang and Levine, 2010). In contrast, adults did
547 not upregulate autophagy-related transcripts and mainly upregulated chaperonins during
548 recovery to preserve cellular function rather than clearing highly damaged cells (Colinet et al.,
549 2010a; Frydenberg et al., 2003; Koštál and Tollarová-Borovanská, 2009). We note that *D.*
550 *melanogaster* larvae are not susceptible to all stressors; they are more heat-tolerant than adults
551 (Freda et al., 2019), likely because they feed in fruits that can become substantially hotter than
552 air temperatures experienced by adults (Feder et al., 1997). We therefore do not think larvae are
553 more cold-susceptible simply because they are undergoing rapid cellular growth, division, and
554 differentiation compared to adults. Rather, it would appear that each stage has adapted to
555 opposing thermal extremes: heat in larvae and cold in adults.
556
557 *Transcriptomic time course and constituent genes differ across life stage*

558 Larvae rapidly differentially regulated a relatively large number of transcripts both during and
559 following cold exposure. These changes likely include active regulation in response to cellular
560 damage, as evidenced by the aforementioned autophagy response. We also observed differential
561 regulation of lipid metabolism in larvae. Fatty acids are important in energy storage (as part of
562 triacylglycerides) and membrane fluidity (as part of phospholipids) (Denlinger and Lee, 2010).
563 Larvae downregulated several desaturases (e.g. *Desat1, CG8630, CG9743*), suggesting that they
564 are not increasing the abundance of unsaturated fatty acids in phospholipids to maintain
565 membrane fluidity at low temperatures (Ohtsu et al., 1998; Overgaard et al., 2005). However, the
566 downregulation of several enzymes associated with fatty acid catabolism (e.g. *ACOX1*) and
567 synthesis (e.g. *ACC, acsl, bgm*) is consistent with restructuring of lipid metabolism to potentially
568 support growth or recovery from stress (Sinclair and Marshall, 2018).
569
570 In contrast, adults had relatively muted transcriptomic responses during cold exposure, with a
571 limited (in number of transcripts) but robust (in the degree of differential expression) response
572 during recovery. The best-characterized gene expression response to temperature stress, hot or
573 cold, is upregulation of Hsps and other chaperonins during recovery after exposure to a stressor
574 (Colinet et al., 2010b; Philip and Lee, 2010; Yocum, 2001). This was the most prominent adult
575 response in our study as well, with no detectable changes in Hsp expression during cold
576 exposure. As mentioned above, it is likely that the relative stability of gene expression during

stress in adults reflects less severe perturbations from homeostasis and more restricted cellular damage.

Implications for genetic decoupling across development

Though the transcriptome is only one metric of physiology, the scale of the differences across stages in this study suggests that allelic variants in many genes could strongly affect environmental sensitivity of one stage, while having little effect on other stages. Our results are entirely consistent with empirical studies that repeatedly show little to no genetic correlation in environmental (thermal) sensitivity across metamorphosis in insects (Dierks et al., 2012; Freda et al., 2017; Freda et al., 2019; Gilchrist et al., 1997; Loeschcke and Krebs, 1996; Tucić, 1979). In combination, these results suggest that strong genetic decoupling of environmental sensitivity is relatively common for organisms with complex life cycles, likely facilitating adaptation/acclimation of different life stages to different thermal environments.

Being so widespread, differences in stage-specific thermal tolerance might not appear so surprising. However, temperature is fundamental to limiting species' spatial distributions (Bale, 2002; Bale et al., 2002), and thus thermal performance must be constrained in some ways. Though the results of our RNAi knockout experiments suggest that cross-stage pleiotropy for environmental sensitivity is not widespread, we expect that such pleiotropy exists, and will constrain the limits of thermal flexibility across life stages. For example, genetic modifications to increase Hsp70 copy number (and subsequently expression) affected both larval and adult thermal tolerance in *D. melanogaster* (Krebs and Bettencourt, 1999). In that instance, the genetic differences between modified and non-modified lines was relatively extreme (12 extra gene copies). However, there is some evidence for cross-stage effects of naturally segregating genetic variants in plants. Quantitative Trait Locus (QTL) studies in rice have identified QTL associated with cold tolerance at multiple developmental stages, though most QTL only affect a single developmental stage (Yang et al., 2020).

