Increased abundance of frost mRNA during recovery from cold stress is not essential for cold tolerance in adult Drosophila melanogaster.

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Increased abundance of *Frost* mRNA during recovery from cold stress is not essential for cold tolerance in adult *Drosophila melanogaster*

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ABSTRACT

Frost is a candidate gene associated with the response to cold in Drosophila melanogaster because Frost mRNA accumulation increases during recovery from low temperature exposure. We investigated the contribution of Frost expression to chill-coma recovery time, acute cold tolerance, and rapid cold hardening (RCH) in adult D. melanogaster by knocking down Frost mRNA expression using GAL4/UAS-mediated RNA interference. In this experiment, four UAS-Frost and one tubulin-GAL4 line were used. We predicted that if Frost is essential for cold tolerance phenotypes, flies with low Frost mRNA levels should be less cold tolerant than flies with normal levels of cold induced Frost mRNA. There was no correlation between cold-induced Frost abundance and recovery time from chill-coma in either male or female flies. Survival of 2 h exposures to sub-zero temperatures in Frost knockdown lines was not lower than that in a control line. Moreover, a low temperature pre-treatment increased survival of severe cold exposure in flies regardless of Frost abundance level during recovery from cold stress, suggesting that Frost expression is not essential for RCH. Thus, cold-induced Frost accumulation is not essential for cold tolerance measured as chill-coma recovery time, survival to acute cold stress and RCH response in adult D. melanogaster.

Keywords: RNAi, Frost, cold tolerance, rapid cold hardening, chill-coma, acute cold stress
**Introduction**

Temperature influences the distribution and abundance of insects (Chown & Nicolson, 2004). At low temperatures, insects lose the ability to move, a reversible state termed chill coma, and the time taken to recover from chill coma is commonly used as an index of cold tolerance (see review MacMillan & Sinclair, 2011). Species that are killed by cold exposure that is not associated with ice formation are termed chill-susceptible (Denlinger & Lee, 2010). In many insect species, exposure to a short term, non-lethal cold stress increases tolerance of a subsequent, more extreme cold stress, a process called rapid cold-hardening (RCH) (Denlinger & Lee, 2010). However, the molecular mechanisms underlying variation and plasticity in cold tolerance are still not well understood.

The genetic model organism *Drosophila melanogaster* has been used to understand the mechanisms underlying chill susceptibility in insects because its cold tolerance varies clinally in the wild, changes with artificial selection and is phenotypically plastic (Hoffmann, 2010; Hoffmann *et al.*, 2003). Genes with increased expression following cold exposure are expected to contribute to repair or avoidance of injury resulting from cold exposure. For example, *smp-30* is thought to be related to cold tolerance because *smp-30* mRNA accumulates in *D. melanogaster* after cold acclimation at 15 °C (Goto, 2000) and there is an association between genetic variation at *smp-30* and chill-coma recovery in a wild population (Clowers *et al.*, 2010). Similarly, clinal variation in sequence at the *hsr-omega* locus is associated with variation in chill coma recovery (Anderson *et al.*, 2005; Rako *et al.*, 2007). However, these correlational studies do not necessarily establish a causal relationship between gene expression, or the function of the
proteins they encode, and cold tolerance.

Frost (Fst) is a candidate cold tolerance gene in *D. melanogaster* (Goto, 2001) that is expressed in the Malpighian tubules and midgut of unstressed adult flies (Wang *et al.*, 2004). Frost mRNA does not accumulate during cold exposure, but Fst abundance increases during the first few hours of recovery from cold stress in most life stages of *D. melanogaster* (Bing *et al.*, 2012; Sinclair *et al.*, 2007). Although the role of the Frost protein is still not clear, it appears to be a stress-related disordered protein (Bing *et al.*, 2012) that is secreted into extracellular spaces (Goto, 2001).

