

## RESEARCH ARTICLE

# CRISPR-induced null alleles show that *Frost* protects *Drosophila melanogaster* reproduction after cold exposure

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## ABSTRACT

The ability to survive and reproduce after cold exposure is important in all kingdoms of life. However, even in a sophisticated genetic model system like *Drosophila melanogaster*, few genes have been identified as functioning in cold tolerance. The accumulation of the *Frost* (*Fst*) gene transcript increases after cold exposure, making it a good candidate for a gene that has a role in cold tolerance. Despite extensive RNAi knockdown analysis, no role in cold tolerance has been assigned to *Fst*. CRISPR is an effective technique for completely knocking down genes, and is less likely to produce off-target effects than GAL4-UAS RNAi systems. We have used CRISPR-mediated homologous recombination to generate *Fst*-null alleles, and these *Fst* alleles uncovered a requirement for FST protein in maintaining female fecundity following cold exposure. However, FST does not have a direct role in survival following cold exposure. *FST* mRNA accumulates in the Malpighian tubules, and the FST protein is a highly disordered protein with a putative signal peptide for export from the cell. Future work is needed to determine whether FST is exported from the Malpighian tubules and directly interacts with female reproductive tissues post-cold exposure, or whether it is required for other repair/recovery functions that indirectly alter energy allocation to reproduction.

**KEY WORDS:** Genome editing, CRISPR-Cas9, Cold tolerance, Chill coma, Intrinsically disordered protein, Insect

## INTRODUCTION

In temperate, polar and alpine environments, insects and other small ectotherms risk internal ice formation and death when exposed to low temperatures. In addition, low temperatures can cause a range of types of sublethal damage, reducing the fitness or reproductive output of ectotherms that survive these thermal challenges. Ectotherm cold tolerance strategies are well described (Lee, 2010; Sinclair et al., 2015), and a number of low molecular weight cryoprotectants are associated with surviving sub-zero temperatures, such as glycerol, trehalose and proline, and ice-binding proteins, such as antifreeze proteins (Zachariassen and Kristiansen, 2000; Bale and Hayward, 2010; Storey and Storey, 2013). In addition, the role of maintaining ion and water balance (and recovering homeostasis post-cold

exposure) in cold hardiness is reasonably well understood (Overgaard and Macmillan, 2017). Although genes covering a broad range of functions appear to be associated with increased cold tolerance, surprisingly, few of these genes have been shown to have a function in cold tolerance. Candidate genes associated with increased cold tolerance are identified in studies on evolutionary adaptation (e.g. Collinge et al., 2008; Dennis et al., 2015), selection (e.g. Telonis-Scott et al., 2009; Williams et al., 2014) or phenotypic plasticity (MacMillan et al., 2016). Thus, insect cold tolerance is an ecologically important trait for which the mechanistic link from genotype to phenotype is poorly understood.

*Drosophila* (*Sophophora*) *melanogaster* has been used for a range of studies of the mechanisms and evolution of insect cold tolerance. *Drosophila melanogaster* is chill-susceptible, i.e. is killed by exposure to cold at temperatures above its freezing point (Czajka and Lee, 1990). Mortality after brief intense cold exposures appears to be caused by cellular injury, while longer exposures to low temperatures disrupt ion and water balance (Sinclair and Roberts, 2005). Repeated sublethal cold exposures reduce fecundity and fitness of *D. melanogaster* females, likely because of reallocation of energy stores from reproduction to repair and survival (Marshall and Sinclair, 2010; Zhang et al., 2011). Cold tolerance of *D. melanogaster* is plastic: variation among natural populations indicates evolutionary adaptation (Hoffmann et al., 2001); acclimation responses improve cold tolerance over a period of days (Watson and Hoffmann, 1996); and rapid cold-hardening (RCH) improves tolerance response after a brief exposure to cold (Czajka and Lee, 1990). This RCH also preserves reproductive behavior (Shreve et al., 2004). The molecular underpinnings of *Drosophila* cold tolerance have been explored extensively via microarrays (Qin et al., 2005; Zhang et al., 2011), quantitative trait loci (QTL) (Morgan and Mackay, 2006; Gerken et al., 2015), RNA sequencing (MacMillan et al., 2016) and proteomics (Sørensen et al., 2017), yet surprisingly few genes are consistently upregulated or associated with low temperatures.

The most prominent of the cold-related genes identified in *D. melanogaster* is *Frost* (*Fst*), which increases in abundance after (but not during) cold exposure in all life stages (Goto, 2001; Sinclair et al., 2007; Bing et al., 2012) (note: we refer to the gene as *Fst*, the mRNA as *FST* and the protein as FST). Basal *FST* mRNA expression is most abundant in the Malpighian tubules (Chintapalli et al., 2007), although the site of expression induced by cold exposure has not been investigated. The position of *Fst* in an intron of the diuretic hormone gene *Dh-44* potentially links *Fst* with the ion and water balance consequences of cold exposure in *Drosophila* (MacMillan et al., 2015b; Terhzaz et al., 2015), although *FST* and *DH-44* mRNA expression do not appear to be co-regulated (Bing et al., 2012). The *Frost* (FST) protein sequence is rich in polar and charged amino acids, with the motif 'PEEST' occurring multiple times. The lack of non-polar amino acids suggests that FST may be

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**List of symbols and abbreviations**

BSA	bovine serum albumin
Cas9	CRISPR-associated system 9
CCRT	chill coma recovery time
CCRT <sub>80</sub>	time taken for 80% of flies to recover from chill coma
CD	circular dichroism
CDS	coding DNA sequence
cDNA	complementary DNA
chiRNA	chimeric RNA
CRISPR	clustered regularly interspaced short palindromic repeats
C <sub>t</sub>	cycle threshold
<i>Fst</i>	the <i>Drosophila</i> gene <i>Frost</i>
<i>Fst</i> <sup>del</sup>	fly line with a deletion of the <i>Fst</i> locus
<i>Fst</i> <sup>ins</sup>	fly line with an insertion into the <i>Fst</i> locus
<i>FST</i>	messenger RNA transcribed from <i>Frost</i>
FST	protein encoded by <i>Frost</i>
GAL4	yeast transcription activator protein
LDH	lactate dehydrogenase
LEA	late embryogenesis abundant
<i>loxP</i>	locus of X-over P1
Lt <sub>80</sub>	lethal time for 80% of flies at 0°C
Lt <sub>80-2h</sub>	lethal temperature for 80% of flies after a 2 h exposure
MT	Malpighian tubules
pFusA	Golden Gate plasmid cloning vector
QTL	quantitative trait locus/loci
RCH	rapid cold-hardening
RNAi	RNA interference
RT-qPCR	reverse transcriptase quantitative polymerase chain reaction
SCP	supercooling point
UAS	upstream activation sequence

an intrinsically disordered protein (i.e. it has no stable secondary or tertiary structure), similar to the drought- and cold-protective dehydrins and late embryogenesis abundant (LEA) proteins found in plants (Tunnacliffe and Wise, 2007; Hinch and Thalhacker, 2012; Graether and Boddington, 2014). Dehydrins protect enzymes and membranes from freeze or thaw damage *in vitro*, and DNA from radical damage (reviewed in Graether and Boddington, 2014). The potential role(s) of disordered proteins in insect low temperature tolerance have not been confirmed, nor has the structure of FST been explored.

The presence of consistent allelic variation along a thermal cline (Hoffmann et al., 2012), coupled with the high, consistent and conserved upregulation after cold exposure (Reis et al., 2011; Bing et al., 2012), and the fact that *Fst* resides on QTL associated with several different measures of cold tolerance (Morgan and Mackay, 2006; Gerken et al., 2015) implies that there is a significant temperature-related selective pressure on *Fst*. However, in spite of this consistent evidence that *Fst* is associated with cold exposure in *D. melanogaster*, the gene's function remains obscure. Two studies have attempted a functional analysis of *Fst* using an RNAi knockdown of *FST* mRNA. Strikingly, these studies have yielded conflicting results. Colinet et al. (2010) reported that RNAi-mediated *FST* knockdown with the GAL4/UAS system increased *D. melanogaster* chill coma recovery times (CCRT) and mortality following cold stress. In contrast, Udaoka et al. (2013) found no effect of *FST* knockdown on *D. melanogaster* CCRT or survival at low temperatures, using four GAL4/UAS RNAi *Fst* lines. These conflicting results could result from the different genetic backgrounds of the mutant lines – the GAL4 driver lines have different backgrounds, and Colinet et al. (2010) used several different GAL4 driver lines; similarly, the different RNAi constructs have distinct off-targets (Kulkarni et al., 2006). Indeed, the different

UAS constructs used by Udaoka et al. (2013) gave a range of knockdown efficiency, which did not correlate with a variation in cold tolerance. We propose that CRISPR-Cas9 technology is a more appropriate technology to generate complete *Fst* knockout, with minimal off-target effects. Furthermore, the assays used in both of these studies examine the phenotype of flies during and immediately after cold exposure, even though *FST* mRNA abundance increases only after removal from cold (Sinclair et al., 2007). Thus, we propose that a functional analysis of the role of *Fst* should (1) include treatments that induce *FST* expression prior to cold exposure and (2) examine the role of *Fst* in the post-cold-exposure biology of *D. melanogaster*.

