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Gene transcription during exposure to, and recovery from, cold and desiccation stress in *Drosophila melanogaster*

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Abstract

We exposed adult male *Drosophila melanogaster* to cold, desiccation or starvation, and examined expression of several genes during exposure and recovery. *Frost* was expressed during recovery from cold, and was up-regulated during desiccation. Desiccation and starvation (but not cold) elicited increased expression of the senescence-related gene *smp-30*. *Desat2* decreased during recovery from desiccation, but not in response to starvation or cold. *Hsp70* expression increased after 1 h of recovery from cold exposure, but was unchanged in response to desiccation or starvation stress, and *Hsp23* levels did not respond to any of the stressors. We conclude that *D. melanogaster*'s responses to cold and desiccation are quite different and that care must be taken to separate exposure and recovery when studying responses to environmental stress.

Keywords: cross-tolerance, rapid cold-hardening response, starvation, heat shock proteins.

Introduction

Among the many environmental stresses that terrestrial insects encounter, low temperatures and desiccation are thought to be particularly closely linked (Ring & Danks, 1994; Williams *et al*., 2004), both in the nature of the stress and in the adaptations that allow insects to survive them. At temperatures below 0 °C, ice has a lower vapour pressure than liquid water or biological fluids. This makes the environment desiccating and leads to dehydration in overwintering insects (Danks, 2000). In addition, insects that survive freezing utilize osmotic dehydration to convert a cold stress into a desiccation stress at the cellular level (Sinclair & Wharton, 1997). There is also considerable cross-tolerance between cold and desiccation (Klok & Chown, 1998; Ramløv & Lee, 2000; Bayley *et al*., 2001; Block, 2002; Williams *et al*., 2004), and at least one cold tolerance strategy utilized by soil invertebrates (cryoprotective dehydration) is based upon this cross-tolerance (Holmstrup *et al*., 2002).

The relationship between responses to cold and desiccation stress is not as clear in species such as *Drosophila melanogaster* that succumb to cold injury at higher temperatures than those at which they freeze (Czajka & Lee, 1990; Bale, 1996; Sinclair, 1999). This nonfreezing cold injury (NFCI) seems to result from two main sources – acute injury is probably a result of membrane phase transitions (Quinn, 1985; Drobnis *et al*., 1993; Ramløv, 2000), while chronic cold injury seems to be a consequence of lethal ion equilibration across membranes (Kostal *et al*., 2004). In many species, including adult *D. melanogaster*, NFCI may be mitigated somewhat by the rapid cold-hardening (RCH) response, whereby tolerance to acute cold stress can be increased by a brief pretreatment at a milder cold temperature (for example, 1 h at 0 °C) (Lee *et al*., 1987; Czajka & Lee, 1990; Kelty & Lee, 1999). The RCH response in *D. melanogaster* is not associated with an increase in inducible Hsp70 or an increase in carbohydrate cryoprotectants (Kelty & Lee, 1999, 2001), although Overgaard *et al*. (2005) have recently shown increases in polyunsaturated fatty acids, probably leading to an increase in membrane fluidity, during a pretreatment that induces RCH.

The proximal causes of mortality from desiccation stress are not well understood. In the extreme example of anhydrobiosis, membrane phase transitions can allow cell contents to leak, and proteins may cross-link and lose activity (Crowe *et al*., 2002; Goyal *et al*., 2005; Watanabe, 2006). Like cold tolerance, desiccation tolerance can be increased by pre-exposure to a nonlethal water stress. For example, in the soil nematode *Aphelynchus avenae*, exposure to 97% relative humidity (RH) causes accumulation of high levels of trehalose (Womersley & Smith, 1981), which acts to preserve membrane and protein integrity (Crowe *et al*., 1992). Similarly, pre-exposure to low relative humidity can substantially increase desiccation tolerance in some

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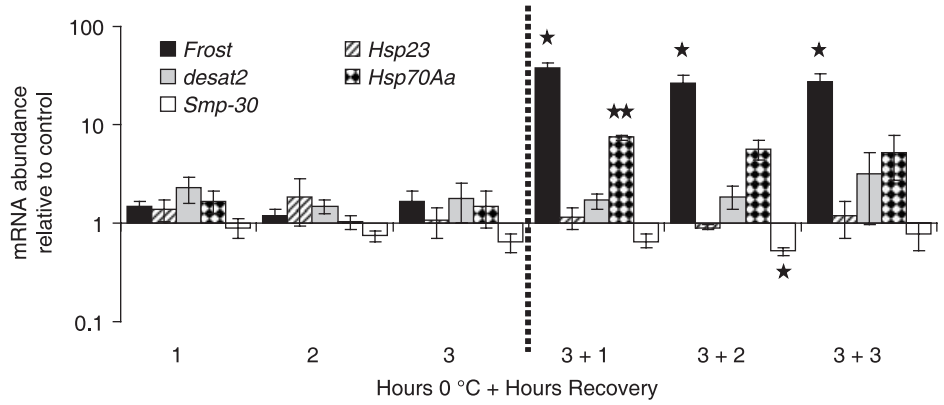