Quantitative Trait Loci studies suggest that Frost is associated with variation in chill-coma recovery in female *D. melanogaster* (Morgan & Mackay, 2006; Norry *et al.*, 2007). However, sequence variation at the Frost locus and its promoter region are not associated with clinal variation of chill-coma recovery time in Australian populations (Hoffmann *et al.*, 2012; Rako *et al.*, 2007). Knock-down of Frost with RNA interference (RNAi) increased the recovery time from chill coma after exposure to 0 °C for 10 h (Colinet *et al.*, 2010); however, the contribution of Frost expression to survival following acute cold stress and the RCH response has not been examined.

Here, we assess the role of Frost by examining the effect of reducing Frost transcript levels on several cold tolerance phenotypes of *D. melanogaster*. We used tublin-GAL4/UAS-mediated RNAi (Dietzl *et al.*, 2007; Duffy, 2002) to reduce the abundance of Frost mRNA. We then assayed recovery time from chill coma, survival after acute cold stress, and RCH by examining survival after acute cold stress. We predicted that, if Frost is essential for cold tolerance in *D. melanogaster*, flies with low Frost mRNA levels would be less cold tolerant than flies with normal Frost mRNA levels.
after cold exposure.

Results

Abundances of *Frost* mRNA were measured with real-time PCR. *Frost* mRNA accumulations significantly increased in response to cold stress in all five control lines (*tub-GAL4/+* and */+/UAS-Fst*) of both male and female *Drosophila melanogaster* (Fig. 1).

In male flies, *Frost* expression after cold exposure was suppressed by RNAi in three of four *tub-GAL4>UAS-Fst* lines: *tub-GAL4>UAS-Fst1*, *tub-GAL4>UAS-Fst2* and *tub-GAL4>UAS-Fst4* (Fig. 1A). In female flies, the level of mRNA *Frost* was not significantly increased after cold exposure in three *tub-GAL4>UAS-Fst* lines: *tub-GAL4>UAS-Fst2*, *tub-GAL4>UAS-Fst3* and *tub-GAL4>UAS-Fst4* (Fig. 1B).

We examined the effect of reduction of *Frost* mRNA accumulation on recovery time from chill-coma. *Frost* knockdown resulted in significantly increased chill-coma recovery time of both male and female flies in only the *tub-GAL4>UAS-Fst2* line (Fig. 2). On the other hand, both male and female *tub-GAL4>UAS-Fst4* showed shorter recovery times than their corresponding */+UAS* control line (Fig. 2). Chill-coma recovery time did not differ among the four */+UAS-Fst* lines in male flies, but in female flies */+UAS-Fst3* showed significantly shorter recovery times than */+UAS-Fst1* and */+UAS-Fst2* (Fig. 2). There was no significant correlation between cold-induced *Frost* mRNA abundance and recovery time from chill-coma in either males (Fig. 3, $r_s = 0.20$, $p = 0.58$) or females ($r_s = -0.067$, $p = 0.84$). However, the *tub-GAL4>UAS-Fst2* lines had unusually slow recovery time, and if these points were removed, there was a positive correlation between recovery time and relative level of *Frost* abundance in male flies ($r_s = 0.74$, $p < 0.05$), although the correlation remained non-significant in female flies ($r_s = 0.34$, $p =$..
If increasing _Frost_ mRNA abundance during recovery from cold stress is essential for tolerance to acute cold stress, we would expect _Frost_ knockdown flies to show lower survival after exposure to acute cold stress than control flies. Male _tub-GAL4>UAS-Fst2_ flies had significantly greater survival than _tub-GAL4/+_ individuals after acute exposure to -3 and -4 °C (Fig. 4A). The survival rates of males of the _tub-GAL4>UAS-Fst1_ and _tub-GAL4>UAS-Fst3_ lines were not significantly different from that of _tub-GAL4/+_ at all temperatures. In female flies, _tub-GAL4>UAS-Fst3_ had significantly lower survival than _tub-GAL4/+_ at -2 °C (Fig. 4C). However, the survival rates of _tub-GAL4>UAS-Fst2_ and _tub-GAL4>UAS-Fst 4_ lines were significantly higher than _tub-GAL4/+_ at -3 and -4 °C. Survival after exposure to -4 °C was higher for all _tub-GAL4>UAS-Fst_ lines compared to _tub-GAL4/+_. In both males and females, there was no difference in survival among _+/UAS-Fst_ lines at any test temperature (Figs 4B, 4D).

