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

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Abstract:

The ability to thermal adaptation can involve altering the reproductive traits and physiologic response. In insects, it reported that the higher levels expression of Heat shock proteins (Hsps) and *Frost* (*Fst*) genes during the recovery phase of cold shock and suggested higher levels of expression of these genes help repair the injury caused by cold stress. The changes in the lipids, trehalose, and glycogen can also protect from cold stress. Therefore, we examined the levels of lipid and expression patterns of *Hsps* and *Frost* genes under cold stress or the 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 both the FSB and FCB populations during the recovery phase post-cold stress compared to the no shock condition. However, we did not find a significant change in the transcript levels of these genes between FSB and FCB under both the cold stress or under the benign condition. Additionally, we noticed the higher levels of total lipid levels in the females cold shock treated compared to no shock treated females. Suggesting 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 changes in the external environment. Therefore, insects have evolved a number of mechanisms to protect themselves when subjected to external environmental fluctuations. Thermal stress (hot

or cold) is known to affect multiple traits, including fecundity, egg viability, mating ability, and pre- and post-copulatory traits, and it also reportedly affects the physiology of the insect (reviewed by [1,2,3,4,5]. A number of studies suggested that thermal stress causes damage to various molecules like proteins and lipids and damage to cell membranes, protein transport machinery, etc [6,7,8,9]. Insects are known to have evolved a range of mechanisms to mitigate and prevent the damage caused due to cold or heat stress. Change in lipid composition imparting more fluidity to cell membranes is one of the significant changes that is often employed [10].

Multiple proteins involved in repairing protein damage, including misfolding, aggregation, and denaturation are, known as heat shock proteins, play a crucial role. Heat shock proteins (Hsps) mitigate the effects of heat stress by protecting proteins and enzymes and facilitating their proper functioning under stressful conditions [11,12]. Also, an increase in the synthesis of several other metabolites like glycerol [13], glycogen, and cryoprotectants like trehalose proline. These metabolites help insects to survive under thermal stress [14]. Various insects like flesh fly [15], stick insects [16], field crickets [17], and corn borer [18], amongst others, have been used to study the mechanisms that are involved in coping with environmental stress. These insects employ a number of mechanisms that could be distinct yet overlapping. *D. melanogaster* has been a favorite model to address questions pertaining to underlying mechanisms of cold tolerance. Results from these studies suggest that *Drosophila* also employs mechanisms like lipid modification, increase in expression of heat shock proteins, synthesis of other metabolites like proline, glycogen, etc. to survive under thermal stress [13,7,19,20].

To investigate the underlying mechanisms of evolved cold stress resistance, we used a cold shock selected population 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. To examine the molecular and biochemical mechanism of cold stress resistance, we assessed the total lipid content and transcripts levels of *Hsp22, Hsp23, Hsp40, Hsp68, Hsp70Aa* (*Hsp70*), *Hsp83*, and *Fst* genes associated with cold stress response in the cold selected and their control populations.

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 shock and no shock treatment, flies were transferred into an empty glass vial, and the cotton plug

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

Lipid estimation

This assay was performed to assess the lipid levels in FSB and FCB flies, after 50 generations of selection for increased resistance to cold shock. Lipid estimation was performed following the protocol described in [21] with minor modifications. Ten sets were made for each gender and treatment or population, and each set had a group of five flies. Each set of flies was transferred to a clean 2 mL microcentrifuge tube. Ten such replicates for a given treatment, gender or populations were set up. These tubes of flies were incubated at 65°C for 48 h, bodyweight for each group of five flies was recorded using a fine microbalance. These flies were transferred back to a microcentrifuge tube. Following this, 1.5 mL of diethyl ether was added to each tube.

These tubes were placed on a shaker for mild agitation for 24 h at 25°C. The lipids are extracted out into the ether. After 24 h, Diethyl ether was discarded, and flies were again dried at 70°C for 12 h. Bodyweight after lipid extraction was measured using the fine microbalance. Absolute lipid content was calculated as the difference between body weight before and after lipid extraction. Lipid content per fly was calculated by dividing this difference by the number of flies per sample. Fractional lipid content was measured by dividing the lipid content by the bodyweight of the given sample. Fractional lipid content was analyzed using a three-factor mixed model analysis of variance (ANOVA) with selection regime (FSB vs. FCB) and treatment (Cold shock vs. no shock) as fixed factors crossed with the block (1-5) as random factors. Multiple comparisons using Tukey's HSD were performed. All the analyses were done at $\alpha = 0.05$ level of significance using JMP PRO 16.

