Evolution of cross-tolerance in *Drosophila melanogaster* as a result of increased resistance to cold stress

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Running short title: Evolution of cross-tolerance

Keywords: Cross-tolerance, desiccation resistance, egg viability, mating frequency, reproductive behavior, starvation resistance

Abstract

Cold stress is a critical environmental challenge that affects an organism's fitness-related traits. In *Drosophila*, increased resistance to specific environmental stress may lead to increased resistance to other kinds of stress. In the present study, we aimed to understand whether the increased cold stress resistance in *Drosophila melanogaster* can facilitate their ability to tolerate other environmental stresses. For the current study, we used successfully selected replicate populations of *D. melanogaster* against cold shock and their control population. These selected

populations have evolved a number of reproductive traits, including increased egg viability, mating frequency, male mating ability, ability to sire progenies, and faster recovery for mating latency under cold shock conditions. In the present work, we investigated egg viability and mating frequency with and without heat and cold shock conditions in the selected and their control populations. Additionally, we also examined resistance to cold shock, heat shock, desiccation, starvation, and survival post-challenge with *Staphylococcus succinus* subsp. *succinus* PK-1 in the selected and their control populations.

We found 1.25 times increase in egg viability in the selected population at 24-30 h post-cold shock compared to control populations. The mating frequency of selected populations increased by 1.57 times under cold shock conditions compared to the control population. At 24-30 h post cold shock, the egg viability of the selected populations had increased 0.3 times than the control populations. After being subjected to heat shock, the mating frequency of the selected populations was increased 0.57 times greater than the control populations. A higher number of males (0.87 times) and females (1.66 times) of the selected populations survived under cold shock conditions relative to their controls. Similarly, more males (0.31 times) and females (0.98 times) of the selected populations survived under heat shock conditions than the controls. Desiccation resistance had slightly increased in the females of the selected populations relative to their control, but we observed no change in the case of males. Starvation resistance has decreased in males and females of the selected populations compared to their controls.

Our findings suggest that the increased resistance to cold shock correlates with increased tolerance to heat stress and decreased tolerance to starvation.

Introduction

Empirical studies on *Drosophila* revealed that the evolution of increased resistance to specific environmental stress might confer an advantage or a disadvantage on their ability to tolerate other environmental stresses[1,2,3]. A number of studies confirmed the relationship between multiple stress conditions[1,3,4,5,6,7]. For instance, heat tolerance and resistance to starvation correlate with desiccation resistance and cold tolerance independently[1,3,4,5,6,7]. Insects experiencing transient environmental stress for a single generation are protected from other

environmental stresses. For example, exposing *D. melanogaster* to mild cold stress can increase their ability to tolerate heat stress[8]. Cold hardening in *D. melanogaster* confers protection against heat stress[9]. Le Bourg's[8] study reported that the subjection of *D. melanogaster* to mild starvation followed by a 2-6 h delay to cold stress, could facilitate their ability to resist cold stress. Mild desiccation experience in springtail can increase their ability to tolerate cold stress. Short-term environmental stress experience in insects can provide their ability to withstand other stresses. For example, exposing *D. melanogaster* to mild cold stress can increase their ability to tolerate heat stress[8]. Cold hardening in *D. melanog*aster confers protection against heat stress[9]. Le Bourg's[8] study reported that the subjection of *D. melanogaster* to mild starvation followed by a 2-6 h delay to cold stress could facilitate their ability to resist cold stress. Mild desiccation experience in springtail can increase their ability to tolerate cold stress[10]. Similarly, *Folsomia candida* house flies exposed to anoxic conditions at 27°C show greater tolerance when exposed to -7°C[7,10,11,12,13].

A number of laboratory selection studies against the stresses reported cross-tolerance between multiple stresses[4,7,14,15,16], such as increased heat tolerance in lines selected for the increased resistance to desiccation or heat knockdown time[4, 7]. Similarly, increased cold shock tolerance was observed in the lines selected for resistance to chill-coma recovery, heat shock, or desiccation[7]. Additionally, a positive correlation in desiccation resistance was observed in the lines selected for chill-coma recovery or cold shock resistance[15,16]. An additional unique mechanism required to adapt to a specific environmental stress might conflict with a mechanism of adaptation to other stresses, thereby leading to trade-offs across stress resistance traits[17,18]. For example, Hoffmann et al.[19] documented the flies selected for increased starvation resistance had decreased resistance to cold stress. In contrast, those lines selected for increased cold resistance show reduced starvation resistance[2]. However, none of the studies investigate how the evolution of cold stress resistance in the context of egg viability and mating frequency post cold shock can impart cross-resistance with other stresses.

In the present study, we aimed to assess the cross-tolerance in the context of egg viability and mating frequency with and without cold and heat shock conditions in the selected and their control populations. We also investigated resistance to cold shock, heat shock desiccation, starvation, and survival post-challenge with *Staphylococcus succinus* subsp. *succinus* PK-1 (*Ss*)

in the selected and their control populations. Our study populations consisted of 5 selected and 5 control populations of *D. melanogaster*, and experiments were conducted over 57-70 generations of selections.

Results

Experiment 1A: Effect of cold shock on egg viability and mating frequency

To assess the response of the selection regime in the context of egg viability and mating frequency, we measured the egg viability with and without cold shock conditions in the Freeze shock Selected populations derived from the BRB populations (FSB) and Freeze Shock Control populations derived from the BRB populations (FCB). Egg viability data of 0-6 and 24-30 h were analyzed with a three-factor mixed model analysis of variance (ANOVA) with a generalized linear model (GLM) considering selection regime (FSB vs. FCB), treatment (Cold shock vs. No shock), as fixed factors crossed with the random block (Block 1-5). We did not find a significant effect of selection on egg viability data of 0-6 h. However, treatment had a significant effect on the egg viability, suggesting that cold shock treatment drastically reduces more than 90% egg viability compared to no shock treatment (**Fig. 1A; Table S1A)**. We did not observe a significant interaction between selection and treatment **(Table S1A)**. For 24-30 h egg viability, we observed a significant effect of selection, treatment, and selection × treatment interaction on the egg viability **(Table S1B).** Using Tukey's HSD test, we confirmed that the post cold shock egg viability of FSB populations increased by 1.25 times more than the FCB populations (**Fig. 1B)**. We also measured the mating frequency post cold shock to determine whether the increased egg viability could be because of more mating frequency in the FSB populations compared to the FCB populations. Therefore, we measured the mating frequency from the same set up of the experiment that was used to assess the egg viability. We observed that the selection had a significant effect on mating frequency, suggesting that FSB populations had higher mating frequency compared to FCB populations (**Fig. 1C)**. We also observed that treatment had a significant effect on mating frequency, indicating that cold shocked populations had more matings compared to unshocked populations. We found the significant effect of selection × treatment interaction on mating frequency (**Table S1C**). Multiple comparisons using Tukey’s HSD test confirmed that post cold shock FSB populations had ~1.57 times more mating

frequency compared to FCB populations (**Fig. 1C)**. No shocked FSB populations had roughly 7% more mating frequency compared to unshocked FCB populations (**Fig. 1C)**. This result suggests that the basal rate of mating frequency also increased in the FSB populations (**Fig. 1C**). This result indicates that evolved responses to cold shock in the context of egg viability and mating frequency persist across many generations of selection in the selected populations. These results are in agreement with previous reports[22,24].

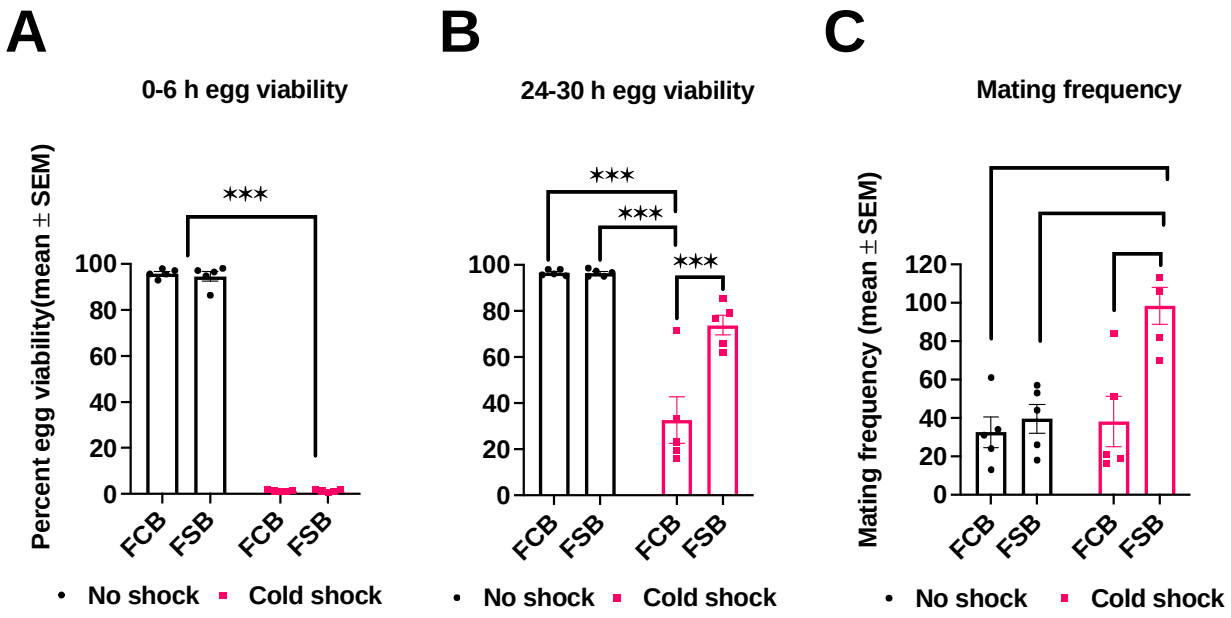

Figure 1. Heightened egg viability and mating frequency in the selected populations post cold shock. (**A** and **B**) Egg viability measurement for each replicate population of FSB and FCB at 0-6 h (**A**) and 24-30 h (**B**) post cold shock and no shock, eggs were transferred at a density of ~200 eggs/plate to plates containing 1.2% agar from the food plates. Forty-eight hours later hatched eggs were counted from 1.2% agar plates for each replicate populations of FSB and FCB and followed by percent egg viability was computed. n = 1 plate (200 eggs/plate) for each FSB and FCB populations and for each treatment condition.

