Cold stress upregulates the expression of heat shock and *Frost* genes, but the evolution of cold stress resistance is apparently not mediated through either heat shock or *Frost* genes in the cold stress selected populations of *Drosophila melanogaster*

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Abstract:

The ability to thermal adaptation can alter insects' reproductive traits and physiologic response. A number of studies in *Drosophila* documented the higher expression levels of *heat shock proteins* (*Hsps*) and *Frost* (*Fst*) genes during the recovery phase of cold shock treatment and suggested that higher expression levels of these genes and lipids can protect from cold stress. However, these genes' expression and cold adaptation have not been well studied. Therefore, understanding the molecular and biochemical basis of cold adaptation, we examined the levels of lipid and expression patterns of *Hsp22, Hsp23, Hsp40, Hsp68, Hsp70, Hsp 83,* and *Fst* genes under the cold stress or benign condition in the populations of *Drosophila melanogaster* selected for increased resistance to cold shock.

We observed the significant up-regulation of *Hsp22, Hsp23, Hsp40, Hsp68,* and *Fst* genes in the FSB and FCB populations during the recovery phase post cold shock compared to the no shock condition. However, there was not a significant change in the transcript levels of these genes between FSB and FCB under both the cold stress or no shock condition. Additionally, we noticed higher total lipid levels in the females from cold shock treatment than those from no shock treatment. This finding suggests that cold selected populations have different mechanisms to sustain cold stress.

Keywords: Heat shock proteins, reproductive traits, cold shock resistance, lipid

INTRODUCTION

Insects are present in diverse habitats ranging from hot springs to cold deserts. Insects are ectothermic organisms and, therefore, cannot regulate internal temperature when temperature

47 changes in the external environment. Therefore, insects have evolved a number of mechanisms to
48 protect themselves when subjected to external environmental fluctuations. Thermal stress (hot or
49 cold) is known to affect multiple traits, including fecundity, egg viability, mating ability, and pre-
50 and post-copulatory traits, and it also reportedly affects the physiology of the insect (reviewed by
51 [1,2,3,4,5]. Multiple studies suggested that thermal stress causes damage to various molecules like
52 proteins and lipids and damage to cell membranes, protein transport machinery [6,7,8,9]. Insects
53 are known to have evolved a range of mechanisms to mitigate and prevent the damage caused due
54 to cold or heat stress. Change in lipid composition imparting more fluidity to cell membranes is
55 one of the significant changes that is often employed [10].
56
57 Multiple proteins involved in repairing protein damage, including misfolding, aggregation, and
58 denaturation are, known as heat shock proteins, play a crucial role. Heat shock proteins (Hsps)
59 mitigate the effects of heat stress by protecting proteins and enzymes and facilitating their proper
60 functioning under stressful conditions [11,12]. Also, an increase in the synthesis of several other
61 metabolites like glycerol [13], glycogen, and cryoprotectants like trehalose proline. These
62 metabolites help insects to survive under thermal stress [14]. Various insects like flesh fly [15],
63 stick insects [16], field crickets [17], and corn borer [18], amongst others, have been used to study
64 the mechanisms that are involved in coping with environmental stress. These insects employ a
65 number of mechanisms that could be distinct yet overlapping. *D. melanogaster* has been a favorite
66 model to address questions pertaining to underlying mechanisms of cold tolerance. Results from
67 these studies suggest that *Drosophila* also employs mechanisms like lipid modification, increase
68 in expression of heat shock proteins, synthesis of other metabolites like proline, glycogen, etc. to
69 survive under thermal stress [13,7,19,20].

However, the expression pattern of *Hsps* and *Fst* genes has not been well studied in the evolved population against cold stress to understand the molecular basis of cold adaptation. To assess the molecular and biochemical mechanism of evolved cold stress resistance, we used cold shock selected populations of *Drosophila melanogaster* (*D. melanogaster*) that has evolved for a number of traits, including better survival, increased egg viability, more mating frequency, higher male mating ability, and ability to sire more progeny, we measured the total lipid content and transcripts levels of *Hsp22, Hsp23, Hsp40, Hsp68, Hsp70Aa* (*Hsp70*), *Hsp83*, and *Fst* genes under the cold stress or no shock condition.