Finally, to examine the contribution of _Frost_ for survival enhanced by RCH, the survival after exposure to acute cold stress (-4.5 C for 2 h) was compared to that in pre-cold treated flies. In male and female flies, survival after exposure to -4.5 °C for 2 h was significantly affected by line and type of treatment, but there was no significant line x treatment interaction (Table 2). RCH increased survival after exposure to -4.5 °C for 2 h in male flies of all the control lines, _tub-GAL4>UAS-Fst3_ and _tub-GAL4>UAS-Fst4_ lines but not in _tub-GAL4>UAS-Fst1_ and _tub-GAL4>UAS-Fst2_ (Fig. 5A). In control groups, the survival of _tub-GAL4>UAS-Fst2_ was significantly higher than other lines. An RCH response was observed in all lines in female flies and _+/UAS-Fst4_ showed a stronger response than other _+/UAS_ lines and _tub-GAL4/+_ (Fig. 5B).
Discussion

There are several candidate genes associated with cold tolerance in *Drosophila melanogaster* (Hoffmann *et al.*, 2003; Qin *et al.*, 2005) but the physiological role of those candidates in cold tolerance, and the relationship between gene expression after cold stress and cold tolerance remains unclear. In the present study, we explored the role of *Frost*, one of these candidates, using RNAi-mediated expression knockdown. We were able to obtain three lines of flies that did not show a significant increase of *Frost* mRNA accumulation during recovery from cold stress, and we would predict that if *Frost* is essential to cold tolerance, these *Frost* knockdown flies should show longer chill-coma recovery time, less tolerance to acute cold stress and a loss of the RCH response. However, our results do not support these predictions, suggesting that *Frost* expression is not essential to recovery from chill-coma, survival after acute cold stress or the RCH response.

If a higher *Frost* expression level induces shorter chill-coma recovery time, we would expect a negative correlation between *Frost* mRNA abundance and chill coma recovery time. However, we did not detect a significant relationship between *Frost* mRNA abundance and chill coma recovery time in female flies, and the relationship was significantly positive in male flies. Rako *et al.* (2007) suggest that variation at the *Frost* locus is not related to recovery time from chill coma in Australian populations and Udaka *et al.* (2010) showed that variation of recovery time does not coincide with expression levels of *Frost* using lines selected for chill coma recovery time. Thus, there is little
evidence that chill-coma recovery time is dependent on an increase of \textit{Frost} mRNA accumulation. However, chill-coma recovery time is affected by the duration of cold exposure and temperature (MacMillan & Sinclair, 2011), and \textit{Frost} was identified as a candidate gene following a longer exposure to 0 °C (20 h) in a QTL study by Norry \textit{et al.} (2008). Thus, the role of \textit{Frost} in chill coma recovery may only become apparent at longer exposures than we used in the present study.

Our \textit{UAS-Fst4} line was derived from the same stock as those used by Colinet \textit{et al.} (2010). However, while Colinet \textit{et al.} (2010) found delayed recovery from chill-coma in this line, \textit{Frost} knockdown in \textit{tub-GAL4>UAS-Fst4} did not cause the delay of recovery time in the present study. Colinet \textit{et al.} (2010) used \textit{actin-GAL4} and \textit{tub-GAL4} as a driver and the \textit{tub-GAL4} driver has different genetic background from the \textit{tub-GAL4} line we obtained from Bloomington \textit{Drosophila} Stock Centre (BDSC). Thus, this genotypic variation of \textit{tub-GAL4} line may cause the discrepancy in recovery time in \textit{tub-GAL4>UAS-Fst4}. Additionally, in experiments using RNAi, off-target effects, which a non-target gene mRNA accumulation is reduced by binding short interference RNA, can be problematic (Ma \textit{et al.}, 2006). \textit{UAS-Fst1}, \textit{UAS-Fst2} and \textit{UAS-Fst3} have the same construct that produces the same hairpin RNA (Table 3) and the sequence of this RNA has one predicted off-target. The construct of \textit{UAS-Fst4} is different from other three \textit{UAS-Fst} lines and has no predicted off-target. Therefore, the delay of recovery from chill-coma in \textit{tub-GAL4>UAS-Fst4} might be caused by off-target effect.

