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

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Abstract

In insect species like *Drosophila melanogaster*, the ability to evolve greater resistance or evolution of certain traits under specific environmental conditions 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, no known studies have assessed the life-history associated cost with evolved reproductive traits and increase egg viability in cold shock selected population. To explore this, we used replicate populations of *D. melanogaster* that have evolved reproductive traits and egg viability in response to increased resistance to non lethal cold shock. To assess life-history cost; we measured longevity, life time fecundity, Larvae to adults development time, and larvae to adults survival. We found that there were no significant differences in longevity, life time fecundity, larvae to adults survival, and male body weight between the selected and control populations. However, selected populations have significantly longer pre adults developmental time compared to their control population. 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 in the aspect of faster recovery of egg viability and reproductive traits post cold-shock. It quite possible the cost of the evolution of reproductive traits and egg viability in response to cold shock resistant is paid in terms of reduced resistance to other stresses

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 investigated the evolution of life-history traits in response to thermal variation. *D. melanogaster* being widely distributed offers a great model to study the evolution of life-history traits 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 investigated 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 documented reduced longevity in females (but not in males) in populations selected for increased resistance to freeze shock. However, Bubliy and Loeschcke (2005) did not notice any difference in longevity and development time in populations of *D. melanogaster* selected for increased cold tolerance. Thus, the correlated evolution of life-history traits in response to cold stress has been fairly variable.

In this study, our aim in probing the life-history costs, if any, of increased resistance to cold stress in terms of increased reproductive traits and egg viability (Singh et al., 2015, Singh et al. 2016, Singh and Prasad 2016) in populations of *D. melanogaster* selected for cold shock resistance. 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.

MATERIALS AND METHODS:

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 explained previously (Singh et al. 2015). Briefly, after 35 generation of the laboratory adaption of BRB 1-5, one FSB population and one FCB population were established 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

126 are included in block 1, similarly FSB 2 and FCB 2 are included in block 2 and so on. FSB
127 and FCB populations are large outbred populations maintained under standard laboratory
128 environment (25°C temperature, 50–60% relative humidity, 12 hour light: 12 hour dark cycle,
129 on a 13-day discrete generation cycle). On day 12 post egg collection, flies (flies are roughly
130 2-3 days old as adults and mated) are moved into empty, clean, dry glass vials (30 mm
131 diameter × 90 mm length). After that flies belonging to the FSB populations are subjected to -
132 5^{o}C temperature in ice-salt-water slurry for one hour. FCB populations, on the other hand, are
133 held at 25^{o}C for one hour. Subsequently, all populations are quickly moved into a separate
134 Plexiglass cage (25 cm length × 20 cm width × 15 cm height) having a fresh food plate. After
135 24 hour a fresh food plate is given to flies in order to collect eggs to initiate next generation.
136 For each population, 20 vials are collected at density of 70 eggs per vial containing ~ 6 ml of
137 a fresh food.
138
139
140 **Standardization**
141 To account the non-genetic parental effects (Rose 1984), flies from the selected populations
142 and from their controls were reared for one generation in common rearing environment. This
143 method is referred to as standardization and these flies are known as standardized flies. A
144 detail of the standardization of the protocol has been described earlier in Singh et al. (2015).
145 Shortly, to control eggs density, for each selected and their control populations, 20 vials were
146 established at density of 70 eggs per vials in ~6 ml food, reared at standard laboratory
147 conditions (12 hours light:12 hours dark). On day 12 after egg collection, (roughly 2-3 days
148 old as adult flies) ~ 1200-1400 flies of each population were transferred separately in a
149 Plexiglass cage and provided a fresh food plate. These flies were further used for experiment
150 egg collection.

151

152 **Cold shock treatment for experiments**

153 Detailed account of the cold shock protocol has been described in our previous study (Singh
154 et al., 2015). In short, on day 12 post egg collection, (by this time flies were roughly 2-3 days
155 old as adult and mated flies) 25 pairs of males and females were moved to clean, dry glass
156 vials under mild carbon dioxide anesthesia. The cotton plug was inserted deep into the vial
157 such that the flies were allowed to stay in a confined space in vial (1/3 of the vial). The flies
158 were kept in an incubator to recover from carbon dioxide anesthesia for half an hour. The
159 vials containing flies were then kept for one hour in ice-salt-water slurry maintained at -5°C.
160 Post cold shock, flies were quickly shifted to Plexiglass cages (14 cm length × 16 cm width ×
161 13 cm height. The cage was provided with a food Plate and was kept under standard
162 laboratory conditions (Singh et al., 2015). The control treatment flies were handled similar
163 way, except that the vials containing flies, were kept in a water bath that maintained at 25°C
164 for one hour.

165

166 **Experimental details**

167 **Experiment: 1.1: Longevity assay**

168 The longevity assay was performed after 24 generations of selection. Eggs were collected
169 from standardized flies at a controlled egg density of 70 eggs/vial provisioned with ~ 6 ml of
170 fresh banana-yeast-jaggery food (hereafter referred to as “food”). Twenty four such type of
171 vials were set up for each of the FSB (1-5) and FCB (1-5) populations. On day 12 after egg
172 collection, flies were sorted (25 mating pairs per vial) under mild carbon dioxide anesthesia.
173 After sorting, flies were divided into two sets: (a) set first for cold-shock treatment (both male
174 and female flies were exposed to cold shock for one hour) and (b) set second for no-shock
175 treatment (neither males and nor females were exposed to cold-shock).