MATERIAL AND METHODS

Maintenance and derivation details of the experimental populations (FSB and FCB) are described previously [1,2,3,4,5] Experimental flies were generated from standardized flies, as explained previously in [1,2]. Briefly, flies from both FSB and FCB regimes were not subjected to any selection for one generation. Eggs were collected from these standardized flies at a controlled density of 70 eggs per vial. Eighteen vials were established for each of the FSB and FCB populations. Flies that emerged from these vials were used for the experiments. Gene expression was measured only in males, while both males and females were used for lipid estimation.

Fly rearing for lipid estimation

To understand the fractional levels of lipid levels, assay was performed after 33 generations of selection for increased resistant cold shock. On the 12th-day post egg collection, vials were randomly divided into two treatments – (a) cold shock and (b) no shock treatment. For both cold

93 shock and no shock treatment, flies were transferred into an empty glass vial, and the cotton plug
94 was pushed deep into the bottom, leaving one-third of the vial space for flies. After that, flies were
95 subjected to either of the treatments following the protocol described in the previous report [1,2,5].
96 For each treatment, gender, and population, four sets of vials with each containing a density of 50
97 males or 50 females have been subjected to cold shock or no shock treatment. After the treatment,
98 flies were immediately transferred to a Plexiglas cage containing a fresh food plate. Twenty-four
99 hours post cold shock treatment, a fresh food plate was given to the flies for one hour to lay the
100 stored eggs, and followed by this, and another fresh food plate was provided for four hours. These
101 plates with eggs were incubated for 18 hours to hatch eggs, and later from this plate, 30 first instar
102 larvae were collected with a wet paintbrush and cultured into vials with 6 mL of food. For a given
103 population and treatment, ten such vials were set up. These vials were carefully monitored for
104 eclosion. Flies were collected within two hours of eclosion and were immediately flash-frozen
105 using liquid nitrogen. Fifty males and 50 females per treatment and per population were frozen.
106 These flies were stored at -80°C until assayed for lipid content.
107

108 **Lipid estimation**

109 This assay was performed to assess the lipid levels in FSB and FCB flies, after 50 generations of
110 selection for increased resistance to cold shock. Lipid estimation was performed following the
111 protocol described in [21] with minor modifications. Ten sets were made for each gender and
112 treatment or population, and each set had a group of five flies. Each set of flies was transferred to
113 a clean 2 mL microcentrifuge tube. Ten such replicates for a given treatment, gender or populations
114 were set up. These tubes of flies were incubated at 65°C for 48 h, bodyweight for each group of
115 five flies was recorded using a fine microbalance. These flies were transferred back to a

116 microcentrifuge tube. Following this, 1.5 mL of diethyl ether was added to each tube. These tubes
117 were placed on a shaker for mild agitation for 24 h at 25°C. The lipids are extracted out into the
118 ether. After 24 h, Diethyl ether was discarded, and flies were again dried at 70°C for 12 h.
119 Bodyweight after lipid extraction was measured using the fine microbalance. Absolute lipid
120 content was calculated as the difference between body weight before and after lipid extraction.
121 Lipid content per fly was calculated by dividing this difference by the number of flies per sample.
122 Fractional lipid content was measured by dividing the lipid content by the bodyweight of the given
123 sample. Fractional lipid content was analyzed using a three-factor mixed model analysis of
124 variance (ANOVA) with selection regime (FSB vs. FCB) and treatment (Cold shock vs. no shock)
125 as fixed factors crossed with the block (1-5) as random factors. Multiple comparisons using
126 Tukey's HSD were performed. All the analyses were done at $\alpha = 0.05$ level of significance using
127 JMP PRO 16.
128
129 **Rearing of fly for gene expression assays**
130 To determine the transcript levels of *Hsps* and *Fst* genes in males of the FSB and FCB. This assay
131 was performed after 50 generations of selection for increase resistance cold shock. On the 9-10th
132 day post-egg collection, males were collected as virgins and were held in groups of ten per vial
133 (10 vials per treatment). Therefore, each treatment for a given population had 100 males. Two days
134 later, *i. e.* on the 12th-day post-egg collection, males were randomly divided into two treatment
135 groups – a) Cold Shock and b) No-Shock, and treatment was given as described in Singh et al.
136 2021. Soon after the treatment, flies were transferred to a Plexiglas cage. The transcript level of
137 genes was measured using qRT-PCR of the flash-frozen flies during the recovery time post cold
138 shock, *i.e.,* 4 h or 12 h. Transcript levels of genes were analyzed using the Nonparametric Kruskal-