(C) The number of mating pairs were were observed every 30 min intervals till 36 h post cold shock from each replicate populations of FSB and FCB and data are represented as summed of the matings observed across 0-36 h. n = 5 cages (100 mating pairs of male and female) for FSB and FCB populations for cold shock and no shock treatments. Statistical significance of **Fig**. **1A-C** was evaluated employing a three-factor mixed model analysis of variance (ANOVA) using selection and treatment as fixed factors crossed with block as a random factor. Multiple

comparisons were performed with Tukey's multiple comparisons test. Data are represented as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001.

Experiment 2: Effect of heat shock on egg viability and mating frequency

To examine the cross-tolerance, we measured the egg viability and mating frequency in FSB and FCB populations under heat shock and no shock conditions. Egg viability data of 0-6 and 24-30 h were analyzed using three-factors mixed model ANOVA with GLM, applying selection (FSB vs. FCB), treatment (Heat shock vs. No shock) as fixed factors crossed with block as a random factor. For 0-6 h egg viability, we did not find a significant effect of selection and selection × treatment interaction on egg viability (**Table S2B)**. However, we observed the significant effect of treatment on egg viability (**Table S2B)**, suggesting that heat shocked FSB and FCB populations had tremendously low egg viability (~10%) compared to no shocked FSB and FCB populations (**Fig. 2A**). For 24-30 h egg viability, we found a significant effect of selection, treatment, and selection × treatment interaction on egg viability (**Table S2B**). The significant effect of treatment indicates that at 24-30 h post heat shock, the egg viability was reduced by 34% in the FCB and 43% in the FSB populations than in the unshocked FCB and FSB populations, respectively. Multiple comparisons using Tukey's HSD test confirmed that the post heat shock FSB populations had ~27% more egg viability than the FCB populations (**Fig. 2B**).

We also measured mating frequency with and without heat shock conditions in the FSB and FCB populations, and its data were analyzed using a three-factor mixed model ANOVA with GLM, considering selection (FSB vs. FCB), treatment (Heat shock vs. No shock) as fixed factors, and block as a random factor. We observed a significant effect of selection on mating frequency (**Table S2C**). Indicated the FSB populations were estimated to be 46% more mating frequency than the FCB populations (**Fig. 2C**). However, we did not notice any significant effect of treatment and selection × treatment interaction on mating frequency (**Table S2C**).

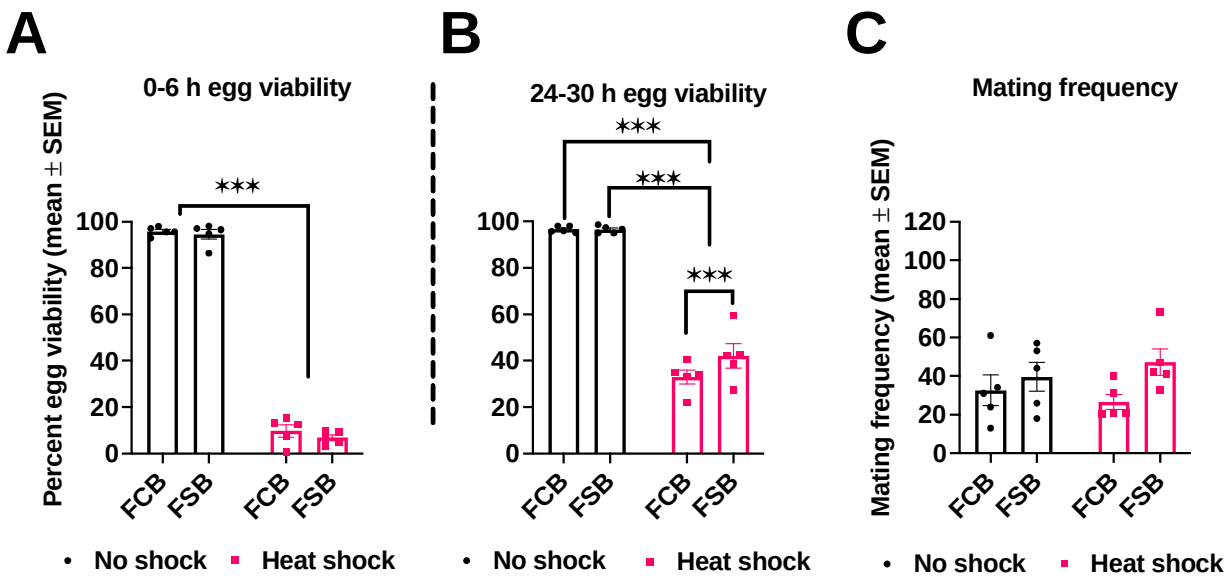

Figure 2. Increased egg viability and mating frequency in the selected populations. (**A** and **B**) Egg viability measurement for each replicate populations of FSB and FCB at 0-6 h (**A**) and 24-30 h (**B**) post heat shock and no shock, a group of ~200 eggs/plate were transferred to a Petri plate containing 1.2% agar from the standard food plate. Forty-eight hours later, a number of hatched eggs were counted from 1.2% agar plates for each of the FSB and FCB populations and followed by percent egg viability was computed. n = 1 plate (200 eggs/plate) for each replicate population of FSB and FCB populations, and for heat shock and no shock treatments. (**C**) Post heat shock, mating frequency was monitored every 30 min intervals till 36 h from each of the FSB and FCB populations. n = 5 cages (100 males and females/cage) for selection regime and treatment group. The statistical significance of **Fig. 2 A-C** was evaluated by a three-factor mixed model ANOVA employing selection and treatment as fixed factors crossed with block as a random factor. Multiple comparisons were performed with Tukey's test. Data are represented as mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Experiment 3A: Effects of cold shock on adults' survival

To probe the effect of the selection regime on cold tolerance, we exposed 2-3 days old virgin males of FSB and FCB populations to -5.6°C for 1 h, and virgin females exposed to -5°C for 1 h. Twenty-four hours post cold shock, the survival data of males and females, were analyzed using a two-factor mixed model ANOVA employing selection (FSB vs. FCB) as a fixed factor and block as a random factor. We detected a significant effect of selection on males' survival post

cold shock (**Table S3A)**. Indicates that 24 h post cold shock, FSB males had 0.87 times more survival than the FCB males **(Fig. 3A)**. We also observed a significant effect of selection on females' survival 24 h post cold shock **(Table S3B)**, suggesting that 1.66 times more FSB females survived relative to FCB females (**Fig. 3A)**.

Experiment 3B: Effects of heat shock on adults' survival

To examine heat-tolerance, virgin male and female flies of the FSB and FCB populations were exposed to heat shock conditions as described in the methods. Twenty-four hours after heat shock, we counted the total number of dead male and female flies from FSB and FCB populations. The survival data were analyzed using the two-factor mixed model ANOVA applying selection (FSB vs. FCB) and treatment as fixed factors, and block as a random factor. We found the significant effect of selection on males' survival (**Table S3C).** Twenty-four hours after heat shock, survival of the FSB males increased by 0.31 times more than the FCB males (**Fig. 3B)**. Similarly, we also observed a significant effect of selection on females' survival data of 24 h post heat shock (**Table S3D).** Indicating that 24 h post heat shock, FSB females had 0.98 times more survival than the FCB females (**Fig. 3B)**. These findings suggest that the increased resistance to cold stress in *D. melanogaster* can provide their ability to resist heat stress.

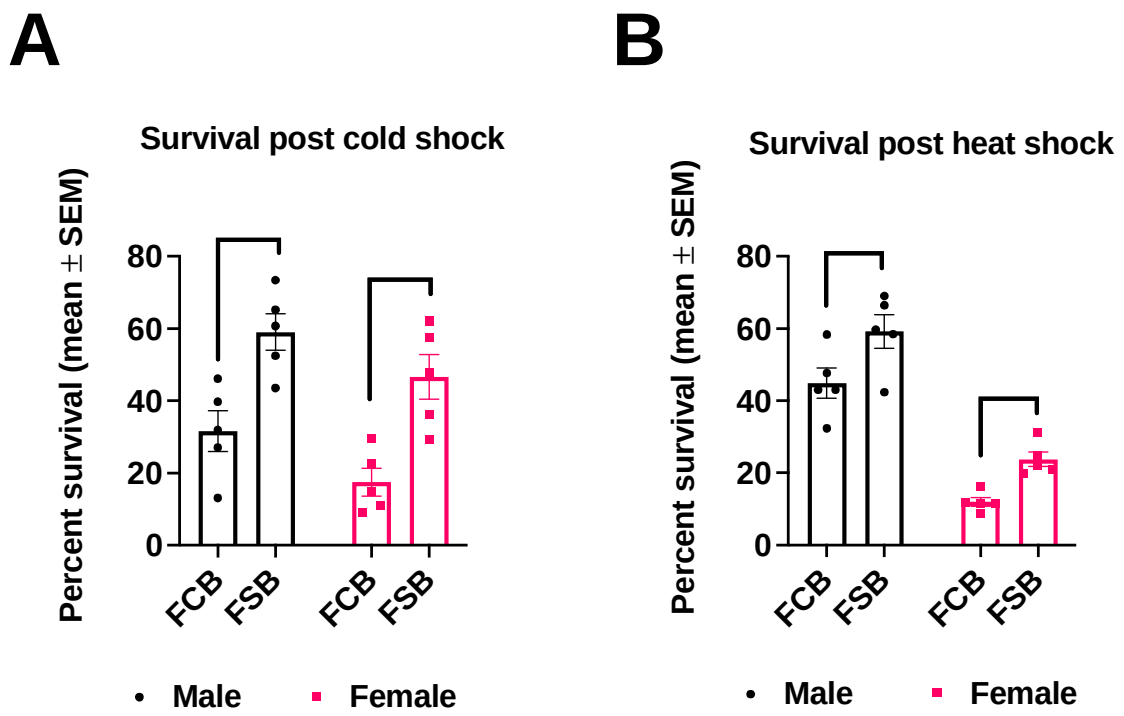

Figure 3. Increased survival in the selected populations post cold and heat shock. **(A)** Two-three days old virgin male flies of the FSB and FCB populations were exposed to -5.6°C for 1 h, and 2-3 days old virgin females were exposed to -5°C for 1 h. Twenty-four hours post cold shock number of dead male and female flies were counted from each replicate populations of FSB and FCB, and percent survival was computed for them. (**B)** Two-three-3 days old virgin males of the FSB and FCB populations were exposed to 38.9°C and virgin female flies were exposed to 39.2°C. Twenty-four hours after heat shock number of dead male and female flies were counted from each replicate populations of FSB and FCB, and percent survival was computed for them. n = 300 male and female flies at a density of 100 males or females /cage were used for each replicate populations of the FSB and FCB. The statistical significance of **Fig. 3A** and **B** was evaluated by employing two-factors mixed model analysis of variance. Data are represented as mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Experiment 4: Adult survival post-challenge with a Ss

