

# Phototransduction genes are up-regulated in a global gene expression study of *Drosophila melanogaster* selected for heat resistance

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**Abstract** The genetic architecture underlying heat resistance remains partly unclear despite the well-documented involvement of heat shock proteins (Hsps). It was previously shown that factors besides Hsps are likely to play an important role for heat resistance. In this study, gene expression arrays were used to make replicate measurements of gene expression before and up to 64 hours after a mild heat stress treatment, in flies selected for heat resistance and unselected control flies, to identify genes differentially expressed in heat resistance–selected flies. We found 108 genes up-regulated and 10 down-regulated using the Affymetrix gene expression platform. Among the up-regulated genes, a substantial number are involved in the phototransduction process. Another group of genes up-regulated in selected flies is characterized by also responding to heat shock treatment several hours after peak induction of known Hsps revert to nonstress levels. These findings suggest phototransduction genes to be critically involved in heat resistance, and support a role for components of the phototransduction process in stress-sensing mechanisms. In addition, the results suggest yet-uncharacterized genes responding to heat stress several hours after treatment to be involved in heat stress resistance. These findings mark an important increase in the understanding of heat resistance.

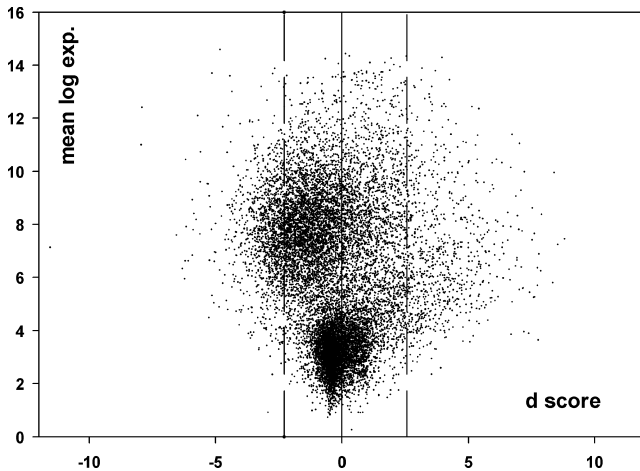
## INTRODUCTION

High temperature is an important environmental factor that has a significant impact on reproduction and survival of organisms. Heat thus influences the distribution and abundance of species in time and space (Cossins and Bowler 1987). In most organisms, heat resistance can be improved by a mild heat hardening, which is mainly due to the induction of heat shock proteins (Hsps), whose protective role in heat resistance has been demonstrated, eg, in *Drosophila melanogaster* overexpressing Hsp70 (Feder et al 1996; Krebs and Feder 1998) and Hsp22 (Morrow et al 2004). Less understood is the genetic basis underlying variations in basal (ie, noninduced) heat resistance

as observed both across species and within species. Although Hsps may also contribute to such variations, it is clear that the protective effects associated with Hsps come with a cost in terms of detrimental effects on growth, fertility, development, and survival (Krebs and Feder 1997; Krebs and Loeschcke 1994; Williams et al 2003). Moreover, studies performed on *Drosophila* indicate that the protective effect of heat hardening is sustained long after Hsp70 expression returns to prestress level (Krebs and Feder 1997; Dahlgaard et al 1998), suggesting that factors other than Hsps may contribute to protection against heat. Indications of connections between stress in general and low metabolic rate (Hoffmann and Parsons 1991) and the stress theory of aging (Parsons 1995) support this view, and it is thus likely that genes involved in basal and induced heat resistance are not necessarily the same.

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**Fig 1.** Distribution of  $d$  scores for all genes. The plot presents normalized average expression values plotted against the  $d$  values obtained by the SAM algorithm (Tusher et al 2001) for all genes on the array. Each dot represents the statistical summary, based on the 18 paired comparisons of set B, for 1 gene. Dots on the outside of the 2 broken lines ( $d$  score, 2.56 and  $-2.28$ ) represent genes that are considered differentially expressed at a 1% FDR level.