139 Wallis test with Dunnett's multiple comparisons test. All the analyses were done at $\alpha = 0.05$ level
140 of significance using GraphPad Prism 9.
141
142 **RNA extraction**
143 Flies were homogenized in TRI reagent (Sigma-Aldrich) using a motorized pestle. RNA extraction
144 was performed following the protocol and protocol described in Litwinoff et al. 2018. Extracted
145 RNA was suspended in 30µL of DEPC treated water. Remnants of genomic DNA contamination
146 were removed from the sample using RNase-Free DNase digestion and followed by RNA quality,
147 and quantity was checked using NanoDrop 2000 Spectrophotometer (Thermo Scientific).
148 Absorbance was measured at 260 nm and 280 nm. Absorbance 260/280 (A260/280) was
149 calculated, and samples with A260/280 > 1.9 were further used. An equal amount of RNA of all
150 samples was converted into cDNA using reverse transcriptase enzyme and random hexamers
151 (Superscript III First-Strand Synthesis kit). The obtained cDNA was diluted ten-fold and used to
152 measure the gene expression at mRNA levels.
153
154 **Quantitative Real-Time PCR (qRT-PCR).**
155 Transcript levels of *Hsp22, Hsp23, Hsp40, Hsp68, Hsp70Aa* (*Hsp70*), *Hsp83*, and *Fst* genes were
156 measured. Expression of a housekeeping gene *Actin* or *ribosomal protein S20* **(***Rps20)* was used
157 as an internal control. Primer sequences for all the genes were given in Table 2. Transcript levels
158 of genes were measured using qRT-PCR with Fast SyBr green (Thermo Scientific) on Eppendorf
159 Mastercycler. All the samples were run in duplicates. The cycle threshold (C_T) values were
160 obtained, and the expression of each gene of interest was normalized using the expression of
161 internal control. The following calculations were done:

$$\Delta C_T \text{ (Normalized gene expression)} = C_T \text{ (Gene of Interest)} - C_T \text{ (Housekeeping gene)}$$

ΔC_T values under cold-shocked and non-shocked conditions were obtained for FSB and FCB. These ΔC_T values were further used to derive gene expression differences between FSB and FCB under either shocked or non-shocked conditions. Therefore, for all genes, cold-shocked/no shock FSB or cold-shock FCB was compared with no-shock FCB. This was denoted as $\Delta\Delta\ C_T$ following:

$$\Delta\Delta C_T = \Delta C_T \text{ (cold shock or no shock FSB or cold-shock FCB)} - \Delta C_T \text{ (no-shock FCB)}$$

The difference in normalized gene expression between FSB and FCB ($\Delta\Delta\ C_T$) was used to calculate the fold change in the expression of genes (Pfaffl 2001).