Rearing of fly for gene expression assays

To determine the transcript levels of *Hsps* and *Fst* genes in males of the FSB and FCB. This assay was performed after 50 generations of selection for increase resistance cold shock. On the 9-10th day post-egg collection, males were collected as virgins and were held in groups of ten per vial (10 vials per treatment). Therefore, each treatment for a given population had 100 males. Two days later, *i. e.* on the 12th-day post-egg collection, males were randomly divided into two treatment groups – a) Cold Shock and b) No-Shock, and treatment was given as described in Singh et al. 2021. Soon after the treatment, flies were transferred to a Plexiglas cage. The transcript level of genes was measured using qRT-PCR of the flash-frozen flies during the recovery time post cold shock, *i.e.*, 4 h or 12 h. Transcript levels of genes were analyzed using

the Nonparametric Kruskal-Wallis test with Dunnett's multiple comparisons test. All the analyses were done at $\alpha = 0.05$ level of significance using GraphPad Prism 9.

RNA extraction

Flies were homogenized in TRI reagent (Sigma-Aldrich) using a motorized pestle. RNA extraction was performed following the protocol and protocol described in Litwinoff et al. 2018. Extracted RNA was suspended in 30µL of DEPC treated water. Remnants of genomic DNA contamination were removed from the sample using RNase-Free DNase digestion and followed by RNA quality, and quantity was checked using NanoDrop 2000 Spectrophotometer (Thermo Scientific). Absorbance was measured at 260 nm and 280 nm. Absorbance 260/280 (A260/280) was calculated, and samples with A260/280 > 1.9 were further used. An equal amount of RNA of all samples was converted into cDNA using reverse transcriptase enzyme and random hexamers (Superscript III First-Strand Synthesis kit). The obtained cDNA was diluted ten-fold and used to measure the gene expression at mRNA levels.

Quantitative Real-Time PCR (qRT-PCR).

Transcript levels of *Hsp22*, *Hsp23*, *Hsp40*, *Hsp68*, *Hsp70Aa* (*Hsp70*), *Hsp83*, and *Fst* genes were measured. Expression of a housekeeping gene *Actin* or *ribosomal protein S20* **(***Rps20)* was used as an internal control. Primer sequences for all the genes were given in Table 2. Transcript levels of genes were measured using qRT-PCR with Fast SyBr green (Thermo Scientific) on Eppendorf Mastercycler. All the samples were run in duplicates. The cycle threshold (C_T) values

were obtained, and the expression of each gene of interest was normalized using the expression of 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

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

Lipid Content

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

DISCUSSION

The study investigated the underlying mechanisms of evolved cold tolerance. To measure these mechanisms, we examined (a) transcript level of several *Hsp22, Hsp23, Hsp40, Hsp68, Hsp70Aa* (*Hsp70*), *Hsp83*, and *Fst* genes under the cold shock or the benign condition, and (b) lipid

content (b). This study presents evidence of heightened transcripts levels of Hsps and *Fst genes* measured at 4h or 12h of recovery post cold shock compared to no shock conditions. However, no significant alteration in transcript levels of Hsps or *Fst gene* between FSB and FCB populations. This study reports the higher levels of lipid in the cold shock females of the both FSB and FCB population compared to no shock condition.

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

Heat shock proteins play a crucial role in mitigating the damage. We assessed the expression of several *Hsp22, Hsp23, Hsp40, Hsp68, Hsp70Aa* (*Hsp70*), *Hsp83*. In addition to Hsps, we also examined expression patterns of the *Fst* gene. *Fst* is linked with cold adaptation even though its molecular function is yet to be determined [25,26]. The expression of all the genes that were assayed was heightened under cold shocked conditions compared to non-shocked conditions suggesting that these genes are involved in the cold shock response. This finding agrees with the results of previous reports [25,26]. However, we did not find a significant change in transcript

levels of either Hsps or *Fst* in the FSB populations relative to FCB populations. These results are contrary to the previous studies [26,27]. There could be various reasons for the observed results. Firstly, our selection maintenance protocol is different from [26]. Secondly, the time (post-recovery) at which measured the expression of these genes. Expression of Hsps is shown to be temporally regulated [25,26,27]). Colinet et al. [26] found differences in gene expression when measured after 2 h or 4 h of recovery. We selected the time points - 4 h, and 12 h during recovery post cold shock since the previous report documented the apparent differences in behavioral and fitness-related traits between the FSB and FCB populations at these time points. Therefore, there is a possibility that measurement of gene expression at different time points during and post-treatment might show differences in the gene expression. An additional point of caution is that we assayed only the levels of mRNA, not the functional proteins. It is quite possible that the levels of the proteins of these genes could be higher in the FSB populations even when the RNA levels are not different between FSB and FCB populations.