*D. melanogaster* is a particular suitable model organism to study heat resistance, because there is a long tradition for investigation of thermal stress (Hoffmann et al 2003) and a vast amount of genetic information associated with this species. Global gene expression studies in flies have proven successful for providing new insights in various biological processes, mostly based on response to treatments, but recently also based on differences in complex phenotypic traits between selection and control lines (Toma et al 2002; Pedra et al 2004; Kristensen et al 2005, 2006). The aim of the present study has been to increase the understanding of basal heat resistance in *D. melanogaster* and in particular to describe characteristic changes occurring at the level of gene expression. Based on the previous findings mentioned above, it is assumed that genes other than *Hsps* will respond to heat resistance selection by differential regulation. Here, we have used Affymetrix Genechips to measure gene expression in flies selected for heat resistance and control flies arising from the same background population. Heat resistance was significantly increased in the selected flies as measured by survival rate following heat shock (Bubliy and Loeschcke 2005). Using 5 replicate measurements of selected vs control flies, we have found 108 genes to be up-regulated and 10 genes to be down-regulated following selection for heat resistance. These findings are supported by re-analysis of a different time series experiment (Sørensen et al 2005) with measurements taken before and after heat stress treatment in selected and control flies. Here, approximately 16% of all genes showed differential expression following selection. The results show that a substantial number of genes involved in the phototransduction

process respond to heat stress selection by up-regulation. Furthermore, we point to the existence of a set of genes, induced by heat stress treatment several hours after *Hsps* return to prestress levels, that also respond to heat resistance selection by up-regulation. This study thus demonstrate the usefulness of investigating heat resistance using global gene expression technology and contribute significantly to the understanding of heat resistance.

## MATERIALS AND METHODS

### Origin of experimental flies

The selection and control lines used in this experiment originated from a mass population of *D. melanogaster* from different geographical regions and background that were mixed in order to increase the genetic diversity in the starting material. The mass population was created in September 2002 by mixing 600–700 flies from each of several pre-existing stocks from the laboratory. The origin of flies and the set up of the mass population is described in more detail (Bubliy and Loeschcke 2005). The population was maintained as 1 interbreeding population for 4 generations after which a number of selection regimens were established. Here, flies from 2 regimens were used (control and heat selection). Independent replicate lines were established for each selection regimen and maintained at population sizes of 300 pairs. Flies were kept under standard laboratory conditions at 25°C and a 12/12 hour light/dark cycle on standard agar-sugar-yeast-oatmeal medium. Flies from the heat resistance–selected regimen were heat shocked every second generation when 5 days of age. The flies were hardened for 30 minutes at 36°C and after 1-hour recovery heat-shocked for 1 hour at 38°C. The heat shock temperature was gradually increased to 38.5°C for 65 minutes between selection events to maintain selection intensity (50%) as the lines improved the resistance due to the selection. For the array experiment, the lines had passed 20 generations, ie, 10 selected generations. The flies were allowed 1 generation without selection before flies were sampled for the experiment.

### Experiment design

The experiment was composed of 2 sets: set A with focus on heat resistance selection and set B with focus on heat-induced genes. In set A, gene expression was measured in 5 independent heat resistance–selected replicate lines and 5 control lines without treatment ( $2 \times 5$  chips). In set B (for further details, see Sørensen et al 2005), gene expression was measured before ( $-1$  hour) and after (0.25, 1, 2, 4, 8, 16, 32, and 64 hours) a 36°C heat treatment for 1 hour (from  $-1$  to 0 hour) in 2 independent heat resis-

**Table 1** Experiment design

Set A										
5×	Heat	–1								
	Control	–1								
Set B										
2×	Heat	–1	¼	1	2	4	8	16	32	64
	Control	–1	¼	1	2	4	8	16	32	64

Set A: Five measurements of gene expression in heat resistance–selected flies and control flies. Set B: Gene expression was measured on flies given a 1-hour heat shock (from –1 to 0 hour) at the given time points (hours after treatment). The flies were either selected (Heat) or not selected (Control) for heat resistance. Measurements at time point –1 hour overlap between the sets.

tance–selected replicate lines and in 2 control lines (2 × 2 × 9 chips, 4 of these chips (those at –1 hour) overlap with the measurements of set A). The data has been submitted to the Gene Expression Omnibus database with series records GSE5146 and GSE5147. Both sets were processed simultaneously, ie, flies from set B at –1 hour were frozen simultaneously with flies from set A. Each sample, consisting of 10 female flies collected from a mix of several bottles within each line and raised under standard conditions, was flash-frozen in liquid nitrogen and stored at –80°C until ribonucleic acid (RNA) extraction.