Given the polygenic architecture of environmental tolerance in general (Healy et al., 2018) and thermal tolerance specifically (Barghi et al., 2019; Freda et al., 2017; Sanghera et al., 2011), it's unlikely that further, detailed analysis of single-locus pleiotropy will fully address questions

608 about the limits of stage-independent adaptations to environmental stressors. Rather, comparative
609 studies leveraging existing variation in stage-specific adaptation or selection studies generating
610 relevant phenotypic variation would seem to be the most promising avenues for further research.
611
612
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617
618 **Competing Interests**

619 The authors have no competing interests to declare.
620
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627
628 **Data Availability**

629 All raw transcriptomic datasets from this study are available in NCBI BioProject PRJNA783562
630 (currently embargoed). All code and other data not include in the supplement are available at
631 https://github.com/gjragland/Stage-specific-expression.
632
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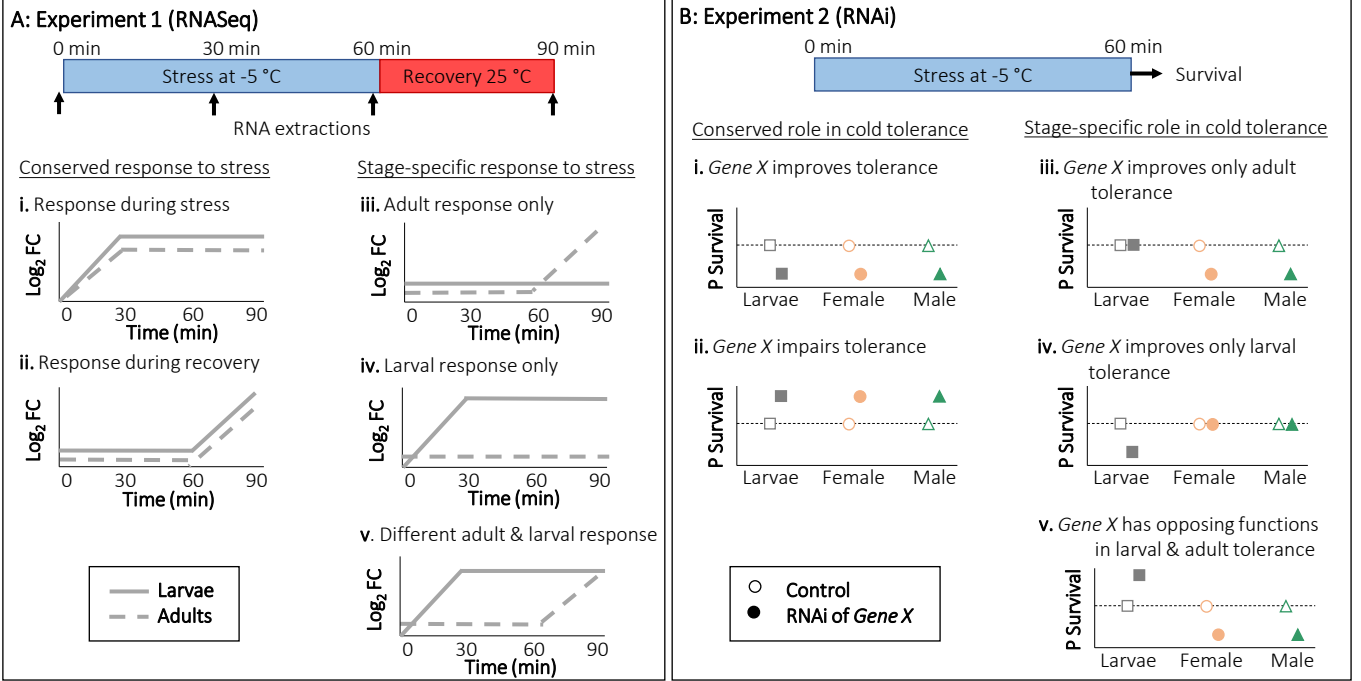

