Evolution of reproductive traits have no life-history associated cost in *Drosophila melanogaster* selected for cold resistance

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Abstract

In insect species like *Drosophila melanogaster*, ability to evolve greater resistance or evolution of certain traits under specific environmental condition leads to energy trade-offs with other important life-history traits. A number of studies from multiple fields have documented the life-history associated cost. However, very few laboratory selection studies on increased resistance to cold stress have assessed the life-history associated cost.

For this study, we used replicate populations of *D. melanogaster* that have been selected for increased resistance to non lethal cold-shock and their control populations. We have previously reported that the selected populations have evolved faster reproductive recovery and higher egg-viability post cold shock. In this study we investigated the potential life-history costs of these evolved traits. To assess life-history cost, we measured longevity and life time fecundity with and without cold-shock. Development time and dry body weight were measured from the progeny of parents that were given cold-shock or no-shock treatments.

We found that there were no significant differences in longevity, life time fecundity, and male body weight between the selected and control populations. However, females from the selected populations were bigger in size compared to the control populations. These findings suggest that there is no life-history cost associated with the evolution of greater resistance to cold-shock.

INTRODUCTION

A number of ecological factors including temperature are known to vary across time and space and as a result, organisms experience different types of unfavorable environmental conditions during their lifespan. These environmental stresses can be major drivers of evolution of life-history of organisms in nature (Hoffman and Parsons, 1991, reviewed in Parsons, 2005).

Temperature is one of the fundamental ecological features of an organism's environment. Organisms can respond to extreme temperatures in various ways, like changes in their behavior patterns and physiology or life-history traits (Hoffmann and Parsons, 1991, Patton and Krebs, 2001, Fasolo and Krebs, 2004). Resources used for coping with stress are unavailable for other functions under limited resource conditions, which can lead to trade-offs across important life-history traits such as somatic maintenance and reproduction (Stearns, 1992). For example, one important way in which organisms cope with immediate changes in temperature (heat-shock and cold-shock) is by expressing heat shock proteins (HSPs). Expression of these proteins is extremely costly and is known to affect reproduction (Krebs and Loeschcke, 1994). Thus, temperature shock can affect various important life-history traits (Huey and Berrigan, 2001, Hochachka and Somero, 2002, Sinclair et al., 2003, Angilletta, 2009). Deviation from ambient temperature (where absolute fitness of an organism is maximum) drastically affects various life-history and related traits of insects (Lee and Denlinger, 1991, Voituron et al., 2002, Hoffmann et al., 2003) such as fecundity, male fertility, lifespan (Denlinger and Yocum, 1998, Bubliy and Loeschcke, 2005, Rohmer et al, 2004, reviewed in Hance, 2007, Lieshout et al., 2013, Nguyen et al., 2013, Singh et al., 2016), reproduction (Singh et al., 2016, Singh and Prasad,

2016), mating ability (Singh et al., 2015), development time (Trotta et al., 2006, Austin and Moehring, 2013) and motility (Angilletta et al., 2002)

Several studies have addressed the evolution of life-history traits in response to temperature variation. *D. melanogaster* being widely distributed offers a great model to study the evolution of life-history in response to temperature variation across latitudes and altitudes. In general, a number of traits vary progressively across populations inhabiting various latitudes. This pattern of results suggests that life-history evolution in populations of *Drosophila* is primarily being driven by environmental differences and that the populations are adapting to local environment, most probably, including temperature, which is an important component of the environment. Latitudinal clines have been found in a number of life-history traits such as development time, survivorship, larval competitive ability, fecundity and body size (Stanley and Parsons, 1981, Bouletreau-Merle et al., 1982, James and Partridge, 1995, 1998, reviewed in Hoffmann et al., 2003, Hangartner et al., 2015).

Some experimental evolution studies have addressed the evolution of life-history traits in response to selection for cold stress tolerance (Tucic, 1979, Chen and Walker, 1993, Watson and Hoffmann, 1996, Anderson et al., 2005, Bubliy and Loeschcke 2005, MacMillan et al., 2009). However, such studies are a few (Overgaard et al., 2010). Anderson et al. (2005) found increased female fecundity and decreased male longevity in populations of *D. melanogaster* selected for rapid chill-coma recovery. MacMillan et al. (2009) also found reduced longevity in females (but not in males) in populations selected for increased resistance to freeze shock. However, Bubliy and Loeschcke (2005) did not find any difference in longevity and development time in populations of *D. melanogaster* selected for

increased resistance to cold stress. Thus, the correlated evolution of life-history traits in response to cold stress has been fairly variable.

In the present study, we were interested in probing the life-history costs, if any, of increased resistance to cold stress in terms of increased reproductive behavior and egg viability (Singh et al., 2015) in populations of *D. melanogaster* selected for increased resistance to cold stress. To investigate the underlying life-history cost to increased resistance to cold stress, we assayed various life-history (longevity and life time fecundity) and related traits such as development time, and body weight in the populations of *D. melanogaster* selected for increased resistance to cold stress. These experiments were performed over 24-33 generations of selection.

Experimental protocol

Experimental populations:

Details of the maintenance and derivation of the selected (FSB; Cold shock Selected population derived from BRB population) and their control (FCB; Cold shock Control derived from BRB population) populations has been described in our earlier study (Singh et al. 2015). Briefly, after 35 generation of the laboratory adaption of BRB 1-5, we set up one FSB population and one FCB population from each of the BRB populations, for example, FSB 1 and their corresponding control FCB 1 derived from the BRB 1, similarly FSB 2 and their control FCB 2 established from the BRB2 and so on. Hence, we had five replicate populations for the selected population and five replicate of control populations. Populations carrying the same numerical subscript have originated from same base line population (BRB) and are more close to each other than any other populations. For instance, FSB 1 and FCB 1

are more close (due to the origin from the same ancestral population) to each other than FSB 2 or FCB 2 or any other population. Hence, in our statistical data analysis FSB 1 and FCB 1 are included in block 1, similarly FSB 2 and FCB 2 are included in block 2 and so on.

FSB and FCB populations are large outbred populations maintained under standard laboratory conditions (25°C temperature, 50–60% relative humidity, 12 hour light: 12 hour dark cycle, on a 13-day discrete generation cycle). These populations start eclosing on day 9, and attain peak of eclosion on day 10 post egg collection. On day 12 post egg collection, flies (flies are ~ 2-3 days old as adults and mated) are moved into empty, clean, dry glass vials (30 mm diameter × 90 mm length). After that flies belonging to the FSB populations are subjected to -5°C temperature in ice-salt-water slurry for one hour. FCB populations, on the other hand, are held at 25°C for one hour. Subsequently, all populations are quickly transferred into a separate Plexiglas cage (25 cm length × 20 cm width × 15 cm height) having a fresh food plate. Twenty four hours later a fresh food plate is given to flies in order to collect eggs to initiate next generation. For each population, 20 vials are collected at density of 70 eggs per vial containing ~ 6 ml of a fresh food.

Standardization

To account the non-genetic parental effects (Rose 1984), flies from the selected populations and from their controls were reared for one generation in common rearing environment., For one generation selection was relaxed in the selected populations and both selected and control populations were reared through common rearing protocol. This process is referred to as standardization and these flies are known as standardized flies. Details of the standardization protocol has been described previously in Singh et al. (2015). Briefly, to control eggs density,

for each selected and their control populations, 20 vials were established at density of 70 eggs per vials in ~6 ml food, reared at standard laboratory conditions (12 hours light:12 hours dark). On day 12 post egg collection, (roughly 2-3 days old as adult flies) ~ 1200-1400 flies of each population were transferred separately in a Plexiglass cage and provided a fresh food plate. These flies were further used for experiment egg collection.