176

(a) Cold-shock: For each population, flies contained in 12 vials (each vial contains 25 mating pairs of male and female) were imposed cold-shock (-5^{o}C for one hour) as mentioned in the cold shock protocol. Quickly, 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 moved into a Plexiglass cage and given 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^{o}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 moved into Plexiglas cages and given a fresh food plate. Hence, each population (FSB 1-5 and FCB 1-5) had 3 replicates.

We established three replicate cages 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 48 hours internal and dead flies were aspirated out and computed. 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 common rearing environment or standard laboratory condition (no selection was imposed on FSB and FCB population), 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 after egg collection, 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 Plexiglass cage and provided with a fresh food plate. Twenty four hours post cold-shock, fresh - food plates were given to each cage for 1 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

229 randomized and moved daily within the incubator. Once pupae formed, each vial was
230 manually scanned every 2 hours. Freshly eclosed flies were transferred into empty glass vials,
231 sexed and counted. The flies were then flash frozen using liquid nitrogen and then transferred
232 to -80^{o}C for storage used to assess dry body weight.. Mean larva to eclosion development
233 time was computed for each vial and this vial mean time was considered as the unit of
234 analysis.

235

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

237 In order to measure the dry body weight, we used the same flies from the development time
238 assay (mentioned above). Freshly eclosed flies were flash frozen using liquid nitrogen and
239 stored at -80^{o}C until dry body weight measurement. Five flies of a given sex were grouped
240 together, dried in a hot air oven at 65^{o}C for 48 hours and weighed. For each population,
241 treatment and sex combination, ten such sets were weighed. Thus, a total of 50 males and 50
242 females per population and treatment were used for body weight measurement. Body weight
243 of each group of five flies was considered as the unit of analysis.

244

245 **Experiment 4: Larvae to adults survival:**

246 To investigate larvae to adults survival, we monitored total number of flies eclosed from the
247 cultured larval vial at density of 30 larvae/vial. We calculated percentage of larvae to adult
248 survival using the equation in given a bracket (percentage of larvae to adults survival =
249 (number of eclosed flies in a vial/total number of larvae cultured in a vial)*100).

250

251 **Statistical analysis**

252 Mean longevity, development time, dry body weight of males and females, and larvae to
253 adults survival were analyzed using a three-factor mixed model analysis of variance
254 (ANOVA) treating selection regime (FSB vs. FCB), treatment (cold-shock vs. no-shock) as a
255 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.

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

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:

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

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

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

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,

310 treatment or selection × treatment interaction on female fecundity (Table 2, Figure 2a, and
311 2b). Just like longevity, the absence of any significant effect of treatment on fecundity
312 revealed that cold-shock treatment had no direct effect on lifetime fecundity.

313

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

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

323

324 **Experiment 3: Dry body weight**

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

330

331 **Experiment 4: larvae to adults survival**

332 Mean larvae to adult survivals analysis shown that there was no significant effect of selection
333 or selection × treatment interaction (Table 5, Figure 5).

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336

337 **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

363 cold-shock. However, in our study there was very little cold induced mortality. This is further
364 strengthened by the fact that the lifespan of the FSB and FCB populations that were subjected
365 to cold-shock were not different from the longevity of those populations not subjected to cold
366 shock. Thus, it is not surprising that longevity did not evolve in the FSB compared to FCB
367 populations.
368
369 In several previous studies, fecundity has responded to selection for cold resistance.
370 Anderson et al. (2005) found that at least two of the three replicates in their selection regime
371 evolved lower fecundity. Watson and Hoffmann (1996) found that cold selected populations
372 had lower fecundity. However, we found no difference in the life-time fecundity of in the
373 FSB relative to the FCB populations. This is in agreement with our earlier, short-term
374 measurement of fecundity in these two populations (Singh et al., 2015). Thus, we found no
375 evidence of a trade-off between evolved cold stress resistance and fecundity.
376
377 Increased development time can potentially be a cost in species like *D. melanogaster* that
378 inhabit ephemeral habitats and have to complete their development before the habitat
379 disappears. We did find that the FSB males and females had increased developmental time.
380 However, the magnitude of the increase was very small (~3-4 hours) and hence we are not
381 sure whether this represented a cost. Increased development time represented an adaptation to
382 increase resource storage that helped in coping stressful conditions. During the late third
383 larval instar stage, *D. melanogaster* larvae feed rapidly and increased their weight
384 exponentially (reviewed in Prasad and Joshi, 2003). An increase of ~3-4 hours of feeding
385 time during this period drastically increased the amount of resources stored by the larvae.
386 Accordingly, populations of *D. melanogaster* selected for increased starvation and
387 desiccation stress resistance are known to show increased development time and increased

