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RESEARCH ARTICLE

Divergent transcriptomic responses to repeated and single cold exposures in *Drosophila melanogaster*

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SUMMARY

Insects in the field are exposed to multiple bouts of cold, and there is increasing evidence that the fitness consequences of repeated cold exposure differ from the impacts of a single cold exposure. We tested the hypothesis that different kinds of cold exposure (in this case, single short, prolonged and repeated cold exposure) would result in differential gene expression. We exposed 3 day old adult female wild-type *Drosophila melanogaster* (Diptera: Drosophilidae) to –0.5°C for a single 2 h exposure, a single 10 h exposure, or five 2 h exposures on consecutive days, and extracted RNA after 6 h of recovery. Global gene expression was quantified using an oligonucleotide microarray and validated with real-time PCR using different biological replicates. We identified 76 genes upregulated in response to multiple cold exposure, 69 in response to prolonged cold exposure and 20 genes upregulated in response to a single short cold exposure, with a small amount of overlap between treatments. Three genes – *Turandot A*, *Hephaestus* and *CG11374* – were upregulated in response to all three cold exposure treatments. Key functional groups upregulated include genes associated with muscle structure and function, the immune response, stress response, carbohydrate metabolism and egg production. We conclude that cold exposure has wide-ranging effects on gene expression in *D. melanogaster* and that increased duration or frequency of cold exposure has impacts different to those of a single short cold exposure. This has important implications for extrapolating laboratory studies of insect overwintering that are based on only a single cold exposure.

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Key words: cold tolerance, microarray, chilling injury, stress proteins, immune response

INTRODUCTION

Insects in the field are exposed to repeated bouts of stress (e.g. cold, heat, desiccation) of varying intensity and length, interspersed with periods of recovery (Chown and Nicolson, 2004). Ongoing anthropogenic climate change has led to an increased interest in extending laboratory studies of thermal physiology to the field (see Chown and Gaston, 2008). An important aspect of this for insects is the expansion of research beyond single stress exposures to the impacts of repeated stress (Sinclair et al., 2003). The simplest forms of response to repeated exposure are hardening or pre-treatment responses, whereby the response of an insect to a stress is modified by a prior exposure to a mild stress [e.g. rapid desiccation hardening (Bazinet et al., 2010), the heat-shock response (Feder and Hofmann, 1999) and rapid cold hardening (Lee et al., 1987)]. There is also evidence for cross-tolerance, whereby exposure to one stress, for example heat, improves tolerance of another, such as cold (e.g. Bayley et al., 2001; Le Bourg et al., 2009; Rajamohan and Sinclair, 2008).

Although hardening responses are beneficial, stress exposure in the field often takes the form of multiple (i.e. three or more) bouts of stress (e.g. Sinclair, 2001). Repeated high-temperature exposure in larvae disrupts learning in *Drosophila melanogaster* (Wang et al., 2007), whereas repeated cold exposure of *D. melanogaster* larvae improves the longevity of adults (Le Bourg, 2007). At low temperatures, repeated cold exposure impairs feeding and growth in the sub-Antarctic caterpillar *Pringleophaga marioni* (Sinclair and Chown, 2005), damages Malpighian tubules and increases immune responsiveness in larvae of the arctiid *Pyrrharctia isabella* (Marshall and Sinclair, 2011) and results in high energetic costs and protein damage in larvae of the Antarctic midge *Belgica antarctica* (Teets et al., 2011). In several other freeze-tolerant larvae, repeated freezing appears to result in a loss of freeze tolerance (Bale et al., 2001; Brown et al., 2004). By contrast, survival of long-term cold-stored parasitoid wasp mummies is enhanced by brief returns to higher temperatures [referred to as 'fluctuating thermal regimes', or FTR (e.g. Renault et al., 2004)], whereas *B. antarctica* larvae that remain supercooled accrue little damage from repeated cold exposure (Teets et al., 2011). In *D. melanogaster*, repeated exposure to cold does enhance survival, but at the cost of reproductive output: female flies exposed to five 2 h bouts of –0.5°C had lower reproductive output and a skewed sex ratio compared with those exposed to a single 2 h or 10 h bout at –0.5°C (Marshall and Sinclair, 2010). Thus, although repeated cold exposure is ecologically relevant, responses by insects are variable, and appear to differ from the results obtained from a single cold exposure. Nevertheless, it is not clear whether the difference in response is because there is a difference in accumulated damage or because there are physiological changes in the recovery process among the treatment groups.

There is little or no *de novo* gene transcription during exposure to cold in *D. melanogaster* (Sinclair et al., 2007). Thus, the immediate physiological responses by insects during low-temperature exposure are probably controlled by post-transcriptional regulation. However, several studies have identified candidate genes in *D. melanogaster* that are associated with recovery from cold exposure (e.g. Goto, 2001; Morgan and Mackay, 2006; Qin et al., 2005). Gene transcription during this recovery period likely reflects repair of damage incurred during the cold exposure. However, because insects in the wild are exposed to stress repeatedly, we propose that there is a potential for gene expression changes during recovery to reflect also preparation for future cold exposures.

Repeated cold exposure leads to reduced reproductive output in female *D. melanogaster*, compared with a single short cold exposure (i.e. the most recent cold exposure the fly has experienced) and a long cold exposure [in which flies are exposed to the same amount of cold, but as a single bout (Marshall and Sinclair, 2010)]. This implies that the kind of stress (or the nature of recovery from that stress) changes, depending on the schedule of cold exposure. Such differences are key to interpreting laboratory responses to cold by insects in the field context. Here, we hypothesize that there are significant differences in gene expression that underlie the observed phenotypic effects of different cold-exposure schedules (and therefore the different kinds of stress imposed). We test this hypothesis by examining global gene expression patterns in adult *D. melanogaster* in response to three different types of cold stress. In particular, we measure gene expression 6 h post-exposure, allowing us to examine the longer-term homeostatic perturbations.

