Insect Molecular Biology (2005) **14**(6), 607–613

Cold hardening and transcriptional change in *Drosophila melanogaster*

W. Qin*, S. J. Neal†, R. M. Robertson*, J. T. Westwood† and V. K. Walker*

**Department of Biology, Queen's University, Ontario, Canada;* †*Department of Zoology, University of Toronto at Mississauga, Ontario, Canada*

Abstract

Cold hardening treatment – a brief exposure to low temperatures – can protect certain insects against subsequent exposure to temperatures sufficiently low to cause damage or lethality. Microarray analysis to examine the changes in transcript abundance associated with cold hardening treatment (0 °C for 2 h followed by 30 min recovery at 25 °C) was undertaken in *Drosophila melanogaster* in order to gain insight into this phenomenon. Transcripts associated with 36 genes were identified, a subset of which appeared to be also differentially expressed after heat shock treatment. Quantitative RT-PCR was used to independently determine transcript abundance of a subset of these sequences. Taken together, these assays suggest that stress proteins, including Hsp23, Hsp26, Hsp83 and Frost as well as membrane-associated proteins may contribute to the cold hardening response.

Keywords: cold hardening, heat shock protein, microarray, quantitative RT-PCR.

Introduction

The fruit fly, *Drosophila melanogaster*, is found over a wide geographical area in tropical and temperate regions where temperature and the distribution of water are the two most critical factors that define the confines of the range (Demerec, 1950). *D. melanogaster* has been well known as a model genetic organism for almost a century, and although small, has also been useful for environmental physiological studies. Unlike cold hardy insects, *D. melanogaster* is susceptible to chill injury, showing a developmental threshold at 9–10 °C (Cohet & David, 1980), 'chill coma' or the loss of nerve and muscle excitability at 7 °C (Hosler *et al*., 2000), and extensive mortality at less than –5 °C (Czajka & Lee, 1990). Because ice does not form within the body fluids of *D. melanogaster* until temperatures reach –17 to –20 °C (Czajka & Lee, 1990), these effects are classified as cold shock or chilling injuries depending on their severity.

The detrimental effects of such moderately low temperatures are ameliorated in many insects by a prior cold hardening treatment. Responsive insects can enhance their cold tolerance within minutes or hours (Lee *et al*., 1987). For example, when the pupal stage of *Musca domestica* (house fly) was transferred to –7 °C for 2 h, no insects survived, but survival increased to 80% with exposure to 0 °C for 90 min, prior to the transfer to subzero conditions (Coulson & Bale, 1990). These rapid cold hardened house flies, as well as flesh flies (*Sarcophaga crassipalpis*), accumulated cryoprotectants such as low molecular mass polyhydric alcohols, sugars and glycerol (Chen *et al*., 1997; Denlinger & Lee, 1998), which may help stabilize membrane lipids and cellular proteins. Cold shock or chill-tolerant selected *D. melanogaster* lines showed higher levels of energy reserves, including glycogen, triacylglycerols and proline (Chen & Walker, 1994; Misener *et al*., 2001). Thus the synthesis of polyols, sugars and amino acids that are well known in the success of a variety of overwintering insects (Baust & Lee, 1981; Storey & Storey, 1981; Denlinger & Lee, 1998) may also be important for the survival of cold-susceptible insects following a cold hardening response.

Heat shock proteins (Hsps), which are important for protein folding and chaperoning functions, were induced after a prolonged exposure to 0 °C in *D. melanogaster* (Burton *et al*., 1988). However, Kelty & Lee (2001) reported that levels of the most responsive heat-inducible protein, Hsp70, did not change. No alterations to glycerol and sugar levels were detected either, suggesting that the cold hardening response might not be dependent on significant increases in stress protein and/ or cryoprotectant titres (Kelty & Lee, 2001). Despite these somewhat equivocal results, taken together, the regulation of particular genes would presumably be important for chill tolerance, similar to the adjustments made in the autumn in response to lower temperatures. With the completion of the genome sequence of *D. melanogaster*

doi: 10.1111/j.1365-2583.2005.00589.x

Received 6 April 2005; accepted after revision 3 June 2005. Correspondence: Virginia K. Walker, Department of Biology, Queen's University, Kingston, Ontario, K7L 3N6, Canada. E-mail: walkervk@biology.queensu.ca

(Adams *et al*., 2000) and the availability of DNA microarrays, the way is now clear to address this question on the adaptations elicited by cold hardening, on a genome-wide basis.

Results

Survival and array-based profile of D. melanogaster *subjected to cold hardening and heat shock treatments*

It was important for the analysis that the cold hardening treatment selected (2 h at 0 °C) resulted in no mortality. In pilot experiments using the regime described in the methods, no flies died (0/50), as has been reported in previous studies (Chen *et al*., 1993, 1994).