388 body size (Chippindale et al., 1996, 1998). In this study, increased development time
389 represented an adaptation to acquire necessary resources to cope with cold stress.
390
391 Body weight at eclosion is often used as a proxy for the amount of resources stored by the
392 larvae. Anderson et al., (2005) and Watson and Hoffmann (1996) found no difference in body
393 size of flies selected for increased cold resistance. In this study, FSB females were heavier at
394 eclosion compared to FCB females. This indicated that FSB females were storing
395 extra/specific nutrients to survive cold-shock. However, there was no difference in body
396 weight between FSB and FCB males. Taken together, this indicated that at least in females,
397 increased development time was likely to be beneficial in aspect of increased resource
398 acquisition. It is also to be noted that in our previous study, females suffered more mortality
399 post cold shock relative to males (Singh et al., 2015).
400
401 Absence of any change in lifespan and fecundity of the FSB populations could be because of
402 many reasons. Firstly, the evolved cold-shock resistance ability of the FSB populations might
403 be very cheap. Thus, the resources required to combat the effects of cold stress in our
404 selection regime might be very low. It is a known fact that the flies in our population need to
405 produce active gametes and mate in order to increase egg viability post cold shock.
406 Accordingly, the FSB populations mate more often than the FCB populations post cold-shock
407 (Singh et al., 2015, 2016, Singh and Prasad, 2016). Courtship and mating carry a substantial
408 cost to both males and females (Wedell, 2010). Thus, the costs of evolved cold-shock
409 resistance are expected to be substantial in our selection regime. A second alternative is that
410 the resources are abundant and the FSB populations are able to acquire them as adults. The
411 food used in our selection regime was indeed rich. The larval and adult densities were low.
412 Therefore, it was possible that our flies inhabited resource-rich environment. If this is true,

413 then assays under resource depleted condition should lead to different results. Finally, it is
414 quite possible that the cost of increased cold resistance is paid in a different currency. While
415 we did not find any difference in adult longevity or fecundity, other traits that we have not
416 measured here might have been reduced in the FSB populations. The possible set of such
417 traits include starvation and desiccation resistance.

418

419 **CONCLUSIONS**

420

421 Our findings revealed that there is no life-history trade-offs between increased resistance to
422 cold-shock (in aspect of increased reproductive traits and egg viability post cold shock) with
423 life history traits i.e. the longevity, life time fecundity, larvae to adults survival, and larvae to
424 pre adults developmental time, which indicated that evolved cold stress resistance need not
425 come at a cost of life-history traits. It is quite possible that the cost of increased cold stress
426 resistance is paid in terms of reduced resistance to other stresses.

427

428 **Conflict of Interest**

429 **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)	0.006	0.006	1	4.001	0.004	0.955
Treatment (Trt)	4.264	4.264	1	4.009	24.427	**0.007**

Block (Blk)	13.135	3.284	4	1.650	2.639	0.328
Sel × Trt	0.017	0.017	1	4.002	0.023	0.886
Sel × Blk	7.161	1.790	4	4	2.485	0.199
Trt × Blk	0.698	0.174	4	4	0.242	0.901
Sel × Trt × Blk	2.881	0.720	4	39	5.699	**0.001**

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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Table 5. Effect of cold shock on the larvae to adults survivals (Experiment 4).Summary of results from a three-factor mixed model ANOVA on the mean larvae to adults survivals considering 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	Prob > F
Selection (Sel)	37.556	37.556	1	4	2.449	0.193
Treatment (Trt)	128.000	128.000	1	4	3.578	0.132
Block (Blk)	1270.889	317.722	4	1.918	10.417	0.096
Sel × Trt	107.556	107.556	1	4	5.218	0.084
Sel × Blk	61.333	15.333	4	4	0.744	0.609
Trt × Blk	143.111	35.778	4	4	1.736	0.303
Sel × Trt × Blk	82.444	20.611	4	180	1.013	0.402