A: Experiment 1 (RNASeq)
0 min
30 min
60 min
90 min
Stress at -5 °C
Recovery 25 °C
RNA extractions
Conserved response to stress
Stage-specific response to stress
i. Response during stress
iii. Adult response only
ii. Response during recovery
iv. Larval response only
v. Different adult & larval response
Log2 FC
Time (min)
Larvae
Adults
B: Experiment 2 (RNAi)
0 min
60 min
Stress at -5 °C
Survival
Conserved role in cold tolerance
Stage-specific role in cold tolerance
i. Gene X improves tolerance
iii. Gene X improves only adult tolerance
ii. Gene X impairs tolerance
iv. Gene X improves only larval tolerance
v. Gene X has opposing functions in larval & adult tolerance
P Survival
Larvae
Female
Male
Control
RNAi of Gene X

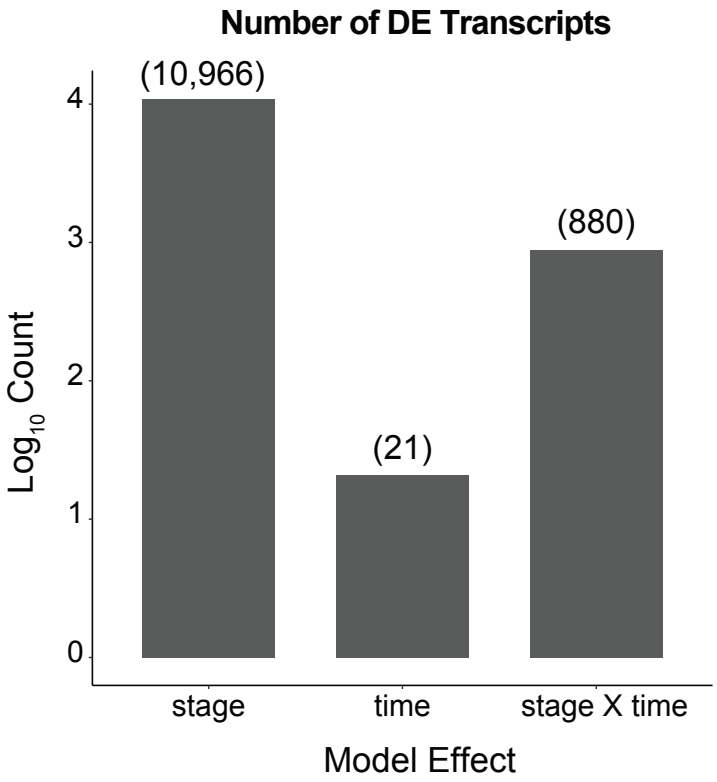


Figure 2. Number of transcripts demonstrating significant (FDR < 0.05) effects of stage (larva or adult), time (t0, t30, t60, t90), or a stage X time interaction. The y-axis is log10 scaled; actual counts are reported above each bar.