The 7000 sequence CDMC DNA microarrays (d7k2) were used for the initial analyses but were replaced with the 12 000 sequence arrays (d12k1) when they became available. Not surprisingly, the results with the two versions of the arrays were concordant. These were used to identify genes encoding transcripts that were differentially abundant in *Drosophila* adult male controls and males subjected to a low temperature treatment that we, and others, have shown to elicit a cold hardening response. In all, four separate hybridizations were done including using 'flipped fluors' on microarray slides containing duplicate DNA spots. Differentially expressed genes were identified using the Statistical Analysis of Microarrays software (Tusher *et al*., 2001). A total of 40 sequences were identified by the software including two sequences representing the same gene. Visual inspection of the raw data reduced the list by two genes based on poor spot quality. This left 37 unique sequences that consistently showed hybridization intensities with Cy-labelled cDNAs from low temperature-treated flies that were different from those derived from control flies (Tables 1 and 2). Of these sequences, RNA appeared to be more abundant for 31 transcripts from cold-treated flies, and less abundant for six transcripts.

After submission of the 37 sequences to FlyBase, 16 could be identified by gene, 16 were similar to other known protein-encoding genes, 17 conceptually translated sequences had domains which allowed their cellular locations to be determined by similarities to sequences in the protein data base, and eight had no significant similarity with other sequences and were designated as unknown sequences. Transcripts that increased in abundance after

Table 1. Sequences corresponding to transcripts that showed an increase in abundance after cold hardening treatments, and their corresponding abundance after heat shock, as identified by microarray analysis. Group refers to similar protein functions or locations. CG numbers can be found in FlyBase, HS (heat shock) and CS (cold shock) values represent the mean $\log_2$ ratios in all the microarray hybridization experiments. –, No significant change

Group	CG number	HS $\log_2$ ratio	CS $\log_2$ ratio	Protein or related function
Stress	CG1242	2.0	1.4	Heat shock protein 83, defense, protein folding, regulates sleep, (*Hsp83*)
	CG4183	3.7	1.2	Heat shock protein 26, defense, protein folding, (*Hsp26*)
	CG4463	4.0	1.0	Heat shock protein 23, defense, protein folding, (*Hsp23*)
	CG11624	–	0.8	Ubiquitin-63E, protein proteolysis, temperature stress, (*Ubi-P63e*)
	CG9434	–	1.0	Frost, (*Fst*)
Membrane	CG3124	–	0.7	G-protein (guanine nucleotide biding protein)?
	CG17144	–	0.8	APT/GTP binding membrane protein?
	CG31085	–	0.9	ABC transporter?
	CG18609	–	1.0	Membrane protein for fatty acid elongation?
	CG10939	–	0.6	SRY interacting protein 1, Na^+/H^+ regulation (ion transport), (*Sip1*)
	CG9355	–	0.6	dusky, cuticle (membrane) protein, (*dy*)
	CG10912	–	1.3	Membrane protein?
	CG9568	–	1.2	Membrane protein?
	CG13510	–	1.0	Membrane protein?
	CG3814	–	0.8	Membrane protein?
	CG15347	–	0.8	Membrane protein?
	CG10934	−0.6	0.7	Membrane protein?
Mitochondrial and energy	CG8026	–	0.5	Mitochondrial acyl carrier protein 1?
	CG9160	–	0.5	Mitochondrial acyl carrier protein 1, (*mtacp1*)
	CG8778	2.2	1.2	Enoyl-CoA hydratase
Expression	CG10203	–	0.8	RNA binding, splicing factor, (*xl6*)
	CG1098	−0.6	0.8	Serine/threonine protein kinase?
	CG4143	–	0.6	Multiprotein bridging factor 1, transcription co-activator (*mbf1*)
Other	CG8838	–	0.6	ATP/GTP binding protein?
	CG5011	–	0.7	Proline-rich protein
	CG3132	–	0.8	Glycoside hydrolase family, can be associated with cell death, (*Ect3*)
	CG15745	–	0.7	Glycoside hydrolase family?
	CG32038	–	1.1	Unknown
	CG8012	–	0.8	Unknown
	CG4710	0.8	0.7	Unknown
	CG3345	–	0.5	Unknown

Table 2. Sequences corresponding to transcripts that showed a decrease in abundance after cold hardening treatment, as identified by microarray analysis. CG numbers can be found in FlyBase. Cold shock (CS) values represent the mean $\log_2$ ratios in all the microarray hybridization experiments