Cold stress is known activates the immune system in *D. melanogaster*[20]. Therefore, we challenged the male and female flies of the FSB and FCB populations with a natural pathogen of *Drosophila*, i.e., gram-positive bacteria, *Ss*[21] to determine the effect of selection regime on

adults' immunity, The survival data of male and female flies post-infection was analyzed using Cox proportional hazards methods. Analysis of the results indicates no significant difference in survival between the FSB and FCB populations **(Fig. 4A** and **B; Table S4)**. We found no significant effect of selection and sex on the survival of male and female flies post-infection with *Ss* (**Table S4)**.

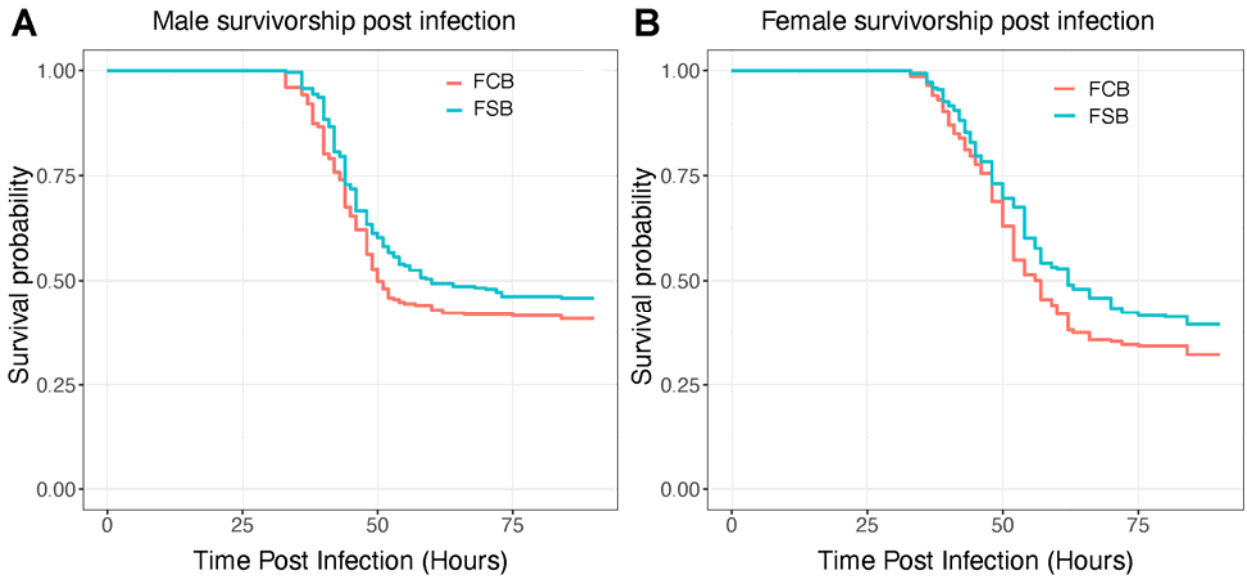

Figure 4. Immune response has not altered in the selected populations.

(A and **B)** Age of 2-3 days old virgin males and females of the FCB or FSB population were challenged with Ss. The number of dead flies were recorded every 3 h intervals till 24h post-infection, and followed by the number of dead flies monitored every 1 h intervals till 90 h post-infection. n = 7 vials of 10 males or females/vial for each of the 5 replicate populations of FSB and FCB. Therefore, a total of ~350 males and females flies were assayed from the FSB and FCB populations. Statistical significance was confirmed by using the Cox proportional-hazards model (**Fig. 4A** and **B)**. Data are represented as the proportion of survival.

Experiment 5: Desiccation resistance

To determine the effect of the selection regime against desiccation resistance, we exposed males and females of FSB and FCB populations to desiccation conditions. The data were analyzed using Cox proportional hazard model. We observed the FSB females had slightly higher

desiccation resistance than the FCB females (**Table S5B; Fig. 5B**). However, in the case of males, we did not find the significant effect of selection (**Table S5A; Fig. 5A**).

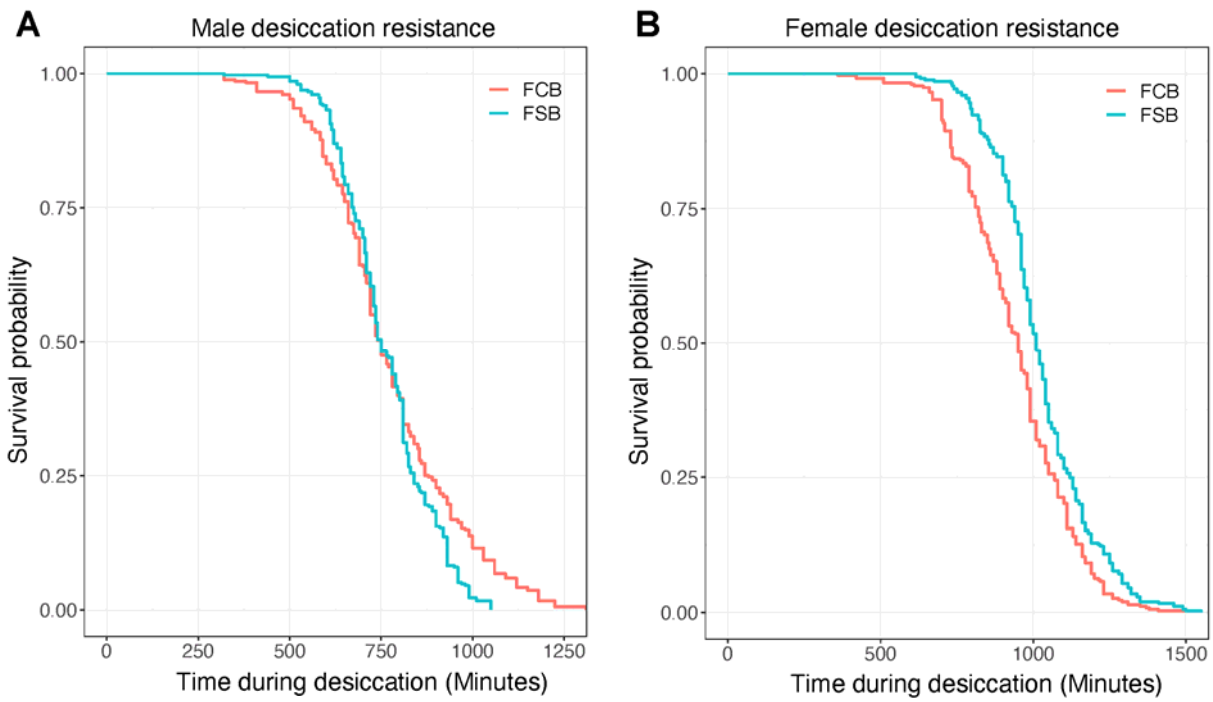

Figure 5. No alteration in desiccation resistance in the selected populations

(A) We assayed starvation resistance on virgin males aged 2-3 days old as adults, males of the FSB and FCB populations were transferred to the food-less vials at a density of 10 flies/vial, and cotton plugs were pushed deep into the vials so that flies can stay in the 1/3 area of the vial. Then 6g of silica gel was added on the top of the cotton plug, and the mouth of vials were sealed with parafilm. A number of dead flies were traced every 30 min intervals until the last flies died in a given vial. **(B)** Just like males, females of the FSB and FCB population were kept under the same desiccation environment as defined for the males. A number of dead flies were counted every 30 min intervals until the last flies died in a given vial. n = 7 vials of 10 males or females/vial for each of the 5 replicate populations of FSB or FCB were used for assay. Statistical significance of **Fig. 5A** and **B** were evaluated by using the Cox proportional-hazards model. Data are represented as the proportion of survival.

Experiment 6: Starvation resistance

To assess the cross-tolerance against starvation in selected populations, those who had increased survival under cold shock conditions. We measured the starvation resistance in the FSB and FCB populations, and the survival data were analyzed using the Cox proportional-Hazard model. We observed a significant effect of selection on males' and females' starvation resistance (**Table S6A** and **B)**, suggesting that the FSB males had significantly reduced starvation resistance compared to FCB males (**Fig. 6A)**. Like FSB males, FSB females also had significantly decreased starvation resistance than the FCB females (**Fig. 6B)**. These results suggest that increased cold shock resistance comes with a cost; as a result, there is a trade-off with resistance to starvation.