Here we report a functional analysis of *Frost* in *D. melanogaster*. First, we show that *FST* expression after cold exposure is most abundant in the Malpighian tubules. Second, we use a recombinant FST protein to demonstrate that recombinant FST is indeed an intrinsically disordered protein, and that its disorder does not change appreciably at low temperatures. Finally, we use CRISPR-Cas9 to create *Fst*-null alleles, and show that *FST* expression has no impact on several measures of cold tolerance or on reproduction under rearing conditions, but is required to maintain female post-cold reproductive output.

**MATERIALS AND METHODS****Rearing conditions and induction of *FST* expression**

Wild-type and mutant *D. melanogaster* were bred and maintained in 35 ml vials containing 10–15 flies at 22°C under 13 h:11 h light:dark and 50% relative humidity. Wild-type Oregon R flies used to localize *FST* mRNA expression were reared on banana-yeast-agar food (Markow and O'Grady, 2005), and CRISPR-modified flies used in cold tolerance and reproductive output assays were reared on a 2:1 water-to-cornmeal diet (10% cornmeal, 1% agar, 6% sugar, 1.5% yeast, 25 mmol l<sup>-1</sup> methyl 4-hydroxybenzoate). For all experiments, newly eclosed flies were sorted under light carbon dioxide anaesthesia and allowed at least 4 days to recover before experiments (Nilson et al., 2006).

To ensure that *D. melanogaster* produced *FST* mRNA, 5- to 7-day-old flies were exposed to an RCH treatment (2 h at 0°C) followed by recovery at 22°C for 3 h (Sinclair et al., 2007; Udaoka et al., 2010). Control (non-RCH) flies were maintained at 22°C for 5 h. During treatments, flies were placed in an empty 35 ml vial. For the cold treatments, these vials were sealed in a zip-lock bag and buried in a slurry of crushed ice and water (0°C).

**Measuring *FST* and *DH-44* expression**

We used reverse transcriptase quantitative PCR (RT-qPCR) to quantify *FST* and *DH-44* transcript abundance from *D. melanogaster* 5 days post-eclosion. To determine where *FST* is normally expressed, we isolated body segments and select tissues from wild-type adult males reared on a banana food diet, following exposure to control or RCH treatments. To confirm success of CRISPR modification (described below), we assayed *FST* abundance in whole flies from the parental strain and four mutant strains (*Fst*<sup>ins-1</sup>, *Fst*<sup>ins-2</sup>, *Fst*<sup>del-1</sup> and *Fst*<sup>del-2</sup>). In addition, because the CRISPR modifications alter an intron of *DH-44*, we determined *DH-44* expression in the same five lines. Details on each of these RT-qPCR experiments are below.

To collect body segments, wild-type flies were snap-frozen in liquid nitrogen immediately after a control or RCH treatment. In a 1.5 ml microcentrifuge tube, we shook 15 to 30 frozen flies vigorously to separate the head, thorax and abdomen. All wings and legs fell off during this process. The small number of thoraces that

were still attached to the abdomen were carefully separated using a razor blade. We pooled 30 heads, 20 thoraces and 10 abdomens for each biological replicate ( $n=7$  per treatment). All samples were stored at  $-80^{\circ}\text{C}$  until RNA extraction.

To collect the Malpighian tubules and whole gut after control or RCH treatment, wild-type flies were exposed to  $\text{CO}_2$  for 10 min and dissected within 15 min in phosphate-buffered saline. In preliminary experiments, we confirmed that light  $\text{CO}_2$  anaesthesia does not affect *FST* expression, regardless of cold treatment. Approximately 100 pairs of Malpighian tubules, 30 guts and three abdomen carcasses were collected for each biological replicate ( $n=4$  per treatment). The gut, Malpighian tubules and the remaining abdomen carcass were stored in RNALater (Ambion, Austin, TX, USA) at  $4^{\circ}\text{C}$  or  $-20^{\circ}\text{C}$  until RNA extraction.

To collect whole flies from the parental strain and CRISPR-induced *Fst* mutants, we transferred male or female flies to 1.5 ml microcentrifuge tubes following exposure to control, RCH or repeated cold treatments, and snap-froze samples in liquid nitrogen. We pooled 15 individuals for each biological replicate ( $n=3$  per treatment, line and sex). All samples were stored at  $-80^{\circ}\text{C}$  until RNA extraction.

We extracted total RNA from samples using TRIzol (Invitrogen, Burlington, ON, Canada), according to the manufacturer's instructions. We synthesized cDNA (qScript Flex cDNA Synthesis kit; Quanta Biosciences, Gaithersburg, MD, USA) from 300 ng of DNase-treated RNA (DNase I Amplification grade; Invitrogen), according to the manufacturer's protocols. We used previously published primers for *Actin-79B* and *Fst* (Sinclair et al., 2007) for localizing *FST* expression. To confirm *FST* and *DH-44* expression in *Fst* mutant lines, we designed custom primers (*Fst* 2 L: 5'-AGTGAATCCAAATGGCAAC-3', *Fst* 2 R: 5'-ATCCTCGG-TGGTCAACTCAG-3', *DH* L: 5'-GACGTGGGAACAGAAGGT-GT-3', *DH* R: 5'-GAAAGCGGTAGGGAAAGGAC-3'), in addition to *Actin-79B* as a housekeeping control (Zhang et al., 2011).

We conducted RT-qPCR using PerfeCTa SYBR Green FastMix (Quanta) on a Bio-Rad CFX96 Real-Time System with a C1000 Thermal Cycler (Bio-Rad, Mississauga, ON, Canada) with the following reaction conditions:  $95^{\circ}\text{C}$  for 3 min, 45 cycles of 10 s at  $95^{\circ}\text{C}$ , 30 s at  $55^{\circ}\text{C}$  and 30 s at  $72^{\circ}\text{C}$ , with melt-curve determination in increments of  $0.5^{\circ}\text{C}$  from  $65^{\circ}\text{C}$  to  $95^{\circ}\text{C}$ . We used the  $\Delta\Delta C_t$  method (Pfaffl, 2001) to calculate relative expression level based on  $C_t$  (cycle threshold) values obtained from the Bio-Rad CFX 96 software, normalizing expression of *FST* and *DH-44* to *ACTIN-79B*. For body segment and tissue samples, we calculated fold-change in *FST* expression relative to *FST* abundance in the head and carcass, respectively, of control flies. Fold-change expression of *FST* and *DH-44* in *Fst* mutant lines were relative to control parental flies. We compared relative expression among treatments, tissues and lines using two-way ANOVAs followed by Tukey's *post hoc* test in R version 3.0.3 (www.r-project.org).

### Cloning, expression, purification and characterization of recombinant FST

*Fst* was made synthetically (Genscript, Piscataway, NJ, USA) and ligated into the pET22B expression vector (Novagen, Gibbstown, NJ, USA) using standard molecular biological methods. The pET22B-*Fst* construct was transformed into *E. coli* BL21(DE3) cells (Novagen), and *Fst* expression was induced with isopropyl  $\beta$ -D-1-thiogalactopyranoside ( $0.4\text{ mmol l}^{-1}$ ) in a culture with an OD600 of  $\sim 0.8$  in Luria-Bertani medium supplemented with  $50\text{ }\mu\text{g ml}^{-1}$  ampicillin. After 3 h incubation, the bacterial cells were centrifuged at  $6000\text{ g}$  for 15 min. Pellets were resuspended in 15 ml

Milli-Q grade water and one protease inhibitor tablet with EDTA (Roche Diagnostics, Indianapolis, IN, USA) was added. Similar to the purification of *Vitis riparia* K<sub>2</sub> dehydrin (Livernois et al., 2009), the suspension was boiled for 20 min to lyse the cells and denature most cellular proteins before being cooled at  $-20^{\circ}\text{C}$  for 10 min. Sodium acetate (pH 5.0) was added to give a final concentration of  $20\text{ mmol l}^{-1}$ , and the pH was adjusted to 5.0 with the dropwise addition of  $0.1\text{ mol l}^{-1}$  HCl. The sample was centrifuged at  $70,000\text{ g}$  for 30 min at  $4^{\circ}\text{C}$  and subsequently passed through a  $0.22\text{ }\mu\text{m}$  syringe filter.