MATERIALS AND METHODS

Wild *Drosophila melanogaster* Meigen (Diptera: Drosophilidae) were collected from the London, ON, Canada, area in 2007 and mass-reared in 35 ml plastic vials in the lab on Tucson food (yeast: yellow cornmeal, 1:1.8), at 22°C, 50% relative humidity and a 12 h:12 h light:dark cycle. Approximately 1000 flies were placed into each population cage, and, after 8 h, eggs were obtained from a Petri dish of food and transferred into new 35 ml plastic vials (~100 eggs per vial). After 10 or 11 days, virgin females were collected under CO_2 anesthesia, transferred in groups of 15 to new 35 ml vials and maintained at 22°C. Cold treatments started 72 h after CO_2 anesthesia to allow time for recovery (see MacAlpine et al., 2011; Nilson et al., 2006).

The experimental design was a simplified version of the experiment conducted by Marshall and Sinclair (Marshall and Sinclair, 2010). Three day old adult virgin female *D. melanogaster* were divided into four groups: the control group maintained at 22°C; a multiple cold-exposure group that was exposed to –0.5±0.25°C (mean±s.d.) for 2 h during every 24 h period for 5 days; a prolonged cold-exposure group exposed to –0.5±0.25°C for 10 h on the fifth experimental day; and a single short cold-exposure group exposed to –0.5±0.25°C for 2 h on the fifth experimental day (Fig. 1). All cold treatments were performed in a low-temperature incubator (MIR153, Sanyo, Bensenville, IL, USA) in 35 ml plastic vials containing ~10 ml rearing medium (15 flies per vial) that were placed upside down in the incubator to prevent flies from becoming stuck in the food. Flies were maintained at 22°C unless undergoing cold exposures. After the cold treatment, the flies were returned to rearing temperatures for 6 h to allow them to recover from the cold and then snap-frozen in liquid nitrogen and stored at –80°C until RNA extraction. This experiment was repeated three times on separate generations to control for any rearing effects in the microarray, and qPCR validation was performed on separate biological replicates collected at the same time as the microarray samples.

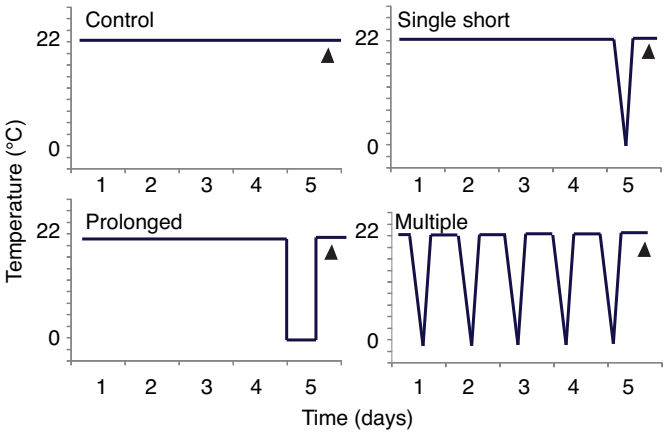

Fig. 1. Experimental design. All experiments were performed on adult, virgin female *Drosophila melanogaster* that were 3 days old on the first experimental day. Each triangle represents a 2 h exposure to –0.5°C, whereas the rectangle represents a 10 h exposure to –0.5°C. Flies were kept at 22°C when not being exposed to cold. Arrowheads indicate sampling points. All samples were collected 6 h after final treatments.

RNA extraction and microarray hybridisation

Total RNA samples used for microarrays were isolated from groups of 30–35 whole, frozen, virgin female *D. melanogaster* using the TRIzol Reagent (Invitrogen, Burlington, ON, Canada) and purified by MEGAclear (Ambion, Streetsville, ON, Canada), according to the manufacturers' instructions. Extracted RNA was resuspended in RNase-free water, and the purity of RNA was assessed by the ratio of absorbance at 260 and 280 nm.

Microarray hybridization and scanning were performed at the Canadian *Drosophila* Microarray Centre (Mississauga, ON, Canada; http://www.flyarrays.com) using custom-designed NimbleGen 4x72K arrays (Roche NimbleGen, Madison, WI, USA), which were built from the FlyBase R4.3 release of the *Drosophila melanogaster* genome. Each array contained 72,000 probes (60 bp long), corresponding to 15,473 *Drosophila* genes. Isolated RNA was reverse-transcribed into double-stranded cDNA with an oligo-dT primer, using the Superscript Double-Stranded cDNA Synthesis Kit (Invitrogen, Carlsbad, CA, USA), and was labeled with either Cy3 or Cy5 (NimbleGen One-Color DNA Labeling Kit). A total of 12 labeled cDNA samples were hybridized to the arrays – three biological replicates from each of the multiple, prolonged and single short cold exposures and the control. All the microarray hybridization, washing and scanning steps were performed according to the instructions of the microarray manufacturer (NimbleGen Gene Expression Analysis User Guide), with the arrays scanned using a Genepix 4000B microarray scanner (Molecular Devices, Sunnyvale, CA, USA).