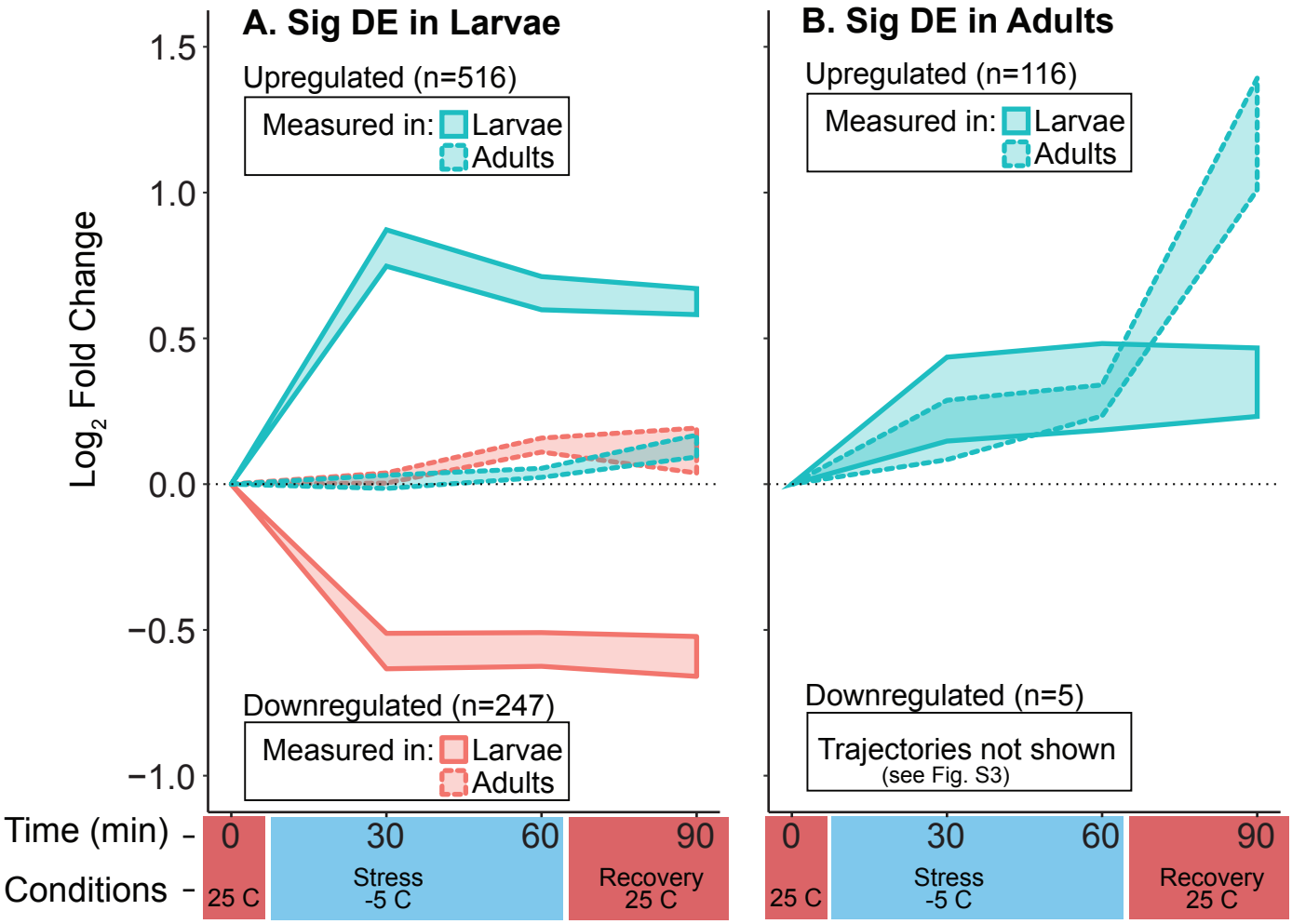


Figure 3. Trajectories of differential expression (DE) during and after cold exposure for transcripts significantly (FDR < 0.05) DE across at least two time points in larvae (A) or adults (B). For comparison, the same transcripts measured in larvae (solid borders) and adults (dashed borders) are ploted. Blue indicates transcripts significantly up-regulated on average in larvae (A) or adults (B), while pink indicates transcripts down-regulated on average in those respecitve life stages. Y-axis values are log2 fold changes at each time point relative to the first (t0) time point. Shaded regions are 95% Confidence intervals (CI) for each group of transcripts designated in the legends. For example, the blue shaded region with a solid boundary in (A) represents 516 transcripts significantly up-regulated (on average) in larvae, while the blue shaded region with a dashed boundary represents those same transcripts measured in adults. Darker red and blue on the x-axis denote the temperature at which individuals were sampled over the time course.