*FST* protein was purified by passing the sample through a 5 ml GE Healthcare HiTrap Q FF column (Baie d'Urfe, QC, Canada); the protein was eluted using a linear gradient from buffer A ( $20\text{ mmol l}^{-1}$  sodium acetate, pH 5.0) to buffer B (buffer A with  $1\text{ mol l}^{-1}$  NaCl) over five column volumes. Fractions containing *FST* were desalted and further purified by using reversed-phase HPLC. Fractions containing pure *FST* were pooled, lyophilized to dryness and stored at  $-20^{\circ}\text{C}$  until further use.

To determine whether recombinant *FST* is disordered, we used circular dichroism (CD) experiments to measure the Stoke's radius of recombinant *FST*. The CD data were collected using a Jasco-815 CD spectropolarimeter (Easton, MD, USA). The protein was dissolved in  $10\text{ mmol l}^{-1}$  sodium phosphate, pH 7.4 at a concentration of  $5.5\text{ }\mu\text{mol l}^{-1}$ . A quartz cuvette with a 2 mm path length (Hellma, Concord, ON, Canada) containing the recombinant *FST* sample was scanned from 250 to 190 nm, with averaging over eight accumulations at  $4^{\circ}\text{C}$  and  $25^{\circ}\text{C}$ . To determine the Stoke's radius of *FST*, a sample of  $200\text{ }\mu\text{g}$  *FST* was injected onto a previously calibrated Superdex G75 gel filtration column. The samples were eluted at a flow rate of  $0.8\text{ ml min}^{-1}$  using a  $50\text{ mmol l}^{-1}$  sodium phosphate, pH 7.4,  $150\text{ mmol l}^{-1}$  NaCl buffer.

We determined whether *FST* can protect enzyme activity during freezing using a lactate dehydrogenase (LDH) cryoprotection assay as performed previously (Hughes and Graether, 2011). The samples were immersed five times in liquid nitrogen for 30 s and thawed by immersion in a circulating water bath at  $4^{\circ}\text{C}$  for 5 min. Enzyme activity was determined on a Cary 100 spectrophotometer (Varian, Mississauga, ON, Canada) by measuring absorbance at 340 nm to follow the disappearance of NADH. Data from the assays are plotted as percent recovery of LDH activity versus additive concentration. The results were fitted to:

$$\% \text{LDH recovery} = \frac{a}{1 + e^{\frac{-(x-x_0)}{b}}} \quad (1)$$

where  $x$  is the additive concentration,  $x_0$  is the percent recovery in the absence of the additive, and  $a$  and  $b$  are fitted variables.

### CRISPR-induced homologous recombination in *Fst*

We generated four *Fst* mutant lines (two independent insertion alleles *Fst*<sup>ins-1</sup> and *Fst*<sup>ins-2</sup>, and two independent deletion alleles *Fst*<sup>del-1</sup> and *Fst*<sup>del-2</sup>) in *y<sup>1</sup> Df(1) w<sup>67c23.2</sup> (yw) D. melanogaster* using CRISPR-Cas9-mediated homologous recombination (Gratz et al., 2013). Each mutant line was produced by independent gene disruptions/homologous recombination events. pU6-BbsI-chiRNA was used to construct two plasmids expressing chiRNA (chimeric RNA) guides that recognize the *Fst* sequences A (GCCTTGGTG-GCAGTGGCTTC) and B (GTAAAACCATTTCTAGGGTT) (Gratz et al., 2013). Two repair templates were constructed with ordered assembly of BsaI restriction fragments into pFusA using Golden Gate cloning (Cermak et al., 2011). One repair template had a *miniwhite* gene flanked with *loxP* sites inserted behind the start



codon and only the methionine coding codon, *Fst<sup>ins</sup>* (insertion mutant alleles), and the second template had the *miniwhite* gene flanked by *loxP* sites replacing the coding DNA sequence (CDS), *Fst<sup>del</sup>* (deletion mutant alleles). Deletion mutant alleles lacked the *Fst* gene, while insertion mutant alleles contained the *Fst* gene, but lacked the ability to transcribe it downstream of the *miniwhite* gene. pFusA and PCR products containing the 5' and 3' homology region and the *miniwhite* gene flanked by *loxP* sites were digested with BsaI and purified from an agarose gel. The four DNA fragments were ligated, and plasmid DNA extracted from spectromycin-resistant transformants was screened for repair templates. The repair templates were injected into *y w* flies (parental) at a concentration of 100 ng ml<sup>-1</sup>. Co-injected were 150 ng ml<sup>-1</sup> of *pHsp70-cas9* and for the insertion allele, 150 ng ml<sup>-1</sup> of the plasmid expressing the chiRNA recognizing sequence A, or for the deletion allele 75 ng ml<sup>-1</sup> of each plasmid expressing the chiRNAs. G1 flies were screened for the *w<sup>+</sup>* phenotype. The structure of the alleles (Fig. 1) in four mutant lines (*Fst<sup>ins-1</sup>*, *Fst<sup>ins-2</sup>*, *Fst<sup>del-1</sup>* and *Fst<sup>del-2</sup>*) was confirmed by PCR (product presence/absence and size of PCR product).

### Cold-tolerance assays

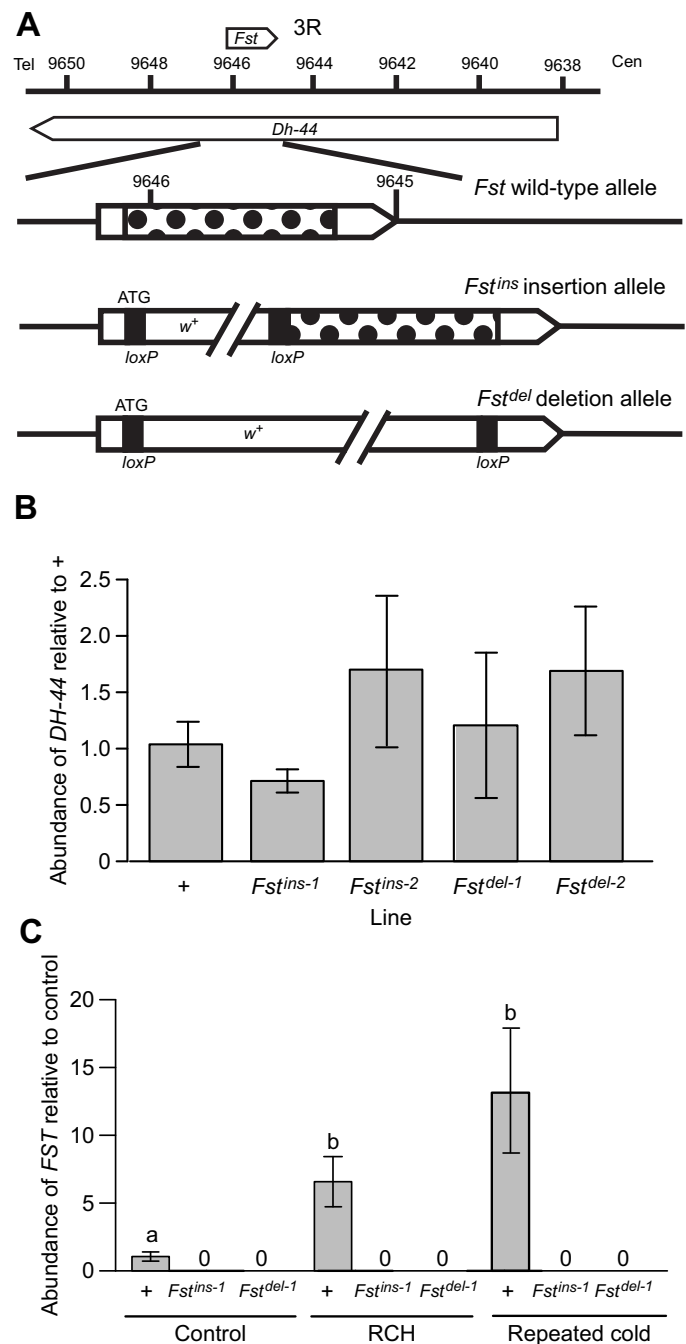
We evaluated the cold tolerance of parental and all four *Fst* mutant lines (*Fst<sup>ins-1</sup>*, *Fst<sup>ins-2</sup>*, *Fst<sup>del-1</sup>* and *Fst<sup>del-2</sup>*) by determining the supercooling point (SCP), survival of acute and chronic cold exposure, and CCRT (Sinclair et al., 2015). We measured acute cold survival and CCRT in all lines, in flies that had never experienced prior cold exposure (control), and in flies that had undergone an RCH treatment. We measured SCP and chronic cold survival in control flies only. We used replicates (groups) of 15 flies per line and sex in each experimental assay described in this section.