CG number	CS $\log_2$ ratio	Protein or related function
CG2118	–0.7	Mitochondrial CoA carboxylase
CG2086	–1.9	draper (lubricant for extra cellular matrix), (*drpr*)
CG4193	–2.4	deadhead (electron transport), membrane protein ? (*dhd*)
CG7390	–1.0	Senescence marker protein-30, Ca^{2+} binding signalling protein, (*smp-30*)
CG5403	–0.8	retained (transcription activation), (*retn*)
CG9885	–0.7	decapentaplegic (signal transduction), (*dpp*)

low temperature exposure were classified into five groups based on gene description or potential functions as described (Table 1). The largest group, 12/31 genes (39%), were classified as membrane-related protein genes. Five (14%) were placed in the stress gene group. Because four of these genes (*Hsp23*, *Hsp26*, *Hsp83* and *Ubi-P63e*) with increased transcript levels after our cold-hardening protocol are also known to be up-regulated after heat shock at 36.5 °C, microarrays hybridized with RNA from heat-treated males were compared with those subjected to cold hardening (Tables 1 and 2; S. J. Neal and J. T. Westwood, unpublished observations). As has been seen in other work (Pauli *et al*., 1992), Hsp transcripts including the three *Hsp* genes of interest in this study, substantially increased in abundance after heat shock ($\log_2$ ratios of 2–4). Transcripts corresponding to the other two stress-associated genes identified after cold-hardening treatment were also slightly increased after transfer to 36.5 °C (data not shown).

Quantification of mRNA using real time RT-PCR

The expression of selected genes (9/31 putative up-regulated and 5/6 putative down-regulated sequences) after cold hardening treatment was examined by real time RT-PCR in order to have a second assay of specific RNA abundance. As there is not an *a priori* reason to favour the results of one type of experiment over another, assessments of transcript abundance were averaged (Table 3).

The two independent methods of assessment were not concordant for an unknown gene, CG32038, and transcripts could not be amplified even with two different sets of primers for *dhd* (CG4193) (Table 4). This latter gene is reportedly expressed only in females (Svensson *et al*., 2003) and thus a lack of amplification product with the PCR primers would have been expected. However, the apparent abundance of the corresponding message was reduced under cold hardening conditions, according to the microarray findings. To verify the DNA sequence on the d12K1 array, the cDNA (clone LD23983) was sequenced and found to be correct (result not shown). Examination of the *dhd* sequence, however, showed 100% identity to 39 bp of the *msk* gene (AF251145), which encodes a Ran binding protein 7 that likely binds the extracellular matrix. It also showed 96% identity to 47 bp of the *tst* gene (AJ276896), which encodes a helicase Ski2 protein. Of these two, a minor decrease in the hybridization intensity (mean $\log_2$ ratio of –0.2) was seen for *msk* in cold-hardened males. This study suggests that the abundance and tract of sequence identity of *msk* mRNA in control flies was sufficient to hybridize to the *dhd* sequence on the microarray, in the absence of competition from the corresponding *dhd* mRNA. The decrease that was erroneously initially attributed to *dhd*'down-regulation', could reflect a decrease in *msk* transcript abundance. This hypothesis should be tested by real time PCR assays with *msk* primers.

Table 3. Sequences corresponding to transcripts that showed changes in abundance after cold hardening treatment as assessed by microarray and real time RT-PCR. The gene symbol is listed, if known. N/A, non-amplifiable; see Results

		Transcript levels		
CG Number	Gene symbol	by microarrays[a]	by real-time RT-PCR[b]	Average[c]
CG1242	*Hsp83*	2.6	4.5 ± 1.1	3.6
CG4183	*Hsp26*	2.3	1.7 ± 0.1	2.0
CG4463	*Hsp23*	2.0	1.2 ± 0.1	1.6
CG9434	*Fst*	2.0	2.3 ± 0.8	2.2
CG10912		2.4	1.5 ± 0.2	2.0
CG9568		2.3	1.2 ± 0.2	1.8
CG13510		2.0	1.3 ± 0.2	1.6
CG8778		2.3	2.1 ± 0.3	2.2
CG32038		2.1	0.9 ± 0.1	1.5
CG2118		0.6	0.7 ± 0.2	0.6
CG2086	*drpr*	0.3	0.5 ± 0.2	0.4
CG7390	*smp-30*	0.5	0.8 ± 0.1	0.6
CG5403	*retn*	0.6	0.7 ± 0.2	0.6
CG4193	*dhd*	0.2	N/A	–

[a]The mean $\log_2$ ratio (x) shown in Tables 2 and 3 was converted to a transcript ratio, where ratio = 2^x.
[b]The mean values ± standard deviation of the transcript abundance of at least three separate determinations.
[c]The relative levels of mRNA encoded by each sequence and as assessed by the two independent methods was averaged (see Results).