There are several ways organisms cope with stressful conditions. Further investigation into these mechanisms would better answer the mechanistic basis of evolved cold resistance in FSB populations. Future research into lipid composition (saturated vs. unsaturated lipids), other metabolites like glycogen, glycerol and proline [13,19] could help us find a clear answer. Microarray profiles [27,28,29]) of other cold-adapted lines in *D. melanogaster* have found a large number of other genes to be involved in cold stress. We also propose a future investigation into these genes to understand the mechanistic basis of the evolved response.

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Author's 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.

Conflict of interest

All of the authors declare no conflict of interest.

Resource Availability

Lead Contact, further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Karan Singh (karan.iiser@gmail.com) and N. G. Prasad (prasad@iisermohali.ac.in).

Table 1: Summary of the results from the 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. p-values in the bold case are statistically significant. Estimated denominator DF (Satterthwaite method) was very low. Hence F ratio and *p* values 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**

Table 2: Primer sequences for genes

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	
frost	Forward	CGATTCTTCAGCGGTCTAGG	92
	Reverse	CTCGGAAACGCCAAATTTTA	
RpS20	Forward	CCGCATCACCCTGACATCC	134
	Reverse	TGGTGATGCGAAGGGTCTTG	

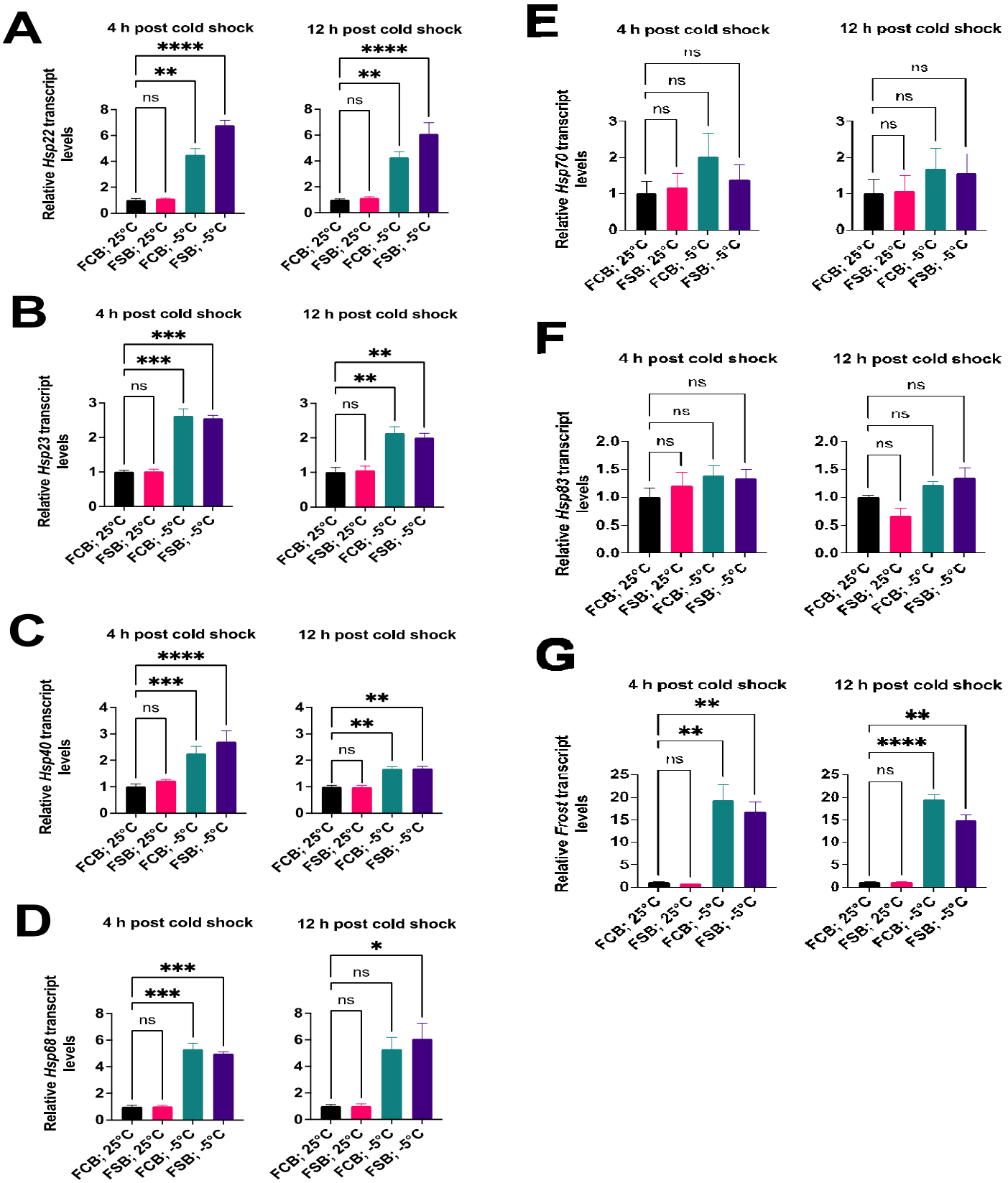
A
4 h post cold shock
12 h post cold shock
Relative Hsp22 transcript levels
B
Relative Hsp23 transcript levels
C
Relative Hsp40 transcript levels
D
Relative Hsp68 transcript levels
E
Relative Hsp70 transcript levels
F
Relative Hsp83 transcript levels
G
Relative Frost transcript levels
ns
**
***
****
*
FCB; 25°C
FSB; 25°C
FCB; -5°C
FSB; -5°C