We measured the SCP of individual adult flies cooled in an aluminium block cooled at a rate of 0.1°C min<sup>-1</sup> as described elsewhere (Jakobs et al., 2015). We compared SCP among the acclimation regimes and sexes using a two-way ANOVA and Tukey's *post hoc* test in R.

We determined tolerance of acute cold treatments by exposing males and females of each line to subzero temperatures for 2 h. We placed groups of control and pre-treated adult flies of each line and sex into 1.5 ml microcentrifuge tubes, which we placed in a pre-cooled aluminium block whose temperature was controlled by 50% methanol circulated from a programmable refrigerated bath (Lauda Proline 3530, Würzburg, Germany). Exposure temperature ranged from -0.5°C to -5.5°C, encompassing 0–100% mortality. Following the 2 h treatment, we transferred flies to food vials at 22°C, and the proportion of flies that survived (i.e. were standing) for each sample was recorded after 24 h. We determined tolerance of prolonged exposure to 0°C by placing groups of 15 adult flies of each line and sex in food vials in an ice-water slurry for 12 to 48 h, followed by transfer to 22°C. The proportion of flies that survived was recorded after 24 h at 22°C.

We determined CCRT in groups of flies placed at 0°C in an ice-water slurry bath for 12 h (Sinclair et al., 2015). We recorded the time required for recovery (to the nearest minute) of flies of each line and sex in three separate trials. A fly was considered 'recovered' once it was standing. If, after 1 h, a fly was not standing, it was placed in 22°C overnight. If the fly could not stand after 24 h at 22°C, it was considered as not recovered.

For acute cold and chronic cold analysis, we determined the effect of line, sex and treatment on survival using generalized linear models with binomial error distributions and logit link function. Interaction terms that were non-significant were removed to improve



**Fig. 1. CRISPR-mediated homologous recombination knockout lines.**

(A) Schematic depiction of mutant *Fst* alleles. *Fst* is encoded on the antisense strand of *Dh-44* intron 2. Numbers indicate the genome sequence nucleotide positions for the right arm of the third chromosome. The coding DNA sequence (CDS) of *Fst* is indicated by polka dot shading. In the insertion allele, *w<sup>+</sup>* (flanked by *loxP* sites) is inserted immediately downstream of the *Fst* start codon. In the deletion allele, *w<sup>+</sup>* (flanked by *loxP* sites) replaces the *Fst* CDS. 3R, right arm of chromosome 3; Tel, telomere; Cen, centromere; ATG, start of the CDS. (B) *Fst* insertion and deletion mutant alleles do not alter *DH-44* expression. Mean  $\pm$  s.e.m. fold-change in *DH-44* expression is normalized to *Actin-79B*, and relative to control parental line (+). (C) *FST* expression is induced by RCH and repeated cold in the parent strain, but absent in mutant lines. *Fst<sup>ins-2</sup>* and *Fst<sup>del-2</sup>* mutants not shown, but also show no *FST* expression, similar to *Fst<sup>ins-1</sup>* and *Fst<sup>del-1</sup>*. Mean  $\pm$  s.e.m. fold-change in *FST* expression is normalized to *Actin-79B*, and relative to control parental line (+). Different letters indicate significant differences in expression relative to control (untreated) parental strain flies, based on two-way ANOVA followed by Tukey's *post hoc* tests. We used three biological replicates (15 pooled individuals) for data in B and C.

model fit, which was tested with Akaike's information criterion (Venables and Ripley, 2002). For CCRT analysis, we determined the effect of line, sex and treatment (and their interactions) on recovery time with a Cox proportional hazards model with trial number as a random effect using the 'coxme' package in R. We used hazard ratios (odds ratios) to determine which mutant lines differed from the parental strain. We calculated the lethal temperature for 80% of flies after 2 h ( $LT_{80-2h}$ ) from the acute cold model, the lethal time for 80% of flies at 0°C ( $Lt_{80}$ ) from the chronic cold model, and the time at which 80% of flies have recovered from chill coma ( $CCRT_{80}$ ) from the CCRT model, commonly measured parameters in insect cold tolerance literature (Sinclair et al., 2015).

### Reproductive output post-cold exposure

To determine the effects of cold exposure on both fertility and sterility, we exposed flies from the parental line and three *Fst* mutant lines (*Fst<sup>ins-1</sup>*, *Fst<sup>ins-2</sup>* and *Fst<sup>del-1</sup>*) to −2°C for 2 h (acute cold), 0°C for 8 h (chronic cold) or 2 h at 0°C every day for 5 days (repeated cold). We excluded *Fst<sup>del-2</sup>* mutants because they had poor breeding capacity under normal rearing conditions. None of the flies were pre-treated with RCH, but we expect all of these cold exposures to induce *Fst* expression in wild-type flies (Zhang et al., 2011).

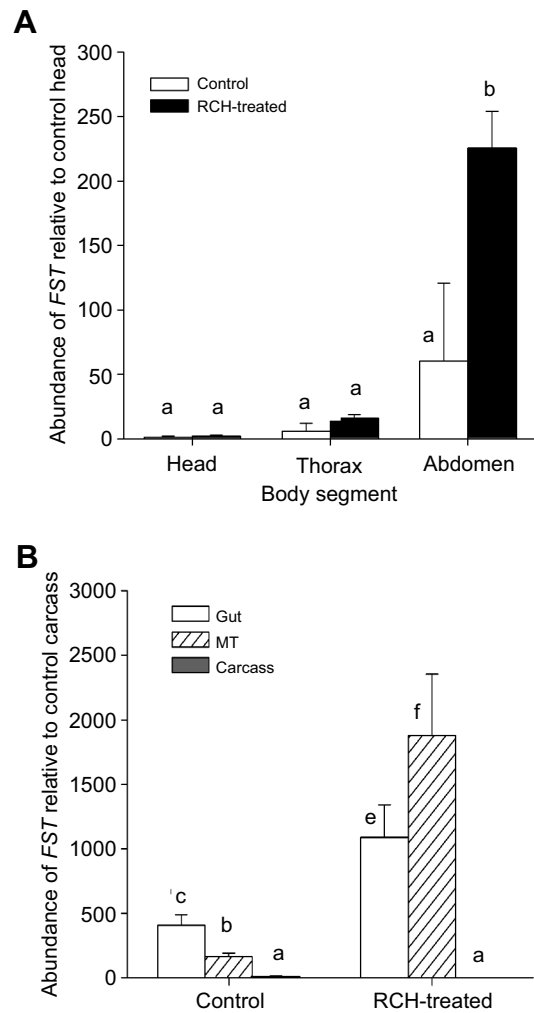
We determined the impact of cold exposure on immediate female reproductive output after methods described previously (Dillon et al., 2007; Marshall and Sinclair, 2010). Briefly, after cold exposure, we transferred 10 virgin females from each treatment to individual food vials at 22°C, along with two control (not cold-exposed) virgin males of the same line. The flies were removed from the vials after 2 days, and if, upon removal, the female could not stand, she was recorded as dead. The vials were monitored daily for emergence of offspring, which were removed, counted and sexed each day until eclosion ceased. Sex ratio was calculated as the proportion of females out of total offspring per vial.