### RNA extraction and hybridization

Flies from each sample were homogenized and RNA extraction and cRNA labeling was performed as described (Sørensen et al 2005). Briefly, double-stranded cDNA was prepared from 5 µg of total RNA using the SuperScript Choice System (Life Technologies), according to the manufacturer's instructions, except using an oligo-dT primer containing a T7 RNA polymerase promoter site. Biotin-labeled cRNA was prepared using the BioArray High Yield RNA Transcript Labelling Kit (Enzo). Unincorporated nucleotides were removed using RNeasy columns (Qiagen). Labeled cRNA was hybridized to Affymetrix *Drosophila* Genechips (version 1) as described (Dyrskjot et al 2003). The 2 replicates of the full time series were, for capacity reasons, hybridized in 2 rounds, which was taken into account in the paired analysis, ie, measurements from 1 hybridization were not paired with measurements of the other hybridization.

### Statistical analysis

Raw data was GC-RMA normalized using the Bioconductor application for R (Gentleman et al 2004). We used unpaired (set A) or paired (set B) SAM (Tusher et al 2001) on full datasets to identify differentially expressed genes. The SAM approach uses the *t*-test–derived statistical parameter *d*, which for paired data is defined as follows:

$$d = \frac{\sum_k \frac{x_{j(k)} - x_{j(-k)}}{K}}{s_0 + \sqrt{\frac{\sum_k \left[ x_{j(k)} - x_{j(-k)} - \sum_k \frac{x_{j(k)} - x_{j(-k)}}{K} \right]^2}{K(K-1)}}$$

where  $j(k)$  and  $j(-k)$  denote the pairing of measurements. In the present analysis, heat-selected and control flies were paired for the same hybridization group at the same time points.  $K$  denotes total number of pairings, 18 in this case, and  $s_0$  is a calculated factor set to minimize the coefficient of variation of  $d$ .

Analyses were done using the multi-experiment viewer TMeV (Saeed et al 2003). We used the EASE application (Hosack et al 2003) to correlate differentially expressed genes with functional categories defined by the Gene Ontology (GO) consortium (Ashburner et al 2000).

## RESULTS

### Identification of genes differentially expressed in heat resistance–selected flies

Gene expression was measured in flies selected for heat resistance and nonselected control flies using Affymetrix high-density oligo arrays. It was shown previously that selection of these flies significantly increased the survival rate following heat shock from 0.16 in control flies to 0.66 in selected flies (Bubliy and Loeschcke 2005). The experimental setup (Table 1) consists of 2 sets of measurements. Set A represents 5 replicates each of heat resistance–selected flies and nonselected control flies, and set B represents a time series experiment in 2 replicates with measurements taken before and after a mild heat stress treatment (36° for 1 h) at several time points in heat resistance–selected flies and control flies. The data represented in set B were previously analyzed for the effect of heat shock treatment on gene expression (Sørensen et al 2005) and are reanalyzed here with respect to selection effects.

To identify genes differentially expressed in the heat

resistance-selected flies, statistical analysis was applied to the data defined by set A using the SAM approach (Tusher et al 2001) which allows control of the false discovery rate (FDR). At a 5% FDR, 120 probe sets corresponding to 118 genes were differentially expressed, 108 genes were up-regulated, and 10 genes were down-regulated (Table 2). A paired SAM analysis of the measurements defined by set B confirmed this for 96 of the 108 up-regulated genes and for 6 of the 10 down-regulated genes. The much higher power obtained by the 18 paired comparisons arising from the measurements in set B (2 replicates of 9 time points measured in control and selected lines) yielded 888 up-regulated probe sets and 1333 down-regulated probe sets at a 1% FDR, corresponding to approximately 16% of all genes present on the array. Despite this, the distribution of the test statistic *d* of the SAM algorithm supports the finding of more up-regulated than down-regulated genes in the analysis of set A, because down-regulated probe sets have a tendency to lie much closer to the 1% FDR limit than up-regulated genes (Fig 1).