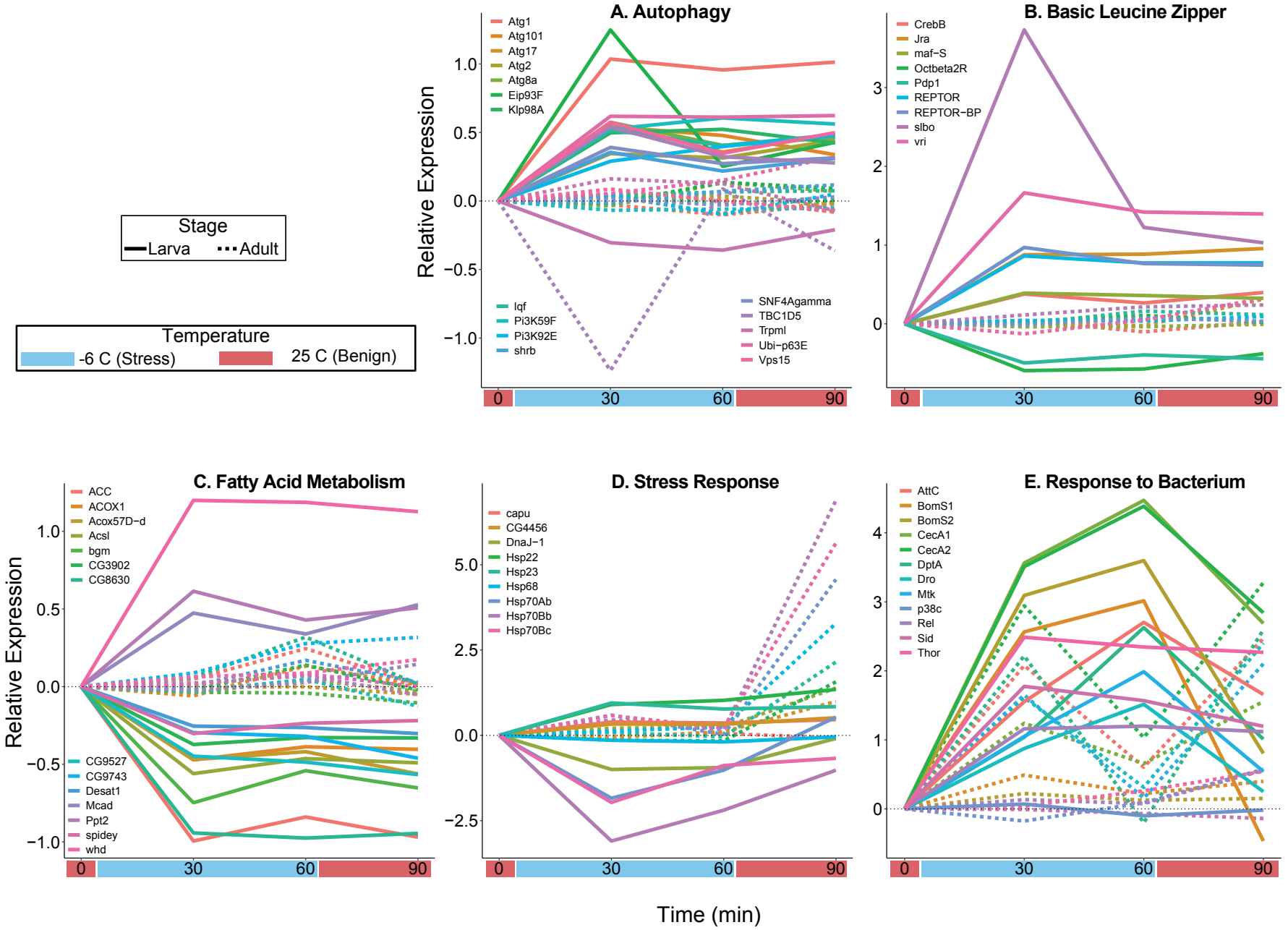


Figure 4. Trajectories of differential expression during and after cold exposure for transcripts in select functional categories enriched in the set of all transcripts significantly differentially expressed across at least two time points in larvae or adults. Y-axis values are log2 fold changes at each time point relative to the first (t0)