Figure 1. Relative abundance of five transcripts in *Drosophila melanogaster* during exposure to, and recovery from, cold shock at 0 °C. The dotted vertical line separates exposure and recovery treatments. Note the logarithmic *y*-axis, where values > 1 indicate up-regulation and values < 1 indicate down-regulation of the transcript. Asterisks indicate values that are significantly different from 1 (*$P < 0.05$; **$P < 0.01$). Mean ± SEM presented, $n = 3$ in all cases.

Collembola (Sjursen *et al.*, 2001), although we are not aware of any such studies that have been conducted on higher insects. Trehalose and other sugars accumulated during desiccation stress can also act as cryoprotectants (Ring & Danks, 1994).

A long-standing focus on the immediate mechanisms associated with insect survival of environmental stress means that the process of recovering from that stress has often been neglected. Recovery from a stress is important not only in the context of surviving the stress, but also because, as for the heat-shock response, a period of recovery from a milder stress can allow the synthesis of proteins that protect against more severe stress (Feder & Hofmann, 1999). Recovery from cold or desiccation stress is considerably less well understood than responses during the stress. Kristiansen & Zachariassen (2001) examined the redistribution of ions after freezing in larvae of the wood fly, *Xylophagus cinctus*, and found that redistribution of solutes occurred reasonably quickly and without apparent osmotic stress. Folk & Bradley (2004) investigated recovery from desiccation in adult *D. melanogaster* and found that nutrition during rehydration determined recovery time. Hayward *et al.* (2004) found that different sets of heat-shock protein genes were up-regulated during desiccation and recovery in the flesh fly, *Sarcophaga crassipalpis*, suggesting that the physiological effects of dehydration and rehydration are different.

In this study, we used quantitative real-time PCR to explore the patterns of expression of several candidate genes during and after exposure to cold or desiccation in adult male *D. melanogaster*. Because desiccation treatment in this species is necessarily confounded with starvation, we included a starved control treatment. We also measured candidate gene expression in heat-shocked flies, whose responses are better understood. We investigated five genes: *Frost*, *Smp-30*, *Hsp23*, *Hsp70Aa* (a transcript of an inducible form of HSP70) and *Desaturase2*. *Frost* and *Smp-30* (synonymous with *DCA*) have been directly implicated in cold exposure in *Drosophila* (Goto, 2000, 2001) and microarray data suggest that *Frost* expression increases during desiccation (Vanier & Gibbs, in preparation). The inducible heat shock protein *Hsp23 has* been implicated in cold stress recovery in *S. crassipalpis* and *D. melanogaster* (Yocum *et al.*, 1998; Qin *et al.*, 2005), while *Hsp70* is up-regulated in response to a variety of environmental stresses, including during recovery from cold shock in some *Drosophila* species (Goto & Kimura, 1998), although not during rapid cold-hardening in *D. melanogaster* (Kelty & Lee, 2001; Nielsen *et al.*, 2005). Finally, *Desaturase 2* is a Δ9-desaturase with inducible expression in *D. melanogaster* (Dallerac *et al.*, 2000), which we included because of its possible relationship to cold tolerance in *D. melanogaster* (Greenberg *et al.*, 2003). We used a design that allowed us to examine changes in gene expression during exposure to nonlethal cold, desiccation and starvation stress and during a period of recovery from the stress, allowing us to determine the role of gene expression changes during these two phases.