The high confidence seen from the FDR was not associated with a dramatic increase or decrease in gene expression in the heat resistance-selected flies. Rather, most significant genes responded to the selection with minute changes of less than 2-fold magnitude. Both up and down regulated genes are located randomly throughout the genome (not shown).

#### Phototransduction genes are up-regulated in selected flies

The differentially expressed genes defined by set A fall into a diverse set of functional categories (Table 2). By focusing on groups of functionally related genes as defined by the GO consortium (Ashburner et al 2000), we used the EASE application (Hosack et al 2003) to identify GO categories that have an overrepresentation of genes among the differentially regulated genes. The most overrepresented categories are related to vision, with phototransduction having the relatively highest number of significantly overexpressed genes. Nine of 27 genes in the phototransduction GO category are up-regulated in the heat resistance-selected flies (Table 2). The probability of finding this number of genes or more based on random selection of 27 genes among all genes represented on the array is less than  $3 \times 10^{-10}$  as calculated by Fisher's exact test, indicating that the phototransduction process has responded to the selection by up-regulation. For the paired analysis of set B, we found 20 phototransduction genes to lie within the 1% FDR limit of up-regulated genes (Fig 2 and supplemental Table 3). A similar low *P* value of  $9 \times 10^{-6}$  was found for muscle contraction genes of which 8 were found up-regulated (Table 2).

**Table 2** Genes differentially expressed in heat resistance-selected flies

Gene symbol	Cytoband position	Differentially expressed in:		
		(a)	(b)	(c)
Up-regulated				
Phototransduction				
inaD	59B1	x	x	
ninaE	92B8	x	x	
Rh4	73C5	x	x	
Rh6	88F6	x		x
Rh3	92C5	x	x	
ninaA	21D3	x		x
ninaC	27F5	x	x	
Arr1	36E3	x	x	
Gβ;76C	76C3	x	x	
Muscle contraction				
sis	62C2			
Mhc	36A8			
wupA	16F7			
Tm2	88E12			
Rya-r44F	44F5		x	
CG30084	52C7			
Ca-α	35F1		x	
CG5023	92D2			
Signal transduction				
nrm	80A1			
skf	47D7			
HLHmβ	96F10			x
CG2082	83C1			
CAP	46F9			
Development				
Msp-300	25C8			
CG17739	48F8			
Prm	66D14	x		
CG6416	66D9	x		
Proteolysis and peptidolysis				
CG1304	19E5		x	x
CG31267	89F1			
CG7829	99C5			x
CG8329	67B10			x
Synaptic transmission				
comt	1E1			
GluClα	92B2			
CG31272	86C4			
Lcch3	13F13			
Transcription				
Six4	77E6			x
scrt	64A2			
CG32940	85B3			
CG6689	86D7			x
Defense response				
Def	46D7			
hig	45B7			
CG17323	37B3			
Carbohydrate metabolism				
CG14935	33B2	x		
CG8690	44C4			
Fatty acid metabolism				
CG16904	85E11			
v(2)k05816	23C5	x		
Oxidative phosphorylation				
Vha68-1	34A5			x
CG6020	77C3			

Table 2 Continued.