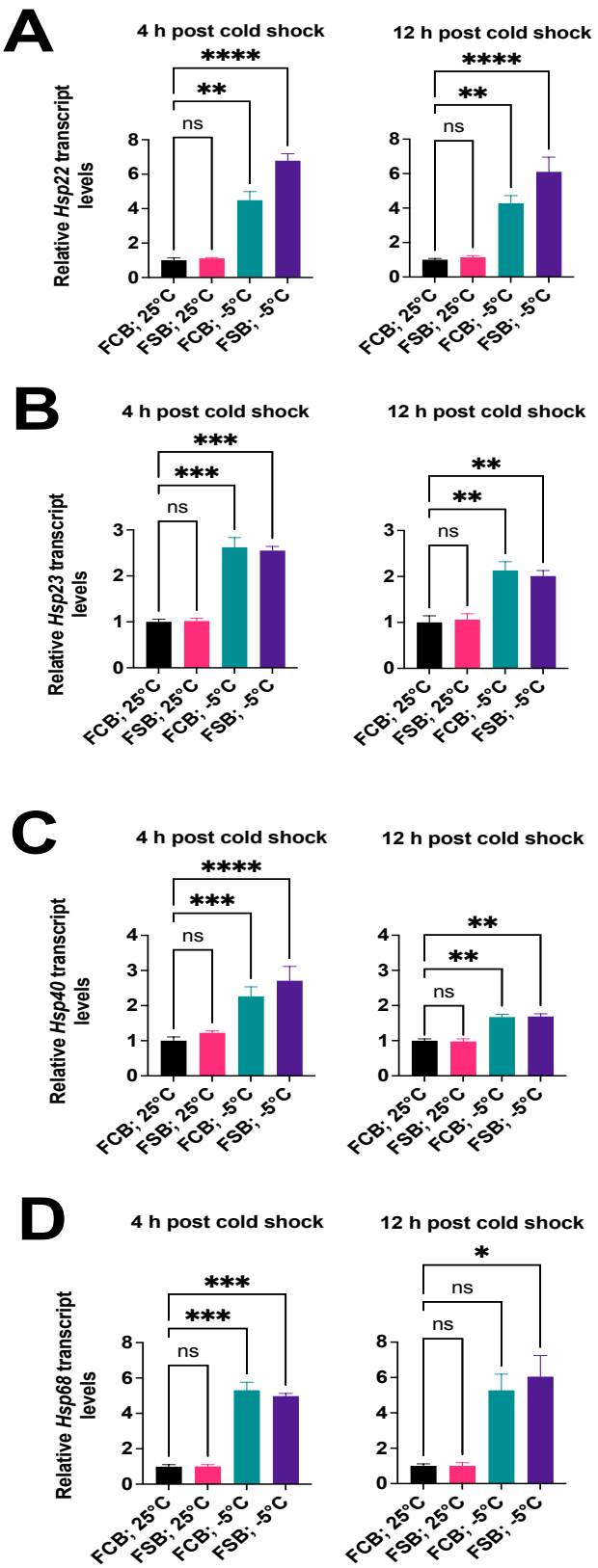
$$\text{Fold Change} = 2\text{^-}(\Delta\Delta C_T)$$

For the analysis, mean fold change across five blocks was calculated relative to the FCB no shock, expression for the given gene between FSB and FCB. The difference in normalized gene expression between FSB and FCB ($\Delta\Delta\ C_T$) was used to calculate the fold change in the expression of genes follow previous report [22].

RESULTS

Transcript levels of genes

184 Transcript levels of *Hsp22, Hsp23, Hsp40, Hsp68, Hsp70Aa* (*Hsp70*), *Hsp83*, and *Fst* were
185 assessed 4 h or 12 h post Cold shock (-5°C) or No shock (25°C) (Fig. 1A-G). Expression of *Hsp22,*
186 *Hsp23, Hsp40, Hsp68,* and *Fst* genes that we assayed was significantly higher under Cold-shock
187 than No-shock conditions in both the FSB and FCB populations (data not shown), indicating that
188 these genes are associated with cold shock response. However, the study aims to compare the
189 expression of these genes between the FSB populations and FCB populations to address questions
190 about the evolution of gene expression patterns at mRNA levels. The pattern was similar across
191 the two-time points (4 h or 12 h) of recovery after cold shock. These results indicate no substantial
192 difference in the expression of these genes between FSB and FCB populations.
193
194
195