Results

Changes in expression of our candidate genes in response to three environmental stresses (Figs 1, 2 and 3) were one to two orders of magnitude lower than the marked increase in expression of the Hsps following heat shock (Fig. 4). Changes in gene expression were not observed during 3 h exposure to 0 °C, but were apparent during recovery from cold exposure (Fig. 1). By contrast, gene expression changed during both exposure to and recovery from desiccation and starvation (Figs 2 and 3). Threshold cycle (Ct) values of technical replicates of both control and treatment samples did not differ by more than 0.9 cycles in any case,

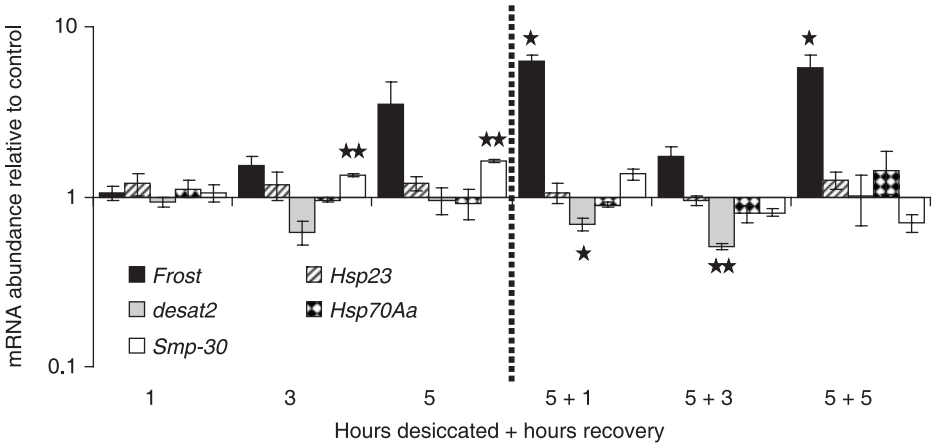

Figure 2. Relative abundance of five transcripts in *Drosophila melanogaster* during exposure to, and recovery from, desiccation. The dotted vertical line separates exposure and recovery treatments. Note the logarithmic *y*-axis, where values > 1 indicate up-regulation and values < 1 indicate down-regulation of the transcript. Asterisks indicate values that are significantly different from 1 (**P* < 0.05; ***P* < 0.01). Mean ± SEM presented, *n* = 3 in all cases.

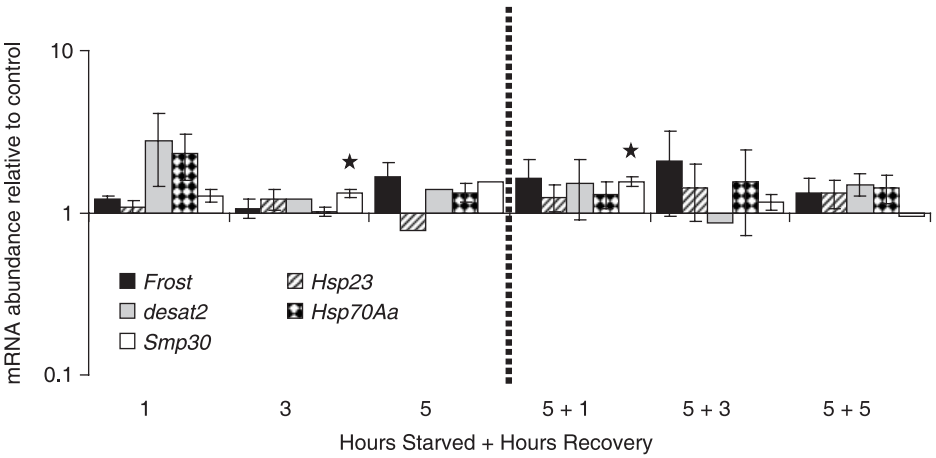

Figure 3. Relative abundance of five transcripts in *Drosophila melanogaster* during exposure to, and recovery from, starvation in a vial containing agar. The dotted vertical line separates exposure and recovery treatments. Note the logarithmic *y*-axis, where values > 1 indicate up-regulation and values < 1 indicate down-regulation of the transcript. Asterisks indicate values that are significantly different from 1 (*P* < 0.05). Mean ± SEM presented, *n* = 3 in all cases, except for 3 and 5 + 3 (*desat2*) and 5 + 5 (*smp-30*) time points, where *n* = 2.

and the average difference in Ct among technical replicates was 0.18 cycles.

Frost was up-regulated in association with both cold and desiccation stress (Figs 1 and 2). There was a trend of 1.5- and 3.5-fold increases in *Frost* expression at the 3 h and 5 h time points during exposure to desiccation (Fig. 2). A power analysis indicated that sample sizes of 7 and 13 would be required to detect this effect. *Frost* expression was significantly increased approximately sixfold at the 5 + 1 and 5 + 5 h time points, and would have been significant with *n* = 4 at the 5 + 3 h time point during recovery from desiccation (Fig. 2). *Frost* expression was up-regulated 26–40-fold during recovery from cold, but not during cold exposure itself. No changes in *Frost* expression were detected in response to starvation or heat stress (Figs 3 and 4).

