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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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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 ºC (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 ºC 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-Fst*2 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 ($r_s = 0.34$, $p =$

113 0.39).

114 If increasing *Frost* mRNA abundance during recovery from cold stress is
115 essential for tolerance to acute cold stress, we would expect *Frost* knockdown flies to
116 show lower survival after exposure to acute cold stress than control flies. Male *tub-*
117 *GAL4>UAS-Fst*2 flies had significantly greater survival than *tub-GAL4/+* individuals
118 after acute exposure to -3 and -4 ºC (Fig. 4A). The survival rates of males of the *tub-*
119 *GAL4>UAS-Fst*1 and *tub-GAL4>UAS-Fst*3 lines were not significantly different from
120 that of *tub-GAL4/+* at all temperatures. In female flies, *tub-GAL4>UAS-Fst3* had
121 significantly lower survival than *tub-GAL4/+* at -2 ºC (Fig. 4C). However, the survival
122 rates of *tub-GAL4>UAS-Fst*2 and *tub-GAL4>UAS-Fst* 4 lines were significantly higher
123 than *tub-GAL4/+* at -3 and -4 ºC. Survival after exposure to -4 ºC was higher for all *tub-*
124 *GAL4>UAS-Fst* lines compared to *tub-GAL4/+*. In both males and females, there was no
125 difference in survival among +/*UAS-Fst* lines at any test temperature (Figs 4B, 4D).

126 Finally, to examine the contribution of *Frost* for survival enhanced by RCH, the
127 survival after exposure to acute cold stress (-4.5 C for 2 h) was compared to that in pre-
128 cold treated flies. In male and female flies, survival after exposure to -4.5 ºC for 2 h was
129 significantly affected by line and type of treatment, but there was no significant line x
130 treatment interaction (Table 2). RCH increased survival after exposure to -4.5 º C for 2 h
131 in male flies of all the control lines, *tub-GAL4>UAS-Fst3* and *tub-GAL4>UAS-Fst4* lines
132 but not in *tub-GAL4>UAS-Fst1* and *tub-GAL4>UAS-Fst2* (Fig. 5A). In control groups,
133 the survival of *tub-GAL4>UAS-Fst2* was significantly higher than other lines. An RCH
134 response was observed in all lines in female flies and +/*UAS-Fst4* showed a stronger
135 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

159 evidence that chill-coma recovery time is dependent on an increase of *Frost* mRNA
160 accumulation. However, chill-coma recovery time is affected by the duration of cold
161 exposure and temperature (MacMillan & Sinclair, 2011), and *Frost* was identified as a
162 candidate gene following a longer exposure to 0 ºC (20 h) in a QTL study by Norry *et al.*
163 (2008) . Thus, the role of *Frost* in chill coma recovery may only become apparent at
164 longer exposures than we used in the present study.
165 Our *UAS-Fst4* line was derived from the same stock as those used by Colinet *et*
166 *al.* (2010). However, while Colinet *et al.* (2010) found delayed recovery from chill-coma
167 in this line, *Frost* knockdown in *tub-GAL4>UAS-Fst4* did not cause the delay of recovery
168 time in the present study. Colinet et al. (2010) used *actin- GAL4* and *tub-GAL4* as a driver
169 and the *tub-GAL4* driver has different genetic background from the *tub-GAL4* line we
170 obtained from Bloomington *Drosophila* Stock Centre (BDSC). Thus, this genotypic
171 variation of *tub-GAL4* line may cause the discrepancy in recovery time in *tub-*
172 *GAL4>UAS-Fst4*. Additionally, in experiments using RNAi, off-target effects, which a
173 non-target gene mRNA accumulation is reduced by binding short interference RNA, can
174 be problematic (Ma *et al.*, 2006). *UAS-Fst1*, *UAS-Fst2* and *UAS-Fst3* have the same
175 construct that produces the same hairpin RNA (Table 3) and the sequence of this RNA
176 has one predicted off-target. The construct of *UAS-Fst4* is different from other three *UAS-*
177 *Fst* lines and has no predicted off-target. Therefore, the delay of recovery from chill-
178 coma in *tub-GAL4>UAS-Fst2* might be caused by off-target effect.
179 We also examined the contribution of *Frost* to the response to acute cold stress,
180 measured by survival. Two of the *tub-GAL4>UAS-Fst* lines that did not show an increase
181 of *Frost* mRNA abundance after cold stress had higher survival following exposure to -3

182 or -4 ºC, or both for 2 h than *tub-GAL4*>+ line. Although we did not examine the level of
183 *Frost* expression at all test temperatures, a previous study showed that increase of *Frost*
184 mRNA accumulation is induced by a 2 h exposure at -4.5 ºC (Colinet & Hoffmann,
185 2012). Thus, increased expression of *Frost* is not associated with higher tolerance to
186 acute cold stress. Colinet &, Hoffmann (2012) also found that acclimated flies that had
187 higher acute cold tolerance had lower *Frost* mRNA abundance. We conclude that high
188 expression of *Frost* during recovery from cold stress does not play an essential role in
189 survival following acute cold stress.