Scanned images from the microarrays were quantified using NimbleScan v2.5 software (Roche NimbleGen). The expression value of each gene was calculated as the $\log_2$ ratio of relative signal intensities to exacted background. The Robust Multi-Array Average (RMA) algorithm was used to normalize data from the 12 arrays (Irizarry et al., 2003), and the quality of hybridization was assessed by using NimbleScan with the default software settings. Microarray hybridization was of good quality: quality-control analyses indicated a low spatial bias of signal intensity and low non-specific binding during hybridization. The microarray data are deposited in ArrayExpress (accession number: E-MEXP-3194).

Analysis of microarray data

ArrayStar software (v3.0.2, DNAStar, Madison, WI, USA) was used to analyse the microarray data generated by the Nimblescan software. The data were normalized using the Robust Multi-array Average (RMA) algorithm (Irizarry et al., 2003). To identify differentially expressed genes, normalized $\log_2$ expression ratios of transcripts from the *D. melanogaster* transcriptome after multiple, prolonged and single short cold exposures were each compared with control gene expression, and the fold-change was used to describe the degree of up- or down-regulation of each gene. A moderated *t*-test was performed to confirm the statistical significance of differential expression of transcripts from the three biological replicates (Mutch et al., 2002). We designated genes as candidates if they were up- or down-regulated more than twofold in cold-exposed *D. melanogaster* relative to animals under control conditions, with a *P*-value of <0.05 in the moderated *t*-test. In addition, an ANOVA was performed to determine whether expression of the candidate genes differed significantly among the treatment groups rather than just compared with controls. Some candidate genes did not differ among treatment groups, but they were still considered candidates because they were significantly up- or down-regulated in response to cold exposure when compared with the control.

Gene ontology and pathway analysis were investigated with the Database for Annotation, Visualization, and Integrated Discovery (DAVID) v6.7 (http://david.abcc.ncifcrf.gov/). Gene annotation was assigned using the FlyBase R4.3 release, and then converted into DAVID IDs. The whole *D. melanogaster* genome was set as background, and a modified Fisher's exact test (EASE score) was used to examine the significance of candidate gene enrichment in annotation terms. Annotation categories having EASE scores of <0.05 were considered to be significant and examined further. For the pathway analysis, DAVID was used to identify candidate genes involved in pathways from the KEGG_PATHWAY (http://www.kegg.com/kegg/pathway.html).

Quantitative real-time PCR validation

To validate the changes in RNA transcript abundance detected by the microarrays, the expression of identified candidate genes was quantified with three new biological samples by quantitative real-time PCR (qPCR). The 11 most significantly up- and down-regulated genes that were differentially expressed after multiple cold exposures in comparison with the control flies, but not in the flies after sustained and single short cold exposures, were selected for qPCR validation. *TotA*, the gene that was up-regulated in all three cold treatments, was also selected for validation. The gene *Actin79B* was used as a reference gene to normalize data of target genes. Primers for the candidate genes (see supplementary material Table S1) were designed with NCBI primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) or taken from previous publications and checked with Primer3 (http://primer3.sourceforge.net/). Primer pairs were predicted to produce unique amplicons no more than 300 bp in length, with a G–C content higher than 50% and avoiding self-complementarity. cDNA was reverse-transcribed from a total of 5 µg RNA (Invitrogen, Carlsbad, CA, USA) and amplified using the SYBR Green PCR Master Mix (Applied Biosystems, Streetsville, ON, Canada).

The qPCR reactions were cycled up to 45 times as follows: 95°C for 15 min, 55°C for 30 s, 72°C for 30 s in a Rotor-Gene 6000 cycler (Corbett, San Francisco, CA, USA). Standard curves of target genes were generated using five different concentrations of mixed samples, and threshold cycle (Ct) values were calculated from Corbett Rotor-Gene 6000 Application Software version 1.7 (Build 87). For all the tested genes, the correlation coefficient of Ct values (R) was >0.99, and reaction efficiencies were close to 1 (indicating that each cycle results in a doubling of cDNA) from each gene. The relative expression ratio of target genes was calculated and normalized by REST 2009 software version 1 (http://rest.gene-quantification.info).

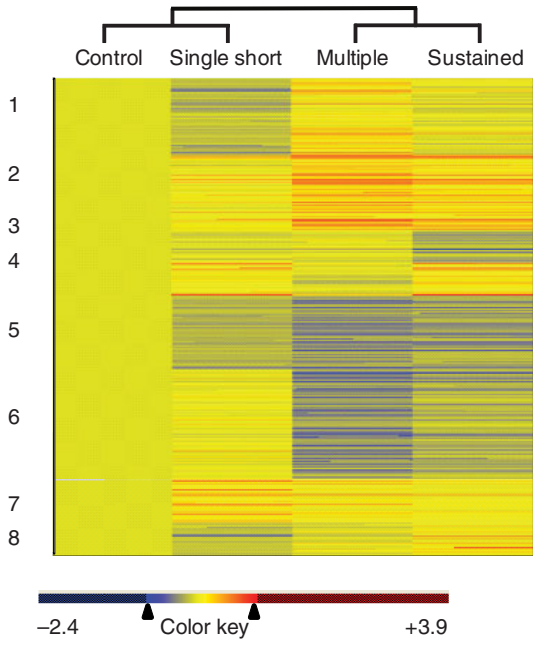

Fig. 2. Heat map representing differential expression of the examined transcripts in female *D. melanogaster* after repeated exposure (five 2 h daily exposures to –0.5°C), prolonged exposure (one 10 h exposure to –0.5°C), a single short cold exposure (one 2 h exposure to –0.5°C) and in the control (maintained at 22°C). The color key corresponds to the scale of transcript mean $\log_2$ expression ratio relative to the control condition on the heat map. Blue indicates genes down-regulated after treatments, yellow indicates genes with no expression changes, red represents genes up-regulated after treatments, and grey indicates empty probes or missing data. Numbers on the left side indicate the eight groups clustered by the k-means method.