Hsp70Aa was up-regulated sevenfold after 1 h of recovery from cold exposure. Sample sizes of 5 and 11 would have been necessary to detect the fivefold up-regulation observed at the 3 + 2 and 3 + 3 h time points, respectively. *Hsp70Aa* expression did not change significantly during or after desiccation or starvation (Figs 2 and 3), and abundance increased approximately 570-fold after a heat shock (Fig. 4). Expression of *Hsp23* did not change in response to any of the environmental stressors we applied, except for a 15-fold increase after heat shock (Figs 1–4). We detected a significant 0.7- to 0.5-fold down-regulation of *Desat2* transcription during recovery from desiccation (Fig. 2), but transcription of this gene did not change significantly during or after cold exposure or starvation, or during heat shock (Figs 1, 3 and 4), and would

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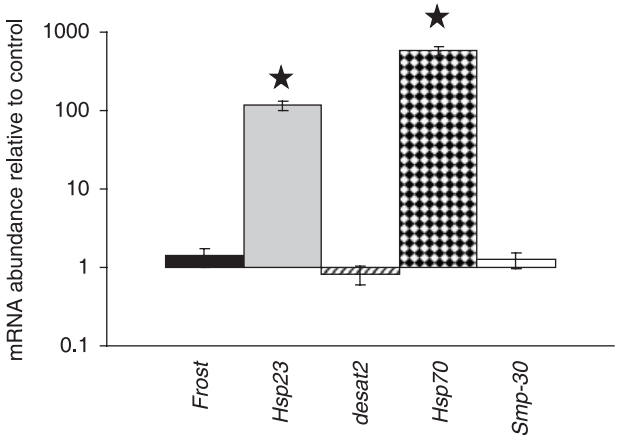

Figure 4. Relative abundance of five transcripts in *Drosophila melanogaster* after a 1 h heat shock followed by 1 h of recovery at room temperature. Note the logarithmic *y*-axis, where values > 1 indicate up-regulation and values < 1 indicate down-regulation of the transcript. Asterisks indicate values that are significantly different from 1 ($P < 0.05$). Mean ± SEM presented, $n = 3$ in all cases.

have required a sample size of 7–27 to allow the observed effect to be detected.

Smp-30 was down-regulated 0.5-fold after 2 h recovery from cold, and a power analysis indicates that an *n* of 5–7 may have been sufficient to detect the 0.6–0.7-fold down-regulation observed after 2 and 3 h exposure and after 1 h recovery from cold (Fig. 1). By contrast, *Smp-30* was up-regulated 1.3- and 1.6-fold after 3 and 5 h desiccation, respectively (Fig. 2). The trend for up-regulation continued after 1 h of recovery from desiccation (a sample size of four would have been required to detect this 1.4-fold increase), but turned abruptly into a trend for down-regulation of 0.8- and 0.7-fold after 3 and 5 h recovery from desiccation, which would have been significant with $n = 4$ and 5, respectively (Fig. 2). Significant 1.3- and 1.5-fold increases in *Smp-30* expression were also detected after 3 h of starvation and after 1 h of recovery from starvation (Fig. 3). At other time points during starvation, a power analysis indicated that $n = 4$ would have been sufficient to detect a difference from 1. This change in *Smp-30* was the only change in expression detected in starved flies (power analyses indicate that sample sizes greater than 10 would be necessary to detect any other observed differences), implying that the increased expression of *Frost* in the desiccation treatment was a response to desiccation, rather than concurrent starvation.

Discussion

Both direct and indirect methods have suggested a role for *Frost* in cold stress for *D. melanogaster* (Goto, 2001; Qin *et al*., 2005; Morgan & Mackay, 2006), as well as during exposure to desiccation stress (Vanier & Gibbs, in preparation). Here, we confirm that *Frost* is up-regulated in response to both cold and desiccation (but not starvation). Whereas *Frost* expression is somewhat elevated during the process of desiccation, and markedly at two time points during recovery, cold-induced up-regulation of *Frost* expression occurs only during recovery. Thus, *Frost* is a poor candidate for involvement in the rapid cold-hardening response (Lee *et al*., 1987), which is induced in *D. melanogaster* without a recovery period at room temperature (Nilson *et al*., 2006). Nevertheless, the involvement of *Frost* in recovery from cold is intriguing; recovery from cold (chill–coma recovery) is a trait that has been well-explored by evolutionary geneticists, but whose underlying mechanisms are not well-understood (Hoffmann *et al*., 2003; Sinclair & Roberts, 2005). There is a trend towards increased expression of *Frost* during desiccation, which suggests that up-regulation begins during stress exposure, in contrast to cold exposure. We are unable to explain the relatively lower (but still significant) levels of *Frost* expression at the 5 + 3 h recovery time point relative to the more robust response at the other time points (Fig. 2). The function of the protein encoded by *Frost* is unknown, although sequence similarities indicate that it is a mucin-like protein, and appears to have a secretory signal peptide, suggesting that it is secreted into the haemolymph (Goto, 2001).