Discussion

When temperature changes, multicellular organisms respond by altering metabolic rate, intracellular pH, ion concentration, as well as membrane composition and gene expression. Past research has established that a significant elevation of

Table 4. Primers used for the real time RT-PCR amplification of selected sequences identified in the *D. melanogaster* microarray analysis. Most primers were named after the CG number found in FlyBase; LP and RP refer to forward and reverse primers, respectively. RA refers to a specific transcript of the gene as defined in FlyBase. GenBank accession numbers are links to the gene sequence data at http://www.ncbi.nlm.nih.gov

Primer name	Sequence	GenBank number
CG1242LP1	5′-GGAGTTCCGTGCTCTGCTCTTC	NM_079175
CG1242RP1	5′-CTGGAATGAGGTCCTCGCAGTT	
CG10912LP1	5′-ATGCTTCGGTCTCGGTCAGTTC	NM_137443
CG10912RP1	5′-GATTCTCACCGCTCAGGCACTT	
CG8778LP1	5′-ACTGCTGCTAGCGACACGAAGA	NM_136961
CG8778RP1	5′-AACACTCGTGCCGTGAAAATGA	
CG9568LP1	5′-TGCGTCTTCAACGAAACGAATG	NM_135423
CG9568RP1	5′-GGCGGATCCCACGATTGTATAG	
CG4183LP1	5′-GGTGGACGACTCCATCTTGGTC	NM_079273
CG4183RP1	5′-CTTGTAGCCATCGGGAACCTTG	
CG32038LP1	5′-CAACTACTCCGTGCTGCTCTCG	NM_168344
CG32038RP1	5′-ATTGGTCTGGGCATGGTCTGAT	
CG9434LP1	5′-CATCATGGTCATCATGGCAACA	NM_079570
CG9434RP1	5′-CACATCATCCTCGGTGGTCAAC	
CG13510LP1	5′-TGGCACCTACTCAAGCCAACAA	NM_137856
CG13510RP1	5′-TGGAGGGGTACAAGAGGAGTCG	
CG4463LP1	5′-GAATCCCTACTTGGCCCTGGTT	NM_079275
CG4463RP1	5′-ACACATCCATGCAGACCTGGAA	
CG2086RALP1	5′-GGACCCGCCTGCGATATAATTT	NM_167911
CG2086RARP1	5′-TTCCATGCCGTAGAATCCAGGT	
CG4193LP1	5′-GTGAAGGCGTAAGCAGCAATCC	NM_078491
CG4193RP1	5′-ACGAACACAAATGAGGCGAACA	
CG4193LP2	5′-TGTTCGCCTCATTTGTGTTCGT	
CG4193RP2	5′-GCTGCACTGCATCTCCTGCTAC	
CG2118RALP1	5′-TGGCACCGTGGAGTTCATTCTA	NM_143639
CG2118RARP1	5′-CAATGCGAATCTGCCATTCAAC	
CG5403LP1	5′-ACAGTTCGCCCGTTTCCACTAA	NM_176254
CG5403RP1	5′-AGGATTGAGCTTGCCACTGGAC	
CG7390RALP1	5′-CTGGGATGTCAAGGCCAAGAAG	NM_079629
CG7390RARP1	5′-GCCGAATCTTCCTCAGATCGAA	
DmAct5CLP1	5′-AGCGCGGTTACTCTTTCACCAC	K00667
DmAct5CRP1	5′-GTGGCCATCTCCTGCTCAAAGT	

temperature results in a specific heat shock response and synthesis of a set of highly conserved heat shock proteins (Craig, 1985). In contrast, the cellular response to low temperature has not been as well studied. Cold hardening is a phenomenon that is recognized in several flies (Chen *et al.*, 1993, 1994; Czajka & Lee, 1990) that leads to a dramatic reduction in lethality if these insects are subsequently transferred to −5 °C or −7 °C. It is not understood what metabolic changes lead to this increased survival, however, some common mechanisms for cold adaptation have been found in a variety of organisms, including alterations in membrane fluidity and protein translation machinery (Thieringer *et al.*, 1998).

When microarrays were hybridized with transcripts expressed during the cold hardening response, only 37 messages, representing a relatively small number of the 12 000 *D. melanogaster* sequences, appeared to be differentially abundant. More than one-third of those that were more abundant during the cold hardening response appear to encode membrane proteins. Perhaps this could have been expected, as changes in the phospholipid composition of membranes is a common adaptive response to temperature variation (Haque & Russell, 2004), with low temperature exposure frequently associated with plasma membrane damage (Steponkus, 1984). In some microorganisms, a decrease in temperature results in an elevated synthesis of membrane-bound desaturase and an increased stability of this enzyme, believed to protect membrane function (Fulco & Fujii, 1980).

Most of the transcriptional changes induced by the cold hardening response lead to an increase in mRNA levels, but a few mRNAs were reduced in abundance. Of these, transcript levels for *smp-30* (CG7390) decreased after 0 °C exposure (Tables 2 and 3), but previously had been reported to increase during 15 °C acclimation in *D. melanogaster* (Goto, 2000). The encoded protein regulates intracellular Ca^{2+} homeostasis, with down-regulation associated with the decline in cellular functions during ageing in mammals (Fujita & Maruyama, 1998; Fujita *et al.*, 1998). This appears to be correlated with increased reactive oxygen species and lipopolysaccharide-induced free radicals (Jung *et al.*, 2004). Thus it is possible that the reduced levels of *smp-30* mRNA, rather than reflect an adaptive response to low temperature, actually reflect the damage that can occur if flies are kept at these temperatures for prolonged periods. Because chilling injury has been ascribed to oxidative stress (Jahnkhe *et al.*, 1991; Walker & McKensie, 1993; Prasad *et al.*, 1994), and injury to the mitochondria and electron transport system could generate free radicals, it is interesting that some of the sequences identified in this study were associated with mitochondria or stress responses.