RESULTS

Candidate gene identification

In this work, changes in the levels of transcripts in 8 day old virgin female *D. melanogaster* resulting from multiple, prolonged or a short cold treatment(s) relative to control treated flies were examined using DNA microarrays. The mean $\log_2$ differential expression ratio of genes from treatments compared with the control ranged from –2.4 to 3.9 (Fig. 2). Hierarchical clustering indicates that the expression patterns of genes from multiple and prolonged cold exposures cluster together, and the single short group clusters with the control (Fig. 2).

Substantially more candidate genes were identified from flies that received multiple or prolonged cold exposures than from flies that received a single short cold exposure. Seventy-two genes in *D. melanogaster* were identified as candidate genes that were differentially expressed compared with the control during recovery from multiple cold exposures (P<0.05) (Fig. 3). Fifty-nine genes were differentially expressed in flies that received multiple cold exposures (42 up-regulated and 17 genes down-regulated), but not in those that experienced sustained and single short cold exposures (Fig. 3;

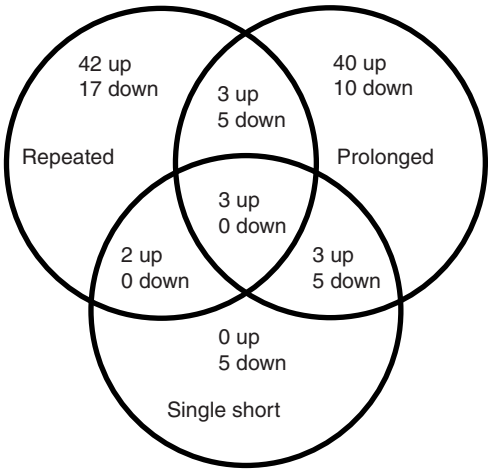

Fig. 3. The number of candidate genes identified in virgin female *D. melanogaster* after repeated (five daily 2 h exposure to –0.5°C), prolonged (one 10 h exposure to –0.5°C) and single short cold (one 2 h exposure to –0.5°C) exposures. The candidate genes were selected with fold changes of expression values >2 compared with that of the control, and *P*-values <0.05 after a moderated *t*-test (supplementary material Tables S2–S5).

supplementary material Table S2). Expression of 20 of these genes in repeatedly exposed flies differed significantly from expression in other cold treatments (ANOVA: $P<0.05$; supplementary material Table S2). After prolonged and single short cold exposures, 69 and 18 genes, respectively, were differentially expressed relative to the control (Fig. 3; supplementary material Tables S3, S4).

There was some overlap among the three treatments in candidate cold-related genes (Fig. 3, Table 1). Eight candidate genes that were differentially expressed after multiple cold exposures were also differentially expressed after prolonged cold exposure; two candidate genes differentially expressed after multiple cold exposures were also differentially expressed after a single short exposure; and eight candidate genes after prolonged cold exposure were also differentially expressed in response to a single short cold exposure. There were three genes that were differentially expressed after all three treatments (Fig. 3, Table 1).

Functional analysis of candidate genes

Nine functional terms were significantly enriched (*P*-values lower than 0.05) in the genes differentially expressed after multiple cold exposures, including five terms (encompassing 32% of the candidate genes from this treatment) associated with muscle structure and function, and two associated with egg development (Table 2). Eight functional terms were significantly enriched in the genes differentially expressed after prolonged cold exposure, including two terms associated with the insect immune system (‘innate immunity’ and ‘defense response’), stress response genes, two terms (‘glycosidase’ and ‘carbohydrate metabolic processes’) that could be associated with carbohydrate metabolism and two terms (‘chorion’ and ‘eggshell formation’) associated with egg production (Table 3). No gene ontology (GO) terms were enriched in flies exposed to a single 2 h bout of cold. A total of 11 functional terms were significantly enriched from the genes differentially expressed in one or more cold treatments, including ‘secreted’ and ‘immune response’ (Table 4). For the pathway analysis, a total of 13 candidate genes from different cold treatments were identified as being associated with KEGG cell signaling pathways and metabolic pathways in *D. melanogaster* (supplementary material Table S5).

Quantitative real-time PCR validation

To validate the microarray results, the abundance of seven up-regulated transcripts and five down-regulated transcripts in female *D. melanogaster* after multiple cold exposures was quantified by qPCR in new biological replicates. Expression of genes was significantly correlated between the microarray and qPCR measurements ($R_9=0.86$, $P<0.001$) (Fig. 4), suggesting that the direction and rank of gene expression are consistent among biological replicates and techniques.

DISCUSSION

Here, we show that the gene expression profile after cold exposure in female *Drosophila melanogaster* is dependent on the frequency and timing of stress, rather than the accumulated time of cold exposure. Given that organisms in the field can encounter repeated bouts of cold and other stress (Sinclair et al., 2003), the distinct gene expression profile following repeated cold exposure implies that the biological responses to repeated stress must be considered when extrapolating from laboratory studies to the field.