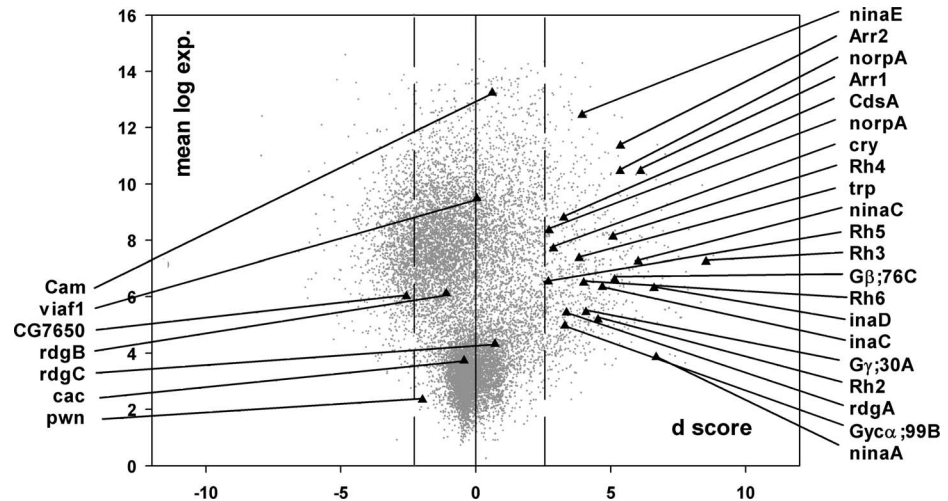
Gene symbol	Cytoband position	Differentially expressed in:		
		(a)	(b)	(c)
Ribonucleotide metabolism				
Adk1	68F8	x		
CG3321	88B8			
Transport				
Obp18a	18B1			
Obp44a	44A8			
Amino acid transport				
CG31547	83B1	x		
Carbohydrate transport				
CG1208	83C4			
Cytochrome p450				
Cyp6g2	48F1			
Cytoskeletal anchoring				
Pax	37E1			
Cytoskeletal organization and biogenesis				
Act79B	79B3			
EGF receptor signaling pathway				
fus	52B5			
Heme biosynthesis				
Alas	60A13			
Hormone metabolism				
7B2	83A5			
Lipoprotein receptor				
CG31221	92A6			
Mannose binding				
CG8343	42A10			x
<i>Myo</i> -inositol biosynthesis				
Inos	43C4			
Nitrogen metabolism				
CG7910	84E10			
Organic anion transport				
CG3382	58D1			
Protein transport				
CG32677	9E2			
Protein biosynthesis				
RpS19	14F5			
Potassium ion transport				
lrk3	37A4			
Response to oxidative stress				
fau	86C4			
Response to heat				
CG4461	67B1	x		
Transmembrane protein				
TM4SF	60A7			
Tricarboxylic acid cycle				
CG5214	86D8			
Vitamin/cofactor transport				
CG8957	96F8			
Water transport				
AQP	49F11	x		

Table 2 Continued.

Gene symbol	Cytoband position	Differentially expressed in:		
		(a)	(b)	(c)
Unknown factor				
CG8801	45D1			x
Ket	62C2			
lcs	19F2			
CG11160	10A2			
CG12726	11F4			
CG1678	19F2			
CG4577	21E3	x		
CG11149	25F5			
CG14277	29A1			
CG18511	30A8	x	x	
CG16885	34E1			
CG16885	34E1			
CG31781	36A10			
CG17041	50A1			
CG15617	53D4			
CG13565	60A12			
CG14109	70A8			
CG33169	79F5			
CG32919	87B14		x	
CG13845	94B3			
CG31030	99E2	x		x
CG12158	45D1			
CG17325	37B1			
CG5597	60A8			
CG2950	25B3			
CG14509	99A4			
CG6803	88E6	x		
CG32919	87B13	x		x
CG9813	87E9	x		
CG3556	4C4			
CG18021	60A15			
CG1648	46B10			
CG1136	64A4			
CG32355	66D8			
CG7686	47C3			
CG14775	2B1			
CG5903	86B9	x		
Down-regulated				
Larval serum protein				
Lsp1 $\beta$	21D3			
Lsp2	68F4			
Chorion formation				
Femcoat	4B4			
Mitotic spindle checkpoint				
CG17498	64F2			
Peptidyl-dipeptidase				
Acer	29D1			
Unknown function				
CG4066	87B9			
CG6005	91E3			
CG13084	37E4			
CG13303	102B8			
CG18088	29F7			

All probe sets found differentially expressed in heat resistance–selected flies at 5% FDR based on unpaired comparison of measurements defined by set A. The last three columns refer to genes found differentially expressed in flies with increased starvation resistance in Harbison et al (2005) (a), DDT resistance in Pedra et al (2004) (b), and genes responding late to heat treatment in Sørensen et al (2005) (c).