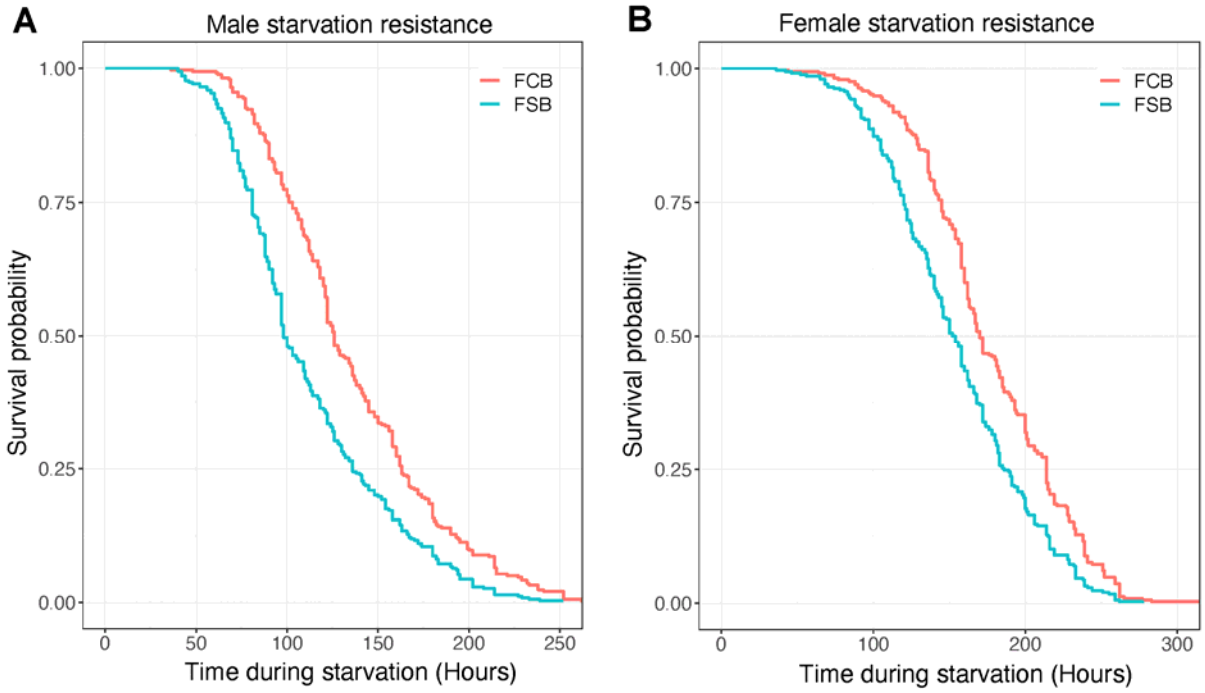

Figure 6. Decreased starvation resistance in the selected populations

(A) To assay starvation resistance in the FSB and FCB populations, 2-3 days old virgin males of FSB and FCB populations were transferred to vials containing 6 mL of 1.24% agar vial at a density of 10 flies/vial. The number of dead flies were monitored every 4 h until the last flies died in a given vial. **(B)** Two-three days aged, virgin females of FSB and FCB populations were transferred to vials containing 6 mL of 1.24% agar vial at a density of 10 flies per vial. A number of dead flies were recorded every 4 h until the last flies died in a given vial. n = 7 vials of 10 males or females/vial for each replicate populations of FSB or FCB. The statistical significance

of **Fig. 6A** and **B** was determined by using the Cox proportional-hazards model. Data are represented as a proportion of survival.

Discussion

This study aimed to explore the evolution of cross-tolerance in the populations of *D. melanogaster* that were selected for increased resistance to cold shock. These selected populations have evolved multiple reproductive traits, including egg viability, mating frequency, male mating ability, male ability to sire more progenies, mating latency, sperm offense ability, and progeny production[22,23,24]. In our present experimental evolution study, we measured egg viability and mating frequency with and without heat and cold shock conditions. We investigated resistance to cold shock, heat shock, starvation, desiccation, and adult survival post-challenge with *Ss* in selected and control populations. We observed the increased egg viability in the selected populations relative to control populations (**Fig. 1B** and **2B**), as documented in the previous study[22,24]. We found that the selected populations had the higher mating frequency with and without heat and cold shock conditions (**Table S1C** and **S2C).** After being subjected to cold and heat shock, selected populations had higher adult survival relative to their control populations (**Fig. 3A** and **B).** Desiccation resistance has slightly increased in females of the selected populations (**Fig. 5A**), which indicates the selection for a single type of environmental stress, leading to improved resistance to other stress. However, in the case of starvation resistance, we found that the selected populations had lower starvation resistance than control populations (**Fig. 6A** and **B**), which suggests that the increased survival of selected populations to cold shock is negatively correlated with the starvation resistance. We discussed each of these observations below in more detail.

At 0-6 h post cold shock, we found approximately 95-97.5% reduction in egg viability (**Fig. 1A**). This could also be because below zero temperatures cause sperm mortality in male seminal vesicles, female seminal receptacles, and spermatheca[25,26]. This result is in line with several other studies that have observed reduced egg viability and sterility in insects upon exposure to extremes of temperature[10,22,24,27,28]. However, we found greater egg viability in the selected populations compared to control populations at 24-30 h after cold shock (**Fig. 1B**), as reported previously[22,24]. There are a number of possible explanations for increased egg viability at 24-30

h post cold shock: (a) The selected populations could be better at protecting their stored sperms/eggs from damage caused by cold shock. For instance, Collett and Jarman[29] have shown that *D. pseudoobscura* females can store sperm for up to 6 months during the cold environment. These stored sperms can be used to fertilize ova in a favorable warm environment. However, this is not the case in our selected populations because a previous report using the same populations confirmed that the females of selected populations relative to their control populations do not store the fertilized eggs or sperms[22]. (b) The selected populations mate more often after heat or cold shock to enhance egg viability. A number of studies documented that high and low temperatures have an impact on the mating behavior[30,31,32,33,34,35]. However, very few studies have addressed the effect of cold shock on mating behavior[22,23,24]. In FSB populations, the mating frequency had increased post heat or cold shock compared to FCB populations (**Fig. 1C** and **2C**). Hence, it is likely that increased mating post heat or cold shock is largely responsible for increase in egg viability. While the pattern of increased mating frequency correlated with increased egg viability post cold shock in the current study as well as in previous reports[22,24], it is interesting that this pattern is seen at a lesser extent under heat shock conditions. This finding indicates that probably some of the other mechanisms underlying resistance to heat and cold stress might be common. This also forms an example of a positive correlation between resistances to two stressors.

We observed that the selected populations had increased survival than the control populations over 24 h post cold shock (**Fig. 3A**). Its important to note that cold shock induces negligible adult mortality in our selection regime. Our results indicate that the FSB populations have evolved the ability to withstand cold stress in terms of increased adult survival along with their ability to maintain higher egg viability after shock at a temperature of -5°C. Multiple laboratory selections studies shown the increased adult survivorship as a correlated response to selection for cold tolerance[2,16,36,37]. In previous reports Singh et al.[22,24], documented that the mortality post cold shock was negligible in our selection regime. However, in the present study, mortality post cold shock is substantial (**Fig. 3A**). These results seem quite contradictory. There are several possible explanations. First, the populations have evolved for a number of generations between these two experiments. Second, in the current study, the flies were virgins when subjected to cold shock, whereas in the previous study, the flies had already mated by the time they were subjected to cold shock. Third, in the present study, the flies were moved into a fresh food vial

soon after eclosion, while in the previous study, the flies remained in the cultured vials (with old, spent food) for two-three days after eclosion. There could be multiple explanations for the superior survivorship of selected populations post cold shock. (a) Chen and Walker[37] report that cold selected lines have higher glycogen and total proteins relative to control lines. Insects are known to store various sugars in order to tolerate cold temperatures[38,39]. It is possible that the selected populations have similarly altered resource storage in terms of carbohydrates, proteins or lipids. (b) Several studies have shown that there are several heat shock proteins that are expressed both during heat and cold stress. It is quite possible that at least some of these genes are expressed at a higher level in our populations. However, these genes are certainly not among the set that we analyzed for the expression differences in our other study [40].

More interestingly, the selected populations also showed higher survival post heat shock compared to control populations (**Fig. 3B**). The present literature depicts some disagreement with regards to cross-resistance between cold and heat stress[41]. Anderson et al.[16] and MacMillan et al.[2] did not find correlated increase in heat shock resistance in populations of *D. melanogaster* selected for faster chill coma recovery or freeze resistance, respectively. Our results are in agreement with those of Kristensen et al.[42], who show that cold selected lines of *D. melanogaster* were more heat tolerant and vice versa. Previous studies in *Drosophila* along latitudinal clines suggest that there is a trade-off between heat and cold tolerance[43]. Our results suggest that heat and cold tolerance might be positively correlated in *D. melanogaster*.

In insects, cold stress can cause somatic injury to the gut and malpighian tubules. This can open up a way for the gut flora to enter the haemocoel and thereby cause an infection[44,45,46,47]. Therefore, in our selected populations, immune activity can potentially evolve. However, we noticed that selected populations had a little bit higher survival (**Fig. 4A** and **B**). However, this difference was not significantly different between FSB and FCB populations in their immunity against *Ss.* One possibility is that the immune response is elicited only in response to the gut flora. In *Drosophila*, evolution against a pathogen can be fairly specific, and the host might not have increased immunity against other pathogens[48,49,50]. Thus, in the present assay, where we use *Ss* as the pathogen, the appropriate immune response might not have been elicited.

We found that the desiccation resistance is slightly increased in females of the selected populations (**Fig. 5B**). Our findings are in line with results from other studies[2,7], which show that increased resistance to cold shock may lead to increased desiccation resistance as a correlated response. However, populations selected for desiccation resistance do not show increased cold tolerance[15]. There is at least one common factor between cold and desiccation resistance that might explain their correlated evolution. Glycogen is known to act as a cryoprotectant[51,52,53]. Chippindale et al.[53] showed that selection for increased desiccation resistance leads to increased glycogen content. Thus, increases in glycogen content through selection on cold shock resistance could in principle, lead to the evolution of increased desiccation resistance. Another possible explanation to increased desiccation resistance could be body size in females, in our other related experiment, we found that females from selected populations had higher body weight relative to females from control populations[24]. However, such an increase, if any, is likely to be sex-specific since we found no change in the desiccation resistance of FSB and FCB males.