Previous genome-wide investigations of low-temperature responses by *D. melanogaster* have, in contrast to many microarray studies, identified surprisingly few candidate genes. These studies have focused on a single cold exposure (e.g. Qin et al., 2005) or populations selected for their ability to recover from a single cold exposure (e.g. Telonis-Scott et al., 2009). For example, 37 candidate genes were identified after 30 min of recovery at 25°C from a cold exposure of 0°C for 2 h (Qin et al., 2005), whereas 94 differentially regulated genes were identified from a comparison of lines of flies selected for chill coma recovery (Telonis-Scott et al., 2009). Our study identified relatively few candidates in association with a single short cold exposure (20, including candidates shared with other cold treatments), but a larger number from prolonged or repeated cold exposures. A core hypothesis of these studies is that differential gene regulation is reflective of responses to physiological perturbation (Dalziel et al., 2009). Thus, our results support the suggestion that an increased total duration of cold exposure results in substantial and long-lasting disruption or modification of homeostasis, which could lead to the observed differences in survival and reproductive output among flies exposed to different cold-exposure regimes (e.g. Marshall and Sinclair, 2010).

Functional analysis of candidate genes using DAVID also implied that the responses of *D. melanogaster* after repeated and single cold exposures were different at the level of gene expression. For example, there were four genes associated with ion binding upregulated after multiple cold exposures, whereas four genes encoding ion-binding proteins were downregulated in the prolonged-exposure group (Table 4). By contrast, there were more immune-related genes differentially regulated in association with single short (10) and prolonged (14) exposures than with repeated exposure (6) (Table 4). The differences in candidate gene functions indicate that, in response to repeated cold exposures, *D. melanogaster* mainly regulate genes to recover muscle activity, whereas an immune response might be more comprehensive after single prolonged or single short cold exposures.

A key difference between our study and previous studies was the use of a 6 h recovery from cold before RNA extraction. Of all of our candidate genes, only three (*Frost*, *Hsp23* and *CG10912*, which has no known function) were shared with a previous study examining expression 1 h after cold exposure (Qin et al., 2005). Three hours after a 3 h cold exposure, the acute gene expression response appears to be in decline (Sinclair et al., 2007). Thus, we expect that, after 6 h, most of the acute gene expression responses to the cold stress

Table 1. Genes differentially expressed in adult virgin female *D. melanogaster* after one or more cold exposures in the three cold treatments (multiple, sustained and single short cold exposures), with a description of associated biological processes or molecular functions, as well as their locations on *D. melanogaster* chromosomes

Differential regulation after cold treatments	Name	Fold change			Biological processes and molecular functions	Chromosome
		Multiple	Sustained	Single short		
Differentially regulated after all three cold treatments	*Turandot A*	15.3 up	4.0 up	3.1 up	Encodes a humoral immune factor that is excreted from the fat body and accumulates in the body fluids	3R
	CG11374	2.4 up	2.2 up	2.1 up	Galactoside binding; urate transmembrane transporter activity	2L
	Hephaestus	2.1 up	2.0 up	2.2 up	mRNA binding; notch signaling pathway; imaginal-disc-derived wing margin morphogenesis	2L
Differentially regulated after multiple and sustained cold treatments	*Turandot C*	9.9 up	2.0 up	1.9 up	Encodes a humoral immune factor	3R
	CG1648	2.8 up	2.1 up	1.0 up	Unknown	2R
	CG40116	2.7 up	2.0 up	1.8 up	Unknown	
	CG41074	2.1 up	2.1 up	1.4 up	Unknown	
	CG15369	2.1 down	2.2 down	1.2 down	Cysteine-type endopeptidase inhibitor activity	X
	Metallothionein D	2.5 down	2.9 down	1.1 down	Metal ion binding	3R
	CG9463	2.6 down	3.3 down	1.6 down	Zinc ion binding; carbohydrate binding	2L
	CG15533	2.6 down	2.6 down	1.1 down	Sphingomyelin phosphodiesterase activity	3R
	Larval serum protein 2	3.5 down	4.5 down	1.2 down	Nutrient reservoir activity; oxygen transporter activity	3L
	CG31532	3.2 up	2.1 up	5.2 down	Unknown	3R
Differentially regulated after multiple and single short cold treatments	*CG40103*	2.0 up	1.7 up	2.1 up	CG40103 has been withdrawn from flybase, but the probe maps to *Argonaute 3*.	
	CG16971	2.0 up	1.4 up	3.1 down	Unknown	3L
Differentially regulated after sustained and single short cold treatments	*Odorant-binding protein 99b*	1.0 up	8.6 up	3.9 up	Olfactory behavior; autophagic cell death	3R
	CG10814	1.0 down	4.8 up	2.0 up	Oxidation reduction; gamma-butyrobetaine dioxygenase activity	2R
	CG15043	1.0 up	4.8 up	2.5 up	Unknown	X
	Attacin A	1.1 up	4.6 up	4.4 up	Antibacterial humoral response; defense response to bacteria	2R
	CG10962	1.0 down	3.4 up	2.1 up	Oxidoreductase activity	X
	Urate oxidase	1.3 down	3.4 up	2.0 up	Urate oxidase activity	2L
	Attacin C	1.3 down	2.5 up	2.0 up	Antibacterial humoral response	2R
	Attacin B	1.0 down	2.3 up	2.0 up	Antibacterial humoral response	2R

Genes were classified based on their GO terms that were identified by DAVID.

would have subsided and that the 6 h time-point represents a new steady-state of gene expression, perhaps reflecting a new homeostatic state or the longer-term processes of recovery from cold stress. For example, we found four genes associated with the regulation of transcription (*Raptor*, *RhoGEF3*, *CG11505* and *CG34415*) up-regulated after a single short cold exposure (supplementary material Table S4). These genes did not experience a change in their expression during a short period of cold recovery (Qin et al., 2005), but they might regulate the response to cold in the long-term.