To determine whether *Fst* knockout affected male fertility immediately after cold exposure, we transferred 10 virgin males from each treatment to individual food vials at 22°C, along with two control (not cold-exposed) virgin females of the same line. The flies were removed from the vials after 2 days, and if, upon removal, the male fly had not stood up, he was recorded as dead. We recorded the presence or absence of offspring for 12 days. A male was considered fertile if the vial contained offspring, and sterile if no offspring eclosed during that time period. We compared reproductive output (total number of offspring from females, offspring sex ratio, proportion of fertile males) among treatments by separate generalized linear models with line and treatment as factors in R (Marshall and Sinclair, 2010).

## RESULTS

### *Frost* is expressed in the Malpighian tubules following cold exposure

Cold exposure of wild-type flies significantly increased the abundance of *FST* mRNA in the thorax and abdomen compared with control (not cold-exposed) wild-type flies, with the highest basal and induced *FST* expression levels in the abdomen (treatment:  $F_{1,41}=14.54$ ,  $P<0.001$ , body segment:  $F_{2,41}=35.10$ ,  $P<0.001$ , treatment×body segment:  $F_{2,41}=11.97$ ,  $P<0.001$ ; Fig. 2A). A Tukey's *post hoc* test indicated that upregulation in response to cold exposure was only statistically significant in the abdomen (Fig. 2A), so the components of this tissue were explored further. In the abdomen, *FST* expression level was higher in the gut and Malpighian tubules than in the remaining tissue in the abdomen carcass (treatment:  $F_{1,23}=14.51$ ,  $P=0.001$ , tissues:  $F_{2,23}=8.43$ ,

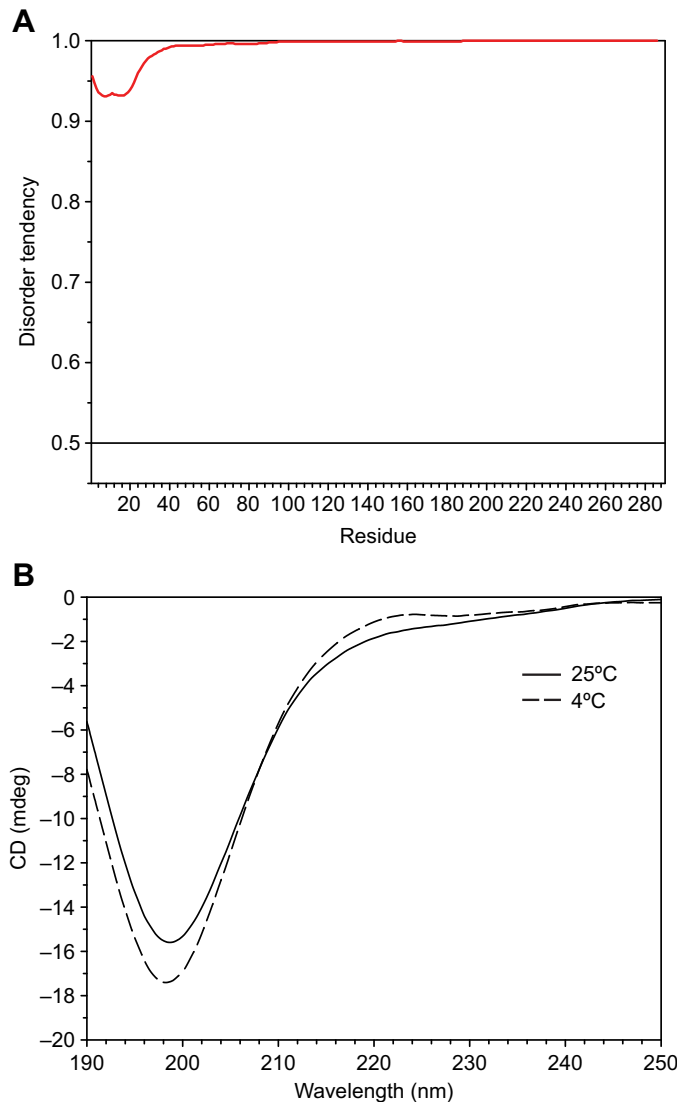


**Fig. 2. Cold-induced upregulation of *Frost* mRNA compared among body segments and tissues in adult male *Drosophila melanogaster*.** Flies were either exposed to a RCH treatment (2 h at 0°C followed by 3 h recovery at 22°C) or were untreated (controls, 5 h at 22°C), and *FST* mRNA abundance was normalized to *Actin79B*. Mean±s.e.m. abundance of *FST* is relative to control head (A) or abdomen carcasses (B). Different letters indicate a significant difference within the same treatment, and between control and cold-treated samples within the same body part/tissue (Tukey's *post hoc* test following two-way ANOVA,  $P<0.05$ ). We used seven biological replicates (30 heads, 20 thoraces or 10 abdomens pooled) for data in A, and four biological replicates (100 pairs of Malpighian tubules, 30 guts and three abdomen carcasses pooled) for data in B. MT, Malpighian tubules.

$P=0.003$ , treatment×tissue:  $F_{2,23}=5.71$ ,  $P=0.012$ ; Fig. 2B). Basal levels of expression in the gut were higher than other tissues, and a statistically significant increase in *FST* expression induced by cold stress was observed in the Malpighian tubules and gut (Fig. 2B).

### *FST* is an intrinsically disordered protein

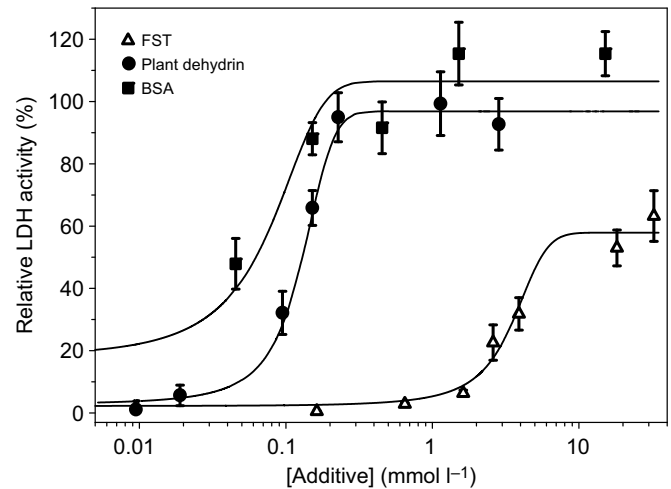
The *FST* protein sequence is enriched in polar and acidic amino acids, especially in Pro, Gly, Glu, Ser and Thr, and lacks hydrophobic amino acids. Based on the MFDp2 structural prediction server (Mizianty et al., 2013), *FST* has a high (>0.5) disorder tendency (Fig. 3A). The N-terminal region, while still highly disordered, is less disordered than the rest of the protein, and appears to correspond to the export signal peptide (residues 1–18) predicted by SignalP (data not shown) (Petersen et al., 2011). Gel



**Fig. 3. Frost is an intrinsically disordered protein.** (A) Disorder prediction by MFDp2. The complete sequence of FST, including the putative export signal, was submitted to the MFDp2 server for analysis. The disorder tendency is plotted on a per residue basis, and is shown as a red line. The solid black line represents the threshold above which a residue is considered to be disordered. (B) Circular dichroic (CD) spectra of FST protein. The structure of FST was examined at 25°C (solid line) and 4°C (dashed line).

filtration data showed that FST has a Stoke's radius of 56 Å, which is consistent with other disordered proteins of this length (Uversky, 2002). The CD spectra of FST at 25°C and 4°C showed a strong negative peak near 200 nm, coupled with the lack of strong negative peaks at 210 and 222 nm (Fig. 3B), providing further evidence for a disordered structure. Interestingly, the slight decrease (more negative) signal near 200 nm at 4°C compared with 25°C indicated that the protein is more disordered at the lower temperature (Fig. 3B).

We examined whether FST, like dehydrin, another disordered stress protein (Hughes et al., 2013), could protect LDH from damage caused by freezing and thawing the enzyme (Fig. 4). While BSA and the plant dehydrin provided effective protection of LDH, the FST protein comparatively was rather weak with no full recovery of enzyme activity (Fig. 4), suggesting that FST is not involved in protecting enzymes from cold stress.