Cold shock treatment for experiments

Detailed account of the cold shock protocol has been described in our previous study (Singh et al., 2015). In short, on day 12 post egg collection, (by this time flies were roughly 2-3 days old as adult and mated flies) flies were moved to clean, dry glass vials (25 mm diameter × 90 mm height) at a density of 50 individuals per vial (25 pairs of males and females) under mild carbon dioxide anesthesia. The cotton plug was inserted deep into the vial such that the flies were allowed to stay in a confined space in vial (one third of the vial). The flies were kept in an incubator to recover from carbon dioxide anesthesia for half an hour. The vials containing flies were then kept in ice-salt-water slurry maintained at -5°C and allowed to stand for one hour. Post cold shock, flies were quickly shifted to Plexiglas cages (14 cm length × 16 cm width × 13 cm height) at a controlled density of 100 pairs of males and females per cage. The cage was provided with a Petri plate containing fresh food and was maintained under standard laboratory conditions (Singh et al., 2015).

The flies from the control treatment were handled identically, except that the dry glass vials containing flies, were transferred to a water bath maintained at 25°C for one hour. The males and females were held together at a density of 100 pairs per cage (Singh et al., 2015).

Experiment: 1.1: Longevity assay

The longevity assay was performed after 24 generations of selection. Eggs were collected from standardized flies at a controlled egg density of 70 eggs/vial provisioned with ~ 6 ml of

fresh banana-yeast-jaggery food (hereafter referred to as “food”). Twenty four such type of vials were set up for each of the FSB (1-5) and FCB (1-5) populations. On day 12 after egg collection, flies were sorted (25 mating pairs per vial) under mild carbon dioxide anesthesia. After sorting, flies were divided into two sets: (a) set first for cold-shock treatment (both male and female flies were exposed to cold shock for one hour) and (b) set second for no-shock treatment (neither males and nor females were exposed to cold-shock).

(a) Cold-shock: For each population, flies contained in 12 vials (each vial contains 25 mating pairs of male and female) were subjected cold-shock (-5°C for one hour) as mentioned in the cold shock protocol. Immediately, after the cold shock, 12 vials were randomly divided into 3 sets referred to as a “replicate”. Each set having 4 vials of flies (100 mating pairs each) were transferred into a Plexiglas cage and provided a fresh food plate. Hence, each population (FSB 1-5 and FCB 1-5) had 3 replicates.

(b) No-shock: For each population, flies contained in 12 vials (each vial contain 25 mating pairs of male and female) were subjected to no-shock treatment (25°C for one hour). Post treatment, 12 vials were quickly randomly divided into three sets that were known as replicate. Each set having 4 vials containing total of 100 mating pairs of male and female flies were transferred into Plexiglas cages and provided a fresh food plate. Hence, each population (FSB 1-5 and FCB 1-5) had 3 replicates.

We set up three cages (replicate) per selection × block × treatment combination (Except block 1 of the FCB population which had 2 replicates for both-shock treatment, due to accidental death of one of the replicates during the assay). Food plate was changed every alternate day and dead flies were aspirated out and counted. Sex of the dead flies was determined under microscope on the basis of sex combs. Mortality was recorded until the last

fly died. Using the mortality data, for each cage, we measured mean longevity of males and females from the selection regime (FSB and FCB), treatment and block. For the analysis of mean longevity, cage means were used as the unit of analysis.

Experiment 1.2: Life time fecundity assay

Fecundity assay was performed along with longevity assay, using the same set of flies. We measured fecundity at every sixth day along with longevity. In order to measure fecundity, fresh food plate was placed in the Plexiglas cage for 6 hours for oviposition. After that, total number of eggs on each plate was counted under the microscope. Subsequently, fecundity per female - number of eggs divided by total number of live females at that time point- was calculated. Average fecundity of the eleven time points was calculated for three replicates for each of the FSB 1-5 and FCB 1-5 populations, and treatments. We also computed median and maximum longevity for each cage populations. The aging rate data was analyzed using the Gompertz model.

Experiment 2: Development time (first instar larva to eclosion)

Development time was assayed after 33 generations of selection. Followed by one generation of relaxation of selection known as standardization, 12 vials each were set up for FSB 1-5 and FCB 1-5 populations at a density of 70 eggs per vial. On day 12 post egg collection (by this time point flies were roughly 2-3 days old as adults), vials containing flies were randomly divided into two sets for - (a) cold-shock (b) no-shock treatment. For both ‘cold-shock’ and ‘no-shock’ treatments, flies were transferred into empty glass vials at density of ~70 flies and the cotton plug was pushed deep up to the bottom one-third of the vial. After that, the flies were subjected to cold shock or no shock treatments, following the protocol as mentioned above. Immediately after cold-shock treatment, flies (200 males and 200 females) were transferred to Plexiglas cage and provided with a fresh food plate. Twenty four hours

post cold-shock, fresh - food plates were given to each cage for one hour to lay stored eggs. After that another set of fresh plates were given for four hours. The second set of plates containing eggs were then incubated at standard laboratory conditions for 18 hours to allow eggs to hatch and first instars larvae to emerge. The larvae were collected (using a moist brush) into vials with 6 ml of a fresh food. For each population and treatment combination, 10 replicate vials were set up (each containing 30 larvae in 6 ml of food). The vials were incubated at standard laboratory conditions. The positions of the vials were randomized and moved daily within the incubator. Once pupae formed, each vial was manually scanned every 2 hours. Freshly eclosed flies were transferred into empty glass vials, sexed and counted. The flies were then flash frozen using liquid nitrogen and then transferred to -80°C for storage (and used to assess dry body weight- see below). Mean larva to eclosion development time was calculated for each vial and this vial mean time was used as the unit of analysis.

Experiment 3: Measurement of dry body weight of male and female flies

In order to measure the dry body weight, we used the same flies from the development time assay (mentioned above). Freshly eclosed flies were flash frozen and stored at -80°C until dry body weight measurement. Five flies of a given sex were grouped together, dried in a hot air oven at 65°C for 48 hours and weighed in a high precision electronic balance (Sartorius CPA225D). For each population, treatment and sex combination, ten such sets were weighed. Thus, a total of 50 males and 50 females per population and treatment were used for body weight measurement. Body weight of each group of five flies was used as the unit of analysis.

Statistical analysis

Mean longevity, development time and dry body weight of males and females were analyzed using a three-factor mixed model analysis of variance (ANOVA) treating selection regime (FSB vs. FCB), treatment (cold-shock vs. no-shock) as fixed factors crossed with a random

block (1-5). The sexes were analyzed separately. Fecundity per female was analyzed using a three-factor mixed model ANOVA treating selection regime (FSB vs. FCB) and treatment (cold-shock vs. no-shock) as fixed factors crossed with block as a random factor. All the analyses were done at α=0.05 level of significance using Statistica (for Windows, version 10, Statsoft). Multiple comparisons were carried out employing Tukey's HSD.