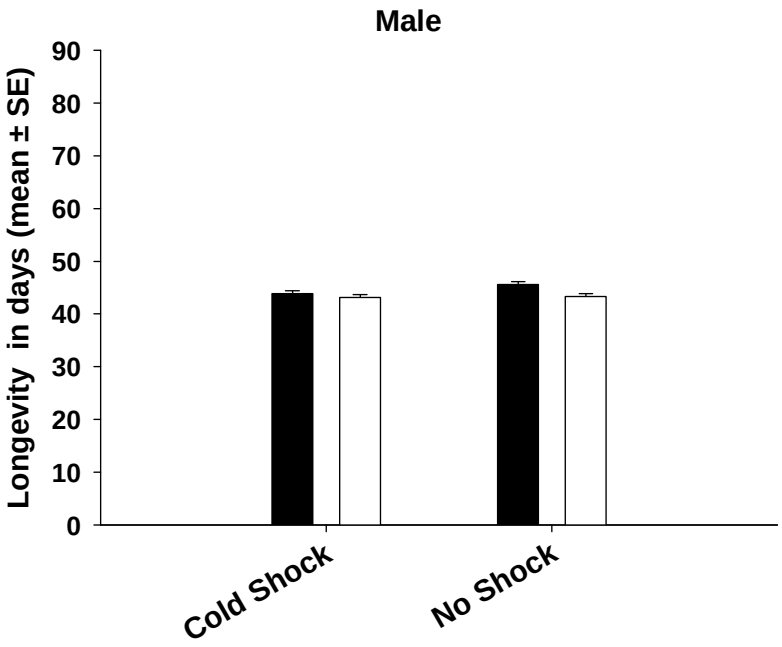
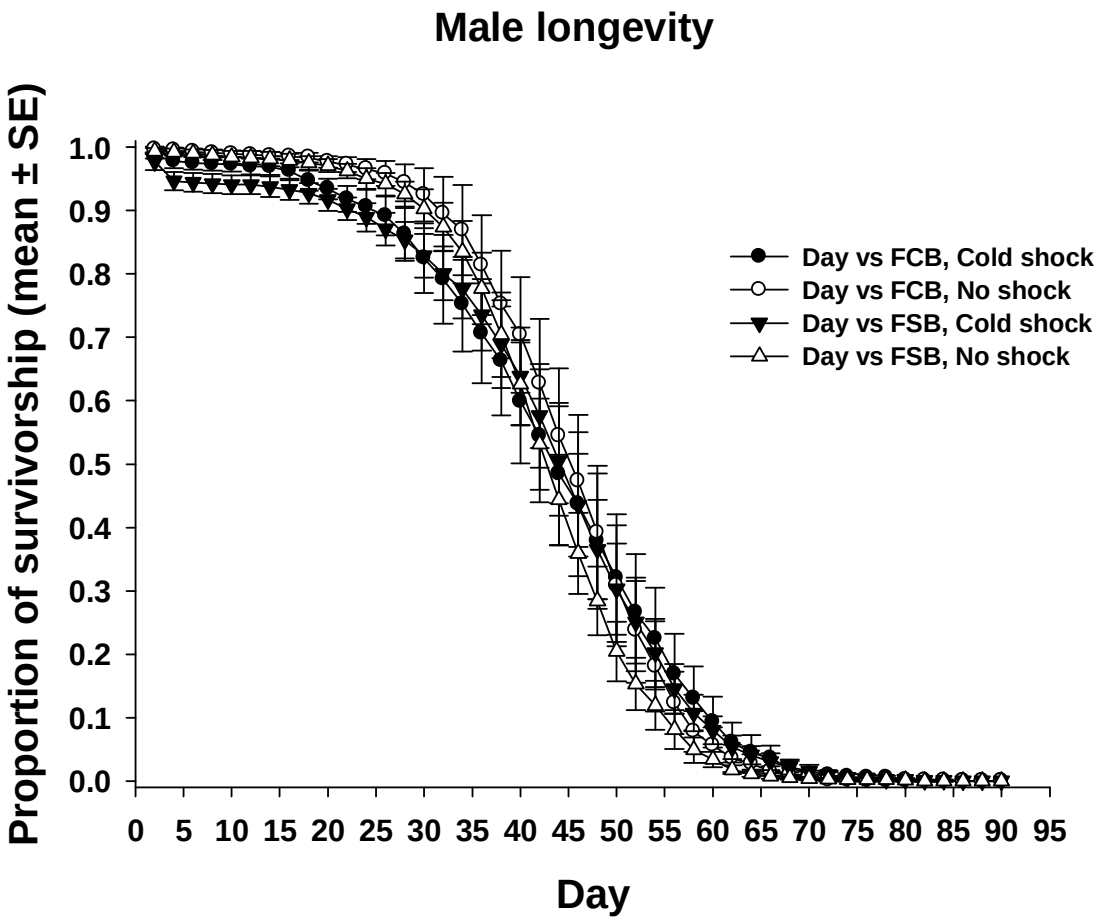


Figure 1a: Mean longevity of the FSB and FCB males after being subjected to cold-shock or no-shock treatment (Experiment 1.1). Selection, treatment or selection × treatment interaction did not have significant effect on male mean longevity. Open bars represent the FSB and closed bars represent the FCB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



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

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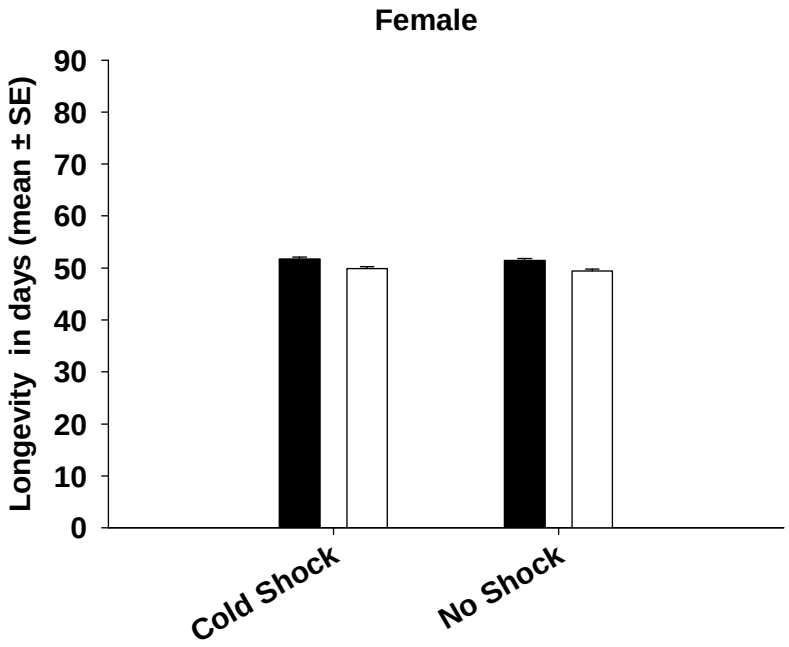