A
4 h post cold shock
12 h post cold shock
Relative Hsp22 transcript levels
B
Relative Hsp23 transcript levels
C
Relative Hsp40 transcript levels
D
Relative Hsp68 transcript levels
FCB; 25°C
FSB; 25°C
FCB; -5°C
FSB; -5°C
ns
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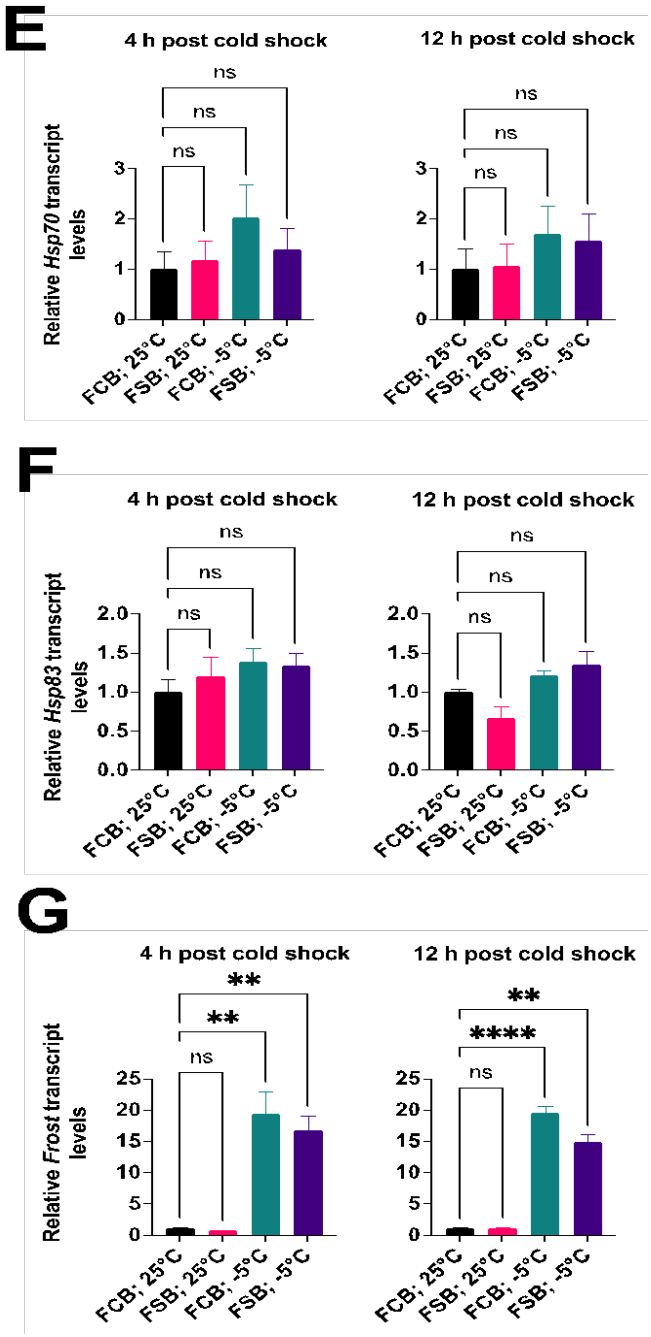
E
4 h post cold shock
12 h post cold shock
Relative Hsp70 transcript levels
F
Relative Hsp83 transcript levels
G
Relative Frost transcript levels
FCB; 25°C
FSB; 25°C
FCB; -5°C
FSB; -5°C
ns
**
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216 **Figure 1: *Expression of Hsps and Fst* up regulated post cold shock treatment. (A-G)** Relative
217 transcripts levels of *Hsp22* **(A),** *Hsp23* **(B),** *Hsp40* **(C),** *Hsp68* **(D),** (*Hsp70* **(E),** *Hsp83* **(F***), and* **(G)** *Fst*
218 were measured at 4 h or 12 h post cold shock or no shock treatment to the FSB and FCB populations.
219 Transcript levels of *Hsp22, Hsp23, Hsp40, Hsp68, and Fst were significantly in both the FSB and FCB*
220 *populations at 4 h or 12 h post cold shock compared to no shock treatment*. qRT-PCR values were
221 normalized to *Actin* and plotted relative to FCB no-shock. Statistical significance (Fig. **A- G**) was
222 evaluated by Nonparametric, Kruskal-Wallis test with Dunnett's multiple comparisons test, Data are
223 represented as means ± SEM in bar graphs from different experiment. *$p < 0.05$, **$p < 0.01$, ***$p < 0$.
224