Rates of aging

Age dependent and age independent rate of aging was measured using the method used by Mueller et al. (1995) and Jafariet al. (2007). Raw survivorship data were used to calculate 'proportion survival' values with subsequent calculation of running average of the proportion survival data, r_x.

$$r_x = (p_x + p_{x+2})/2 \qquad (1)$$

Where, p_x is the proportion of individuals surviving at a given age x. Since mortality was monitored every alternate day, x and $x+2$ are two successive age intervals noticed. The hazard rate that is the probability of death per unit time, μ_x at age x was computed employing the following equation:

$$\mu_x = (r_x - r_{x+2})/ r_x \qquad (2)$$

According to the Gompertz equation, the mortality rate at age x is given by,

$$\mu_x = ae^{bx} \qquad (3)$$

Where, a and b represent age-independent and age-dependent rate of aging respectively. Log-hazard rate was regressed against age intervals; the intercept and the least square slope gave the estimates of *Gompertz a* and *Gompertz b* respectively. The derived parameters were analyzed using three factor mixed model ANOVA with selection regime (FSB vs. FCB), treatment (cold shock vs. no shock) as fixed factor crossed with random blocks (1-5).

RESULTS

Experiment 1.1: Longevity assay for male and female

Male and female longevity was assessed in terms of mean, median and maximum longevity. Analyses revealed that the results were similar regardless of the measure used. After 24 generations of selection, there was no significant effect of selection, treatment or selection × treatment interaction on male or female mean longevity (Table 1a, b Figure 1a, b, c, and d). Interestingly, the absence of any significant effect of treatment indicated that flies subjected to cold-shock treatment as well as the flies that were not subjected to cold-shock,.which proved that cold shock had no direct effect on mean longevity.

We found a significant effect of treatment on the *Gompertz a* (age independent mortality rate) and b (age dependent mortality rate) parameters among males. The FSB and FCB males subjected to cold-shock showed significantly higher age independent mortality but a significantly lower age dependent mortality compared to the males not subjected to cold-shock (Table 1c). The net effect of these two factors was that the average (and median) lifespan of the males subjected to cold-shock and those not subjected to cold-shock was not different. There was no effect of selection or a selection × treatment interaction on the Gompertz parameters. Among the females, none of the factors affected the Gompertz parameters (Table 1d). Thus, we found no evidence for any significant change in mean longevity or rates of aging as a correlated response to selection for increased resistance to cold-shock.

Experiment 1.2: Life time fecundity

The mean number of eggs laid per female in each of the FSB and FCB populations and treatments were computed by averaging across the 11 time points of fecundity measurement and used it as the unit of analysis. We did not observe significant effects of selection,

treatment or selection × treatment interaction on female fecundity (Table 2, Figure 2a, and 2b). Just like longevity, the absence of any significant effect of treatment on fecundity revealed that cold-shock treatment had no direct effect on lifetime fecundity.

Experiment 2: Development time (first instar larva to adult eclosion)

Unlike longevity and fecundity, selection did affect mean development time. Mean development time of males showed a significant effect of selection. (Table 3a, Figure 3a). Starting as first instar larvae, FSB male took about 2-4 hours more to emerge as adults compared to FCB males (Figure 3a). Female mean development time analysis showed that there was significant effect of selection (Table 3b, Figure3b). However, none of the other effects were significant. Just like the males, FSB females also took~3-6 hours more to emerge as adults compared to FCB females (Figure3b). Again, the cold shock experienced by the parents had no effect on offspring development time (no significant treatment effect).

Experiment 3: Dry body weight

Male mean dry body weight analysis revealed that there was no significant effect of selection, treatment or selection × treatment interaction (Table 4a, Figure 4a). In case of female dry body weight, found significant main effect of selection (Table 4b). However, there was no significant effect of treatment or selection × treatment interaction (Table 4b). Mean body weight of FSB females was about ~0.01 mg higher than that of FCB females (Figure 4b).

DISCUSSION

In this study, we assessed mean longevity, rates of aging, developmental time and dry body weight in the FSB and FCB populations with and without cold shock. Neither longevity nor fecundity was different between the FSB and FCB populations. However, we found that males and females from the FSB populations took significantly more time to develop (from

first instar larvae to adult) relative to the FCB populations. Females from the FSB populations were heavier than females from the FCB populations. However, there was no difference in male body size between the FSB and FCB populations. Taken together, our finding suggests there is no evidence for a trade-off between the ability to resist cold stress and important life-history traits.

The correlation between cold-shock resistance and longevity is variable across studies. MacMillan et al. (2009), using a selection protocol very similar to the present study found that females of the cold-shock selected populations had decreased longevity compared to females of the control populations whereas no such difference was visible in the males. According to Anderson et al. (2005) populations selected for faster chill-coma recovery had reduced lifespan compared to controls. In the contrary Norry and Loeschcke (2002) observed that cold adapted populations lived longer at 14°C and shorter at 25°C compared to control populations. Bubliy and Loeschcke (2005) found no change in female longevity between populations selected for cold resistance and their controls. In populations directly selected for increased lifespan, increased cold resistance evolved as a correlated response in adults and pupae of *D. melanogaster* (Luckinbill, 1998). In contrast to all these studies, we found that selection for resistance to cold-shock had no effect on lifespan or rates of aging. There were several possible differences including the base population used for selection, the definition of ‘cold stress’, the assay protocols, *etc*. between these studies that preclude a direct comparison of results. More importantly, other studies, typically selected for increased survivorship post cold-shock. However, in our study there was very little cold induced mortality. This is further strengthened by the fact that the lifespan of the FSB and FCB populations that were subjected to cold-shock were not different from the longevity of those populations not subjected to cold

shock. Thus, it is not surprising that longevity did not evolve in the FSB compared to FCB populations.

In several previous studies, fecundity has responded to selection for cold resistance. Anderson et al. (2005) found that at least two of the three replicates in their selection regime evolved lower fecundity. Watson and Hoffmann (1996) found that cold selected populations had lower fecundity. However, we found no difference in the life-time fecundity of in the FSB relative to the FCB populations. This is in agreement with our earlier, short-term measurement of fecundity in these two populations (Singh et al., 2015). Thus, we found no evidence of a trade-off between evolved cold stress resistance and fecundity.

Increased development time can potentially be a cost in species like *D. melanogaster* that inhabit ephemeral habitats and have to complete their development before the habitat disappears. We did find that the FSB males and females had increased developmental time. However, the magnitude of the increase was very small (~3-4 hours) and hence we are not sure whether this represented a cost. Increased development time represented an adaptation to increase resource storage that helped in coping stressful conditions. During the late third larval instar stage, *D. melanogaster* larvae feed rapidly and increased their weight exponentially (reviewed in Prasad and Joshi, 2003). An increase of ~3-4 hours of feeding time during this period drastically increased the amount of resources stored by the larvae. Accordingly, populations of *D. melanogaster* selected for increased starvation and desiccation stress resistance are known to show increased development time and increased body size (Chippindale et al., 1996, 1998). In this study, increased development time represented an adaptation to acquire necessary resources to cope with cold stress.

Body weight at eclosion is often used as a proxy for the amount of resources stored by the larvae. Anderson et al., (2005) and Watson and Hoffmann (1996) found no difference in body size of flies selected for increased cold resistance. In this study, FSB females were heavier at eclosion compared to FCB females. This indicated that FSB females were storing extra/specific nutrients to survive cold-shock. However, there was no difference in body weight between FSB and FCB males. Taken together, this indicated that at least in females, increased development time was likely to be beneficial in aspect of increased resource acquisition. It is also to be noted that in our previous study, females suffered more mortality post cold shock relative to males (Singh et al., 2015).