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692 **Figure 1c:** Mean longevity of the FSB and FCB females after being exposed to cold shock or
693 no shock treatment (Experiment 1.1). Selection, treatment or selection × treatment interaction
694 did not have significant effect on female mean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

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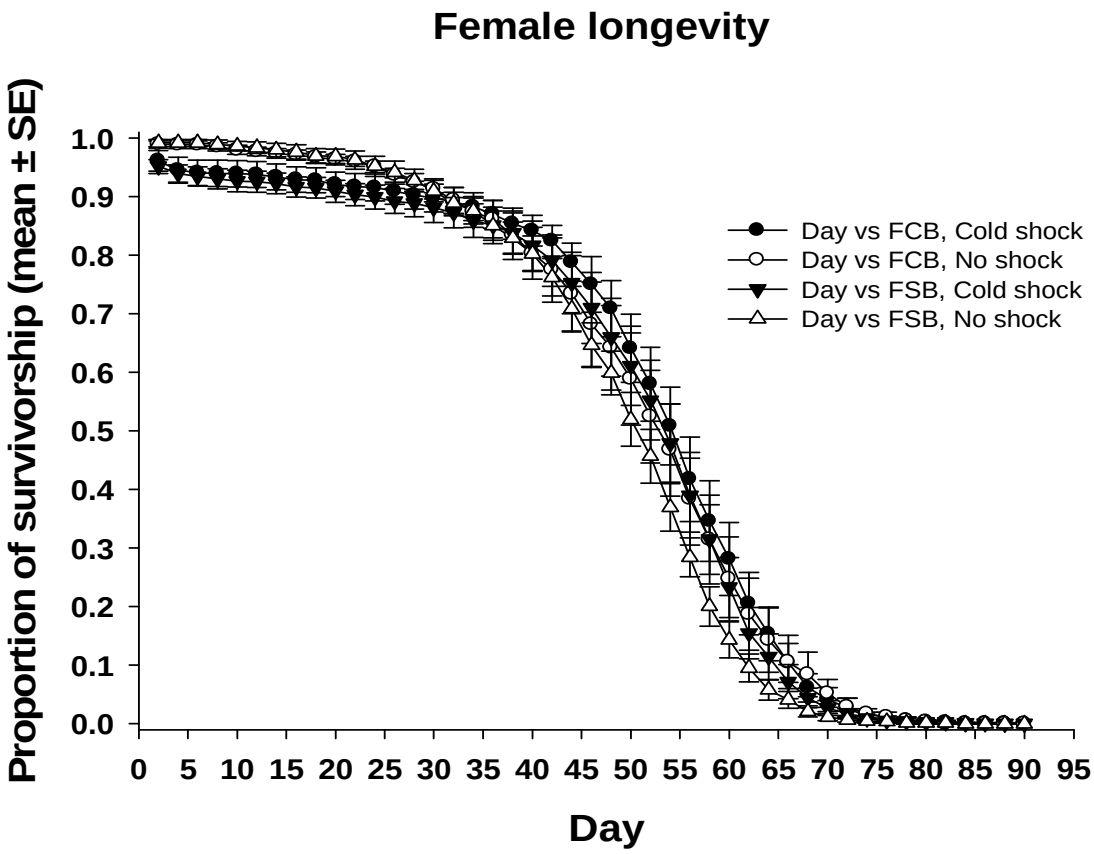

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701 **Figure 1d:** Female survivorship across ages (Experiment 1.1). Longevity was assayed after
702 adult flies were subjected to cold-shock or no-shock treatment. There was no difference in the
703 mean, median and maximum longevity of the FSB and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

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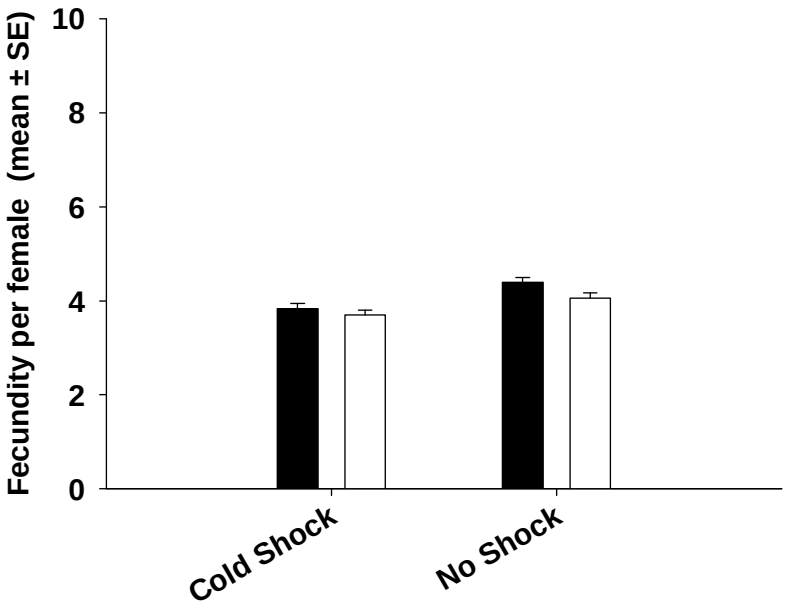