225 **Lipid content**

226 We did not find a significant selection effect on fractional lipid content in both males (Table 1; Fig. 2A)
227 and females (Table 1; Fig. 2B). A significant effect of treatment (Cold-shock vs. No-shock) was found in
228 females ($p = 0.038$). Lower fractional lipid content under the No-shock condition was observed in females.
229 None of the other interactions were found to be significant.

230 001.
231
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238 **Table 1:** Summary of the results from the three-factor mixed model ANOVA on fractional lipid content
239 in (a) male, and (b) female with selection regime (FSB and FCB) and treatment (cold shock and no shock)
240 as fixed factors crossed with blocks (1-5) as a random factor. p-values in the bold case are statistically
241 significant. Estimated denominator DF (Satterthwaite method) was very low. Hence F ratio and *p* values
242 are unavailable for this effect.

Trait	**Effect**	**SS**	**MS Num**	**DF Num**	**DF Den**	***F* ratio**	***p***
(a)	Selection (Sel)	0.016	0.016	1	4.000	4.018	0.116
Male	Treatment (Trt)	0.002	0.002	1	4.000	0.350	0.586
fractional	Block (Blk)	0.043	0.011	4	3.526	1.526	0.360
lipid	Sel × Trt	3×10^{-4}	3×10^{-4}	1	4.000	0.120	0.746
content	Sel × Blk	0.016	0.004	4	4.000	1.465	0.360
	Trt × Blk	0.023	0.006	4	4.000	2.158	0.237
	Sel × Trt × Blk	0.011	0.003	4	180.000	0.845	0.498
(b)	Selection (Sel)	0.012	0.012	1	4.000	3.612	0.130
Female	Treatment (Trt)	0.004	0.004	1	4.000	9.272	**0.038**
fractional	Block (Blk)	0.009	0.002	4	0.300	.	.
lipid	Sel × Trt	0.002	0.002	1	4.000	0.399	0.562
content	Sel × Blk	0.014	0.003	4	4.000	0.604	0.681
	Trt × Blk	0.002	4×10^{-4}	4	4.000	0.075	0.986
	Sel × Trt × Blk	0.022	0.006	4	180.000	2.814	**0.027**