Transcripts encoding a subset of the stress-related proteins found after heat shock, Hsp23, Hsp26 and Hsp83, increased in abundance after the cold hardening regime. These three Hsps are also transcribed in *D. melanogaster* ovarian nurse cells. Interestingly, Zimmerman *et al.* (1983) suggested that this expression may allow these cells to enter a developmental arrest characteristic of the preblastoderm, during heat stress, and later resume normal transcription. Similarly, Yiangou *et al.* (1997) observed a significant increase in Hsp83 transcript levels after prolonged treatment of *Drosophila auraria* at 0 °C. Burton *et al.* (1988), however, reported the induction of all the major Hsps in *D. melanogaster* at the same temperature. Because Hsps are chaperone proteins that facilitate the refolding of damaged proteins, this is likely a useful function presumably for heat or cold-denatured proteins and could protect these insects from cold exposure. Hsps have also been noted in cold hardy diapausing *S. crassipalpis* and the gypsy moth, *Lymantia dispar* (Yocum *et al.*, 1991; Denlinger *et al.*, 1992; Denlinger, 2002), but not in *Lucilia sericata* induced to enter diapause in the absence of low temperature treatment (Tachibana *et al.*, 2005). In addition to serving a

developmental and chaperone function, Hsp83 has been implicated in the sleep response, protecting flies against sleep deprivation stress (Shaw *et al*., 2002). Ubiquitin, a protein that binds nuclear proteins can also be induced by heat stress (Niedzwiecki & Fleming, 1993). Similar to the three Hsps, we have shown that the level of its corresponding transcript also increases after cold hardening (Tables 1 and 3).

Frost (*Fst*) is one of the few genes that has been previously associated only with cold stress. The cold hardening regime used here resulted in lower *Fst* mRNA levels than have been seen after lengthier low temperature exposure (e.g. 0 °C for 8 h), as noted in Northern blots (Goto, 2001). However, our observations demonstrate that this known cold shock transcript also increases in abundance during the cold-hardening treatment response. Although the function of *Fst* is unidentified, its sequence suggests that it is a mucin-like protein; taken together these two studies argue that this gene product likely plays an important role in the cold hardening and cold injury response.

Cycling body temperatures that can approach 0 °C during torpor are characteristic of certain mammalian hibernations (Van Breukelen & Martin, 2002) and thus a comparison of the physiological changes in these organisms with the insect cold hardening response may be useful. During hibernation, there is a shift from carbohydrate to fatty acid oxidation, and in cold hardened flies, sequence CG8778, which likely encodes enolyl-CoA hydratase, appears to be up-regulated (Tables 1 and 3). In hibernating animals Ca^{2+} regulation is maintained, there is an increased concentration of ubiquitin conjugates, antioxidant defenses are increased and stress proteins are synthesized (van Breukelen & Martin, 2002). We have shown here that representatives of sequences encoding proteins involved in these several pathways can be identified by microarray analysis in cold hardened insects as well. It suggests to us that cold tolerance and the cold hardening response in insects and hibernation in animals may share some surprisingly similar molecular adaptations, and the way is now clear to analyse these in *Drosophila*.

Experimental procedures

Insect rearing

A *D. melanogaster* stock (*cn bw dp cl* strain), which originated from flies kept in continuous laboratory culture for over 50 years, was marked with several eye colour and wing mutations to ensure that it was not inadvertently contaminated with feral lines. Flies were cultured in a standard yeast sucrose medium, at 25 °C in a 12 h light/12 h darkness (L/D, 8 am to 8 pm) photoperiod. Newly emerged adults were removed from bottles after eclosion and transferred to fresh medium on which they were allowed to feed and oviposit for 1–2 days. Adult males (≤ 2 days post eclosion) were selected and transferred to fresh food bottles for five more days before being subjected to cold treatment.

Temperature treatment and RNA isolation

Adult males (6–7 days post eclosion) were treated for 2 h at 0 ± 0.5 °C, followed by a 30 min recovery at 25 °C (during the light portion of their photoperiod). Control flies were kept at 25 °C. Similarly, for heat shock experiments, males (6–7 days post eclosion), were heat shocked at 30 min at 36.5 °C, followed by a 30 min recovery at 25 °C. Low and high temperature treated and unshocked control flies were collected, snap frozen in liquid nitrogen and stored at –70 °C. Some flies were allowed to recover for 1.5 h at 25 °C and their viability was assessed. A recovery period was included to ensure that all flies survived the 0 °C treatments. As well, this 30-min period allowed RNA polymerases to complete any transcripts that were initiated during the cold-hardening treatment treatment. It was assumed that 'recovery' transcripts would not accumulate in sufficient titres to be detected by microarray analysis, but not tested.