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709 **Figure 2a:** Mean life time fecundity per female (Experiment 1.2). Fecundity was measured at
710 eleven time points once in every 6 days and mean of eleven time points for fecundity was
711 computed. Selection, treatment or selection × treatment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

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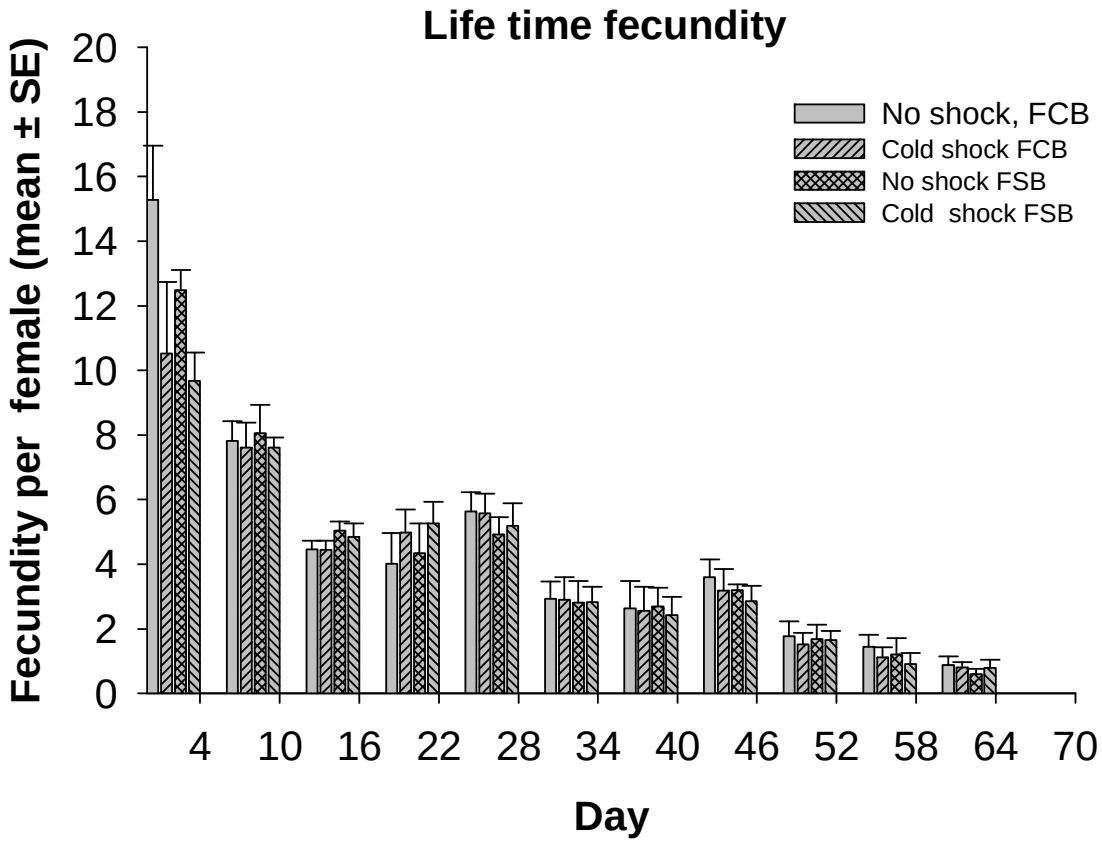

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719 **Figure 2b:** Life time fecundity per female (Experiment 1.2). Fecundity was measured at
720 eleven time points once in every 6 days. Mean fecundity per female for each population and
721 treatment was computed for eleven time points. Results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