243

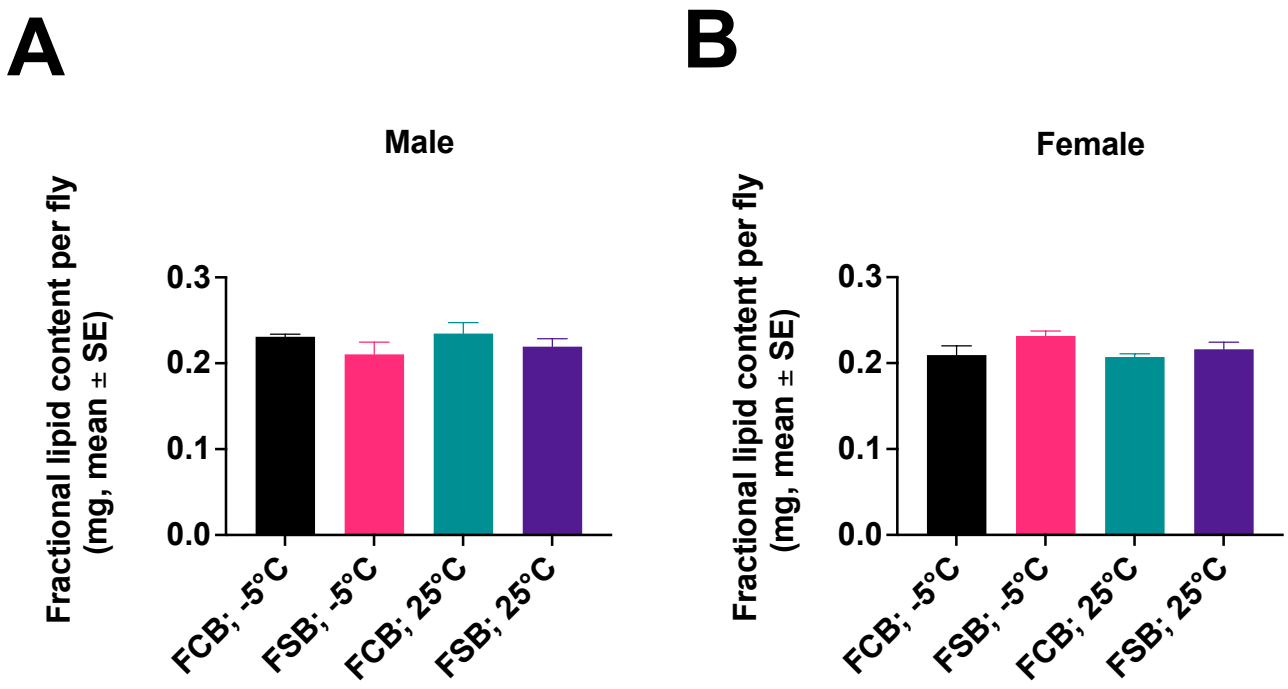

Figure 2: Effect of cold shock on the fractional lipid levels. (A) Fractional lipid content in the males of the FSB and FCB populations was assessed under the cold shock or no shock condition. We did not find significant effects of selection, treatment or selection × treatment interaction. Open bars represent FSB and closed bars represent FCB populations. (**B**) Similarly fractional lipid content was measured in the females of the both FSB and FCB population under the cold shock or no shock conditions. We did not find significant effects of selection or selection × treatment interaction. Open bars represent FSB, and closed bars represent FCB populations. Statistical significance (Fig. **A** and **B**) was evaluated using three-factor mixed model ANOVA on fractional lipid content in (**A**) male, and (**B**) female with selection regime (FSB and FCB) and treatment (cold shock and no shock) as fixed factors crossed with blocks (1-5) as a random factor. Data are represented as means ± SEM in bar graphs from different experiment. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

255 **DISCUSSION**

256 The study investigated the underlying mechanisms of evolved cold tolerance. To measure these
257 mechanisms, we examined (a) transcript level of several *Hsp22, Hsp23, Hsp40, Hsp68, Hsp70Aa* (*Hsp70*),
258 *Hsp83*, and *Fst* genes under the cold shock or the benign condition, and (b) lipid content (b). This study
259 presents the evidence of heightened transcripts levels of *Hsps* and *Fst genes* measured at 4 h or 12 h of
260 recovery post cold shock compared to no shock conditions. However, no significant alteration in transcript
261 levels of *Hsps* or *Fst gene* between FSB and FCB populations. This study reports the higher levels of lipid
262 in the cold shock females of the both FSB and FCB population compared to no shock condition.

263

264 A number of physiological and biochemical changes are expected to occur when organisms experience
265 stress, whether biotic or abiotic. Different kinds of stresses can elicit different responses. Cold stress often
266 causes damage to proteins, and lipids in the cellular machinery. [23] showed that lipids play a significant
267 role in maintaining cold tolerance in different species of *Drosophila*. This study did not find a difference
268 in total lipid content between selected and control populations. However, we did not investigate the types
269 of lipid species. Previous reports suggested that an increase in the unsaturated fatty acid content protects
270 against cold stress [24]. Therefore, it's possible that type of lipid might have evolved in the FSB populations
271 without significantly changing fractional lipid content.