Because of its role as an inducible enzyme associated with fatty acid biosynthesis, *desat2* is a natural candidate for studies of cold and desiccation tolerance, particularly considering the work of Overgaard *et al*. (2005), who suggested that membrane desaturation may be the mechanism of the RCH response. *Desat2* is involved in the synthesis of cuticular hydrocarbons (Dallerac *et al*., 2000; Takahashi *et al*., 2001), which provide the primary barrier to evaporative water loss through the cuticle, and are therefore likely to be associated with desiccation tolerance. In addition, Greenberg *et al*. (2003) suggested that different alleles of *desat2* were associated with population differences in cold, desiccation and starvation tolerance, although this conclusion has since been challenged (Coyne & Elwyn, 2006a,b; Greenberg *et al*., 2006). We did not detect an association between *desat2* expression and either cold or starvation in our data, although its expression decreased during recovery from desiccation.

Qin *et al*. (2005) reported a decrease in abundance of *smp-30* mRNA after cold shock, which matches our findings after 2 h of recovery. This observation does not necessarily conflict with Goto's (2000) description of *smp-30* (*DCA*) as a gene that is up-regulated during longer-term acclimation to low temperatures, as the 15 °C treatment used in those experiments is very different from a 0 °C cold shock. However, *smp-30* is thought to be associated with Ca^{2+} regulation in both mammals and *D. melanogaster* (Goto, 2000), and might therefore be expected to have a role in regulating Ca^{2+} during cold exposure. Our results suggest that if this is the case, the changes to s*mp-30*

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expression necessary for Ca^{2+} regulation during acclimation to low temperatures and recovery from those low temperatures differ. Interestingly, we found that starvation and desiccation both elicited an up-regulation of *smp-30*. These stresses share the absence of food as part of the treatment, so *smp-30* expression may respond to nutritional state.

Both *Hsp23* and *Hsp70* are well-known to be up-regulated in *D. melanogaster* in response to heat stress (e.g. Michaud *et al*., 1997; Feder & Hofmann, 1999; Fig. 4), and have been implicated in the response to other stresses in insects, including heavy metal exposure, desiccation and anoxia (Bournias-Vardiabasis *et al*., 1990; Ma & Haddad, 1997; Tammariello *et al*., 1999). Hsp70 protein levels have previously been shown not to increase during rapid cold-hardening (0 °C for 2 h) in *D. melanogaster* (Kelty & Lee, 2001). However, levels of Hsp70 transcript do increase after longer cold exposure (24 h at temperatures from +8 to –8 °C) in *Drosophila watanabei*, *Drosophila trapezifrons* and *Drosophila triauraria* (Goto & Kimura, 1998), and after 24 h at –5 °C in the mosquito *Culex pipiens* (Rinehart *et al*., 2006). Hsp70 levels increase during recovery from a 10 h exposure to 0 °C in *D. melanogaster* (Sejerkilde *et al*., 2003). We found a significant increase in *Hsp70Aa* message abundance after 1 h of recovery from cold exposure (Fig. 1), although mRNA abundance was two orders of magnitude lower than that elicited by heat shock (Fig. 4). Hsp70 performs an important chaperone role in cellular stress responses (Feder & Hofmann, 1999) and the response we observed may be associated with refolding proteins that were denatured during the cold exposure.

Exposure to, and recovery from, starvation and desiccation did not elicit an increase in *Hsp70Aa* mRNA (Figs 2 and 3). Although nonlethal, the 5 h of desiccation stress is about half the average lethal time (*c*. 10 h, Gibbs *et al*., 1997; Nghiem *et al*., 2000), and is likely to have caused more damage at the organismal and cellular level than the 3 h at 0 °C (average lethal time > 15 h, Guerra *et al*., 1997). To our knowledge, *Hsp70* expression has not been previously investigated in the context of desiccation stress in *D. melanogaster*, although it has in other dipterans. *Hsp70* expression increases in desiccated nondiapausing pupae of *S. crassipalpis*, but not in diapausing pupae (which already have high expression levels as part of the diapause programme, Hayward *et al*., 2004). In the mosquito *C. pipens*, *Hsp70* expression is unaffected by desiccation (Rinehart *et al*., 2006). Although we designed these primers for *Hsp70Aa*, they should amplify five of the inducible *Hsp70* transcripts (*Hsp70Aa*, *Ab*, *Bb*, *Bbb* and *Bc*), but not *Hsp70Ba*, and thus this result likely represents an integrated *Hsp70* response for the flies. The primers will not amplify the constitutively expressed heat-shock cognate, *Hsc70*. Nevertheless, it is possible that *Hsp70Ba*, or other heat shock protein genes may be associated with recovery from cold and desiccation in *D. melanogaster*, as has been observed during different phases of desiccation and rehydration in *S. crassipalpis* (Hayward *et al*., 2004).