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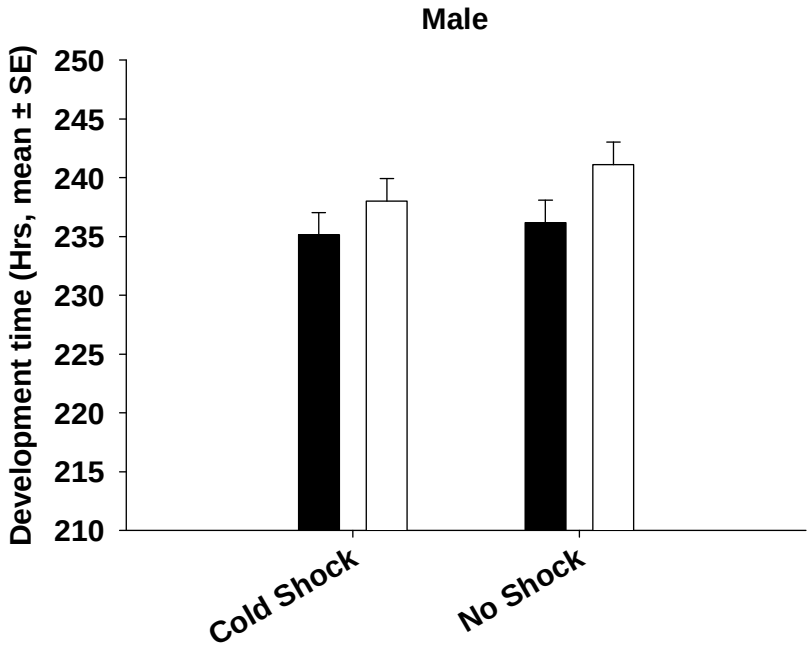
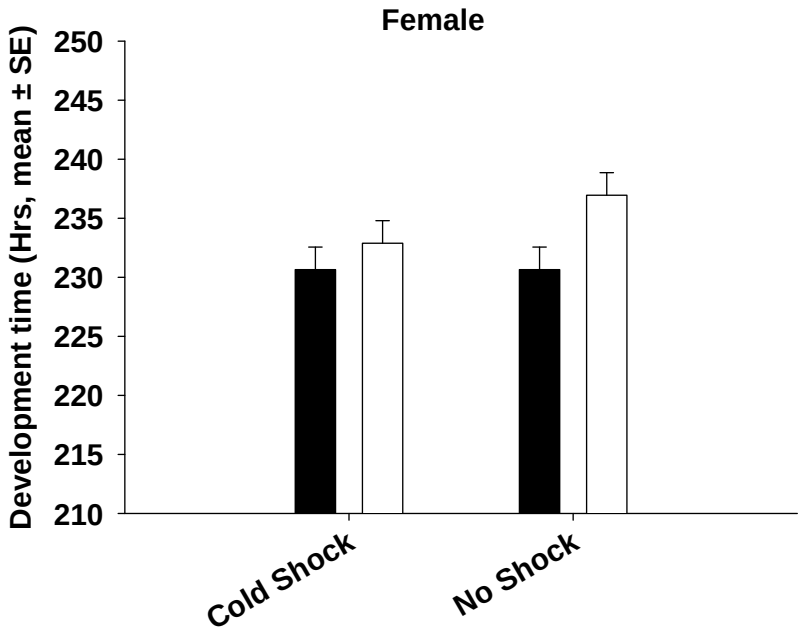


Figure 3a: Mean development time (larva to adult) of the FSB and FCB males when their parents were subjected cold-shock or no-shock treatments (Experiment 2). We found a significant effect of selection regime with the FSB males developing 3-4 hours slower than FCB males. Treatment had no significant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



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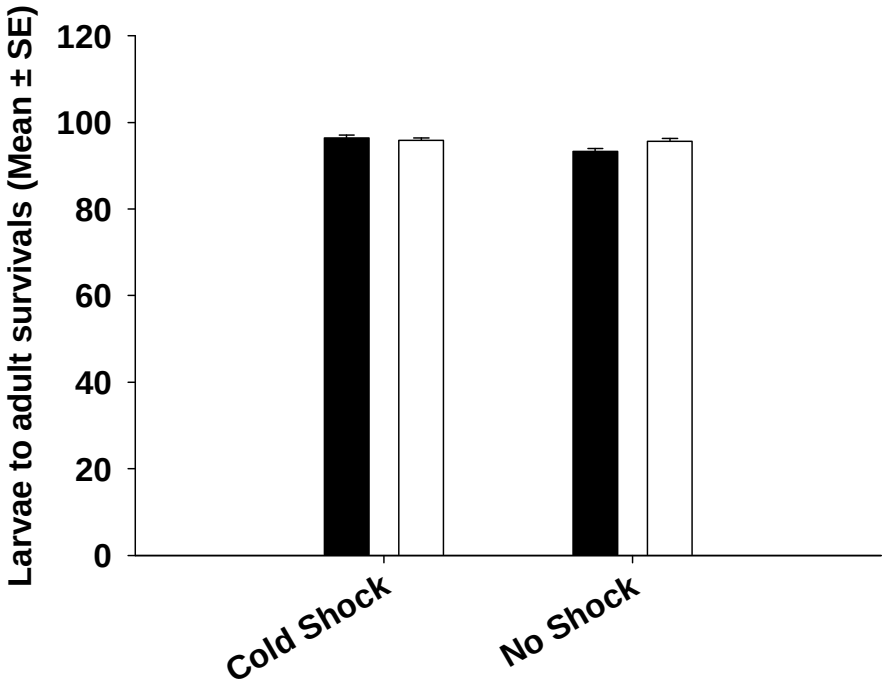