272

273 Heat shock proteins play a crucial role in mitigating the damage. We assessed the expression of several
274 *Hsp22, Hsp23, Hsp40, Hsp68, Hsp70Aa* (*Hsp70*), *Hsp83*. In addition to *Hsps*, we also examined
275 expression patterns of the *Fst* gene. *Fst* is linked with cold adaptation even though its molecular function
276 is yet to be determined [25,26]. The expression of all the genes that were assayed was heightened under
277 cold shocked conditions compared to non-shocked conditions suggesting that these genes are involved in
278 the cold shock response. This finding agrees with the results of previous reports [25,26]. However, we did

279 not find a significant change in transcript levels of either *Hsps* or *Fst* in the FSB populations relative to
280 FCB populations. These results are contrary to the previous studies [26,27]. There could be various reasons
281 for the observed results. Firstly, our selection maintenance protocol is different from [26]. Secondly, the
282 time (post-recovery) at which measured the expression of these genes. Expression of *Hsps* is shown to be
283 temporally regulated [25,26,27]). Colinet et al. [26] found differences in gene expression when measured
284 after 2 h or 4 h of recovery. We selected the time points - 4 h, and 12 h during recovery post cold shock
285 since the previous report documented the apparent differences in behavioral and fitness-related traits
286 between the FSB and FCB populations at these time points. Therefore, there is a possibility that
287 measurement of gene expression at different time points during and post-treatment might show differences
288 in the gene expression. An additional point of caution is that we assayed only the levels of mRNA, not the
289 functional proteins. It is quite possible that the levels of the proteins of these genes could be higher in the
290 FSB populations even when the RNA levels are not different between FSB and FCB populations.
291
292 There are several ways organisms cope with stressful conditions. Further investigation into these
293 mechanisms would better answer the mechanistic basis of evolved cold resistance in FSB populations.
294 Future research into lipid composition (saturated vs. unsaturated lipids), other metabolites like glycogen,
295 glycerol and proline [13,19] could help us find a clear answer. Microarray profiles [27,28,29]) of other
296 cold-adapted lines in *D. melanogaster* have found a large number of other genes to be involved in cold
297 stress. We also propose a future investigation into these genes to understand the mechanistic basis of the
298 evolved response.
299
300 **Ethics declarations**
301 **Ethics approval and consent to participate**
302 Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

All data analyzed or used for this study are available from the DRYAD database.

Competing interests

All the authors declare no competing interests.

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Contributions

KS and NGP conceived the experimental evolution regimes and associated protocols. KS: Conceptualized and designed the study, performed the experiments, analyzed the data, wrote and edited the manuscript. NGP: edited the manuscript, contributed reagents, materials, and analysis tools. All authors have read and approved the final version of the manuscript.

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Abbreviations

FSB: Freeze shock selected line derived from the BRB populations

FCB: Freeze shock control populations derived from the BRB populations

BRB: Blue Ridge Baseline populations

ANOVA: Analysis of variance

h: Hour

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427 **Table 2:** Primer sequences for genes used in this study.

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Gene	Primer	Primer sequence (5' - 3')	Fragment length (bp)
Hsp22	Forward	GCCTCTCCTCGCCCTTTCAC	66
	Reverse	TCCTCGGTAGCGCCACACTC	
Hsp23	Forward	GGTGCCCTTCTATGAGCCCTACTAC	153
	Reverse	CCATCCTTTCCGATTTTCGACAC	
Hsp40	Forward	GAGATCATCAAGCCCACCACAAC	112
	Reverse	CGGGAAACTTAATGTCGAAGGAGAC	
Hsp68	Forward	GAAGGCACTCAAGGACGCTAAAATG	88
	Reverse	CTGAACCTTGGGAATACGAGTG	
Hsp70Aa	Forward	TCGATGGTACTGACCAAGATGAAGG	98
	Reverse	GAGTCGTTGAAGTAGGCTGGAACTG	
Hsp83	Forward	GGACAAGGATGCCAAGAAGAAGAAG	150
	Reverse	CAGTCGTTGGTCAGGGATTTGTAG	
Fst	Forward	CGATTCTTCAGCGGTCTAGG	92
	Reverse	CTCGGAAACGCCAAATTTTA	
RpS20	Forward	CCGCATCACCCTGACATCC	134
	Reverse	TGGTGATGCGAAGGGTCTTG	

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