Figure 1: ***Expression of Hsps and Fst*** **up regulated post cold shock treatment. (A-G)** Relative transcripts levels of *Hsp22* **(A),** *Hsp23* **(B),** *Hsp40* **(C),** *Hsp68* **(D),** (*Hsp70* **(E),** *Hsp83* **(F***), and* **(G)** *Fst* were measured at 4 h or 12 h post cold shock or no shock treatment to the FSB and FCB populations. Transcript levels of *Hsp22, Hsp23, Hsp40, Hsp68, and Fst were significantly in both the FSB and FCB populations at 4 h or 12 h post cold shock compared to no shock treatment*. qRT-PCR values were normalized to *Actin* and plotted relative to FCB no-shock. Statistical significance (Fig. **A- G**) was evaluated by Nonparametric, Kruskal-Wallis test with Dunnett's multiple comparisons test, Data are represented as means ± SEM in bar graphs from different experiment. *p < 0.05, **p < 0.01, ***p < 0.001.

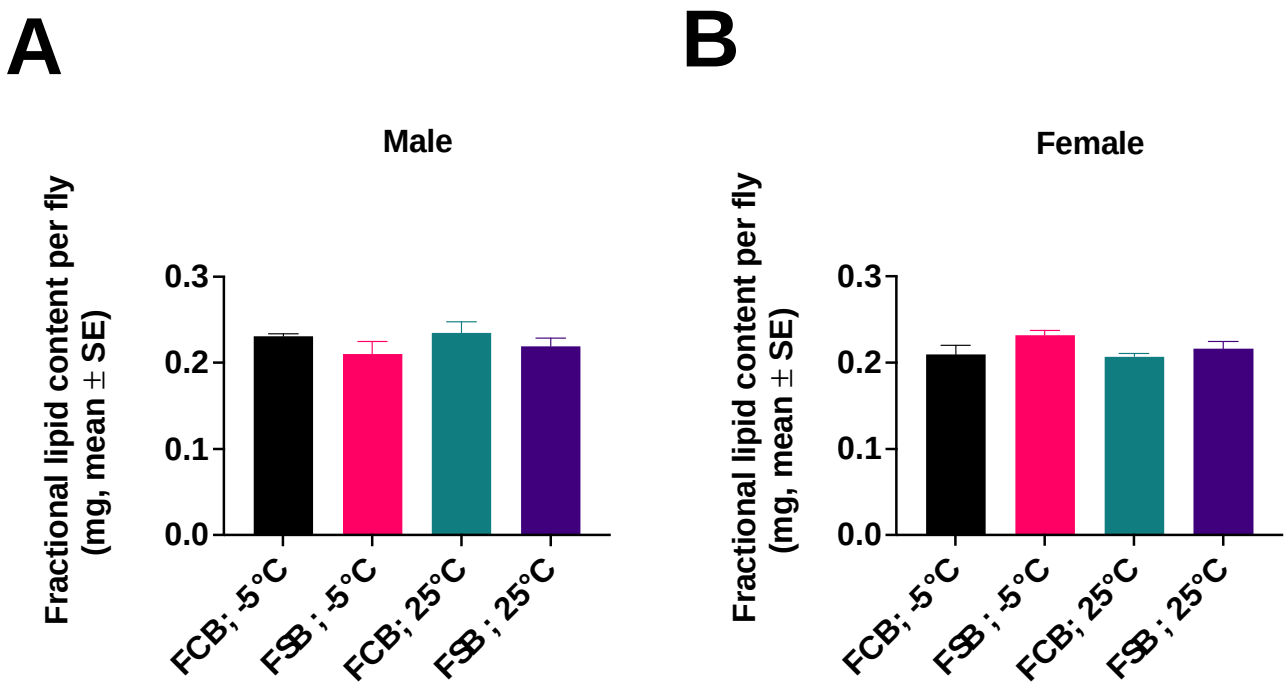

Figure 2: Effect of cold shock on the fractional lipid levels. (A) Fractional lipid content in the males of the FSB and FCB populations was assessed under the cold shock or no shock condition. We did not find significant effects of selection, treatment or selection × treatment interaction. Open bars 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. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

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