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

202 The expression of *Frost* is induced not only by cold stress but also by other
203 stresses, for example desiccation, severe heat stress, hypoxia and dietary shift (Carsten *et*
204 *al.*, 2005; Sinclair *et al.*, 2007; Udaka *et al.*, 2010). *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-proprionic 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 w^{1118} (+) 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: y^1 *w*; P{tub P- GAL4}LL7/TM3,* Sb^1, Bloomington *Drosophila*

228 Stock Centre, BDSC, stock number 5138) was used to drive the expression of the *UAS-*
229 *Fst*. As a control, w^{1118} (+) was crossed to the *tub -GAL4* line and the four *UAS-Fst* lines.
230 To obtain *tub-GAL4>UAS*, *tub-GAL4/+*, and *UAS/+* lines, virgin females and males were
231 collected under CO_2 anesthesia and transferred to 35 ml vials containing food medium.
232 The progeny were sorted, sexed under CO_2 anesthesia within 24 h after eclosion and
233 recovered at 22 ºC for at least 72 h (Nilson *et al.*, 2006). Adult flies were used 5 days
234 after eclosion to measure the expression level of *Frost*, chill-coma recovery, survival after
235 exposure to cold stress, and RCH response.
236
237 *RNA extraction and real-time PCR*
238 To determine the abundance of *Frost* mRNA after cold exposure, ten flies were
239 transferred without anesthesia to empty 50 mL plastic tubes with a sponge plug restricting
240 them to the bottom 5 cm of the tube. The tubes containing flies were immediately
241 exposed to -2 ºC for 2 h in 50:50 methanol:water in a refrigerated bath (Lauda Proline
242 RP3530, Würzburg, Germany) as above and flies were allowed to recover at 22 ºC for 2
243 h. Control groups were kept at 22 ºC. After treatments, flies were transferred to a 1.5 mL
244 microcentrifuge tube and flash-frozen in liquid nitrogen vapour. The samples were stored
245 at -80 ºC until RNA extraction.
246 Total RNA was isolated with TRIzol reagent (Invitrogen, Carlsbad, CA, USA)
247 according to the supplier's instructions. RNA was resuspended in DEPEC-treated water.
248 Genomic DNA was digested with DNase I Amp Grade (Invitrogen), and the RNA was
249 stored at -20 ºC until cDNA synthesis. cDNA was synthesized from 500 ng RNA by using
250 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 $2^{-\Delta\Delta Ct}$ 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 ºC) 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 ºC for 2 h. In pretreatment groups, flies were kept at 0 ºC for 2 h and recovered at 22 ºC for 1 h, followed by exposure to -4.5 ºC for 2 h. After cold exposure, the flies were moved to 6 well plates with a piece of food medium and maintained at 22 ºC. 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 form 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

297 model with binomial error and logit link in SPSS (v. 20; IBM, NY, UAS).

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410

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

412 *melanogaster*.

Temperature (°C) [b]	Male				Female			
	tub-GAL4/+, *tub-GAL4* >*UAS-Fst* [a]		+/ *UAS-Fst* [a]		*tub-GAL4*/+, *tub-GAL4* >*UAS-Fst*		*UAS-Fst*/+	
	F (4[c], 10[d])	*P*	*F* (3, 8)	*P*	*F* (4, 10)	*P*	*F* (3,8)	*P*
-2	1.667	0.233	1.587	0.267	9.399	0.002	0.000	1.000
-3	15.305	< 0.001	1.000	0.441	12.331	<0.001	0.000	1.000
-4	5.285	0.015	1.926	0.204	4.982	0.018	0.706	0.575
-5	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000

413 [a] *tub-GAL4*/ +, four *tub-GAL4>UAS-Fst* , and four + / *UAS-Fst* lines were used. [b] Flies

414 were exposed to each test temperature for 2 h. [c] The number means the degrees of freedom

415 between groups. [d] The number means the degrees of freedom within groups.

416

417 Table 2. Results of generalised linear models of the effect of pre-cold treatment and

418 survival rate after cold stress (-4.5 ºC for 2 h) of adult *Drosophila melanogaster*.

	Male			Female		
	Wald χ^2	d.f.	*P*	Wald χ^2	d.f.	*P*
Treatment[a]	78.475	1	<0.001	94.665	1	<0.001
Line[b]	69.823	8	<0.001	30.578	8	<0.001
Treatment x line	14.090	8	0.079	7.104	8	0.525

[a] Flies were divided into two treatment groups, control and pre-cold treatment group (0 ºC for 2 h and 1h recovery at 22 ºC), and exposed to -4.5 ºC for 2 h to examine RCH responses.

[b] 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.

	Transformant ID	Construct ID	Hairpin length	Inserted chromosome
UAS-Fst1	16604	5629	366	2
UAS-Fst2	17258	5629	366	3
UAS-Fst3	39070	5629	366	2
UAS-Fst4	1020549	110516	422	2

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 (hhtp://www.vdrc.at).

Figure legends

Fig. 1. Relative abundance of *Frost* mRNA without cold treatment (control) and after 2h at -2 ºC followed by 3h at 22 ºC (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/*w^{1118} (+). 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 ºC for 12 h and transferred to 22 ºC 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 2h at -2 ºC followed by 3h at 22 ºC and the expression level was relative to abundance in *tub-GAL4/ +* line without cold treatment (see Fig. 1). Flies were exposed to 0 ºC for 12 h and transferred to 22 ºC to measure

recovery time. Filled grey symbols indicate points corresponding to the *tub-GAL4>UAS-Fst*2 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 ºC with (filled bars) and without (open bars) a pre-treatment (0 ºC for 2 h and 1h recovery at 22 ºC). Survival of a 2 h exposure to -4.5 ºC was measured by transferring to 22 ºC. 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.