TRIzol reagent (Invitrogen, Burlington, ON, Canada) was used to isolate total RNA from the high and low temperature treated *D. melanogaster*, following the manufacturer's instructions. RNAs were resuspended in RNase-free water to a minimum concentration of 4 μg/μl and the RNA quantity and quality were assessed by spectrophotometry where A_{260}/A_{280} ratios were typically above 1.9. The RNA quality was also evaluated by agarose gel electrophoresis of glyoxal-denatured samples (Sambrook & Russell, 2001).

Microarray hybridization and analysis

Microarrays (d7k2 and d12k1) were produced by the Canadian *Drosophila* Microarray Centre (CDMC; http://www.flyarrays.com) Prior to hybridization, the arrays were treated with blocking solution (10 M 1-methyl-2-pyrrolidinone, 430 mM succinic anhydride, and 430 mM sodium borate, pH 8.0) for 15 min, washed twice for 1 min in water, 2 min in 1% SDS, and twice for 1 min in water again and finally rinsed in 95% ethanol prior to drying at 37 °C (Neal *et al*., 2003).

Probes for the microarrays were prepared from 80 μg of total RNA using reverse transcriptase to directly incorporate blue and red cyanine (Cy) dyes into the product cDNA as described by the manufacturer and modified by Neal *et al*. (2003). The Cy3- and Cy5-labelled target cDNAs were combined and precipitated at –20 °C (≥ 1 h) by the addition of an equal volume of isopropanol, centrifuged at > 12 000 ***g*** for 15 min and washed twice with 200 μl cold 70% ethanol. Pellets were briefly air-dried and dissolved in 5 μl RNase-free water. Hybridization solution (75 ml), containing DIG Easy Hyb (Roche Diagnostics, Laval, Quebec) and 37.5 mg each of salmon sperm DNA and yeast tRNA, was added to the samples before they were heat-denatured at 65 °C for 3 min.

Hybridizations were performed at the CMDC using identical conditions as those previously reported (Neal *et al*., 2003), making the present results directly comparable to those from other studies currently underway at the centre. Briefly, relatively stringent hybridization and washing conditions were used (3 × 15 min washes with 1 × SSC, 0.1% SDS at 50 °C and brief room temperature washes in 1 × SSC and 0.1 × SSC). Microarrays were scanned with a ScanArray 4000 system (Perkin Elmer, formerly GSI Lumonics), using both blue and red lasers. Images were quantified with QuantArray software (v3.0, Perkin Elmer), and data were normalized, using GeneTraffic Duo (Stratagene, La Jolla, CA). Low intensity spots were flagged using filters in GeneTraffic on the basis of raw signal intensity and signal-to-background ratio. These

spots were ignored during the analysis. All data from this study are publicly available from the Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo) BLAST (www.ncbi.nlm.nih.gov) was used to link the DNAs to FlyBase (http://FlyBase.net) to determine the function, biological process or cellular component corresponding to each gene.

Real time RT-PCR

A subset of the sequences identified using array-based data as 'up-regulated' or 'down-regulated' genes were selected for independent assessment of mRNA levels using real time RT-PCR. Primers were designed using the Primer3 algorithm (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_http://www.cgi; Rozen and Skaletsky, 2000) in order to obtain expected amplified products of 100–150 bp for each of the 14 genes.

One set of primers for each gene (except CG4193 with two sets of primers; Table 4) was synthesized. *D. melanogaster* actin 5C (DmAct5C) gene-specific primers were used for the amplification of a housekeeping gene amplification product control. All primer sequences are listed in Table 4. A QuantiTect SYBR Green RT-PCR kit (Qiagen, Mississauga, Canada) was used to monitor transcript abundance for each gene. A standard curve was made by serially diluting (0.5, 2, 10, 25, 50 ng) total RNA and amplifying with the DmAct5C primer set. Real time RT-PCRs were performed in 25 μl volumes with 50 ng total RNA using a Cepheid Smart thermocycler (Fisher Scientific, Ottawa, ON) and the following conditions: a 2-temperature cycle repeat (50 °C for 30 min and 94 °C for 15 min) followed by a 4-temperature cycle, repeated 45 times (95 °C for 15 s, 59 °C for 30 s, 72 °C for 30 s, 79 °C for 15 s) using the optics option.

Acknowledgements

We acknowledge Sarah Gonsalves and Jianming Pei for technical assistance with the microarray analysis. The Core Facility for Protein Discovery and Function and an NSERC Discovery grant to V.K.W. allowed the analysis, and W.Q. acknowledges an Ontario Graduate Scholarship.