Starvation resistance has decreased in populations selected for increased resistance to cold shock relative to control populations (**Fig. 6A** and **B**). Our findings are similar to those of MacMillan et al.[2], and Anderson et al.[16], who found lower starvation resistance in populations of *D. melanogaster* selected for increased resistance to cold shock. Interestingly, Bubliy and Loeschcke[7] found decreased cold stress tolerance in populations of *D. melanogaster* selected for increased starvation resistance. Thus, across multiple studies, the correlation between starvation resistance and cold stress tolerance seems to be robust.

These findings indicate that the evolution of cold shock resistance provides resistance to heat stress. At the same time, it negatively correlates with starvation resistance. It suggests that the increased resistance to cold shock comes as a cost paid as decreased resistance to starvation.

Methods

Derivation of selected and control populations

Protocols for the creation and maintenance of the selected and their control, or unselected populations have been described previously[22,24]. In short, we established a large outbred population named BRB in 2010 by mixing of 100 male and 100 female flies from each of the 19

isofemale lines, which were originally established in the lab of Prof. Daniel Promislow from 19 inseminated female flies that were captured from the Blue Ridge mountain, Georgia, USA. In 2010, these 19 isofemale lines were gifted to us. We cultured them for 6 generations on the standard laboratory conditions (25°C temperature, 50–60% relative humidity, 12 h shift of light-dark cycles, banana-yeast-jaggery food) after that, we merged 100 male and female flies from each of the 19 isofemale lines to create a large outbred population referred as 'BRB'. We cultured this BRB population (population size of 2800 flies) for 10 generations at the standard laboratory conditions before dividing it into 5 replicates population known as BRB 1-5. Then we maintained these BRB 1-5 populations for 35 generations on the standard laboratory conditions before creating FSB 1-5 and FCB 1-5 from BRB 1-5. Numbers 1-5 denote that both FSB 1 and FCB 1 originated from BRB 1. Therefore, they are more close to each other than FSB 2 and FCB 2 or any other populations; therefore, in the statistical analysis, we considered them as Block 1. Similarly, FSB 2 and FCB 2 were derived from BRB 2, therefore they are more close to each other than FSB 1 and FCB 1 or any other set of populations. Hence, in the statistical analysis, we treated them as block 2 and so on. For additional details, see the below flow chart (**Fig.7)**.

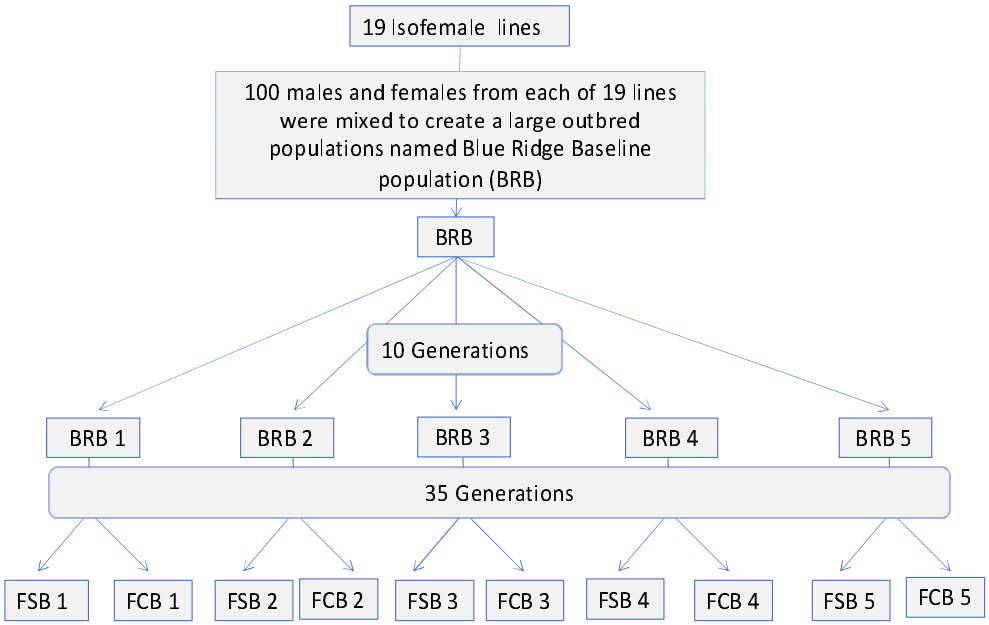

Figure 7. Derivation of the BRB, FSB, and FCB populations.

Cold shock protocol for selection regime

The FSB 1-5 and FCB 1-5 are large outbred populations maintained on 13 days discrete generation cycle under the standard laboratory conditions. On the 12th day post culturing of eggs, 2-3 days old non-virgin, mated flies at a density of ~60-70 flies/vial were housed into an

empty, clean, dry glass vial (30 mm diameter × 90 mm length) and cotton plug was pushed inside the vial so that flies were allowed to stay in the 1/3 area of a vial. Twenty such vials were made for each replicate populations of FSB 1-5 and FCB 1-5. Followed by these vials were held in a water-ice-salt slurry maintained at -5°C, whereas vials containing flies of the FCB 1-5 populations were kept in a water-bath maintained at 25°C for 1 h. Soon after the treatment, flies were transferred to Plexiglas cage with standard food plate at a density of 1200-1400 flies per cage for each populations of FSB 1-5 and FCB 1-5. To initiate the next generation, 24 h after cold shock, a fresh food plate was given to each cage of FSB 1-5 and FCB 1-5 populations for 18 h to collect eggs. For each of the FCB 1-5 populations, 20 vials of ~70 eggs/vial were collected so that the total flies emerged from these vials were roughly 1200-1400 flies per population. Cold shock treatment is known to affect egg viability, and egg viability at 24-30 h after cold shock was ~35-40%. Therefore, to control the density of flies per vial for FSB 1-5 populations, 20 vials of ~100 eggs/vial were collected so that the total number of flies eclosed from these vials were ~1200-1400 files/replicate populations of FSB 1-5. For more information, see below the graphical illustration (**Fig. 8)**.

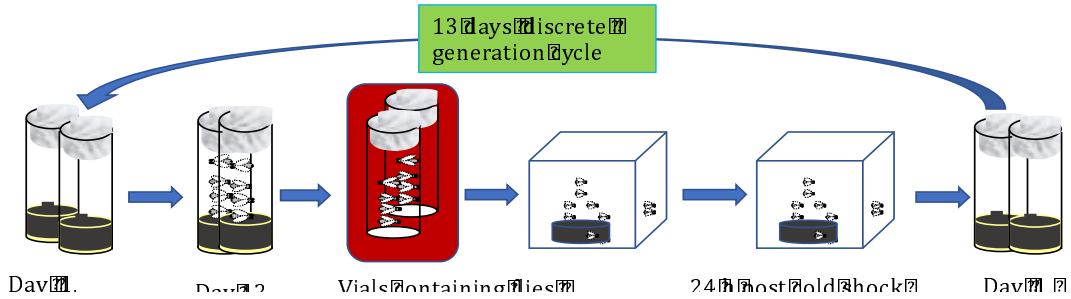

Figure 8. Maintenance of selected and control populations.

Experimental protocol

Standardization of flies

To control over the non-genetic parental effects for selected and control populations [54,55], all populations of FSB 1-5 and FCB 1-5 were reared for one generation under the common standard laboratory conditions as described below, and we did not impose any selection to FSB 1-5 and FCB 1-5 populations for one generation. This process is known as 'standardization' and the flies maintained in this manner are known as standardized flies. To standardize the flies of FSB and FCB populations, we collected eggs at a density of 70 eggs/vial in a vial with 6 mL of banana-yeast-jaggery food (standard food). We collected 20 such vials from each stock population of FSB 1-5 and FCB 1-5. On day 12, after culturing eggs, the flies from a specific population were shifted into a Plexiglas cage containing a standard food plate. In order to collect experimental eggs, on day 13 post egg collection, a standard fresh food plate was given to each of the FSB 1-5 and FCB 1-5 populations, and the flies were allowed to oviposit for 6 h. To raise experimental flies from each of the FSB 1-5 and FCB 1-5 populations, eggs were cultured at a density of 70 eggs/vial in a vial with 6 mL of standard food as specified in the below experiments. All experiments in the present study were conducted over 57 to 70 generations of the selection.

Experiment 1-2: Effect of heat shock or cold shock on the mating frequency and egg viability

To probe the effect of the selection regime on egg viability and mating frequency under heat, cold, and no shock conditions, we collected eggs at a density of 70 eggs/vial in a vial containing 6 mL of food. And 12 such vials were set up from each of the standardized flies of FSB 1-5 and FCB 1-5 populations. On day 12 post culturing of eggs, non-virgin flies were grouped at a density of 25 males and females/vial (n = 4 vials/block/treatment, therefore, total 60 vials for FSB and FCB populations were set up) 20 vials were randomly assigned to one of the following three treatments for FSB and FCB: **(a) Cold-shock:** non-virgin flies were exposed to -5°C temperature for 1 h, as defined above. Post cold shock, flies were quickly transferred to a Plexiglas cage containing a fresh food plate at a density of 100 mating pairs per cage/block (n = 5 cages for the FSB 1-5 and FCB 1-5 populations). **(b) Heat-shock:** Mated flies were exposed to 37.5°C for 1 h. Post heat shock, flies were quickly transferred to a Plexiglas cage containing a fresh food plate at a density of 100 mating pairs per cage/block (n = 5 cages for the FSB 1-5 and FCB 1-5 populations). **(c) No-shock:** Non-virgin flies were exposed to 25°C for 1 h (as described above) and subsequently flies were transferred to a Plexiglas cage at a density of 100 mating pair per Plexiglas cage (n = 5 cages for FSB 1-5 and FCB 1-5 populations). We used the same data of no shock treatment to analyze cold shock vs no shock, as well as heat shock vsno shock treatments. After the cold shock, heat shock, and no shock treatment, we measured the egg viability at 0-6 and 24-30 h from FSB and FCB population. We selected these two periods; (a) 0-6 h and (b) 24-30 h for egg viability measurements (a) because 0-6 h of egg viability would demonstrate the immediate effect of treatment, and (b) because 24-40 h of egg viability, would represent the effect of selection regime because, in their routine maintenance cycle, eggs are collected from 24-42 h after cold shock treatment to start the next generation and therefore, it is a crucial time point for the fitness of the flies. More detail of the experimental design is shown in the illustration (**Fig. 9).**