**Fig 2.** Phototransduction genes. Gray dots represent the background of all genes as illustrated in Figure 1. Phototransduction genes are marked with black triangles and listed as gene symbols.



A number of other studies have identified differentially expressed genes in response to stress inducing treatments (Zinke et al 2002; Landis et al 2004; Harbison et al 2005) and in flies with increased stress resistance (Pedra et al 2004; Harbison et al 2005). When comparing those genes with the genes identified in this study (supplemental Table 3), we find that the number of phototransduction genes differentially regulated in flies selected for dichlorodiphenyltrichloroethane (DDT) resistance (Pedra et al 2004) and in flies with increased starvation resistance (Harbison et al 2005) is remarkably high (9 of 158 and 14 of 477, respectively) (Table 2 and supplemental Table 3), whereas the same is not the case for genes responding to stress-inducing treatments (Zinke et al 2002; Landis et al 2004; Harbison et al 2005).

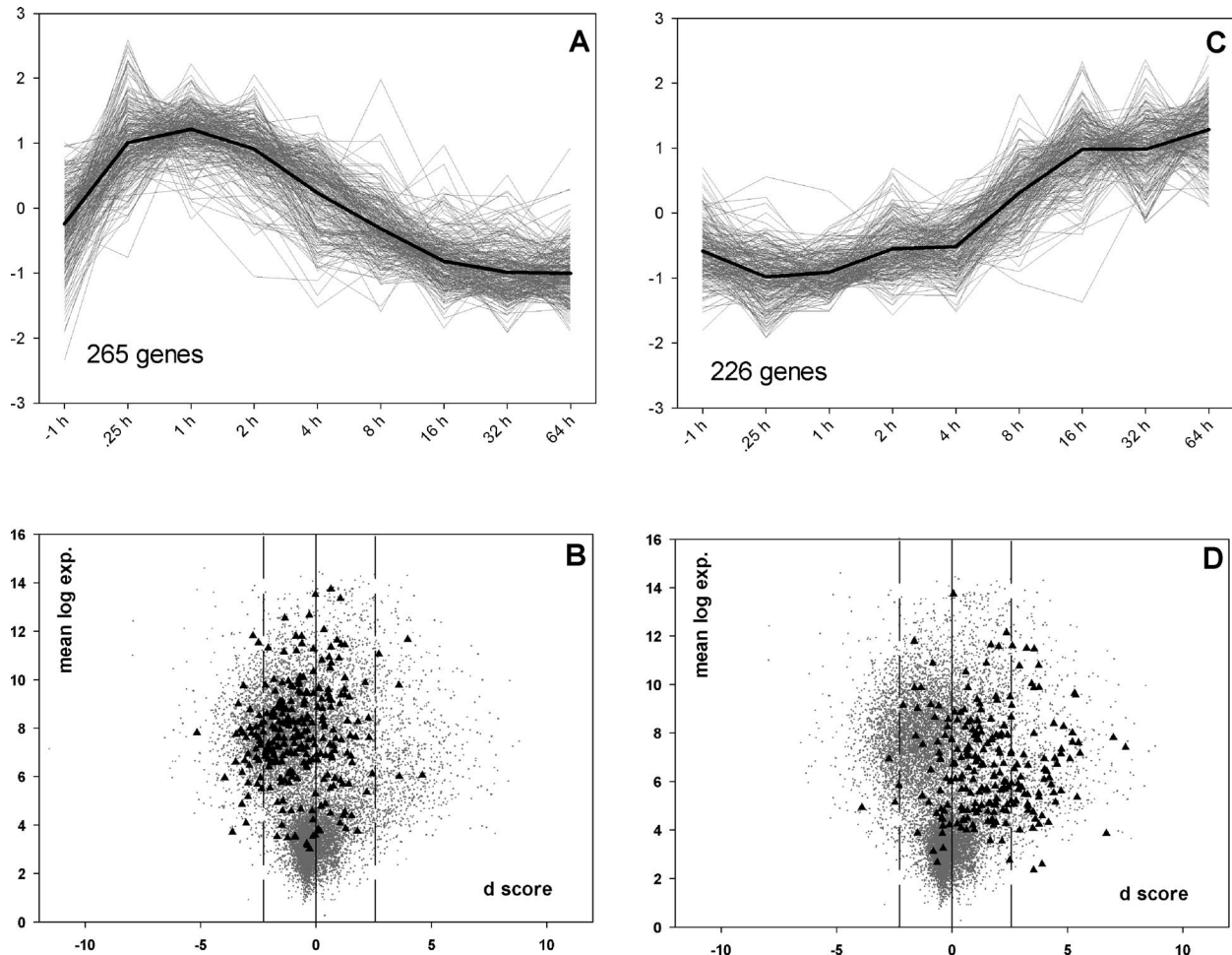
#### Genes responding late to heat treatment are up-regulated in selected flies