The differences in gene expression patterns among flies after single short, multiple and prolonged cold exposures could be related to hypothesized differences between the mechanisms of chronic and acute chilling injury (Sinclair and Roberts, 2005). Recovery at high temperature reduced acute chilling injury in the flesh fly *Sarcophaga crassipalpis* after cold shock but not chronic injury after 10 days of cold (Chen and Denlinger, 1992). In this study, the expression pattern of genes after multiple cold exposures was more similar to the pattern after sustained cold exposure, rather than after a single short cold exposure (Fig. 2). Nevertheless, there are many candidate genes that are not shared between the multiple and prolonged exposures, which implies that the effect of chronic cold stress is more than just the sum of shorter cold exposures, and there are very different patterns of mortality and reproductive output in flies exposed to these two regimes (Marshall and Sinclair, 2010). The re-warming period after cold exposure has similarities to re-perfusion injury in freeze-tolerant species (although possibly not freeze avoiders) (Churchill and Storey, 1989). This process results in oxidative stress in the beetle *Alphitobius diaperinus* (Lalouette et al., 2011) and is associated with the activation and inhibition of apoptosis (Yi et al., 2007). It also requires the re-establishment of ion gradients (Kostal et al., 2006; MacMillan and Sinclair, 2011a). Repeated cycles of any or all of these processes could impose different stresses that are not just cumulative and could explain the differences in candidate genes identified in the prolonged- and multiple-exposed flies. These are discussed in more detail below.

Table 2. Enriched functional terms (P<0.05) identified by DAVID from genes differentially expressed in 8 day old virgin female *D. melanogaster* after five daily 2 h exposures to –0.5°C, but not in flies after a single 10 h exposure or a single 2 h exposure to –0.5°C

Functional term	Term ID	Number of genes up-regulated	Number of genes down-regulated	Percentage of total identified genes
Contractile fiber*	GO:0044449	5	0	8.5
Sarcomere*	GO:0030017	4	0	6.8
Myofibril*	GO:0030016	4	0	6.8
Muscle protein*	KW-0514	4	0	6.8
Chorion*	GO:0042600	4	0	6.8
Actin cytoskeleton	GO:0015629	4	0	6.8
Myosin complex	GO:0016460	2	0	3.4
Mesoderm development	GO:0007498	3	0	5.1
Eggshell formation	GO:0030703	3	0	5.1

*Terms with P<0.001.

Table 3. Enriched functional terms (P<0.05) identified by DAVID from genes differentially expressed in 8 day old virgin female *D. melanogaster* after a single 10 h exposure, but not in flies after five daily 2 h exposures to –0.5°C or a single 2 h exposure to –0.5°C

Functional term	Term ID	Number of genes up-regulated	Number of genes down-regulated
Response to stress	GO:0006950	7	1
Innate immunity	KW-0399	4	0
Defense response	GO:0006952	5	0
Chorion	GO:0042600	3	0
Carbohydrate metabolic process	GO:0005975	1	4
Eggshell formation	GO:0030703	3	0
Glycosidase	KW-0326	0	3

Muscle-related genes are differentially expressed after cold exposure

The genes *Tm2*, *Paramyosin*, *upheld*, *Fhos* and *Myosin light chain 2*, associated with muscle construction, actin binding or motor activity, were significantly up-regulated after multiple cold exposures (supplementary material Table S2). Chilling injury is thought to involve a loss of ion homeostasis in the muscle (Kostal et al., 2006; MacMillan and Sinclair, 2011b), which might lead to damage that must be repaired during rewarming. Multiple damage–repair cycles could lead to either long-term damage or a pre-emptive increase in muscle-repair-related genes. For example, the muscle protein gene *Tm2* is important for actin binding and the organization of muscle thin filaments (Lin and Storti, 1997; Marston and El-Mezgueldi, 2008). The gene *Paramyosin* is involved in motor activity and muscle thick filaments (Levine et al., 1976), and *upheld* is involved in muscle thin filament and myofibril assembly (Nongthomba et al., 2007), all of which could represent ongoing muscle repair or reorganization in response to repeated cold exposure. Rapid cold-hardening protects *S. crassipalpis* muscle from cold-shock-induced changes to neuromuscular resting membrane potential (Kelty et al., 1996) and also decreases cold-induced apoptotic cell death in *D. melanogaster* flight muscle (Yi et al., 2007), whereas cold-exposed *Culex pipiens* mosquitoes substantially restructure midgut actin filaments in response to a cold exposure. However, genes encoding muscle proteins were not up-regulated after sustained and single short cold exposures, suggesting that the changes that we saw were not a general response to cold, although they could reflect longer-term damage to muscles.