Absence of any change in lifespan and fecundity of the FSB populations could be because of many reasons. Firstly, the evolved cold-shock resistance ability of the FSB populations might be very cheap. Thus, the resources required to combat the effects of cold stress in our selection regime might be very low. It is a known fact that the flies in our population need to produce active gametes and mate in order to increase egg viability post cold shock. Accordingly, the FSB populations mate more often than the FCB populations post cold-shock (Singh et al., 2015, 2016, Singh and Prasad, 2016). Courtship and mating carry a substantial cost to both males and females (Wedell, 2010). Thus, the costs of evolved cold-shock resistance are expected to be substantial in our selection regime. A second alternative is that the resources are abundant and the FSB populations are able to acquire them as adults. The food used in our selection regime was indeed rich. The larval and adult densities were low. Therefore, it was possible that our flies inhabited resource-rich environment. If this is true, then assays under resource depleted condition should lead to different results. Finally, it is quite possible that the cost of increased cold resistance is paid in a different currency. While we did not find any difference in adult longevity or fecundity, other traits that we have not

measured here might have been reduced in the FSB populations. The possible set of such traits include starvation and desiccation resistance.

Conclusions

Our study revealed that there was no life-history trade-offs between increased resistance to cold-shock (in aspect of increased reproductive behaviours and egg viability post cold shock) with the longevity and with life time fecundity. We did not find any difference in fecundity and longevity between the FSB and FCB populations. The FSB populations had significantly longer pre-adult development time compared to the FCB populations. The FSB females weighed significantly more than the FCB females but no such difference was observed in the males. These results indicated that evolved stress resistance need not come at a cost of life-history traits such as fecundity and longevity. It is quite likely that the cost of increased cold stress resistance is paid in terms of reduced resistance to other stresses.

Conflict of Interest

All the authors declare "No Conflict of Interest"

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Table 1a. Effect of cold-shock on the mean longevity of males (Experiment 1.1).Summary of results from a three-factor mixed model ANOVA on the male mean longevity using selection (FCB and FSB) and treatment (cold-shock and no-shock) as fixed factors crossed with random block (1-5).

Effect	**SS**	**MS Num**	**DF Num**	**DF Den**	***F* ratio**	***p***
Selection (Sel)	33.439	33.439	1.000	4.006	1.348	0.310
Treatment (Trt)	14.549	14.549	1.000	4.008	0.752	0.435
Block (Blk)	478.234	119.559	4.000	6.177	3.024	0.107
Sel × Trt	9.169	9.169	1.000	4.032	1.972	0.232
Sel × Blk	99.283	24.821	4.000	4.000	5.350	0.067
Trt × Blk	77.398	19.349	4.000	4.000	4.170	0.098
Sel × Trt × Blk	18.559	4.640	4.000	39.000	0.422	0.792

Table 1b. Effect of cold-shock on the mean longevity of females (Experiment 1.1).Summary of results from a three-factor mixed model ANOVA on the female mean longevity using selection (FCB and FSB) and treatment (cold-shock and no-shock) as fixed factors crossed with random block (1-5). *p*-values in bold are statistically significant.

Effect	**SS**	**MS Num**	**DF Num**	**DF Den**	***F* ratio**	***p***
Selection (Sel)	56.044	56.044	1.000	4.007	2.756	0.172
Treatment (Trt)	1.581	1.581	1.000	4.004	0.047	0.839
Block (Blk)	91.967	22.992	4.000	7.012	0.443	0.775
Sel × Trt	0.160	0.160	1.000	4.071	0.084	0.786
Sel × Blk	81.399	20.350	4.000	4.000	10.751	**0.020**
Trt × Blk	133.984	33.496	4.000	4.000	17.697	**0.008**
Sel × Trt × Blk	7.571	1.893	4.000	39.000	0.192	0.941

Table 1c. Effect of cold shock on the age independent and age dependent mortality rates of males (Experiment 1.1). Summary of results from a three-factor mixed model ANOVA on (a) age independent and (b) age dependent mortality rate among males using Selection (FCB and FSB) and Treatment (cold-shock and no-shock) as fixed factors crossed with random block (1-5). *p*-values in bold are statistically significant.

Trait	**Effect**	**SS**	**MS Num**	**DF Num**	**DF Den**	***F* ratio**	***p***
(a) Male	Selection (Sel)	0.479	0.479	1.000	4.021	2.920	0.162
age	Treatment (Trt)	9.979	9.979	1.000	4.021	61.398	**0.001**
independent	Block (Blk)	8.791	2.198	4.000	0.008	126.988	0.960
Mortality	Sel × Trt	0.127	0.127	1.000	4.011	0.410	0.557
	Sel × Blk	0.656	0.164	4.000	4.000	0.531	0.723
	Trt × Blk	0.649	0.162	4.000	4.000	0.525	0.726
	Sel × Trt × Blk	1.236	0.309	4.000	39.00	1.220	0.318
(b) Male	Selection (Sel)	3.9×10^{-5}	3.9×10^{-5}	1.000	4.017	0.340	0.591
age	Treatment (Trt)	7.3×10^{-3}	7.3×10^{-3}	1.000	4.030	112.215	**<0.001**
dependent	Block (Blk)	1.4×10^{-3}	3.5×10^{-4}	4.000	9×10^{-6}	3470.59	1.000
Mortality	Sel × Trt	1.8×10^{-9}	1.8×10^{-9}	1.000	4.011	1×10^{-5}	0.998
	Sel × Blk	4.6×10^{-4}	1.1×10^{-4}	4.000	4.000	0.638	0.663
	Trt × Blk	2.6×10^{-4}	6.4×10^{-5}	4.000	4.000	0.362	0.825
	Sel × Trt × Blk	7.1×10^{-4}	1.8×10^{-4}	4.000	39.00	1.251	0.305

Table 1d. Effect of cold-shock on the age independent and age dependent mortality rates of females (Experiment 1.1).Summary of results from a three-way mixed model ANOVA on (c) age independent and (d) age dependent mortality rate among females using selection (FCB and FSB) and treatment (cold-shock and no-shock) as fixed factors crossed with random block (1-5).

Trait	**Effect**	**SS**	**MS Num**	**DF Num**	**DF Den**	***F* ratio**	***p***
(c) Female	Selection (Sel)	0.005	0.005	1.000	4.008	0.014	0.913
age	Treatment (Trt)	0.240	0.240	1.000	4.007	0.597	0.483
independent	Block (Blk)	0.600	0.150	4.000	1.491	0.366	0.822
Mortality	Sel × Trt	0.092	0.092	1.000	4.008	0.245	0.647
	Sel × Blk	1.534	0.384	4.000	4. 000	1.019	0.493
	Trt × Blk	1.611	0.403	4.000	4.000	1.07	0.475
	Sel × Trt × Blk	1.505	0.376	4.000	39.000	1.74	0.161
(d) Female	Selection (Sel)	1.4×10^{-4}	1.5×10^{-4}	1.000	4.008	0.904	0.395
age	Treatment (Trt)	5.2×10^{-4}	5.1×10^{-4}	1.000	4.012	4.646	0.097
dependent	Block (Blk)	6.9×10^{-4}	1.7×10^{-4}	4.000	1.536	1.204	0.532
Mortality	Sel × Trt	1.3×10^{-4}	1.3×10^{-4}	1.000	4.010	1.038	0.366
	Sel × Blk	6.4×10^{-4}	1.6×10^{-4}	4.000	4.000	1.265	0.413
	Trt × Blk	4.4×10^{-4}	1.1×10^{-4}	4.000	4.000	0.871	0.552
	Sel × Trt × Blk	5.1×10^{-4}	1.3×10^{-4}	4.000	39 .000	1.355	0.267

Table 2. Effect of cold shock on life time fecundity (Experiment 1.2).Summary of results from a three-factor mixed model ANOVA on the life time fecundity using selection (FCB and FSB) and treatment (cold-shock and no-shock) as fixed factors crossed with random block (1-5).