We recently identified genes responding to heat shock treatment using the time series data represented by set B (Sørensen et al 2005). Two major clusters containing 265 genes up-regulated early after heat stress and 226 genes up-regulated late after heat stress (Fig 3A,C) were prominent (Sørensen et al 2005). None of the 265 early up-regulated genes, which includes most known heat-responsive *Hsps* (not shown), were among the 118 genes found to be differentially regulated following heat resistance selection. However, among the 226 late up-regulated genes, we found 13 genes to be up-regulated following heat resistance selection (Table 2). This trend was even more pronounced in plots of SAM *d* values based on the paired analysis of data represented by set B, where bias towards higher *d* values of late up-regulated genes is evident (Fig 3D).

## DISCUSSION

The data presented in the present study identify genes that are differentially expressed in *D. melanogaster* subjected to 10 instances of heat resistance selection taking place every second generation compared to control flies from the same population background. Two parallel set-ups led to identification of a total of 118 and 2221 differentially expressed genes, respectively, of which 102 genes appeared in both analyses. Although all of these genes were found to be significantly differentially expressed at 5% and 1% FDR level, in general the differences were small, usually showing less than 2-fold change. The finding of more up-regulated genes in the analysis of set A combined with a tendency for the *d* value of up-regulated genes to deviate more from the overall *d* value distribution than that of down-regulated genes in the analysis of set B (Fig 1), suggests that up-regulated genes are more consistently differentially expressed, and could indicate that selection pressure has been more severe on these genes, ie, alleles resulting in higher expression of these genes are more frequently present in selected flies.

We found an extensive overrepresentation of genes involved in the phototransduction process among the up-regulated genes, indicating that the heat selection pressure has favored up-regulation of this process. This was observed in both sets, but more pronounced in set B where 21 of 28 phototransduction genes were up-regulated. As for all genes, the up-regulation level was low for most phototransduction genes, but approaching 2-fold for some. In general, the effect of heat selection on phototransduction genes was comparable in magnitude to changes found as the result of light-induced day and night cycles (McDonald and Rosbash 2001). Phototransduction in *Drosophila* is initiated by light-induced isomerization of rhodopsins and mediated through G protein-



**Fig 3.** Expression profiles and distributions of  $d$  scores for heat responsive genes. Profile of genes responding early (A) and late (C) to heat stress treatment ( $36^{\circ}\text{C}$  for 1 hour). Profiles represent normalized average expression values of the measurements defined by set B (Table 1). Distributions of SAM  $d$  values for the early (B) and late (D) heat-responsive genes (black triangles). Gray dots represent the background of all genes as illustrated in Figure 1. Panels A and C are modified from Sørensen et al (2005).