Cold damage has been previously associated with ion regulation in muscles (Kostal et al., 2006; MacMillan and Sinclair, 2011b), and some of these changes are reduced with fluctuating thermal regimes (Kostal et al., 2007). However, genes associated with Na^+ and K^+ ion binding were not differentially regulated after either multiple or sustained cold exposures, and there were no candidate genes that directly encode ATPase subunits (although one gene, *CG32318*, encoding an ATP-binder was down-regulated after a single short cold exposure). By contrast, Qin and colleagues (Qin et al., 2005) identified three genes related to ATP binding and Na^+–H^+ regulation in *D. melanogaster* after cold shock. Hence, their data suggest that transcriptional changes in genes encoding ATP binders or ion transporters could happen quickly during cold recovery and might have returned to basal levels 6 h after cold exposure, when the samples in this study were collected. In addition, many of the changes in muscle ion equilibria are driven by water

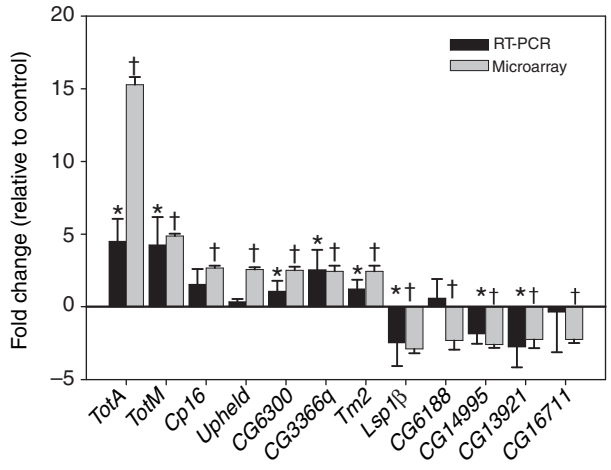

Fig. 4. Expression ratio of 12 candidate genes after multiple cold exposures *versus* the control measured by qPCR (normalized to *Actin79B*). Columns labeled with * (RT-PCR) or † (microarray) indicate genes that are significantly up- or down-regulated relative to the control (P<0.05). Error bars represent the standard error from three biological replicates; note that qPCR and microarrays were performed on different biological replicates. There is a significant positive relationship between the data from the microarray and qPCR (R_9=0.86, P<0.001).

Table 4. Enriched gene ontology terms (P<0.05) identified by DAVID of the genes differentially expressed 6 h after at least one of three cold-exposure treatments

	Repeated		Prolonged		Single short	
Gene ontology terms and IDs	Up	Down	Up	Down	Up	Down
Innate immunity (KW-0399)	2	0	3	0	3	0
Secreted (KW-0964)	2	1	6	1	3	0
Defense response to bacterium (GO:0042742)	1	0	4	0	4	0
Oxidoreductase activity (GO:0016491)	2	0	3	0	3	0
Catalytic activity (GO:0003824)	0	0	3	2	3	0
Metal ion binding (GO:0046872)	2	0	0	2	0	0
Cation binding (GO:0043169)	2	0	0	2	0	0
Organ development (GO:0048513)	1	0	2	0	2	0
Multicellular organismal process (GO:0032501)	1	0	2	0	2	0
Protein binding (GO:0005515)	5	1	6	1	3	0
Membrane-bounded organelle (GO:0043227)	2	0	3	0	1	0

'Single short' indicates a single 2 h exposure to –0.5°C, 'Prolonged' indicates a single 10 h exposure to –0.5°C, and 'Repeated' indicates five daily 2 h exposures to –0.5°C. 'Up' indicates the number of genes within each term that were up-regulated, and 'Down' indicates the number of genes that were down-regulated. Term IDs from DAVID are in parentheses.

and ion movement to the gut from the haemocoel (MacMillan and Sinclair, 2011b), and so ion flux in the muscle cells might be less perturbed than suggested by examining only Nernst potentials across the muscle membrane, and there might be only minimal need for changes in ion homeostasis in muscle cells during recovery. Alternately, the ion-homeostatic responses to cold exposure might be mediated post-transcriptionally (e.g. through phosphorylation) and would therefore not be detected when examining shifts in transcription.

Metabolic and signaling pathways associated with cold exposure

Repeated cold exposures might change the regulation of metabolism in insects. For example, a proteomics approach suggests that fluctuating thermal regimes upregulate pathways associated with glycolysis, the tricarboxylic acid (TCA) cycle and ATP synthesis in the parasitic wasp *Aphidius colemani* (Colinet et al., 2007). Similarly, multiple cold exposure reduces energy reserves in *D. melanogaster* (Marshall and Sinclair, 2010) and the Antarctic midge *Belgica antarctica* (Teets et al., 2011). Here, we show that pathways associated with both lipid and carbohydrate metabolism are differentially regulated in response to prolonged cold exposure, although there are fewer clear metabolic pathways associated with repeated cold exposure (supplementary material Table S5). Several authors have suggested that there might be increased oxidative stress after both single and repeated cold exposure (e.g. Joanisse and Storey, 1998; Lalouette et al., 2011; Rojas and Leopold, 1996), and, after prolonged cold exposure, we observed differential expression of *Glutathione S transferase D2*, which is associated with oxidative stress (supplementary material Table S5).

Several signaling pathways are upregulated in response to cold exposure in insects. For example, the p38 mitogen-activated protein kinase (MAPK) pathway was activated in *S. crassipalpis* within 10 min of exposure to 0°C (Fujiwara and Denlinger, 2007). The up-regulated gene *Phosphotidylinositol 3 kinase 59F* and the down-regulated gene *CG3187* are involved in the p53-signaling pathway, which is associated with repairing DNA damage and regulating apoptosis (Brodsky et al., 2004; Sherr and McCormick, 2002). Upregulation of p53 signaling is consistent with work showing that regulation of apoptosis is a key component of rapid cold hardening in *D. melanogaster*, and we show here that it is particularly responsive to repeated cold exposure (Yi et al., 2007). The Notch signaling pathway is also associated with apoptosis and was differentially regulated in response to cold in our experiment (supplementary material Table S5). In particular, *Hephaestus*, upregulated in all cold-exposure treatments, encodes a protein that regulates Notch signaling and might therefore have other roles in transcriptional regulation (Dansereau et al., 2002). Finally, CG40103, which is upregulated in response to both repeated and single short cold exposures, has been withdrawn from flybase (R5.40), but a BLASTN of the probe maps it to the gene *Argonaute 3*, suggesting that this probe might be detecting upregulation of the *Argonaute 3* transcript (Table 1). *Argonaute 3* is associated with siRNA regulation in *Drosophila* (Nagao et al., 2010), and it might therefore indicate a shift in post-transcriptional modification processes in flies experiencing short cold exposures.