Effect	**SS**	**MS Num**	**DF Num**	**DF Den**	***F* ratio**	***p***
Selection (Sel)	0.807	0.807	1.000	4.002	0.934	0.389
Treatment (Trt)	3.078	3.078	1.000	4.003	5.782	0.074
Block (Blk)	7.899	1.975	4.000	5.661	1.610	0.292
Sel × Trt	0.134	0.134	1.000	4.010	0.778	0.428
Sel × Blk	3.464	0.866	4.000	4.000	5.030	0.073
Trt × Blk	2.132	0.533	4.000	4.000	3.097	0.150
Sel × Trt × Blk	0.689	0.172	4.000	39.000	1.418	0.246

Table 3a. Effect of cold-shock on parents on the male developmental time (larvae to adult eclosion) (Experiment 2).Summary of results from a three-factor mixed model ANOVA on the mean larva to adult development time of males using selection (FCB and FSB) and treatment (cold-shock and no-shock) as fixed factors crossed with random block (1-5). *p*-values in bold are statistically significant.

Effect	**SS**	**MS Num**	**DF Num**	**DF Den**	***F* ratio**	***p***
Selection (Sel)	758.240	758.240	1.000	4.000	17.449	**0.014**
Treatment (Trt)	214.335	214.335	1.000	4.000	0.601	0.482
Block (Blk)	141.786	35.446	4.000	3.993	0.098	0.977
Sel × Trt	54.776	54.776	1.000	4.000	1.404	0.302
Sel × Blk	173.816	43.454	4.000	4.000	1.114	0.460
Trt × Blk	1427.513	356.878	4.000	4.000	9.150	**0.027**
Sel × Trt × Blk	156.012	39.003	4.000	180.000	1.111	0.353

Table 3b. Effect of cold-shock on parent on the female developmental time (larvae to adult eclosion) (Experiment 2).Summary of results from a three-factor mixed model ANOVA on mean larva to adult development time of females using selection (FCB and FSB) and treatment (cold-shock and no-shock) as fixed factors crossed with random block (1-5). *p*-values in bold are statistically significant.

Effect	**SS**	**MS Num**	**DF Num**	**DF Den**	***F* ratio**	***p***
Selection (Sel)	907.888	907.888	1.000	4.000	8.374	**0.044**
Treatment (Trt)	206.835	206.835	1.000	4.000	0.453	0.538
Block (Blk)	950.196	237.549	4.000	1.019	0.855	0.658
Sel × Trt	204.729	204.729	1.000	4.000	0.712	0.446
Sel × Blk	433.683	108.421	4.000	4.000	0.377	0.816
Trt × Blk	1828.026	457.007	4.000	4.000	1.590	0.332
Sel × Trt × Blk	1149.930	287.483	4.000	180.000	1.840	0.123

Table 4a. Effect of cold-shock on the dry body weight of male (Experiment 3).Summary of results from a three-factor mixed model ANOVA on the mean dry body weight of males using selection (FCB and FSB) and treatment (cold-shock and no-shock) as fixed factors crossed with random block (1-5). *p*-values in bold are statistically significant.

Effect	**SS**	**MS Num**	**DF Num**	**DF Den**	***F* ratio**	***p***
Selection (Sel)	8.2×10^{-5}	8.2×10^{-5}	1.000	4.000	0.178	0.694
Treatment (Trt)	7×10^{-4}	7×10^{-4}	1.000	4.000	0.701	0.450
Block (Blk)	6×10^{-3}	1.5×10^{-3}	4.000	6.553	1.050	0.450
Sel × Trt	1.2×10^{-5}	1.2×10^{-5}	1.000	4.000	0.240	0.650
Sel × Blk	1.8×10^{-3}	4.6×10^{-4}	4.000	4.000	9.550	**0.025**
Trt × Blk	4×10^{-3}	1×10^{-3}	4.000	4.000	20.974	**0.006**
Sel × Trt × Blk	2×10^{-4}	4.8×10^{-5}	4.000	180.000	0.145	0.965

Table 4b. Effect of cold shock on the dry body weight of female (Experiment 3).Summary of results from a three-factor mixed model ANOVA on the mean dry body weight of females using selection (FCB and FSB) and treatment (cold-shock and no-shock) as fixed factors crossed with random Blocks (1-5). *p*-values in bold are statistically significant.

Effect	**SS**	**MS Num**	**DF Num**	**DF Den**	***F* ratio**	***p***
Selection (Sel)	6.7×10^{-3}	6.7×10^{-3}	1.000	4.000	32.942	**0.005**
Treatment (Trt)	3×10^{-4}	3×10^{-4}	1.000	4.000	0.287	0.621
Block (Blk)	1.4×10^{-2}	3.4×10^{-3}	4.000	3.756	3.620	0.128
Sel × Trt	2×10^{-4}	2×10^{-4}	1.000	4.000	1.199	0.335
Sel × Blk	8×10^{-4}	2×10^{-4}	4.000	4.000	1.059	0.479
Trt × Blk	3.7×10^{-3}	9×10^{-4}	4.000	4.000	4.828	0.078
Sel × Trt × Blk	8×10^{-4}	2×10^{-5}	4.000	180.000	0.491	0.743

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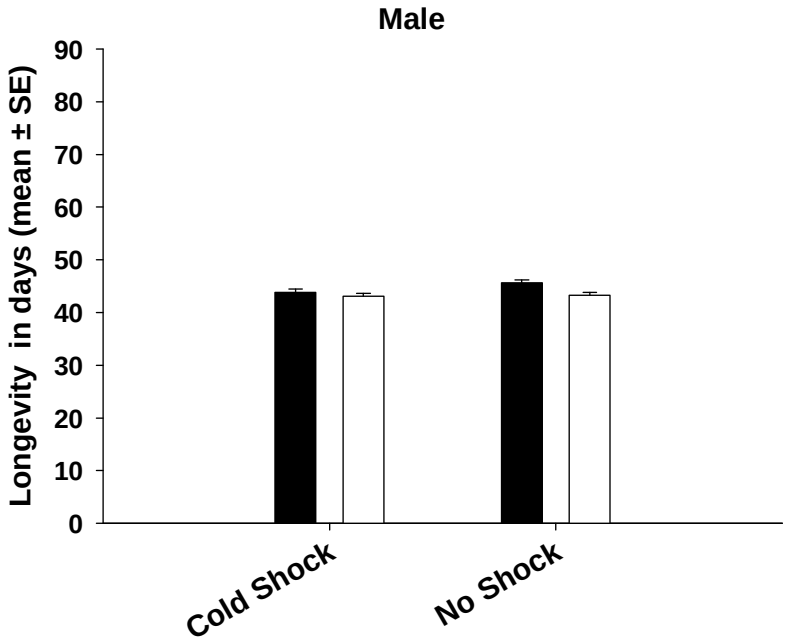

Figure 1a: Mean longevity of the FSB and FCB males after being subjected to cold-shock or no-shock treatment (Experiment 1.1). Selection, treatment or selection × treatment interaction did not have significant effect on male mean longevity. Open bars represent the FSB and closed bars represent the FCB populations.