We also examined the contribution of \textit{Frost} to the response to acute cold stress, measured by survival. Two of the \textit{tub-GAL4>UAS-Fst} lines that did not show an increase of \textit{Frost} mRNA abundance after cold stress had higher survival following exposure to -
or -4 °C, or both for 2 h than \textit{tub-GAL4} line. Although we did not examine the level of \textit{Frost} expression at all test temperatures, a previous study showed that increase of \textit{Frost} mRNA accumulation is induced by a 2 h exposure at -4.5 °C (Colinet & Hoffmann, 2012). Thus, increased expression of \textit{Frost} is not associated with higher tolerance to acute cold stress. Colinet & Hoffmann (2012) also found that acclimated flies that had higher acute cold tolerance had lower \textit{Frost} mRNA abundance. We conclude that high expression of \textit{Frost} during recovery from cold stress does not play an essential role in survival following acute cold stress.

The RCH response was not consistently disrupted by suppression of \textit{Frost} accumulation. As our data and previous studies show, levels of \textit{Frost} mRNA increase during recovery from cold stress (Bing et al., 2012; Colinet et al., 2010; Goto, 2001; Reis et al., 2011; Sinclair et al., 2007) but not during cold exposure (Sinclair et al., 2007). In the present study, the \textit{Frost} expression levels after pre-cold treatment and acute cold stress were not measured, but we assume that accumulation of \textit{Frost} increases during recovery from pre-cold treatment and acute cold stress, following the patterns we saw in these lines. The molecular mechanisms underlying RCH are unclear, but it appears that RCH prevents apoptosis due to cold injury in \textit{D. melanogaster} (Yi et al., 2007). Even if the Frost protein has a role in signaling and apoptosis (suggested by Bing et al., 2012), it is unlikely that the increase of \textit{Frost} mRNA accumulation occurs within a time frame relevant to the RCH response.

The expression of \textit{Frost} is induced not only by cold stress but also by other stresses, for example desiccation, severe heat stress, hypoxia and dietary shift (Carsten et al., 2005; Sinclair et al., 2007; Udaka et al., 2010). \textit{Frost} has also been identified as a
gene involved in immune responses to bacteria, fungi and viruses (Chamilos et al., 2008; De Gregorio et al., 2002). Thus, Frost might be a general stress response gene. In D. melanogaster, mild cold stress increases survival of fungal infection (Le Bourg et al., 2009) and the expression of several immune-related genes increases 6h after exposure to cold stress (-0.5 ºC, 2h) (Zhang et al., 2011). Although there is little information about a relationship between immune responses and cold stress, these results indicate that Frost expression may have a role in the immune system as it relates to cold tolerance. As such, the importance of Frost expression, and the Frost protein, may only be manifest some time after the initial cold exposure, in a manner that is not apparent in the cold tolerance assays we used. Testing this hypothesis will require a deeper understanding of the function of the Frost protein, and exploration of the long-term impact of Frost knockdown.