**Fig. 4. Frost does not protect enzymes from freezing stress.** The recovery of lactate dehydrogenase (LDH) activity was measured after freeze-thaw treatment in the presence of additive. FST, open triangles; plant cold stress dehydrin, closed circles; bovine serum albumin (BSA), closed squares. The error bars represent the s.d. of three to five replicates while the lines were fits of the data to Eqn 1 (see Materials and methods for details).

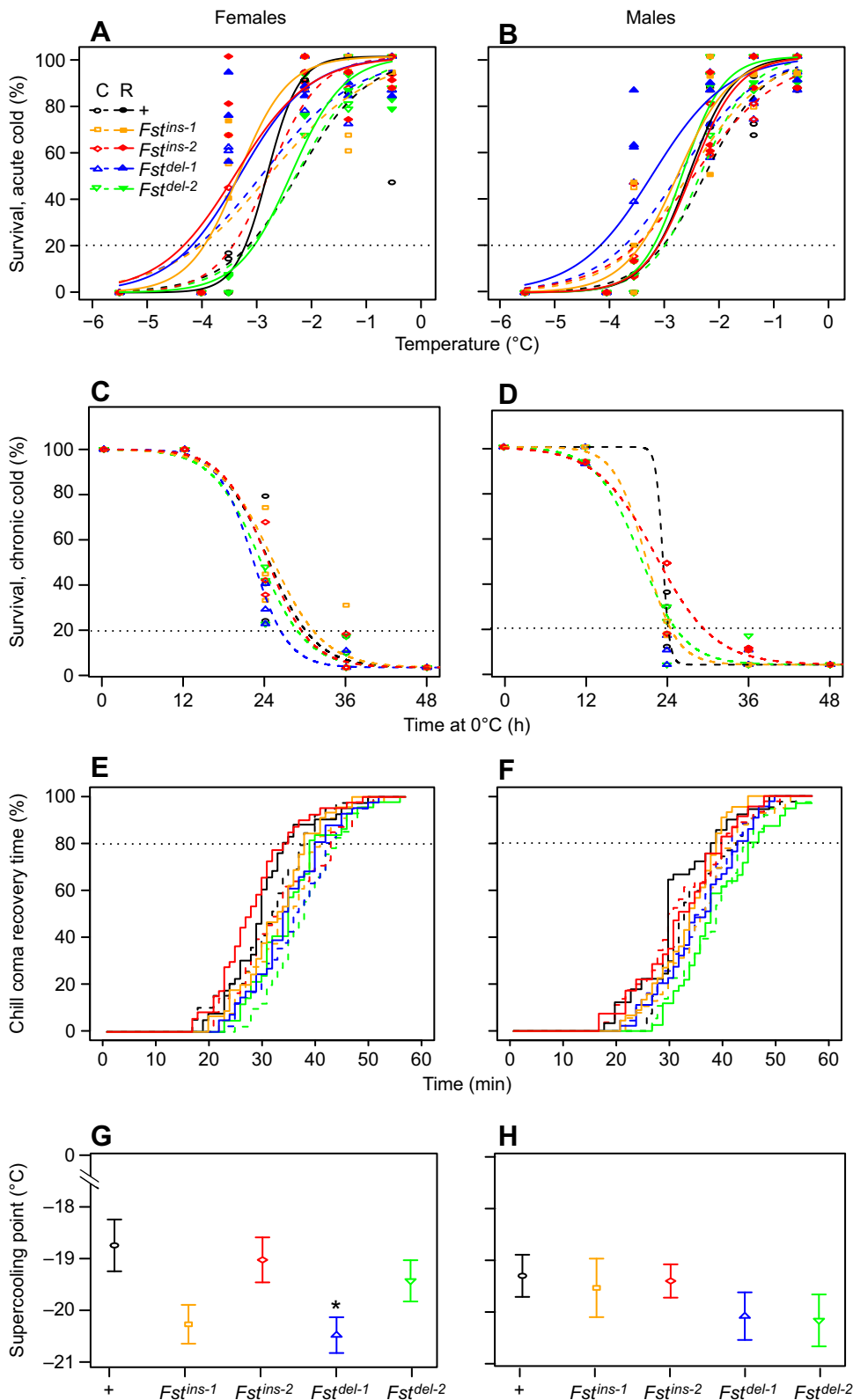
#### CRISPR-mediated homologous recombination in the *Fst* locus prevents *FST* expression

The *Fst* locus is in the second intron of *Dh-44*, and the *FST* transcript is not spliced (Fig. 1). We designed two repair templates to (1) insert the *miniwhite* gene flanked by *loxP* sites behind the start codon of *Fst*, creating an insertion in the *Fst* locus, and (2) delete the CDS of *Fst* upon inserting the *miniwhite* gene flanked by *loxP* sites creating a null deletion in the *Fst* locus. Of the 25 fertile G0 survivors injected with the insertion repair template, four produced *w*<sup>+</sup> progeny, and of greater than 200 fertile G0 survivors injected with the deletion repair template, three produced *w*<sup>+</sup> progeny. The structure of the insertion and deletion *Fst* alleles is shown in Fig. 1A. Insertion and deletion of *Fst* did not alter *Dh-44* expression (line:  $F_{4,18}=1.114$ ,  $P=0.380$ , sex:  $F_{1,18}=2.989$ ,  $P=0.101$ , line×sex:  $F_{4,18}=0.996$ ,  $P=0.435$ ; Fig. 1B). While RCH and repeated cold exposure treatments induced *FST* expression in the parental strain (treatment:  $F_{2,10}=4.550$ ,  $P=0.034$ , sex:  $F_{1,10}=0.768$ ,  $P=0.398$ , treatment×sex:  $F_{2,10}=0.126$ ,  $P=0.883$ ), we did not observe *FST* amplification when performing RT-qPCR from the CRISPR mutant lines (Fig. 1C).

#### *Fst* is not necessary for cold tolerance

CRISPR-mediated homologous recombination in the *Fst* locus did not substantially alter any of our metrics of cold tolerance (summarized in Table S1). There was no significant difference between males and females in acute or chronic cold tolerance (Fig. 5, Table 1). Likewise, a pre-exposure to cold intended to initiate the RCH response did not change acute cold tolerance (Fig. 5, Table 1). Finally, there was no difference in acute or chronic cold tolerance between the parental line and CRISPR-induced mutant lines (Fig. 5, Table 1).

CCRT was marginally affected by sex (Fig. 5, Table 2). Based on the hazard ratio (HR=0.583,  $P=0.021$ ), males recovered more slowly than females, taking an average of 1.7 min longer to reach the CCRT<sub>80</sub> (Tables S1, S2). There was a significant effect of mutant line on CCRT (Fig. 5, Table 2): *Fst*<sup>del-1</sup> (HR=0.527,  $P=0.004$ ) and *Fst*<sup>del-2</sup> (HR=0.453,  $P=0.001$ ) required an average of 4.6 min more than the parental strain to reach the CCRT<sub>80</sub>; however, CCRT of neither of the insertion lines differed from the parental



strain (Tables S1, S2). Despite the main effect of treatment on CCRT in our model (Fig. 5, Table 2), RCH treatment did not change CCRT<sub>80</sub> (HR=1.094, *P*=0.690; Tables S1, S2).

Mean SCP ranged from  $-19.0 \pm 0.3^\circ\text{C}$  in the parental line to  $-20.0 \pm 0.3^\circ\text{C}$  in *Fst<sup>del-1</sup>* (Fig. 5G,H), and although we detected

significant differences among lines ( $F_{4,84}=2.849$ , *P*=0.029), mortality occurred well above the SCP (Fig. 5), likely rendering biologically irrelevant the  $1^\circ\text{C}$  difference we observed. We found no significant difference in SCP between males and females ( $F_{1,84}=0.062$ , *P*=0.804).



**Table 1. Analysis of deviance tables for generalized linear models of survival following acute or chronic cold exposures**

	d.f.	Deviance	Residual d.f.	Residual deviance	P
Acute cold model					
Temperature	1	223.675	298	66.490	<b>&lt;0.001</b>
Sex	1	1.341	297	65.149	0.247
Treatment	4	4.546	293	60.603	0.337
Line	1	2.723	292	57.881	0.099
Chronic cold model					
Time	1	252.458	267	12.905	<b>&lt;0.001</b>
Sex	1	2.057	266	10.848	0.152
Line	4	0.952	262	9.896	0.917

The effect of temperature (acute cold) or time (chronic cold), sex, pre-treatment (control or RCH) and line on survival of *Drosophila melanogaster* exposed to acute or chronic cold. We determined the proportion of survivors in three replicates (per treatment combination) of 15 flies 24 h post-cold exposure. Bold *P*-value indicates a significant effect of the model term on survival.