References

Adams, M.D., Celniker, S.E., Holt, R.A., Evans, C.A., Gocayne, J.D., Amanatides, P.G. *et al.* (2000) The genome sequence of *Drosophila melanogaster*. *Science* **287**: 2185–2195.

Baust, J.G. and Lee, R.E. (1981) Divergent mechanisms of frost-hardiness in two populations of the gall fly, *Eurosta solidaginis*. *J Insect Physiol* **27**: 485–490.

Burton, V., Mitchell, H.K., Young, P. and Petersen, N.S. (1988) Heat shock protection against cold stress of *Drosophila melanogaster*. *Mol Cell Biol* **8**: 3550–3552.

Chen, C.P. and Walker, V.K. (1994) Cold shock and chilling tolerance in *Drosophila*. *J Insect Physiol* **40**: 661–669.

Chen, Z., Jiang, J.C., Lin, Z.G., Lee, W.R., Baker, M.E. and Chang, S.H. (1993) Site-specific mutagenesis of *Drosophila* alcohol dehydrogenase: evidence for involvement of tyrosine-152 and lysine-156 in catalysis. *Biochemistry* **32**: 3342–3346.

Chen, R., Belelli, D., Lambert, J.J., Peters, J.A., Reyes, A. and Lan, N.C. (1994) Cloning and functional expression of a *Drosophila* gamma-aminobutyric acid receptor. *Proc Natl Acad Sci USA* **91**: 6069–6073.

Chen, L., DeVries, A.L. and Cheng, C.H. (1997) Evolution of antifreeze glycoprotein gene from a trypsinogen gene in Antarcticnotothenioid fish. *Proc Natl Acad Sci USA* **94**: 3811–3816.

Cohet, Y. and David, J. (1980) Geographic divergence and sexual-behavior – comparison of mating systems in french and afro-tropical populations of *Drosophila melanogaster*. *Genetica* **54**: 161–165.

Coulson, S.J. and Bale, J.S. (1990) Characterisation and limitations of the rapid cold-hardening treatment response in the housefly *Musca domestica* (Diptera: Muscidae). *J Insect Physiol* **36**: 207–211.

Craig, E.A. (1985) The heat shock response. *CRC Crit Rev Biochem* **18**: 239–280.

Czajka, M.C. and Lee, R.E. Jr (1990) A rapid cold-hardening treatment response protecting against cold shock injury in *Drosophila melanogaster*. *J Exp Biol* **148**: 245–254.

Demerec, M. (1950) *Biology of Drosophila*. New York: John Wiley and Sons, Inc.

Denlinger, D.L. (2002) Regulation of diapause. *Annu Rev Entomol* **47**: 93–122.

Denlinger, D.L. and Lee, R.E. (1998) Physiology of cold sensitivity. In: *Lethal Temperatures in Integrated Pest Management* (Hallman, G.J. and Denlinger, D.L., eds), pp. 55–95. Westview Press, Boulder, CO.

Denlinger, D.L., Lee, R.E., Yocum G.D. and Kukal O. (1992) Role of chilling in the acquisition of cold tolerance and the capacitation to express stress proteins in diapausing pharate larvae of the gypsy moth, *Lymantria dispar*. *Arch Insect Biochem Physiol* **21**: 271–280.

Fujita, T. and Maruyama, N. (1998) Expression and structure of senescence marker protein-30 (SMP30) and its physiological function. *Nippon Ronen Igakkai Zasshi* **35**: 654–657.

Fujita, T., Inoue, H., Kitamura, T., Sato, N., Shimosawa, T. and Maruyama, N. (1998) Senescence marker protein-30 (SMP30) rescues cell death by enhancing plasma membrane Ca^{2+}-pumping activity in Hep G2 cells. *Biochem Biophys Res Commun* **250**: 374–380.

Fulco, A.J. and Fujii, D.K. (1980) Adaptive regulation of membrane lipid biosynthesis in Bacilli by environmental temperature. In: *Membrane Fluidity: Biophysical Techniques and Cellular Regulation* (Kates, M. and Kuksis, A., eds), pp. 77–98. Humana Press, Totowa, NJ.

Goto, S.G. (2000) Expression of *Drosophila* homologue of senescence marker protein-30 during cold acclimation. *J Insect Physiol* **46**: 1111–1120.

Goto, S.G. (2001) A novel gene that is up-regulated during recovery from cold shock in *Drosophila melanogaster*. *Gene* **270**: 259–264.

Haque, M.A. and Russell, N.J. (2004) Strains of *Bacillus cereus* vary in the phenotypic adaptation of their membrane lipid composition in response to low water activity, reduced temperature and growth in rice starch. *Microbiology* **150**: 1397–1404.

Hosler, J.S., Burns, J.E. and Esch, H.E. (2000) Flight muscle resting potential and species-specific differences in chill-coma. *J Insect Physiol* **46**: 621–627.