Expression of *Hsp23*, in contrast to *Hsp70*, did not change during exposure to or recovery from any of the nonheat environmental stresses we imposed. This is surprising, given the key role of small Hsps in environmental stress responses of other Diptera, particularly *S. crassipalpis* (Tammariello *et al*., 1999; Hayward *et al*., 2004). Small Hsps play an important role in preventing protein aggregation (Morrow *et al*., 2006), which is known to be a problem in anhydrobiosis (Goyal *et al*., 2005); perhaps this is not the case under milder desiccation stress.

This study has unusual breadth in its treatment of gene expression responses to environmental stress: we followed expression of five genes at six time points during exposure to and recovery from three different environmental stresses. However, there are several important limitations to this study. First, in order to accommodate a large number of treatment groups and genes, we were forced to economise in the number of replicates ($n = 3$ throughout). As a consequence, we probably lacked statistical power to detect changes in some cases (hence our inclusion of power analyses in the results section). Nevertheless, significant up-regulation in the region of 1–2-fold increases was detected in several cases, suggesting that we did have power sufficient to detect the most emphatic responses. Although the detection of some of the near-significant results would not have changed our conclusions, they would have allowed greater insight into the dynamics of gene expression, and given greater support to our assertion that the expression patterns of *Frost* are qualitatively different in desiccated and cooled flies. We do, however, note that three is not an uncommon sample size among real-time PCR studies, and that our work is unusual in applying a formal statistical test to the data. Second, the stresses were chosen so as not to be potentially lethal, and very different results might be obtained if flies were cooled, starved or desiccated for periods closer to their lethal limits. Such changes may include the detection of hitherto nonsignificant responses during the exposure events, particularly during starvation and desiccation. Third, our data were gathered from whole-fly mRNA extractions. In the case of genes that are up- or down-regulated only in specific cells or organs, this means that whole-body levels of mRNA will underestimate actual gene expression changes. Finally, this is a study of mRNA levels of specific target genes. Unlike global studies of gene expression (e.g. Qin *et al*., 2005), we had to make *a priori* hypotheses about which genes we expected to be important (although in several cases, these hypotheses were drawn from microarray studies). In addition, because expression control is also manifested at the level of translation and protein activation, in many cases mRNA levels poorly reflect protein abundance or activity (Feder & Walser, 2005). Thus, our study has a narrow focus on gene expression, and

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probably makes conservative estimates of changes in genes that are expressed in a tissue-specific manner. A greater understanding of the function of the proteins encoded for these genes, particularly *Frost*, will likely lead to a greater understanding of the nature of the damage caused by these stresses. Such a study could begin with protein expression and characterization, or with the use of loss-of-function mutants.

There are two main differences in the gene expression responses to cold and desiccation. First, there are no changes in expression levels during exposure to cold, while there are several significant (up-regulation of *smp-30* during desiccation and starvation) and near-significant (up-regulation of *Frost* during desiccation) changes during exposure to the other stressors. This difference could result from inhibition of transcription processes at low temperature (Hochachka & Somero, 2002), although without a genome-wide study this possibility remains unsupported. The difference in responses to cold and desiccation that we observed has broader implications for the way in which the organisms respond to the stress, particularly in the context of the RCH response, which seems increasingly unlikely to be directly mediated by gene products synthesized during the pre-treatment period (see also Overgaard *et al*., 2005). Second, there are both qualitative and quantitative differences in the genes whose expression do change: *Frost* is up-regulated in both cases, but expression levels are an order of magnitude higher in response to cold exposure than in response to desiccation. In addition, *smp-30* is up-regulated during desiccation and starvation, but down-regulated during recovery from cold shock (see also Qin *et al*., 2005). *Hsp70Aa* is up-regulated during recovery from cold, but does not respond to desiccation or starvation, while *desat2* is down-regulated during recovery from desiccation, but remains unchanged after cold exposure. Together, this suggests that the responses to (and recovery from) cold and desiccation are qualitatively different, and that in chill-susceptible species such as *D. melanogaster*, there is no strong correspondence in the molecular responses to these two stresses. A separate study in this lab comparing cold tolerance among lines of *D. melanogaster* selected for increased desiccation resistance likewise finds that responses to desiccation and cold are not closely associated (Sinclair *et al*., in press). Similarly, Hoffmann *et al*. (2001) did not find a correlation between chill–coma recovery and desiccation resistance among populations of *D. melanogaster*. However, Bubliy & Loeschcke (2005) found that desiccation selection did impart some improvement to tolerance to chronic cold (50 h at 0 °C) in *D. melanogaster*, although selection for cold tolerance did not improve desiccation survival.