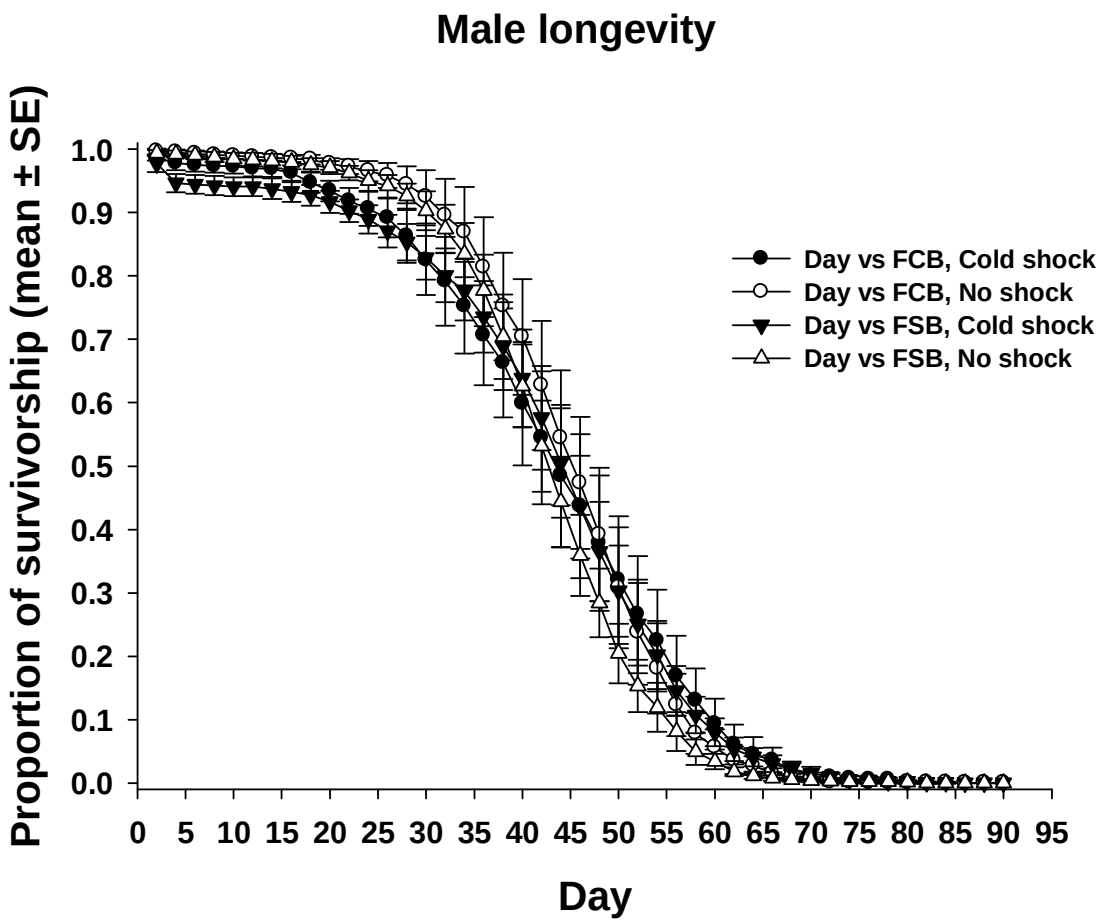

Figure 1b: Male survivorship across ages (Experiment 1.1). Longevity was assayed after adult flies were subjected to cold-shock or no-shock treatment. There was no difference in the mean, median and maximum longevity of the FSB and FCB males. There was no significant difference in the *Gompertz* parameters between the FSB and FCB populations.

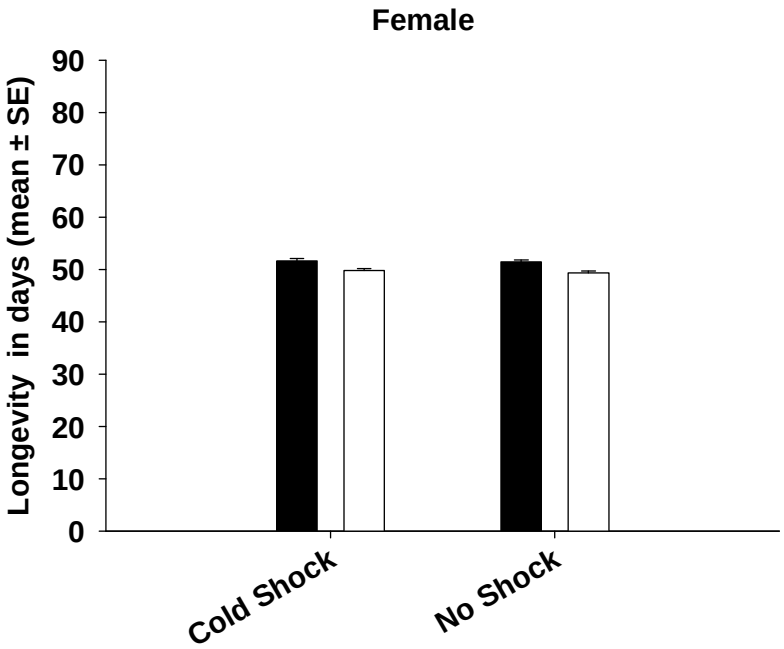

Figure 1c: Mean longevity of the FSB and FCB females after being exposed to cold shock or no shock treatment (Experiment 1.1). Selection, treatment or selection × treatment interaction did not have significant effect on female mean longevity. Open bars represent the FSB and closed bars represent the FCB populations.