To measure the egg viability, at 0-6 and 24-30 h post cold shock, heat shock, and no shock condition, fresh standard food plates were kept in the FSB and FCB cages for flies to lay eggs for 6 h. After that, eggs at a density of ~200 eggs/plate were transferred to a Petri plate containing 1.2% agar from the standard food plate that was given to FSB or FCB populations. Following this, these plates were incubated at standard laboratory conditions as described above

for 48 h; after that, the numbers of hatched eggs were counted to compute the percent egg viability using a formula given in the bracket [Percent egg viability = (Number of hatched eggs/ Total number of eggs) × 100]. We also measured the mating frequency from **Experiment 1A**. Post cold shock, heat shock, and no shock treatment, flies of the FSB and FCB populations were quickly shifted to Plexiglas cages at a density of 100 pairs of male and female flies per cage. n = 1 cage at a density of 100 male and female flies/cage for each FSB 1-5 and FCB 1-5 populations was established. We scanned all cages every 30 minutes until 36 h post treatment to monitor the mating pairs using the same protocol reported previously[22,24]. We then summed the number of mating pairs across all 0-36 h observations for a given cage to estimate the total number of matings and used it as the unit of analysis. More detail of the experimental design is shown in the illustration (**Fig. 9)**.

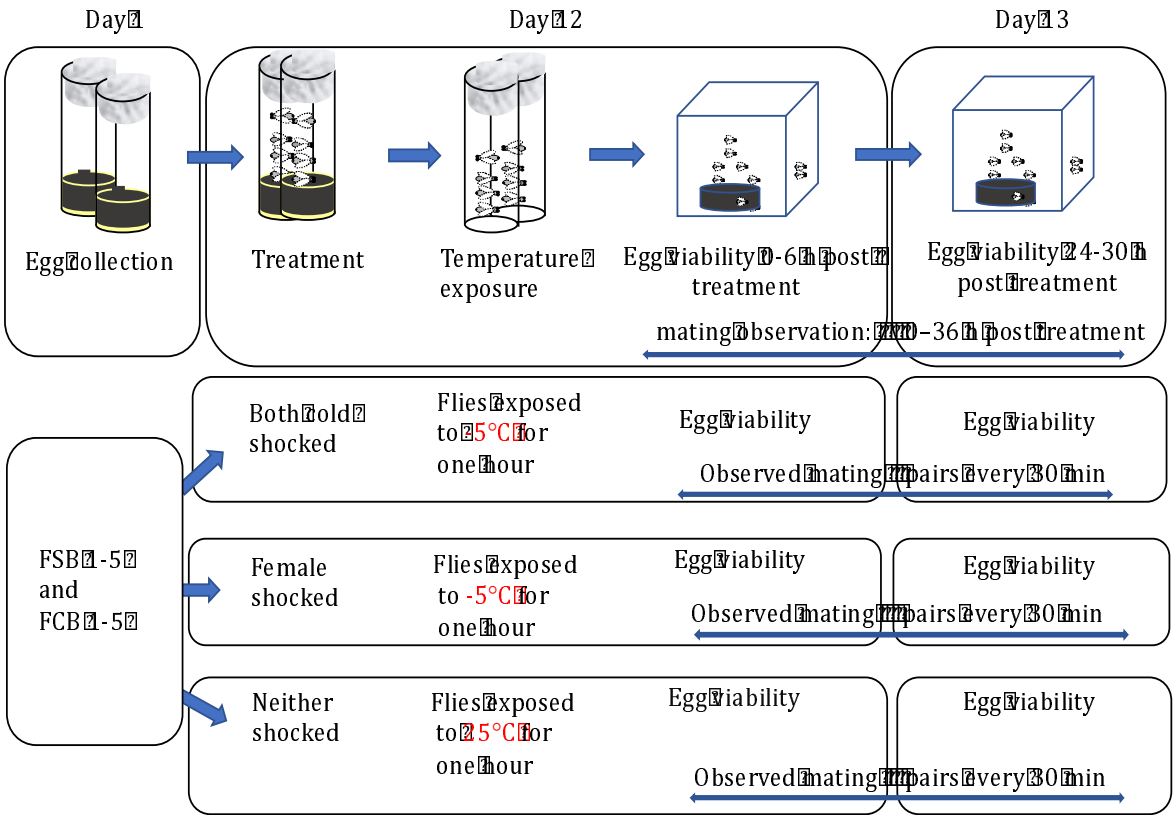

Figure 9. Assay of egg viability and mating frequency.

Experiment 3A: Effect of cold shock on adults' survival

To investigate the effect of selection regime on adult survival post cold shock. We assayed the survival under the cold shock condition after 63 generations of selection. Eggs were cultured in a standard food vial from the standardized flies of FSB 1-5 and FCB 1-5 populations. Twenty-five vials (70 eggs/vial) were set up for each replicate populations of the FSB and FCB. On the 9-10th day post culturing of eggs, virgin male and female flies were collected from the peak of eclsion using light CO_2 anesthesia at a density of 10 male or female flies/vial with 2 mL of standard food. Two-three days old virgin male or female flies were segregated in an empty glass vial at a density of 50 flies per vial, and a cotton plug was pushed deep into a vial so that flies were restricted to the 1/3 area of vial followed by female flies were exposed to -5°C for 1 h, and male flies were exposed to -5.6°C for 1 h (we used -5.6°C for males to get at least 50% mortality post cold shock). Post cold shock, male and female flies were immediately transferred to the plexiglas cage at a density of 100 male or female flies per cage. Twenty-four hours post cold shock, we monitored the adult survival. We selected this time point because 24 h post cold shock is the time when fresh food plates are given to FSB 1-5 and FCB 1-5 populations to collect eggs to initiate the next generation in their regular maintenance cycle and hence, measurement at this time is directly relevant to the fitness of the flies. Twenty-four hours post cold shock, dead flies (if any) were aspirated out of the cage and counted. Mean percent survival for each cage of the FSB and FCB populations were computed and used as the unit of analysis. Statistical significance was confirmed using a two-factor mixed model ANOVA. n = 3 replicate cages at a density of 100 male or female flies/cage for each replicate population of FSB 1-5 and FCB 1-5. Therefore, a total of about 1500 male and female flies were used in the assay from the FSB and FCB populations. For more detail of the experimental design, see the illustration (**Fig. 10)**.

Experiment 3B: High temperature tolerance

To test the heat-shock tolerance in the FSB and FCB populations, we assayed adults' survival post heat shock after 63 generations of selection. Experimental design is the same as it was defined for **Experiment 3A**. Both male and female flies of FSB and FCB populations were handled identically as described for **Experiment 3A** except for the temperature treatment. For heat shock treatment, male flies were exposed to 38.9°C temperature for 1 h in the water-bath. In contrastfemale flies were exposed to 39.2°C temperature for 1 h in the water-bath. Twenty-four hours post heat shock, dead flies (if any) were aspirated out of the cage and computed the percent survival of male and female flies. The mean percent survival of each cage was used as

the unit of analysis. Statistical significance was determined using the two-factor mixed model ANOVA. n = 3 replicate cages at density of 100 male or female flies/cage were assayed for each replicate populations of FSB 1-5 and FCB 1-5. therefore, a total of about 1500 male and female flies were used for assay from FSB and FCB populations. For more detail of the experimental design, see the illustration (**Fig. 10)**.

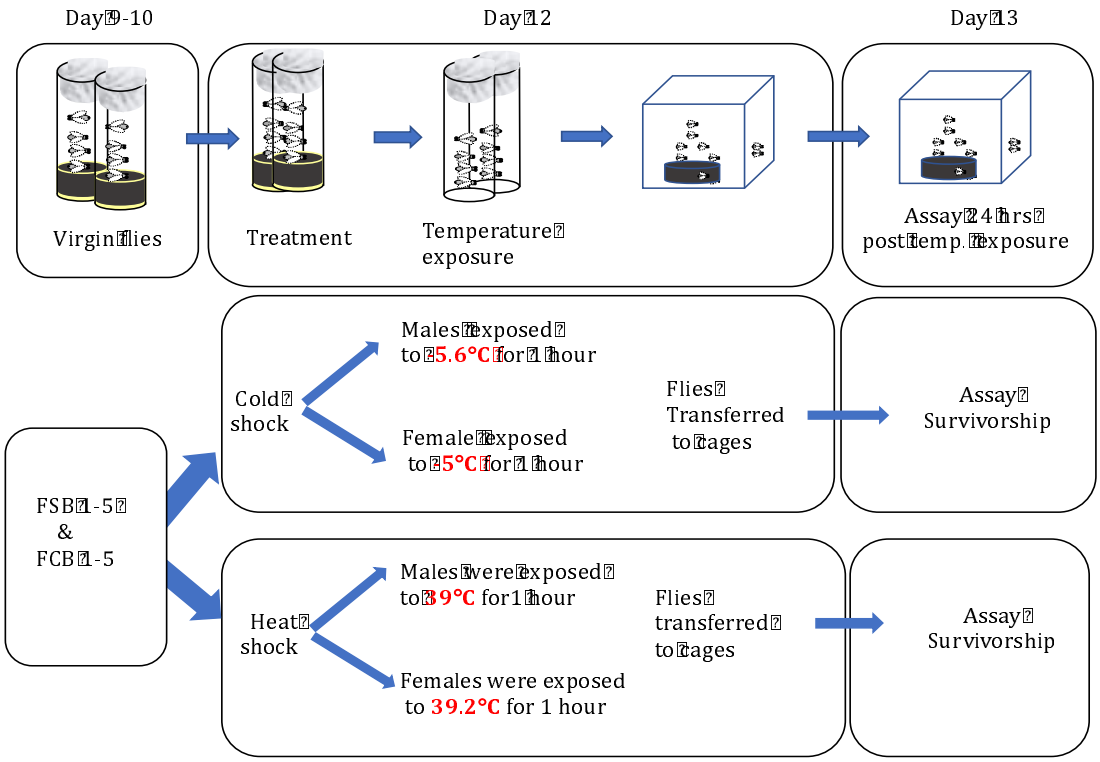

Figure 10. Adults' survival post heat and cold shock.