Jahnkhe, L.S., Hull, M.R. and Long, S.P. (1991) Chilling stress and oxygen metabolizing enzymes in *Zea mays and Zea diploperennis*. *Plant Cell Environ* **14**: 97–104.

Jung, K.J., Ishigami, A., Maruyama, N., Takahashi, R. and Goto, S., Yu, B.P. and Chung, H.Y. (2004) Modulation of gene expression of SMP-30 by LPS and calorie restriction during aging process. *Exp Gerontol* **39**: 1169–1177.

Kelty, J.D. and Lee, R.E. Jr (2001) Rapid cold-hardening treatment of *Drosophila melanogaster* (Diptera: Drosophiladae) during ecologically based thermoperiodic cycles. *J Exp Biol* **204**: 1659–1666.

Lee, R.E., Chen, C.P., Meacham, M.H. and Denlinger, D.L. (1987) Ontogenetic patterns of cold-hardiness and glycerol production in *Sarcophaga crassipalpis.* J. *Insect Physiol* **33**: 587–592.

Misener, S.R., Chen, C. and Walker, V. K. (2001) Cold tolerance and proline gene expression in *Drosophila melanogaster*. *J Insect Physiol* **47**: 393–400.

Neal, S.J., Gibson, M.L., So, A.K. and Westwood, J.T. (2003) Construction of a cDNA-based microarray for *Drosophila melanogaster*: a comparison of gene transcription profiles from SL2 and Kc167 cells. *Genome* **46**: 879–892.

Niedzwiecki, A. and Fleming, J.E. (1993) Heat shock induces changes in the expression and binding of ubiquitin in senescent *Drosophila melanogaster*. *Dev Genet* **14**: 78–86.

Pauli, D., Arrigo, A.P. and Tissieres, A. (1992) Heat shock response in *Drosophila*. *Experientia* **48**: 623–629.

Prasad, T.K., Anderson, M.D., Martin, B.A. and Stewart, C.R. (1994) Evidence or chilling-induced oxidative stress in maize seedlings and a regulatory role for hydrogen peroxide. *The Plant Cell* **6**: 65–74.

Sambrook, J. and Russell, D.W. (2001) Molecular cloning. *A Laboratory Manual*, 3rd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Shaw, P.J., Tononi, G., Greenspan, R.J. and Robinson, D.F. (2002) Stress response genes protect against lethal effects of sleep deprivation in *Drosophila*. *Nature* **417**: 287–291.

Steponkus, P.L. (1984) Role of the plasma membrane in freezing injury and cold acclimation. *Annu Rev Plant Physiol* **35**: 543–584.

Storey, K.B. and Storey, J. (1981) Biochemical strategies of overwintering in the gall fly larva, *Eurosta solidaginis*: Effect of low temperature acclimation on the activities of enzymes of intermediary metabolism. *J Comparative Physiol* **144**: 191–199.

Svensson, M.J., Chen, J.D., Pirrotta, V. and Larsson, J. (2003) The thioredoxinT and deadhead gene pair encode testis- and ovary-specific thioredoxins in *Drosophila melanogaster*. *Chromosoma* **112**: 133–143.

Tachibana, S.I., Numata, H. and Goto, S. (2005) Gene expression of heat-shock proteins (Hsp23, Hsp70 and Hsp90) during and after larval diapause in the blow fly *Lucilia sericata*. *J Insect Physiol* **51**: 641–647.

Thieringer, H.A., Jones, P.G. and Inouye, M. (1998) Cold shock and adaptation. *Bioessays* **20**: 49–57.

Tusher, V.G., Tibshirani, R. and Chu, G. (2001) Significance analysis of microarrays applied to the ionizing radiation response. *Proc Natl Acad Sci USA* **98**: 5116–5121.

Van Breukelen, F. and Martin, S.L. (2002) Molecular adaptations in mammalian hibernators: unique adaptations or generalized responses? *J Appl Physiol* **92**: 2640–2647.

Walker, M.A. and McKensie, B.D. (1993) Role of ascorbate-glutathione antioxidant system in chilling stress. *Hortscience* **17**: 173–186.

Yiangou, M., Tsapogas, P., Nikolaidis, N. and Scouras, Z.G. (1997) Heat shock gene expression during recovery after transient cold shock in *Drosophila auraria* (Diptera: Drosophilidae). *Cytobios* **92**: 91–98.

Yocum, G.D., Joplin, K.H. and Denlinger, D.L. (1991) Expression of heat shock proteins in response to high and low temperature extremes in diapausing pharate larvae of the gypsy moth, *Lymantria dispar*. *Arch Insect Biochem Physiol* **18**: 239–249.

Zimmerman, J.L., Petri, W. and Meselson, M. (1983) Accumulation of a specific subset of *D. melanogaster* heat shock mRNAs in normal development without heat shock. *Cell* **32**: 1161–1170.