The physiological mechanisms of survival of both cold and desiccation stress have been reasonably well-explored (Salt, 1961; Zachariassen, 1985; Lee, 1991; Hadley, 1994; Gibbs, 2002; Gibbs *et al*., 2003; Sinclair *et al*., 2003). However, in the majority of cases, experimental designs have been such that while great attention is paid to exposure conditions, recovery conditions are less well-controlled. Folk & Bradley (2004) showed that selection for desiccation tolerance in *D. melanogaster* affected not only processes during exposure to stress, but also during recovery, suggesting that both sets of processes are under selective pressure, while Hayward *et al*. (2004) showed that *S. crassipalpis* up-regulates different heat shock transcripts during desiccation and rehydration. Often, insects are exposed to low temperatures and removed immediately to room temperature for recovery (see Sinclair & Chown, 2005, 2006 and the present paper for examples from our own work). The fact that the changes in gene expression in response to cold exposure occur primarily during the recovery phase suggests that recovery from cold may be an extremely important aspect of physiological responses to cold by chill-susceptible species, although one that has received scant attention outside measurements of chill–coma recovery (Sinclair & Roberts, 2005). Kelty & Lee (2001) found that repeated cooling on a mock-diurnal thermoperiod resulted in a cumulative increase in cold tolerance in *D. melanogaster*. This cumulative increase could well result from the longer-term effects of gene regulation during recovery that we show here. We are unaware of any equivalent experiments that have studied the effects of repeated desiccation exposure (although repeated desiccation and recovery must occur in the field), and we suggest that this could be a profitable approach for future research to link mechanisms observed in the laboratory with field conditions.

Experimental procedures

Fly rearing

Eggs were collected from a mass laboratory population of *D. melanogaster* (collected in 1998 from Terhune, New Jersey, USA) in cages with plates of *Drosophila* medium [Tucson Stock Center recipe: 0.9% agar, 2.4% cornmeal, 3.9% sugar, 1.4% dried yeast (w/v), 0.3% (v/v) propionic acid] as an oviposition substrate, with larvae and adults reared under constant light (to avoid diurnal effects) at 24 °C in 25 × 95 mm vials containing 10 ml of *Drosophila* medium. Newly emerged adults were collected, sexed under light CO_2 anaesthesia, and sorted into groups of 30 males in fresh vials with *Drosophila* medium. Male flies were used at five days of age, and were not anaesthetized subsequent to the initial sorting.

Exposure to environmental stress

Flies were exposed to environmental stress for three periods of time (1, 2 or 3 h in the case of cold; 1, 3 or 5 h for desiccation and starvation) and given three periods of recovery at room temperature (*c.* 24 °C) in food vials after the longest stress (1, 2 or 3 h after

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Table 1. Primer pairs used for quantitative real-time PCR in this paper. Primers were obtained from the literature or designed from database sequences using PRIMER-3. CG numbers refer to the transcript identities in Flybase (www.flybase.org)

Primer	Sequence	Source
Oligo d(T)	5′-TTT TTT TTT TTT TTT TTT-3′	
Act79B (Left)	5′-CCA GGT ATC GCT GAC CGT AT-3′	CG7478-RA
Act79B (Right)	5′-TTG GAG ATC CAC ATC TGC TG-3′	
Frost (Left)	5′-CGA TTC TTC AGC GGT CTA GG-3′	CG9434-RA
Frost (Right)	5′-CTC GGA AAC GCC AAA TTT TA-3′	
Hsp23 (Left)	5′-GAA TCC CTA CTT GGC CCT GGT T-3′	(Qin *et al*., 2005)
Hsp23 (Right)	5′-ACA CAT CCA TGC AGA CCT GGA A-3′	
Desat2 (Left)	5′-GAC CTG ACC ACC GAC AGA TT-3′	CG5925-RA
Desat2 (Right)	5′-CGC CCT TGT GAA TAT GGA GT-3′	
Hsp70Aa (Left)	5′-AGG GTC AGA TCC ACG ACA TC-3′	CG31366-RA
Hsp70Aa (Right)	5′-CGT CTG GGT TGA TGG ATA GG-3′	
smp-30 (Left)	5′-GAA GGA CCA TTG TTC CCT GA-3′	CG7390-RB
smp-30 (Right)	5′-TGG GTG GTT GGA ATT TTG AT-3′	