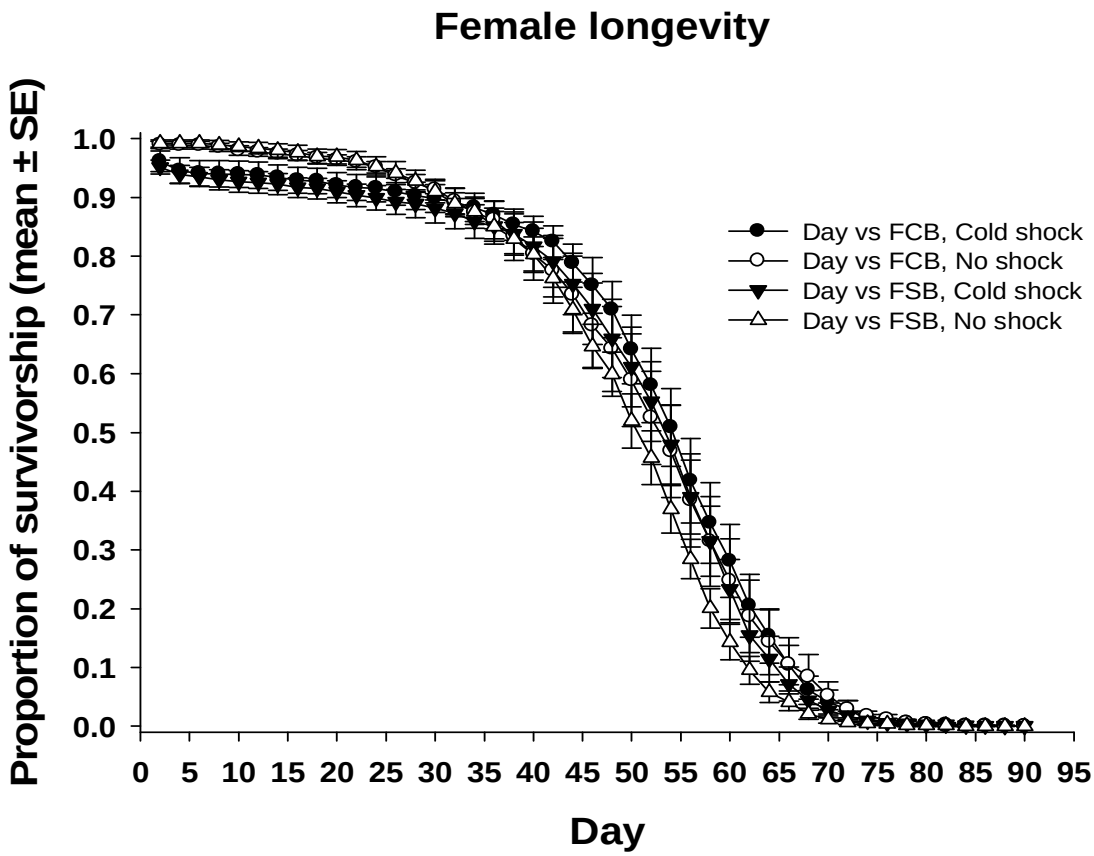

Figure 1d: Female survivorship across ages (Experiment 1.1). Longevity was assayed after adult flies were subjected to cold-shock or no-shock treatment. There was no difference in the mean, median and maximum longevity of the FSB and FCB females. There was no significant difference in the *Gompertz* parameters between FSB and FCB populations.

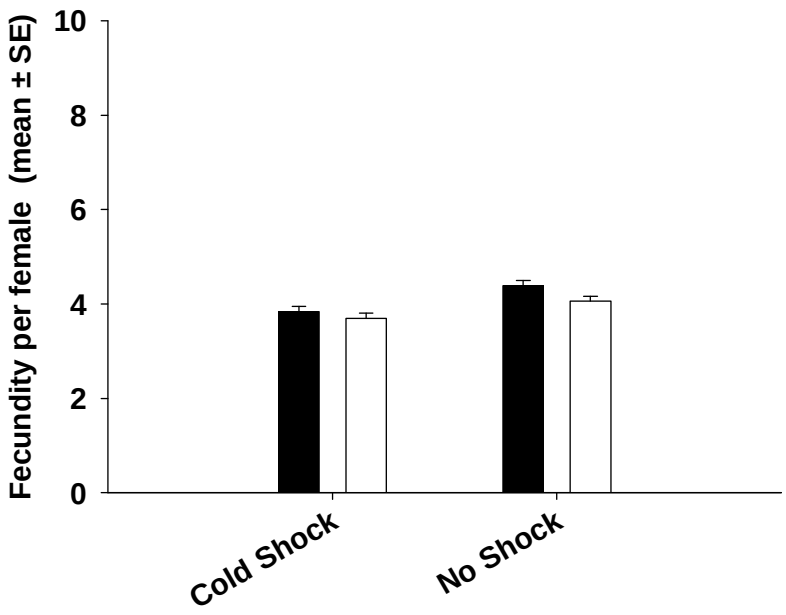

Figure 2a: Mean life time fecundity per female (Experiment 1.2). Fecundity was measured at eleven time points once in every 6 days and mean of eleven time points for fecundity was computed. Selection, treatment or selection × treatment interaction did not have significant effect on fecundity. Open bars represent the FSB populations and closed bars represent the FCB populations.

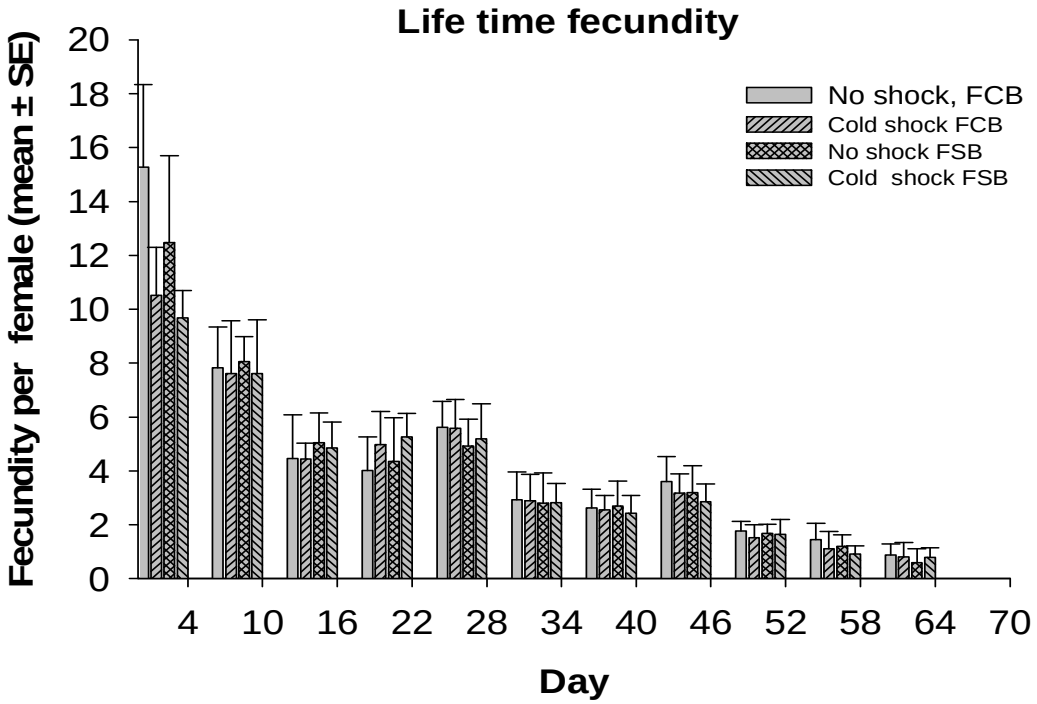

Figure 2b: Life time fecundity per female (Experiment 1.2). Fecundity was measured at eleven time points once in every 6 days. Mean fecundity per female for each population and treatment was computed for eleven time points. Results indicate that fecundity reduces with age. However, none of the other effects were significant.