***Fst*-null mutants have reduced reproductive output following cold exposure**

We were able to identify a clear post-cold exposure phenotype for *Fst* mutants. Offspring production did not differ among lines under rearing (control) conditions (Fig. 6). The number of offspring produced by females from the parental line did not decrease in the 2 days after acute cold (−2°C for 2 h), chronic cold (0°C for 8 h) or repeated cold (0°C for 2 h every day for 5 days) exposures (Fig. 6A, Table 3). By contrast, all of the females carrying CRISPR-induced mutations had reduced offspring production after any of the cold exposures (Fig. 6A, Table 3). There was no effect of cold exposure or mutations at the *Fst* locus on the sex ratio of offspring from cold-exposed females, or the proportion of cold-exposed males that sired offspring (Fig. 6B,C, Table 3). There was a slightly higher proportion of female offspring from *Fst<sup>ins-1</sup>* mutants exposed to repeated cold relative to flies from the same line that were not exposed to cold, or were exposed to acute or chronic cold treatments (Fig. 6B, Table 3).

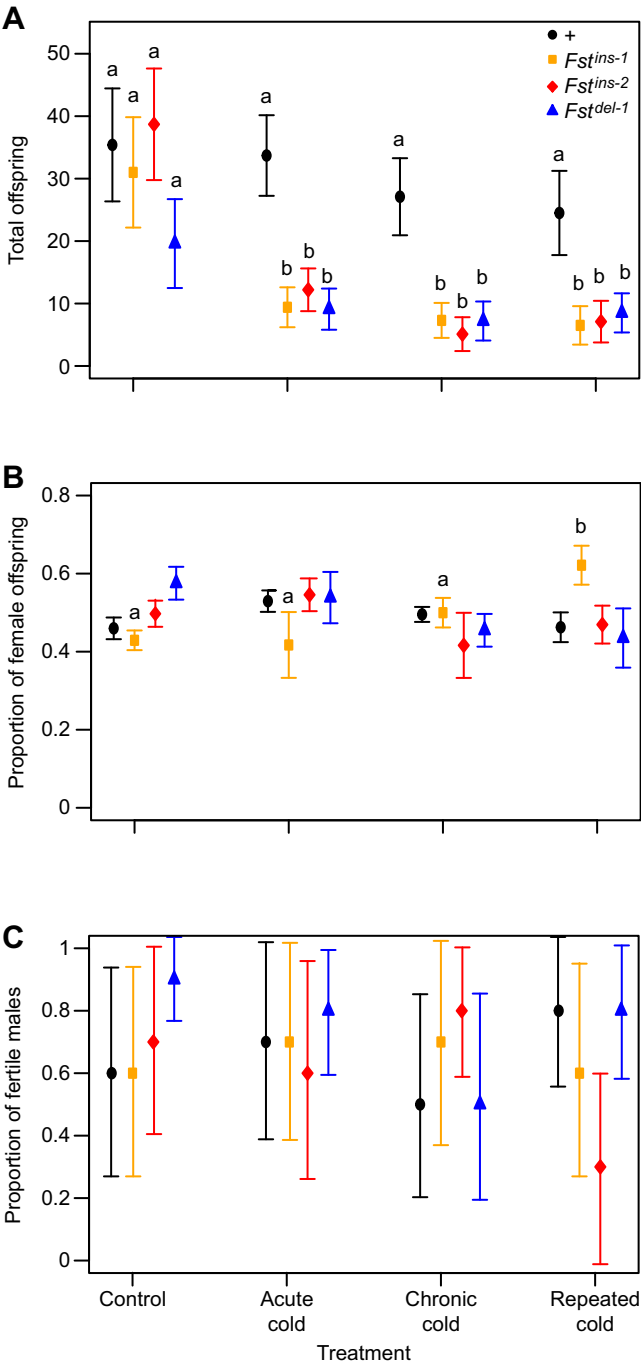
**DISCUSSION**

Understanding how insects survive and recover from cold exposure, and how they maintain reproduction after cold stress, is important for uncovering the mechanisms of evolution and adaptation to seasonal climates. The *Fst* gene shows a large and reliable change in expression in response to cold exposure in *D. melanogaster* and other species in the genus (Goto, 2001; Sinclair et al., 2007; Bing

**Table 2. Cox proportional hazards model of chill coma recovery time (CCRT)**

CCRT model	Wald $\chi^2$	P
Treatment	33.82	<b>&lt;0.001</b>
Line	35.18	<b>&lt;0.001</b>
Sex	16.12	<b>&lt;0.001</b>
Treatment×Line	2.26	0.688
Treatment×Sex	5.33	<b>0.021</b>
Line×Sex	6.14	0.189
Treatment×Line×Sex	3.25	0.517

The effect of pre-treatment (control or RCH), line, and sex on time for adult *D. melanogaster* to recover from cold exposure, based on a Cox proportional hazards model with mixed effects. We determined the CCRT in three replicates (per treatment combination) of 15 flies. Bold *P*-value indicates a significant effect of the model term on CCRT.



**Fig. 6. Cold exposure reduces fecundity of *Fst* mutants.** (A) Total number of offspring from females was lower for all three mutant lines relative to the parental line following acute, chronic and repeated cold exposures. Each point represents the mean (±s.e.m.) offspring number from 10 females over a 2-day period post-treatment. (B) Sex ratio of offspring from females was not affected by cold treatment or mutant line. Each point represents the mean (±s.e.m.) proportion of offspring that were female from three to seven females. (C) Male reproductive output is not affected by cold treatment or mutant line. Each point represents the proportion (±95% confidence intervals) of males (*n*=10) that sired offspring in the 2 days after each treatment. Details of statistical results are in Table 3. Different letters indicate a significant difference between treatments within a line (*P*<0.05), determined by non-overlapping 95% confidence intervals.



**Table 3. Analysis of deviance/variance tables for generalized linear models of total offspring, offspring sex ratio and male fertility**

Total offspring model	d.f.	Deviance	Residual d.f.	Residual deviance	P
Treatment	3	527.21	156	3706.6	<b>&lt;0.001</b>
Line	3	454.44	153	3252.2	<b>&lt;0.001</b>
Treatment×Line	9	240.92	144	3011.3	<b>&lt;0.001</b>
Offspring sex ratio model	d.f.	SS	MS	F	P
Treatment	3	0.006	0.002	0.174	0.914
Line	3	0.014	0.005	0.418	0.741
Treatment×Line	9	0.226	0.025	2.272	<b>0.027</b>
Male fertility model	d.f.	Deviance	Residual d.f.	Residual deviance	P
Treatment	3	3.059	12	8.997	0.383
Line	3	3.452	9	5.545	0.327
Treatment×Line	9	5.545	0	0	0.784

The effect of treatment (acute, chronic, or repeated cold) and line on female fertility (total offspring and offspring sex ratio) and male fertility of adult *D. melanogaster*. Bold *P*-values indicate a significant effect of the model term on total offspring produced by females, sex ratio (proportion of females) in offspring of females, or proportion of fertile males following treatment.

et al., 2012), but the function of *Fst* has remained unclear (Colinet et al., 2010). Here we use CRISPR-Cas9 gene editing to entirely remove the *Fst* gene. We show that preventing *FST* expression does not affect cold tolerance phenotypes, such as acute and chronic cold tolerance and recovery from chill coma, but rather females lacking *Fst* produce significantly fewer offspring in the 2 days following cold exposure. Therefore, we infer that the primary function of *Fst* is the preservation of post-cold-exposure reproductive capacity in female flies. The preservation of post-cold-exposure reproductive capacity is clearly a trait under selective pressure, and would explain the observation of *Fst* allele variation along a thermal cline (Hoffmann et al., 2012) and its presence on QTL associated with cold tolerance (Morgan and Mackay, 2006; Gerken et al., 2016).