3 h exposure for cold; 1, 3 or 5 h after 5 h exposure for desiccation and starvation), giving six treatment + recovery combinations for each stress (1 + 0, 2 + 0, 3 + 0, 3 + 1, 3 + 2 or 3 + 3 h of exposure + hours of recovery for cold, 1 + 0, 3 + 0, 5 + 0, 5 + 1, 5 + 3 or 5 + 5 h for desiccation and starvation), plus a control (kept in food vials at room temperature for the full period of exposure/recovery). After each treatment, flies were rapidly transferred into 2 ml vials and snap-frozen in liquid nitrogen before being stored at –80 °C until use. In parallel groups of flies, we observed 100% survival of all of these environmental stress treatments.

Flies were exposed to low temperatures in food vials. The plug of the food vial was replaced with a rubber stopper, and the vials enclosed in a plastic ziplock bag which was immersed in an ice-water slurry (0 °C). To prevent flies from becoming stuck in the food, cooling and recovery were conducted with the vials in a horizontal position. Flies were desiccated by transferring them to an empty 25 × 95 mm vial, followed by a 1 cm foam plug and *c*. 15 ml of anhydrous $CaSO_4$ (Hammond Drierite Co., Xenia, OH, USA). The tube was then sealed with parafilm. To control for the fact that desiccation necessarily involves removal of food, other flies were starved by transferring them to a 25 × 95 mm vial containing *c*. 10 ml of 1% Agar (Drosophila Agar, Genesee Scientific, San Diego, CA, USA), which provides a source of water, but not nutrients. As a positive control for Hsp up-regulation, flies were also heat-shocked in food vials by immersion in a water bath at 36 °C for 1 h followed by 1 h at room temperature, before being snap-frozen in liquid nitrogen as above.

RNA extraction and real-time PCR

Three groups of 30 flies per treatment-time combination (plus three groups of 30 control flies) were used for real-time PCR, giving three different cDNA extracts per treatment-time combination. Whole flies were homogenized on liquid nitrogen, mixed with 600 µl lysis buffer from RNeasy RNA extraction kits (Qiagen, Valencia, CA, USA) and 6 µl β-mercaptoethanol before being passed 10 times through a sterile 18-gauge needle to complete homogenization. RNA extraction and purification were completed using the manufacturer's instructions. RNA was eluted in nuclease-free water, and quantified and quality-checked using spectrophotometry ($A_{260}/A_{280} > 1.9$; Nanodrop ND-1000, Nanodrop Technologies, Wilmington, DE, USA). 300 ng total RNA was used in the reverse transcription of polyadenylated mRNA to cDNA using the Omniscript kit (Qiagen) and an oligo d(T) primer (Table 1) for semiquantitative real-time PCR (qRT-PCR). Primers were sourced from the literature or designed from transcript sequences from Flybase using Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi), synthesized by IDT (Integrated DNA Technologies, Coralville, IA, USA) and are shown in Table 1. qRT-PCR was performed in duplicate (two separate tubes for each sample of three for each treatment–time–gene combination) with the iQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions on a Bio-Rad iCycler with the following programme: 3 min at 95 °C; 40 repeats of 30 s at 94 °C, 30 s at 58 °C and 1 min at 72 °C followed by a standard melt curve. qRT-PCR of a serial dilution of a single sample of cDNA was conducted for all primers to confirm linearity of response across a wide range of concentrations (data not shown). RT-PCR data were normalized to *Actin-79B* expression and message abundance of each gene compared to that in control samples using the ΔΔ-Ct method, which is a conservative measure of RNA abundance (Pfaffl, 2001). Three different samples from each treatment were analysed with RT-PCR and 95 and 99% confidence intervals calculated for the relative fold change of each gene-treatment-time combination (Sokal & Rohlf, 1981). The confidence intervals were then used to compare the mean ΔΔ-Ct value to 1 (which indicates no change relative to the control). Power analyses were conducted using PROC POWER on SAS STAT (v. 9.1, SAS Institute Inc., Cary, NC, USA).

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