***Experiment 4: Adult survival post-challenge with a Staphylococcus succinus* subsp. *succinus* PK-1**

To investigate the effect of the selection regime on flies ability to survive after a bacterial infection. After 70 generations of selection, experimental flies were generated as defined above for **Experiment 3A**. On day 12 post egg collections, roughly 2-3 days old, 55-60 virgin male and female flies from each replicate populations of FSB 1-5 and FCB 1-5 populations were infected by pricking the lateral thorax with a Minutien pin (0.1 mm, fine Science Tools, Foster City, CA, USA) that was dipped in the slurry of *Ss* which had OD_{600nm} 2[21]. For sham control, the pin was dipped in 10 mM MgSO4 prior to pricking to flies' lateral thorax. The number of dead flies in each vial were monitored at every 3 h intervals until 30 h post-infection. After that, vials were observed every hour till 80 h post-infection. n = ~285-300 males and female flies from the FSB and FCB populations were used for assay. The survival data were analyzed with Cox proportional hazard model.

Experiment 5: Desiccation resistance

Sex-specific desiccation resistance assay was performed for each FSB (1-5) and FCB (1-5) populations. After 57 generations of selection, experimental flies were raised as described above in **Experiment 3A**. Two-three days old**,** virgin male or female flies at a density of 10 flies/vial were transferred from food vials to food-less vials containing ~6g of silica gel (desiccant). The flies were separated from the silica gel by a thin layer of cotton and the open end of each vial was sealed with Parafilm as described previously[56]. Mortality was monitored every half an hour until the last fly died in a given vial. n = 7 replicate vials at a density of 10 male or female flies/vial were established for each replicate populations of FSB and FCB. Therefore, about 285-300 male and female flies were used for assay from FSB and FCB populations. The statistical significance of desiccation resistance was confirmed with the Cox proportional hazard model.

Experiment 6: Starvation resistance

To determine cross-tolerance with starvation resistance in the populations selected for increased resistance to cold shock. We assayed sex-specific starvation resistance after 57 generations of selection following the previously described protocol in Kwan et al.[56] with minor modifications. Experimental flies were generated as described above in **Experiment 3A**. On day 12 post egg collections, 2-3 days old, 10 virgin male or female flies were transferred from food vial to a vial containing 1.24% agar. Flies were transferred into a fresh agar vial (1.24%) every alternate day

until the last fly died in a given vial. We traced mortality every four hours until the last fly died in a given vial. n = 7 replicate vials at a density of 10 male or female flies/vial were set up for each replicate populations of FSB and FCB. Therefore, about ~285-300 male and female flies were used for assay from FSB and FCB populations. The statistical significance of desiccation resistance data were evaluated with Cox proportional hazard model.

Statistical analysis

Experiment 1AB: Effect of heat or cold stress on the mating frequency and egg viability

Egg viability and mating frequency data of **Experiment 1A-C and 2A-C** were analyzed using a three-factor mixed model ANOVA with GLM considering selection regime (FSB vs. FCB), treatment (cold shock vs. no shock) as the fixed factors crossed with block (1-5) as a random factor. Analyses were performed using a generalized linear mixed model with binomial errors and a logit link function (i.e., logistic regression) using the R package lme4. Details of the analysis are given in the supplementary information file. Adult's survival of post heat or cold stress data from **Experiments 3A** and **B** were analyzed using a two-factor mixed model ANOVA considering selection regime (FSB vs. FCB) as a fixed factor crossed with random blocks (1-5). Survival data post infection with *Ss* (**Exp 4**), desiccation resistance (**Exp 5**), and starvation resistance (**Exp 6**) were analyzed using Cox proportional-hazard model in R package Coxme StatSoft.

List of abbreviations

FSB: Freeze shock Selected populations derived from the BRB populations

FCB: Freeze Shock Control populations derived from the BRB populations

BRB: Blue Ridge Baseline populations

ANOVA: Analysis of Variance

Hour: h

Gram: g

Milliliter: mL

Declarations

Ethics approval and consent to participate: Not applicable.

Consent for publication: Not applicable.

Availability of data and material: All data files will be available from the DRYAD database, once the manuscript is accepted.

Competing interests: All the authors declare "No Conflict of Interest."

Funding: This study was supported by the Indian Institute of Science Education and Research Mohali, Govt. of India. The funders had no role in conceptualizations, investigations, study design, data collection, analysis, decision to publish, and manuscript prepratation.

Authors 'contributions

KS: Conceptualized, designed the study, performed the experiments, analyzed the data, wrote an original draft, and edited and revised the manuscript. MAS: Contributed in performing immunity experiments, proofreading the manuscript, and analyzing the data. NGP: Analyzing the data, editing the manuscript, contributing reagents, materials, and analysis tools. All authors have read and approved the manuscript.

Acknowledgments

Karan Singh thanks the Indian Institute of Science Education and Research Mohali and the Ministry of Human Resources Development, Government of India, for financial support in the form of Junior and Senior Research Fellowships. Manas A Samant thanks the Council of Scientific and Industrial Research, Government of India, for financial support in the form of Junior and Senior Research fellowships. We also thank Megha Kothari for her assistance in the laboratory.

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Evolution of cross-tolerance in *Drosophila melanogaster* as a result of increased resistance to cold stress

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Supplementary Information

Table S1A. Immediate effect of cold shock on 0-6 h of egg viability

Cold shock (0-6 h)

anova(fit_ev_cs_0-6 h)

Type III Analysis of Variance Table with Satterthwaite's method

	Sum Sq	Mean Sq	NumDF	DenDF	F value	Pr(>F)
Selection (Sel)	2	2	1	5.2645	0.3608	0.5730
Treatment (Trt)	33185	33185	1	6.0548	5976.7529	**2.671e-10**
Sel:Trt	2	2	1	4.5212	0.3458	0.5846

p-values in bold are statistically significant.

ranova(fit_ev_cs_0-6 h)

boundary (singular) fit: see? is Singular

ANOVA-like table for random-effects: Single term deletions

Model:

Egg.viability ~ Selection + Treatment + (1 | Block) + (1 | Block:Selection) + (1 | Block:Treatment) + Selection:Treatment

	npar	logLik	AIC	LRT	Df	Pr(>Chisq)
<none>	8	-40.977	97.954			
(1 | Block)	7	-40.977	95.954	0.000000	1	0.9999
(1 | Block:Selection)	7	-40.991	95.982	0.028029	1	0.8670
(1 | Block:Treatment)	7	-41.040	96.079	0.125469	1	0.7232

Warning message:

In checkConv(attr(opt, "derivs"), opt$par, ctrl = control$checkConv, :Model failed to converge with max|grad| = 0.172747 (tol = 0.002, component 1

Table S1B. Increased egg viability at 24-30 h post cold shock in the selected populations

>anova(fit_ev_cs_24-30 h)

Type III Analysis of Variance Table with Satterthwaite's method

	Sum Sq	Mean Sq	NumDF	DenDF	F value	Pr(>F)
Selection (Sel)	1881.8	1881.8	1	4.0009	21.281	**0.009927**
Treatment (Trt)	4052.7	4052.7	1	4.0051	45.831	**0.002473**
Sel:Trt	2136.2	2136.2	1	4.0002	24.158	**0.007956**

p-values in bold are statistically significant.

ranova(fit_ev_cs_24)

boundary (singular) fit: see? is Singular

ANOVA-like table for random-effects: Single term deletions

Model: Egg.viability ~ Selection + Treatment + (1 | Block) + (1 | Block:Selection) + (1 | Block:Treatment) + Selection:Treatment

	npar	logLik	AIC	LRT	Df	Pr(>Chisq)
<none>	8	65.531	-147.06			
(1 | Block)	7	-65.531	145.06	0.00059	1	0.9807
(1 | Block:Selection)	7	-65.538	145.07	0.01371	1	0.9068

(1 | Block:Treatment)	7	-65.970	145.94	0.87831	1	0.3487

pairs(emmeans(fit_ev_cs_24, ~Selection*Treatment))

contrast	estimate	SE	df	t.ratio	p.value
FCB cold shock - FSB cold shock	-41.2	6.13	7.97	-6.728	**0.0007**
FCB cold shock - FCB no shock	-64.0	7.66	6.91	-8.357	**0.0003**
FCB cold shock - FSB no shock	-63.9	7.80	7.14	-8.192	**0.0003**
FSB cold shock - FCB no shock	-22.8	7.80	7.14	-2.918	0.0820
FSB cold shock - FSB no shock	-22.7	7.66	6.91	-2.959	0.0798
FCB no shock - FSB no shock	0.1	6.13	7.97	0.016	1.0000

p-values in bold are statistically significant.
Degrees-of-freedom method: kenward-roger
P value adjustment: tukey method for comparing a family of 4 estimates

Table S1C. Higher mating frequency in the selected populations
anova(fit_mr_cs)
Type III Analysis of Variance Table with Satterthwaite's method

	Sum Sq	Mean Sq	Num DF	Den DF	F value	Pr(>F)
Selection (Sel)	292.6	292.6	1	3.9998	22.208	**0.009223**
Treatment (Trt)	390.1	390.1	1	3.9999	29.611	**0.005537**
Sel:Trt	3537.8	3537.8	1	4.0001	268.530	**8.117e-05**

p-values in bold are statistically significant.
ranova(fit_mr_cs)
ANOVA-like table for random-effects: Single term deletions