Experimental procedures

Insects

Flies were reared under 13:11 L:D 22 ºC on banana-yeast-propionic acid medium (Rajamohan & Sinclair, 2008). To knock down Frost mRNA expression, we used RNAi mediated by the GAL4-UAS system. Four UAS-Fst lines (Transform at ID: 16604 [designated as UAS-Fst1], 17258 [UAS-Fst2], 39070 [UAS-Fst3], 102049 [UAS-Fst4]) and the w1118 (+) line, which provides the same genetic background as UAS lines, were obtained from the Vienna Drosophila RNAi Center (VDRC) (Table 3) and the tubulin-GAL4 (genotype: y¹ w*; P(tub P- GAL4)LL7/TM3, Sb¹, Bloomington Drosophila
Stock Centre, BDSC, stock number 5138) was used to drive the expression of the UAS-
Fst. As a control, w¹¹¹8 (+) was crossed to the tub-GAL4 line and the four UAS-Fst lines.
To obtain tub-GAL4>UAS, tub-GAL4/+, and UAS/+ lines, virgin females and males were
collected under CO₂ anesthesia and transferred to 35 ml vials containing food medium.
The progeny were sorted, sexed under CO₂ anesthesia within 24 h after eclosion and
recovered at 22 °C for at least 72 h (Nilson et al., 2006). Adult flies were used 5 days
after eclosion to measure the expression level of Frost, chill-coma recovery, survival after
exposure to cold stress, and RCH response.

RNA extraction and real-time PCR
To determine the abundance of Frost mRNA after cold exposure, ten flies were
transferred without anesthesia to empty 50 mL plastic tubes with a sponge plug restricting
them to the bottom 5 cm of the tube. The tubes containing flies were immediately
exposed to -2 °C for 2 h in 50:50 methanol:water in a refrigerated bath (Lauda Proline
RP3530, Würzburg, Germany) as above and flies were allowed to recover at 22 °C for 2
h. Control groups were kept at 22 °C. After treatments, flies were transferred to a 1.5 mL
microcentrifuge tube and flash-frozen in liquid nitrogen vapour. The samples were stored
at -80 °C until RNA extraction.
Total RNA was isolated with TRIzol reagent (Invitrogen, Carlsbad, CA, USA)
according to the supplier’s instructions. RNA was resuspended in DEPEC-treated water.
Genomic DNA was digested with DNase I Amp Grade (Invitrogen), and the RNA was
stored at -20 °C until cDNA synthesis. cDNA was synthesized from 500 ng RNA by using
Oligo-dT primer (Invitrogen) and SuperScript II Reverse Transcriptase (Invitrogen).
Real-time PCR was performed on a Rotor-Gene 6000 Cycler (Corbett life science, San Francisco, CA, USA) with SYBR Green PCR Master Mix (Applied Biosystems, Foster city, CA, USA). Cycling condition was 95 °C for 10 min followed by 45 cycles of 95 °C for 15 s, 55 °C for 15 s and 72 °C for 30 s, and melting curve analysis was performed. The primers for *Frost* were 5´-CGATTCTTCAGCGGTCTAGG-3´ and 5´-CTCGGAAACGCCAAATTTTA-3´ (Sinclair et al., 2007). *Act79B* was used as a reference gene and the primers were 5´-CCAGGTATCGCTGACCGTAT-3´ and 5´-TTGGATATCCACATCTGCTG-3´ (Sinclair et al., 2007). Abundance of *Frost* mRNA relative to *Act79B* mRNA was calculated using the 2^{-ΔΔCt} method (Livak & Schmittgen, 2001). Real-time PCR was performed on three independent biological replicates.

**Chill coma recovery time**

Chill coma recovery time was measured with three replicates of ten individuals for each sex from each line. Ten flies were placed in a 35 mL vial (25 mm diameter) containing food. The vials were enclosed in sealed plastic bags and maintained on their side in an ice-water slurry (0 ºC) for 12 h. After cold exposure, flies were transferred to 6-well plates and the number of recovered flies was recorded every minute at 22-24 ºC. Flies that could stand were scored as recovered (David *et al.*, 1998).