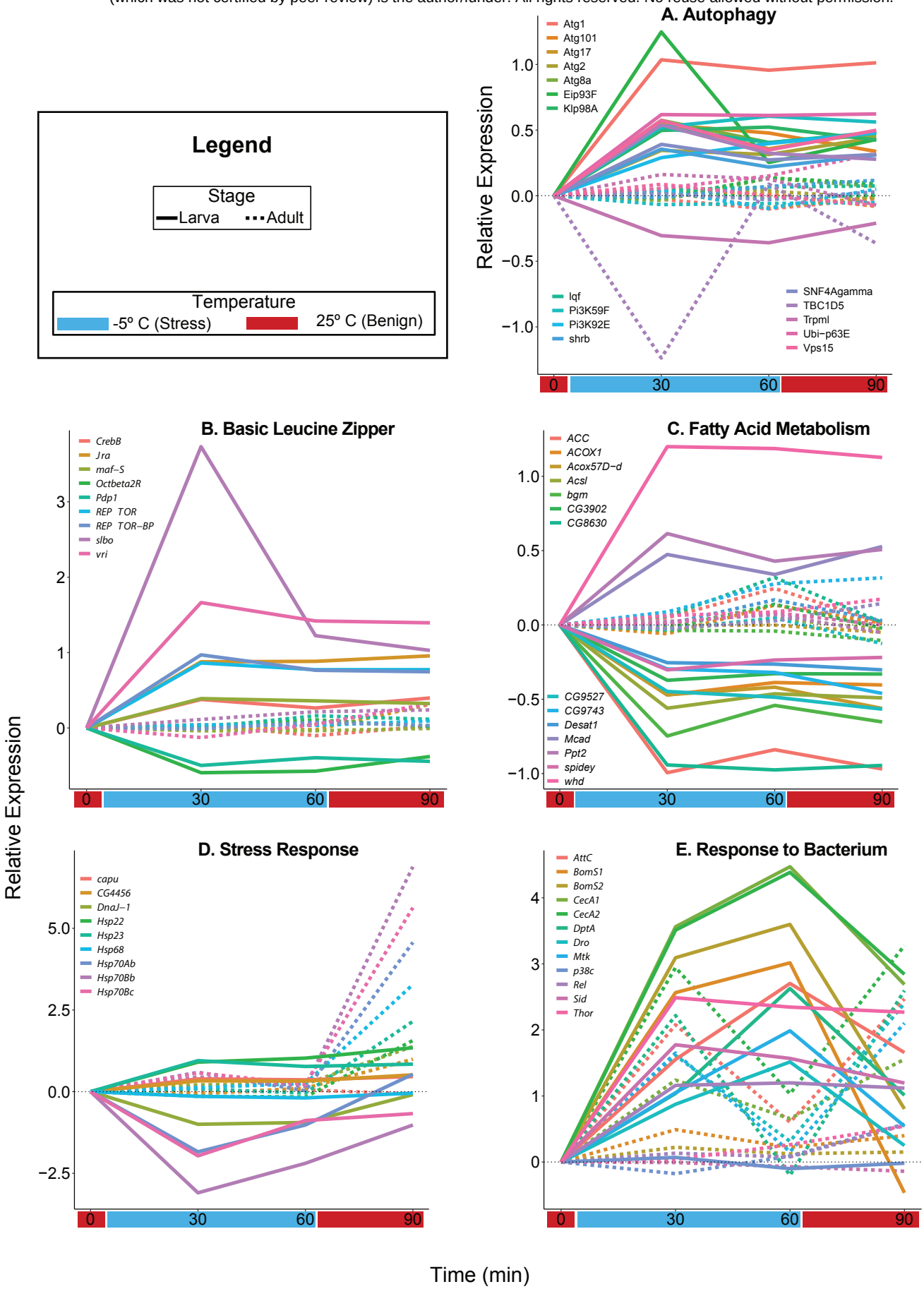
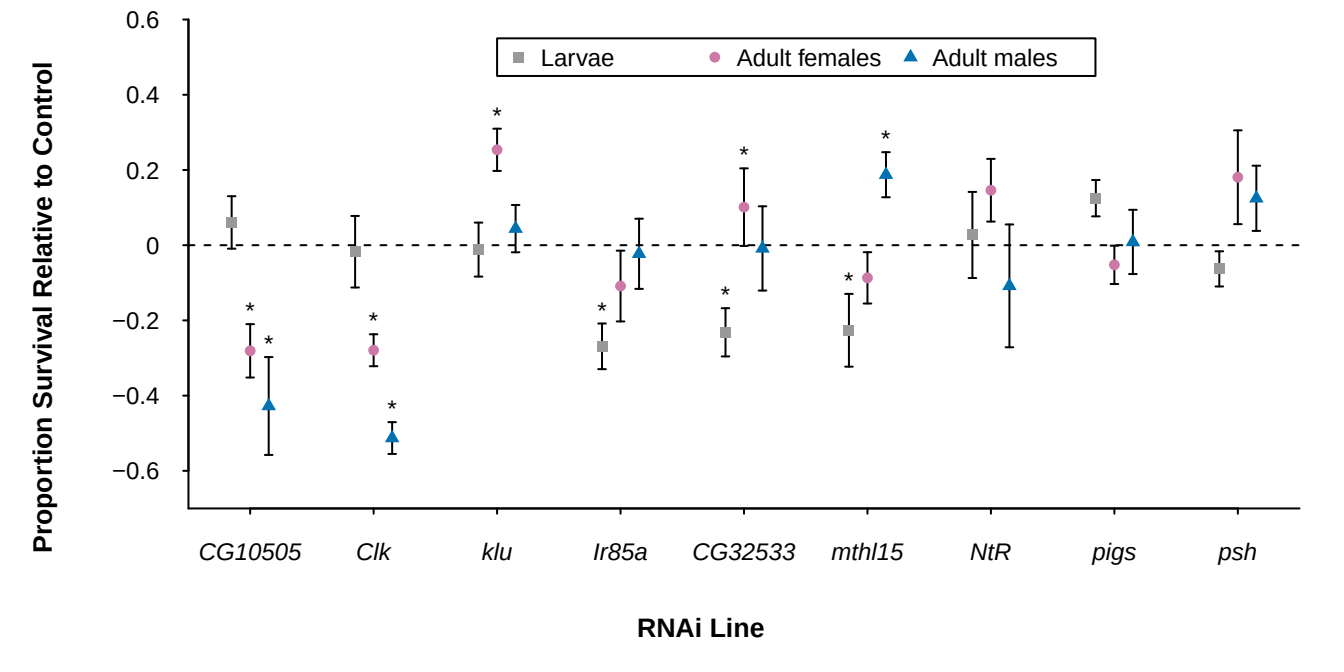


Figure 4. Trajectories of differential expression during and after cold exposure for transcripts in select functional categories enriched in the set of all transcripts significantly differentially expressed across at least two time points in larvae or adults. Y-axis values are log2 fold changes at each time point relative to the first (t0)


Larvae
Adult females
Adult males
Proportion Survival Relative to Control
0.6
0.4
0.2
0
−0.2
−0.4
−0.6
CG10505
Clk
klu
Ir85a
CG32533
mthl15
NtR
pigs
psh
RNAi Line