Model:
Matings ~ Selection + Treatment + (1 | Block) + (1 | Block:Selection)
+ (1 | Block:Treatment) + Selection:Treatment

	npar	logLik	AIC	LRT	Df	Pr(>Chisq)
<none>	8	-67.088	150.18			
(1 | Block)	7	-68.157	150.31	2.1388	1	0.143616
(1 | Block:Selection)	7	-70.436	154.87	6.6979	1	**0.009653**
(1 | Block:Treatment)	7	-69.779	153.56	5.3828	1	**0.020336**

p-values in bold are statistically significant.

pairs(emmeans(fit_mr_cs, ~Selection*Treatment))

contrast	estimate	SE	df	t.ratio	p.value
FCB cold shock - FSB cold shock	-60.2	7.31	4.41	-8.233	**0.0028**
FCB cold shock - FCB no shock	5.6	6.14	4.60	0.913	0.8003
FCB cold shock - FSB no shock	-1.4	9.27	7.74	-0.151	0.9987
FSB cold shock - FCB no shock	65.8	9.27	7.74	7.102	**0.0005**
FSB cold shock - FSB no shock	58.8	6.14	4.60	9.583	**0.0012**
FCB no shock - FSB no shock	-7.0	7.31	4.41	-0.957	0.7788

p-values in bold are statistically significant.
Degrees-of-freedom method: kenward-roger
P value adjustment: tukey method for comparing a family of 4 estimates

Table S2A: Immediate effect of heat shock on egg viability of 0-6 h
>anova(fit_ev_hs_0-6 h)

Type III Analysis of Variance Table with Satterthwaite's method

	Sum Sq	Mean Sq	Num DF	Den DF	F value	Pr(>F)
Selection (Sel)	22	22	1	16	1.2643	0.2774
Treatment (Trt)	37732	37732	1	16	2146.12	**<2e-16**
Sel:Trt	3	3	1	16	0.1989	0.6616

p-values in bold are statistically significant.

ranova(fit_ev_hs_0-6 h)
boundary (singular) fit: see ?isSingular boundary (singular) fit: see ?isSingular boundary (singular) fit: see ?isSingular
ANOVA-like table for random-effects: Single term deletions

Model:
Egg.viability ~ Selection + Treatment + (1 | Block) + (1 | Block:Selection) + (1 | Block:Treatment) + Selection:Treatment

	npar	logLik	AIC	LRT	Df	Pr(>Chisq)
<none>	8	-48.857	113.71			
(1 | Block)	7	-48.857	111.71	0	1	1
(1 | Block:Selection)	7	-48.857	111.71	0	1	1
(1 | Block:Treatment)	7	-48.857	111.71	0	1	1

Table S2B. Selected population have heightened egg viability at 24-30 h post heat shock
>anova(fit_ev_hs_24-30h)
Type III Analysis of Variance Table with Satterthwaite's method

	Sum Sq	Mean Sq	NumDF	DenDF	F value	Pr(>F)
Selection (Sel)	63.00	63.00	1	8.0000	30.290	**0.0005713**
Treatment (Trt)	1186.19	1186.19	1	4.0016	570.328	**1.817e-05**
Sel:Trt	66.62	66.62	1	8.0000	32.029	**0.0004762**

p-values in bold are statistically significant.
ranova(fit_ev_hs_24-30 h)
boundary (singular) fit: see ?isSingular
ANOVA-like table for random-effects: Single term deletions

Model:
Egg.viability ~ Selection + Treatment + (1 | Block) + (1 | Block:Selection) + (1 | Block:Treatment) + Selection:Treatment

	npar	**logLik**	**AIC**	**LRT**	**Df**	**Pr(>Chisq)**
<none>	8	-43.035	102.07			
(1 | Block)	7	-43.037	100.07	0.0044	1	0.947229
(1 | Block:Selection)	7	-43.035	100.07	0.0000	1	0.999801
(1 | Block:Treatment)	7	-48.267	110.53	10.464	1	**0.001217**

p-values in bold are statistically significant.

pairs(emmeans(fit_ev_hs_24-30 h, ~Selection*Treatment))

contrast	**estimate**	**SE**	**df**	**t.ratio**	**p.value**
FCB heat shock - FSB heat shock	-7.2	0.912	8.00	-7.893	**0.0002**
FCB heat shock - FCB no shock	-65.5	2.669	4.49	-24.541	**<.0001**
FCB heat shock - FSB no shock	-65.4	2.669	4.49	-24.504	**<.0001**
FSB heat shock - FCB no shock	-58.3	2.669	4.49	-21.844	**<.0001**
FSB heat shock - FSB no shock	-58.2	2.669	4.49	-21.806	**<.0001**
FCB no shock - FSB no shock	0.1	0.912	8.00	0.110	0.9995

p-values in bold are statistically significant.
Degrees-of-freedom method: kenward-roger
P value adjustment: tukey method for comparing a family of 4 estimates

Table S2C. Increased mating frequency in the selected populations
anova(fit_mr_hs)
Type III Analysis of Variance Table with Satterthwaite's method

	Sum Sq	**Mean Sq**	**Num DF**	**Den DF**	**F value**	**Pr(>F)**
Selection (Sel)	952.2	952.2	1	8	8.1246	**0.02147** *
Treatment (Trt)	1.1	1.1	1	8	0.0094	0.92514
Sel:Trt	231.2	231.2	1	8	1.9727	0.19778

p-values in bold are statistically significant.

ranova(fit_mr_hs)
boundary (singular) fit: see? is Singular
ANOVA-like table for random-effects: Single term deletions

Model:
Matings ~ Selection + Treatment + (1 | Block) + (1 | Block:Selection) + (1 | Block:Treatment) + Selection:Treatment

	npar	logLik	AIC	LRT	Df	Pr(>Chisq)
<none>	8	-68.297	152.59			
(1 | Block)	7	-68.297	150.59	0.000	1	1.0000
(1 | Block:Selection)	7	-68.297	150.59	0.000	1	1.0000
(1 | Block:Treatment)	7	-69.384	152.77	2.173	1	0.1405

Table S3. Summary of the results from a two-factor mixed model ANOVA on adult survival in male (**S3A**) and female (**S3B**) post cold shock. And results from a two-factor mixed model ANOVA and on adult survival in male (**S3C**) and female (**S3D**) post heat shock employing selection regime (FSB and FCB) as the fixed factor crossed with blocks (1-5) as a random factor. *p*-values in bold are statistically significant.

Trait	Effect	SS	MS Num	DF Num	DF Den	*F* ratio	*P*
(**S3A**)	Selection (Sel)	0.566	0.566	1	4	40.209	0.003
Males	Block (Blk)	0.296	0.074	4	4	5.245	0.069
cold shock	Sel×Blk	0.056	0.014	4	20	2.138	0.114
(**S3B**)	Selection (Sel)	0.637	0.637	1	4	52.076	0.002
Females	Block (Blk)	0.268	0.067	4	4	5.486	0.064
cold shock	Sel×Blk	0.049	0.012	4	20	2.240	0.101
(**S3C**)	Selection (Sel)	0.154	0.154	1	4	69.272	0.001
Males	Block (Blk)	0.227	0.057	4	4	25.460	0.004
heat shock	Sel×Blk	0.009	0.002	4	20	0.206	0.932
(**S3D**)	Selection (Sel)	0.105	0.105	1	4	60.146	0.001
females	Block (Blk)	0.026	0.006	4	4	3.737	0.115
heat shock	Sel×Blk	0.007	0.002	4	20	0.630	0.647

Table S4. Estimates and 95% confidence intervals (CIs) for relative hazard rates (i. e. the exponent of the coefficients) corresponding to various fixed parameters of the Cox's proportional hazards model for **survivorship post infection**. Hazard rates are expressed relative to the default level of that fixed factor. The default level for selection regime is FCB, while the default level for sex is female. Hazard rates significantly greater than 1 correspond to poorer survivorship relative to the default level.

Survivorship post infection			
Fixed factors			
Coefficient	**Hazard ratio**	**Lower CL**	**Upper CL**
SelectionFSB	0.8149	0.6604	1.0056
SexMale	0.9528	0.6963	1.3037
SelectionFSB:SexMale	1.0309	0.7578	1.4025
Random factors			
Group	**Variance**		
Block/Sex/Selection	0.0013		
Block/Sex	0.0343		
Block	<0.0001		

Table S5. Estimates and 95% confidence intervals (CIs) for relative hazard rates (i. e. the exponent of the coefficients) corresponding to various fixed parameters of the Cox's proportional hazards model for desiccation resistance of males (A) and females (B). Hazard rates are expressed relative to the default level of that fixed factor. The default level for selection regime is FCB. Hazard rates significantly greater than 1 correspond to poorer survivorship relative to the default level.

(S5A) Male desiccation resistance			
Fixed factors			
Coefficient	**Hazard ratio**	**Lower CL**	**Upper CL**
SelectionFSB	1.1600	0.3261	4.1262
Random factors			
Group	**Variance**		
Block/Selection	1.0324		
Block	0.0714		
(S5B) Female desiccation resistance			
Fixed factors			
Coefficient	**Hazard ratio**	**Lower CL**	**Upper CL**
SelectionFSB	0.5393	0.2760	1.0539
Random factors			
Group	**Variance**		
Block/Selection	0.2770		
Block	0.2889		

Table S6. Estimates and 95% confidence intervals (CIs) for relative hazard rates (i. e. the exponent of the coefficients) corresponding to various fixed parameters of the Cox's proportional hazards model for starvation resistance of males (A) and females (B). Hazard rates are expressed relative to the default level of that fixed factor. The default level for selection regime is FCB. Hazard rates significantly greater than 1 correspond to poorer survivorship relative to the default level.

(S6A) Male starvation resistance			
Fixed factors			
Coefficient	**Hazard ratio**	**Lower CL**	**Upper CL**
SelectionFSB	2.0405	**1.1831**	**3.5192**
Random factors			
Group	**Variance**		
Block/Selection	0.1800		
Block	0.6627		
(S6B) Female starvation resistance			
Fixed factors			
Coefficient	**Hazard ratio**	**Lower CL**	**Upper CL**
SelectionFSB	1.5543	**1.0619**	**2.2749**
Random factors			
Group	**Variance**		
Block/Selection	0.0793		
Block	0.0004		