**Survival of acute cold exposure with and without rapid cold-hardening**

Nine to 15 flies (n= 3 groups per treatment/temperature/sex/line combination) were transferred to 50 mL plastic tubes (28 mm diameter) and a sponge plug was used to restrict the flies to the bottom 45 mm. The tubes containing flies were exposed to a test
temperature (-2, -3, -4 or -5 °C) for 2 h in 50:50 methanol:water in a refrigerated bath. Survival following exposure to test temperatures was measured after 24 h and individuals that could stand up and walk were considered alive.

Rapid cold hardening

To examine RCH responses, flies were divided into control and pretreatment groups and transferred to 50 ml tubes. Control groups were directly exposed to -4.5 °C for 2 h. In pretreatment groups, flies were kept at 0 °C for 2 h and recovered at 22 °C for 1 h, followed by exposure to -4.5 °C for 2 h. After cold exposure, the flies were moved to 6 well plates with a piece of food medium and maintained at 22 °C. Survival was assessed after 24 h. Measurements were made with three to six groups of ten flies for each sex from each line.

Statistical analysis

Relative *Frost* expression was compared between control and cold-treated groups within the same line with Student’s *t*-test on SigmaPlot 10 (Systat Software, Inc., Chicago, IL, USA). Recovery time from chill-coma was compared among lines using the log-rank test followed by Holm-Sidak pairwise test (SigmaPlot 10). Correlation between cold-induced *Frost* mRNA abundances and recovery time from chill-coma was analyzed by Spearman’s rank correlation test by SigmaPlot 10. Survival after acute cold stress was arcsine-square root transformed and compared within the same test temperature by ANOVA and Tukey’s *post hoc* tests (SigmaPlot 10). For the RCH analysis, survival was compared between control and pretreatment and among lines using a generalized linear
Acknowledgments

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References


melanogaster lines that are selected for recovery time from temperature coma. *J Insect Physiol* **56**: 1889-94.


Table 1. Results of ANOVA of survival after acute cold stress in adult *Drosophila melanogaster*.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>tub-GAL4/+</td>
<td>UAS-Fst</td>
</tr>
<tr>
<td></td>
<td><em>tub-GAL4</em> &gt; UAS-Fst</td>
<td>+/ UAS-Fst</td>
</tr>
<tr>
<td></td>
<td><em>F</em> (4, 10)</td>
<td><em>P</em></td>
</tr>
<tr>
<td>-2</td>
<td>1.667</td>
<td>0.233</td>
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<tr>
<td>-3</td>
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<td>&lt; 0.001</td>
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<tr>
<td>-4</td>
<td>5.285</td>
<td>0.015</td>
</tr>
<tr>
<td>-5</td>
<td>1.000</td>
<td>1.000</td>
</tr>
</tbody>
</table>

Table 2. Results of generalised linear models of the effect of pre-cold treatment and survival rate after cold stress (-4.5 °C for 2 h) of adult *Drosophila melanogaster*.

<table>
<thead>
<tr>
<th></th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wald $\chi^2$</td>
<td>d.f.</td>
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<tr>
<td>Treatment$^a$</td>
<td>78.475</td>
<td>1</td>
</tr>
<tr>
<td>Line$^b$</td>
<td>69.823</td>
<td>8</td>
</tr>
<tr>
<td>Treatment x line</td>
<td>14.090</td>
<td>8</td>
</tr>
</tbody>
</table>

$^a$ *tub-GAL4/+*, four *tub-GAL4* > UAS-Fst, and four +/ UAS-Fst lines were used. $^b$ Flies were exposed to each test temperature for 2 h. $^c$ The number means the degrees of freedom between groups. $^d$ The number means the degrees of freedom within groups.
Flies were divided into two treatment groups, control and pre-cold treatment group (0 °C for 2 h and 1 h recovery at 22 °C), and exposed to -4.5 °C for 2 h to examine RCH responses.

Five control lines (tub-GAL4/+ and four +/UAS-Fst lines) and four tub-GAL4>UAS-Fst lines were used.

Table 3. *UAS-Frost* lines used to knockdown *Frost* mRNA in this paper.