dependent activation of phospholipase C (*norpA* gene), leading to the formation of diacyl glycerol and inositol 1,4,5-trisphosphate. Subsequently, members of the transient receptor potential family of light sensitive cation channels (TRP and TRPL) are activated by an unknown mechanism (Hardie and Raghu 2001). The channels are coordinated in the INAD signaling complex also including INAD, protein kinase C (*inaC* gene), rhodopsins, myosin III (*ninaC* gene), and calmodulin (*Cam* gene) (Montell 2003). Several of these key components are up-regulated in the heat resistance–selected flies (Table 2 and Fig 2). A functional link between heat stress resistance and phototransduction is unclear. However, it was recently shown in flies that the TRP and TRPL channels are activated by metabolic stress, anoxia, and ATP depletion (Agam et al 2000). Another finding that provides a link between heat and TRP channels was the isolation of a TRP homolog in mouse that is activated by temperatures above  $43^{\circ}\text{C}$  and by the vanilloid capsaicin known to be responsible for the

“heat” associated with chili peppers (Caterina et al 1997). Several other TRP channels have later been shown to be temperature activated in mouse (Peier et al 2002; Smith et al 2002; Xu et al 2002), and recently 2 TRP homologs in *Drosophila*, *painless* (Tracey et al 2003) and *ANKTM1* (Viswanath et al 2003), have been demonstrated to be activated by heat. In addition to *TRP* and *TRPL*, the *painless* gene is found on the 1% FDR list of up-regulated genes (see supplemental Table 3). It is thus possible that TRP channels could be involved in yet-unrecognized stress-sensing pathways.

As pointed out above, 2 other studies on gene expression in stress-resistant *D. melanogaster* found a marked overrepresentation of phototransduction genes (Pedra et al 2004; Harbison et al 2005). A similar overrepresentation of differentially regulated phototransduction genes was not observed in studies investigating gene expression following stress-inducing treatments (Zinke et al 2002; Landis et al 2004; Harbison et al 2005). Notably, it was found

that 4 phototransduction genes are up-regulated in response to heat stress several hours after the treatment (Sørensen et al 2005), ie, although late, some phototransduction genes also respond to heat treatment. Moreover, phototransduction genes were also found differentially expressed in flies calorie restricted for several days (Pletcher et al 2002). Thus, in summary, these findings strongly indicate that phototransduction gene expression, and thus the phototransduction process, is tightly connected to general resistance to a number of stress factors like heat (Sørensen et al 2005), starvation (Pletcher et al 2002; Harbison et al 2005), and oxidative stressors (Pedra et al 2004).

None of the *Hsp* genes known to be induced by heat treatment was found among the differentially expressed genes despite the indisputable importance of these genes for heat resistance. This could signify that the cost of maintaining alleles leading to a higher basal expression of Hsps may be too high in most natural environments (Sørensen et al 2003), as has been indicated in experiments with genetically modified flies (Feder et al 1996; Krebs and Feder 1998; Morrow et al 2004). Thus, genes acting as a driving force for evolution of basal heat resistance in natural populations are likely to include genes different from the *Hsps*, of which the present study provides a number of candidates. This view is supported by the finding of a general overlap between genes responding to heat treatment after several hours and genes responding to heat resistance selection by up-regulation (Fig 3D). The existence of such genes is suspected, because we (Dahlgaard et al 1998; Sørensen et al 2005) and others (Krebs and Feder 1997) have previously found that the protective effect of heat hardening in flies lasts far beyond the induction of known Hsps. Consequently, we expect that further investigations of these genes will provide new insights into mechanisms of stress resistance in general and heat resistance in particular.

This study demonstrates the effect of combining the suitability of *D. melanogaster* in selection experiments with validated full genome expression platforms to investigate complex traits. Such methods combined with developments in bioinformatics are important tools in the process of clarifying genetic architectures underlying these traits and efficiently complement conventional genetic methods such as QTL mapping. The unmatched possibilities for genetic experiments in publicly available stocks of *D. melanogaster* further enhance this process and will be a powerful supplemental tool for characterizing candidate genes found in the present study.

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