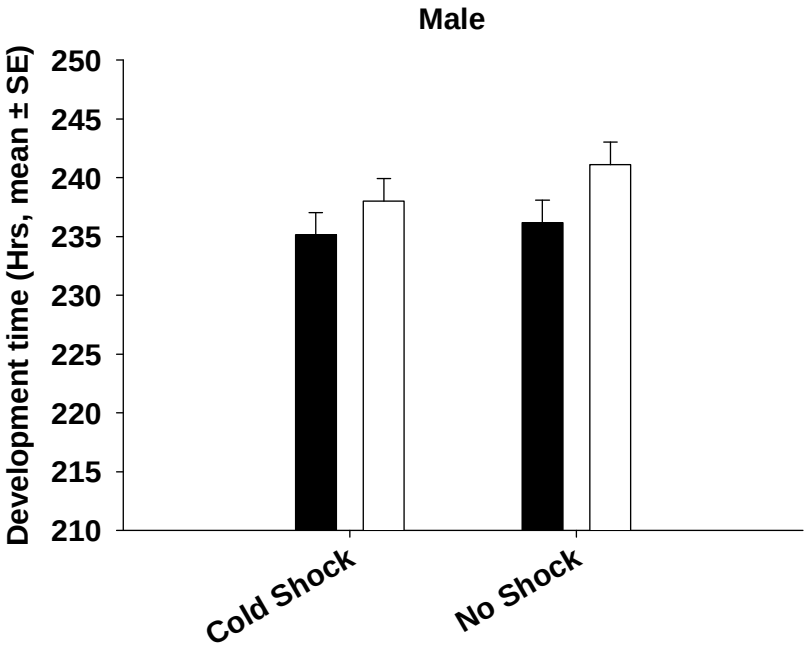

Figure 3a: Mean development time (larva to adult) of the FSB and FCB males when their parents were subjected cold-shock or no-shock treatments (Experiment 2). We found a significant effect of selection regime with the FSB males developing 3-4 hours slower than FCB males. Treatment had no significant effect. Open bars represent the FSB populations and closed bars represent the FCB populations.

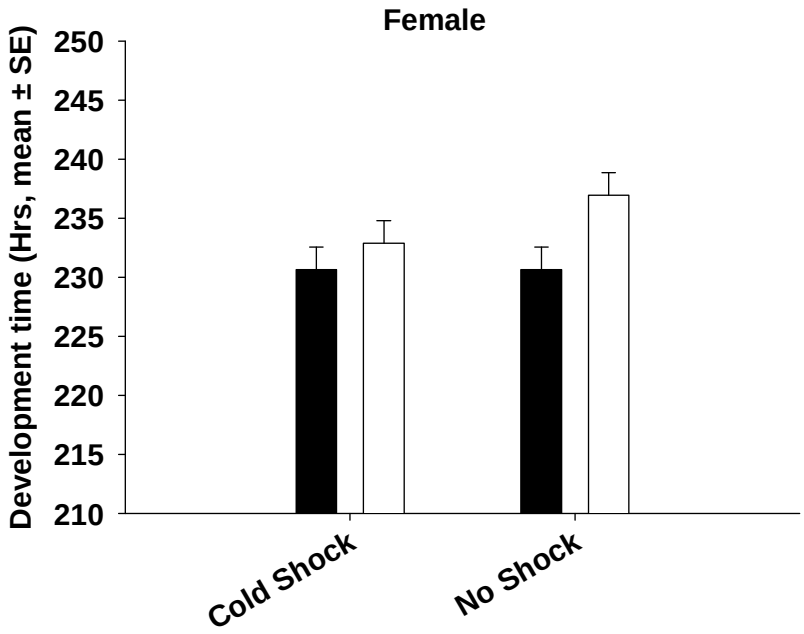

Figure 3b: Mean development time (larva to adult) of the FSB and FCB females when their parents were subjected cold-shock or no-shock treatments (Experiment 2). We found a significant effect of Selection regime with FSB females developing 3-4 hours slower than FCB females. Treatment had no significant effect. Open bars represent the FSB populations and closed bars represent the FCB populations.

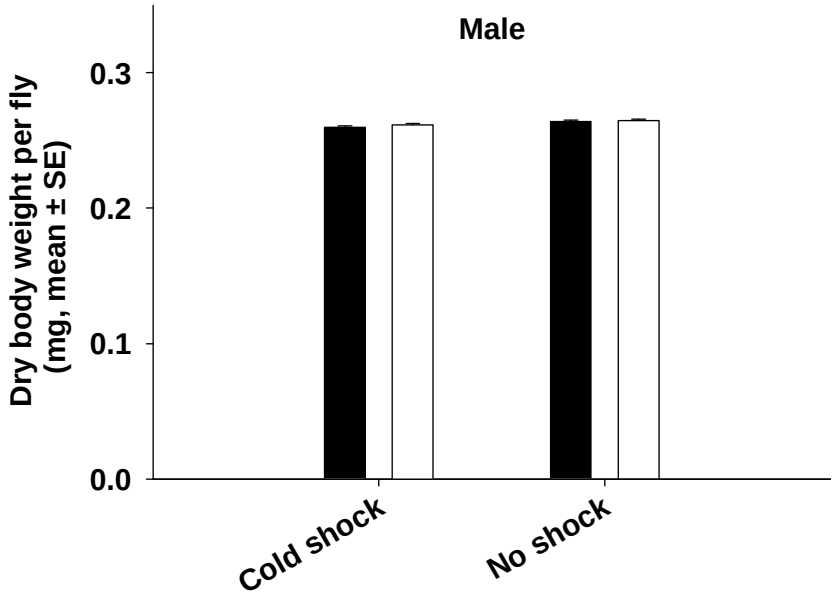

Figure 4a: Dry weight at eclosion of males from the FSB and FCB populations (Experiment 3). Selection, treatment or selection × treatment interaction did not have significant effect on mean dry body weight. Open bars represent the FSB and closed bar represent the FCB populations.

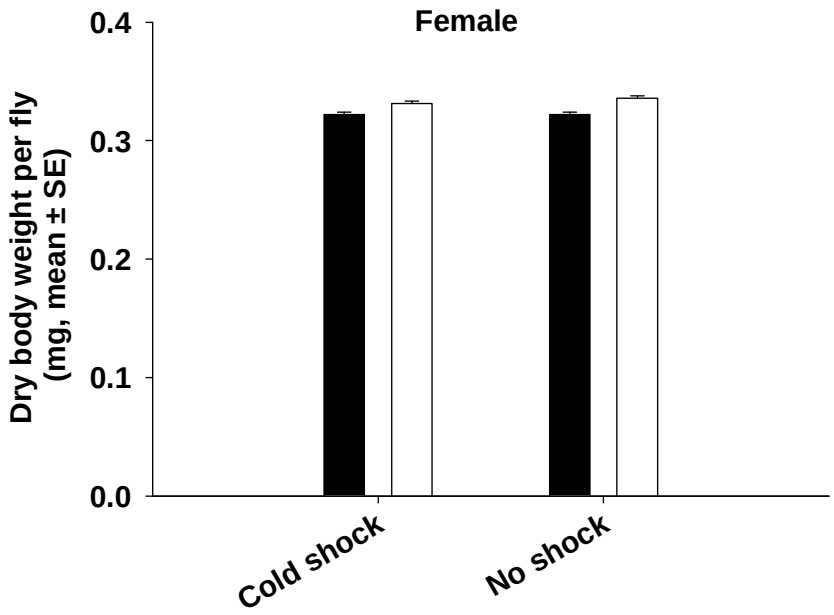

Figure 4b: Dry weight at eclosion of females from the FSB and FCB populations. Selection had significant effect on mean dry body weight (Experiment 3). However, treatment or selection × treatment did not have significant effects on mean dry body weight. Open bars represent the FSB and closed bar represent the FCB populations.