<table>
<thead>
<tr>
<th>Transformant ID</th>
<th>Construct ID</th>
<th>Hairpin length</th>
<th>Inserted chromosome</th>
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<tr>
<td>UAS-Fst1</td>
<td>16604</td>
<td>5629</td>
<td>366</td>
</tr>
<tr>
<td>UAS-Fst2</td>
<td>17258</td>
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<td>366</td>
</tr>
<tr>
<td>UAS-Fst3</td>
<td>39070</td>
<td>5629</td>
<td>366</td>
</tr>
<tr>
<td>UAS-Fst4</td>
<td>1020549</td>
<td>110516</td>
<td>422</td>
</tr>
</tbody>
</table>

*UAS-Fst* lines were obtained from the Vienna Drosophila RNAi Center (VDRC).

The information about *UAS-Fst* lines we used refer to the website of VDRC (http://www.vdrc.at).
Figure legends

Fig. 1. Relative abundance of Frost mRNA without cold treatment (control) and after 2 h at -2 °C followed by 3 h at 22 °C (cold treated) in male (A) and female (B) of Drosophila melanogaster. Expression of Frost was normalized to Actin79B and expressed relative to untreated tub-GAL4/w1118 (+). Mean ± SEM, n = 3. Asterisk indicates a significant difference between cold-treated and control flies within a line (Student’s t-test; p < 0.05).

Fig. 2. Recovery time from chill coma of male (A) and female (B) of Drosophila melanogaster. Flies were exposed to 0 °C for 12 h and transferred to 22 °C to measure recovery time. Underlined genotypes indicate tub-GAL4>UAS-Fst lines where Frost expression after cold stress was suppressed (see Fig. 1). Recovery times from chill-coma for nine groups in both male and female flies were significantly different (log-rank test, P < 0.001) and the same letters above data points indicate lines whose recovery times are not significantly different (Pairwise multiple comparison by Holm-Sidak method, P > 0.05). Data points indicate the median and error bars represent 25% and 75% quartiles. n = 30 – 40.

Fig. 3. The relationship between mean relative Frost mRNA abundance during recovery from cold stress and median chill-coma recovery time in male (triangles) and female flies (circles). The Frost mRNA was measured after 2 h at -2 °C followed by 3 h at 22 °C and the expression level was relative to abundance in tub-GAL4/+ line without cold treatment (see Fig. 1). Flies were exposed to 0 °C for 12 h and transferred to 22 °C to measure
recovery time. Filled grey symbols indicate points corresponding to the tub-GAL4>UAS-
Fst2 line. The data are derived from Fig. 1 and Fig. 2.

Fig. 4. Survival 24 h after 2 h exposure to cold in male (A and B) and female (C and D)
Drosophila melanogaster. Underlines indicate tub-GAL4>UAS-Fst lines where Frost
expression after cold stress was suppressed (see Fig. 1). Survival at points with the same
letters does not differ at a given temperature (ANOVA, see Table 1, Tukey’s post hoc
test, $p > 0.05$). Mean ± SE. $n = 3$ groups of nine - 15 flies at each test temperature.

Fig. 5. Rapid cold-hardening response of control (tub-GAL4/+, +/UAS-Fst) and tub-
GAL4>UAS-Fst lines in male (A) and female (B) Drosophila melanogaster after 2 h
exposure to -4.5 °C with (filled bars) and without (open bars) a pre-treatment (0 °C for 2 h
and 1h recovery at 22 °C). Survival of a 2 h exposure to -4.5 °C was measured by
transferring to 22 °C. Underlines indicate tub-GAL4>UAS-Fst lines where Frost
expression after cold stress was suppressed (see Fig. 1). Asterisks indicate that survival of
pre-treated flies is significantly higher than that of the control group from the same line.
Survival at points with the same letters does not significantly differ (Generalized linear
model, $p > 0.05$). Mean ± SE. $n = 50 – 76$. 