Stress-related genes up-regulated in response to cold exposure

The response of genes encoding heat-shock proteins and other stress-related genes (for example, *Frost*) to cold exposure in insects has been well studied (Clark and Worland, 2008). We observed differential regulation of a number of genes in response to cold stress, including *Hsp23*, which encodes a small heat-shock protein, and *Frost,* which were both upregulated in response to prolonged cold exposure. Three genes (*TotA*, *TotC* and *TotM*) from the Turandot family of stress proteins were also upregulated after cold exposures in our study. Previous work has found that the expression of genes in this family can be induced in *D. melanogaster* by varied stressors, including heat shock, cold shock, septic injury or bacterial infection (Ekengren and Hultmark, 2001). In particular, *TotA* was upregulated in response to all three cold stresses in our study, with a 15-fold up-regulation after multiple cold exposures, whereas *TotM* was only up-regulated in response to multiple cold exposures, and *TotC* was up-regulated after both multiple and prolonged cold exposures. An ortholog of *TotA* is constitutively upregulated in cold-adapted *D.*

subobscura (Laayouni et al., 2007). The function of the *Turandot* genes is not well-understood, but predicted protein sequences suggest that these genes encode 11–14 kDa highly charged proteins, with N-terminal signal peptides that would direct the protein out of the cell (Ekengren and Hultmark, 2001). Our results suggest that the regulation of *Turandot* in response to cold might be slow – we found increased expression of *TotA* in a 6 h cold recovery period, yet a previous microarray study that examined gene expression changes within a 3 h cold recovery period did not find differential regulation of these genes (Qin et al., 2005), and the same is true of studies examining constitutive expression changes in selected lines (De Gregorio et al., 2002; Telonis-Scott et al., 2009).

Immune-related genes were upregulated after a single cold exposure

Surprisingly, 14 candidate genes after prolonged cold exposure and 10 candidate genes after a single short cold exposure were immune-related genes associated with defense against bacterial or fungal infection. In particular, five candidates that were upregulated in our study (*Immune induced molecule 1*, *Immune induced molecule 23*, *AttacinA*, *AttacinB*, *AttacinC* and *Metchnikowin*) encode antimicrobial peptides in *D. melanogaster* and were also upregulated in *D. melanogaster* after septic injury and natural infection (De Gregorio et al., 2002). *PGRP-SC1*, which is upregulated in flies exposed to cold for a single bout of 10 h, might regulate the activation of immune pathways (Bischoff et al., 2006). From this, we hypothesize that *D. melanogaster* activates immune pathways in response to cold stress. This immune upregulation could be because (1) flies have evolved similar cellular responses to cold and infection stress; (2) flies are sensitive to pathogens at low temperatures and therefore respond to cold by pre-emptively up-regulating the immune response; or (3) signaling molecules or pathways that are normally employed during immune responses (e.g. reactive oxygen species) are also produced during cold stress. Larvae of the woolly bear caterpillar that are repeatedly frozen have increased survival to a fungal challenge (Marshall and Sinclair, 2011), and mild cold stress increases survival of fungal infection in *D. melanogaster* (Le Bourg et al., 2009), and so there is mounting evidence for a role for the immune system in insect responses to cold.

Genes associated with reproduction respond to cold exposure

Repeated cold exposure results in a decrease in the fitness of female *D. melanogaster*, which is driven by a reduction in reproductive output, and especially by a shift in the sex ratio of offspring (Marshall and Sinclair, 2010). After multiple and prolonged cold exposures, 4% and 9% of candidate genes, respectively, were associated with reproduction. The Chorion protein families (*Cp16*, *Cp19* and *Cp38*) were up-regulated in the flies after multiple cold exposures, whereas *defective Chorion 1*, *Chorion protein b at 7F* and *Chorion protein c at 7F* were up-regulated and the *Chorion protein 15* was down-regulated by flies after prolonged cold exposures. The Chorion proteins in the vitelline membrane layers play important roles in the assembly and stabilization of the mature eggshell (Noguerón et al., 2000). As vitelline membrane proteins are usually synthesized during the early stages of eggshell development, whereas chorion proteins are synthesized in later stages (Pascucci et al., 1996), cold exposure appears to affect multiple stages of egg development, which could account for at least some of the effects of cold on reproduction. It is unclear whether the upregulation of egg-related genes reflects the repair or replacement of eggs damaged by cold or another role in modifying reproductive output.

CONCLUSIONS

Here, we show that gene expression patterns of *D. melanogaster* in response to exposure to cold vary according to the schedule of exposure and in particular demonstrate that there are substantial differences in responses to multiple and single cold exposure. This adds to a growing body of evidence from studies across taxa that repeated stress must be considered carefully when extrapolating from laboratory experiments to the field. We found overlap of several candidate genes with those identified in previous studies of *D. melanogaster* response to cold, but also established many new candidates, including the Turandot family of stress-responsive genes and a number of genes associated with the immune response, that lead to new questions about the nature of cold exposure and injury in insects.

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