**Frost is not necessary for cold tolerance**

To comprehensively examine the role of *Fst* in cold tolerance, we assayed survival of and recovery from cold in (1) flies that had not been previously cold-exposed (cf. Colinet et al., 2010; Udaka et al., 2013) and (2) flies that had undergone a brief RCH pre-treatment (Udaka et al., 2010) prior to our assays. We expected low levels of *FST* protein in the former group (regardless of RNAi or gene editing), because *Fst* expression increases after, but not during, cold exposure (Sinclair et al., 2007; Udaka et al., 2010). By contrast, our RCH pre-treatment ensured *FST* (and presumably *FST*) accumulation prior to cold tolerance assays, allowing us to evaluate the direct role of *FST* and *FST* expression on responses to a subsequent cold stress. However, there was no variation in acute and chronic cold tolerance between parental and *Fst* mutant lines, regardless of *FST* expression. While two mutant lines had statistically slower recovery from chill coma, the two insertion mutant lines (neither of which expressed *FST*) did not differ from the parental strain. The <5 min increase in CCRT<sub>80</sub> in the two deletion lines is much less than that reported previously (Colinet et al., 2010), and – similar to the conclusions of Udaka et al. (2013) – the inconsistency between lines that do not express *FST* suggests that the effect is not *FST* dependent. Unlike other intrinsically disordered cold-stress proteins (e.g. Hughes et al., 2013), recombinant *FST* did not prevent the loss of LDH activity during freeze–thaw cycles, suggesting the protein has no role in protection of enzymes from freezing damage. We were unable to assess whether *FST* can protect against cold-induced (rather than freezing-induced) protein damage because of the lack of an established cold-denaturation enzyme assay, so we cannot rule out the possibility that *FST* may function at more moderate low temperatures (e.g. 0°C).

However, it is possible that the osmotic effect of freezing on proteins is analogous to the large osmotic shifts that cold exposure engenders in *Drosophila* hemolymph (MacMillan et al., 2015a), although this assay is not normally interpreted in this way. Nevertheless, our whole-animal cold tolerance data suggest that *Fst* and its protein product do not contribute to low temperature survival or recovery rate in *D. melanogaster*.

Previous RNAi studies (Colinet et al., 2010; Udaka et al., 2013) have yielded conflicting conclusions about whether *Fst* is associated with cold tolerance or chill coma in *D. melanogaster*. Here, we use homologous recombination to generate null alleles and definitively show that the gene is not directly required for cold tolerance in this species. We generated multiple independent alleles for both an insertion of *w*<sup>+</sup> directly downstream of the only *Fst* methionine codon (disrupting the gene) and a complete deletion of the *Fst* coding sequence. We could not detect *FST* mRNA in these lines (contrast this with the variable effects of RNAi; Udaka et al., 2013), and all lines behaved similarly in response to cold exposure in our assays. Cold tolerance is variable and dependent on genetic background (Mackay et al., 2012), which is difficult to control for in RNAi experiments where the genetic backgrounds of the driver lines may vary considerably. The advantage of the CRISPR approach is that the parental line used to generate the *Fst*-null alleles is the control, minimizing the effects of genetic background that likely influenced previous RNAi functional studies of *Fst* (Colinet et al., 2010; Udaka et al., 2013) and led to the conflicting conclusions of previous reports.

Because *Fst* is encoded in an intron of *Dh-44*, a gene that may have a role in water balance and therefore cold tolerance (Terhzaz et al., 2015; Overgaard and Macmillan, 2017), we measured *Dh-44* expression patterns in our mutant lines. *Fst* and *Dh-44* expression are not correlated, and we show that the expression of *Dh-44* is unaffected by *Fst* deletion or by insertion of *w*<sup>+</sup> in the *Fst* locus. The lack of altered *Dh-44* expression and the lack of coordinated regulation suggest that *Dh-44* is unlikely to be involved in preservation of post-cold-exposure reproductive capacity. Thus, we are confident that the effect of CRISPR mutation on post-cold-exposure female reproductive capacity is due to the loss of *FST* expression rather than any additional background effects on expression of other physiologically relevant genes.

**What is the function of Frost?**

*Fst* expression is predominantly localized to the Malpighian tubules, which comprise the secretory half of the insect

ionoregulatory system (Dow and Davies, 2006), and are therefore likely important in recovery from cold exposure (Terhzaz et al., 2015). However, in addition to their role in ion homeostasis, Malpighian tubules produce and export many proteins (Marshall, 1973; Dow and Davies, 2006). Protein sequence analysis supports that FST is a secreted protein – it has an N-terminal signal sequence that suggests the protein is exported from Malpighian tubule cells (Goto, 2001) – although we cannot distinguish whether FST is secreted into the gut lumen or hemolymph. We note that this suggests that the action of FST will be fundamentally different from that of the well-characterized dehydrins, which are intracellular (Graether and Boddington, 2014). Although it is possible that FST has a specific protective role in Malpighian tubules, the fact that it is likely secreted, coupled with our observation that the chill coma recovery time of *Fst*-null flies does not differ from their *Fst*-bearing counterparts, suggests that FST has a role elsewhere in the fly. We have not been successful in developing an antibody that detects FST from *Drosophila* extracts, but CRISPR-Cas9 technology could be used to create a modified FST protein that is easily detected through immunoblots or fluorescence to better resolve its distribution in the body.

The expression profile (*Fst* mRNA is abundant only after the fly has been rewarmed), the phenotype of *Fst*-null flies (no effect on acute or chronic cold tolerance), and the failure of recombinant FST to protect LDH in a freeze–thaw assay all suggest that FST is not a directly cryoprotective protein. We do note that the possibility exists that FST could still act as a protective protein. Firstly, the protein could require post-translational modification, such as glycosylation, before it is effective. Secondly, we cannot exclude the possibility that FST has evolved to protect specific proteins and/or membranes, or act as a chelator during cold stress.

We propose that FST protects offspring production following cold exposure through protection of the ovaries from post-cold damage, or via accelerated repair of cold-induced damage. Although we do not have direct evidence for interaction between FST and the ovaries, we can propose two mechanisms by which this might occur. (1) FST protein is exported from Malpighian tubules, travels through the hemolymph to reproductive tissues, where it directly interacts with and protects reproductive tissues or repairs them following cold. (2) FST protein is involved in some other aspect of recovering from cold exposure (e.g. re-establishing ion homeostasis), such that *Fst*-null flies must trade-off energy allocation to the ovaries with the energy demands of repair and recovery elsewhere in the fly. FST is an intrinsically disordered stress-response protein, a group of proteins that fulfill a diverse range of functions, including protecting membranes from cold stress (Clarke et al., 2015) and binding ions (Hara et al., 2005). Therefore, FST could affect recovery from cold exposure in a variety of ways, including direct or indirect impacts on the ovaries; the fertility and number of eggs laid post-cold; longevity of cold-exposed flies; and performance of the offspring.

### Conclusions and future directions

This is the first study that conclusively demonstrates an indirect role of *Fst* in cold tolerance. While FST does not appear to protect *D. melanogaster* during cold exposure, it is important in preserving fecundity following cold stress. The mechanisms by which this disordered protein impacts reproductive output are unknown, but our identification of a clear phenotype for *Fst* justifies further analysis of the structure and function of the FST protein. Given the variation in *Fst* alleles along thermal clines (Hoffmann et al., 2012), natural *Fst* variants may differ in post-cold-exposure reproductive capacity. An analysis of the natural genetic variants associated with

the thermal cline could be performed to determine whether specific alleles have distinct properties or protein expression levels that could be under selective pressure. This analysis could identify regions of the FST protein that are important for its function in preserving reproductive capacity. Thus, having resolved the post-cold protective function of *Fst*, we can begin to work on the more difficult task of understanding how the protein protects reproduction and unravelling the gene's role in adaptation to shifting thermal environments.

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### Competing interests

The authors declare no competing or financial interests.

### Author contributions

This work was conceived by B.J.S. and A.P.-S. H.U., S.A. and B.J.S. designed and conducted the expression localisation experiments, D.M.M. and S.P.G. worked on the recombinant expression and characterization of Frost as a disordered protein. A.P.-S. created the *Fst* alleles. C.E.N., J.T., B.J.S. and A.P.-S. designed and conducted the phenotyping assays. C.E.N., J.T., B.J.S. and A.P.-S. wrote the first draft of the manuscript, to which all authors contributed.

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### Data availability

Data for this paper are available in an Excel spreadsheet (Table S3).

### Supplementary information

Supplementary information available online at <http://jeb.biologists.org/lookup/doi/10.1242/jeb.160176.supplemental>

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