748

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

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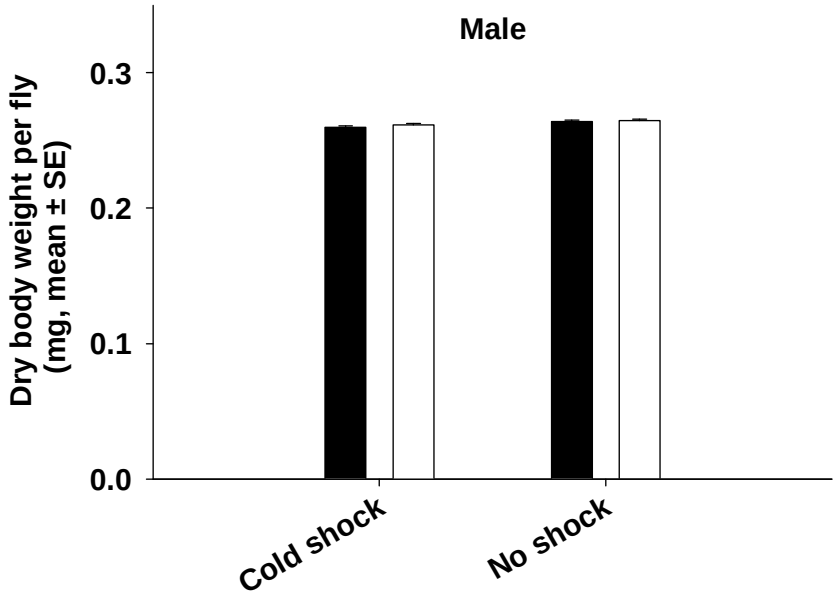
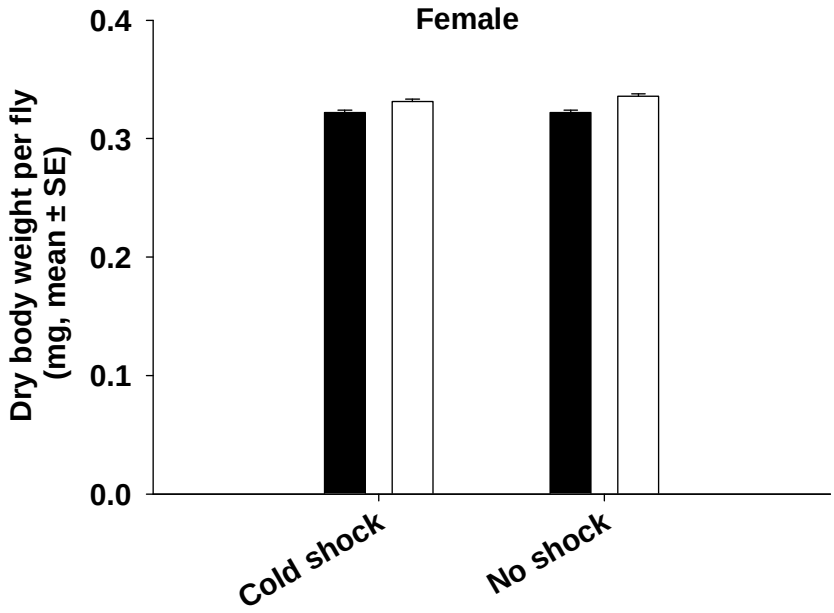


Figure 4a: Dry weight at eclosion of males from the FSB and FCB populations (Experiment 3). Selection, treatment or selection × treatment interaction did not have significant effect on mean dry body weight. Open bars represent the FSB and closed bar represent the FCB populations.



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

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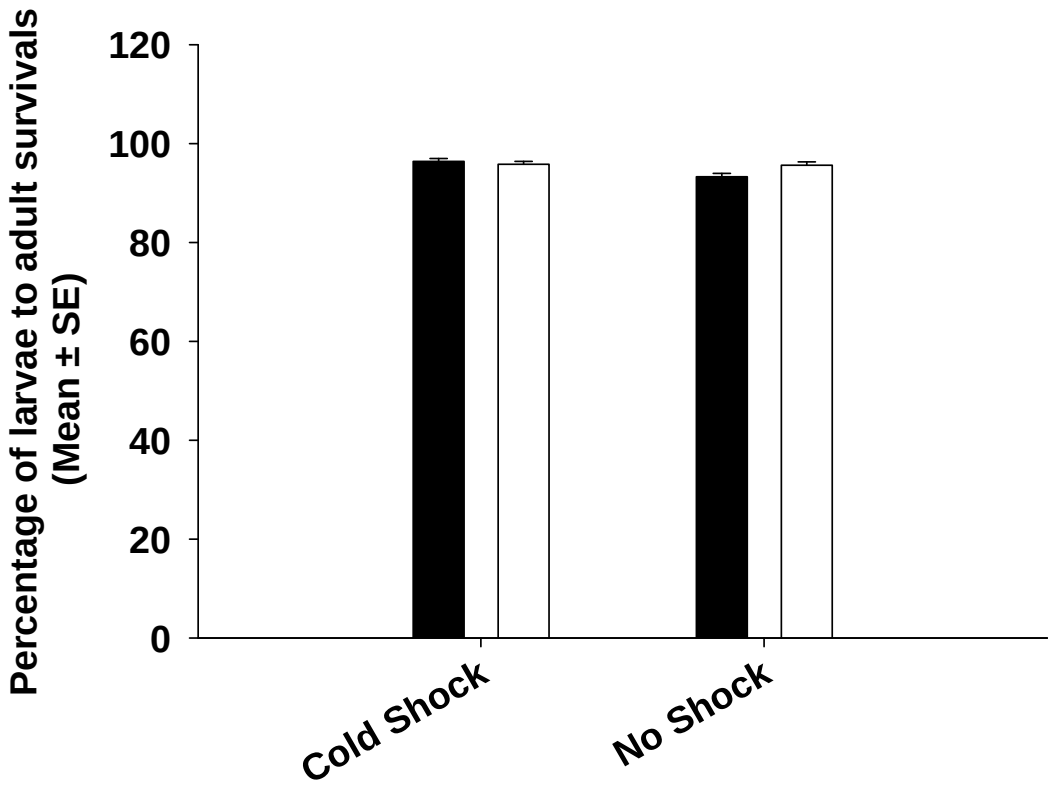

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779 **Figure 5:** Larvae to adults survival from the FSB and FCB populations (Experiment 4).

780 Selection, treatment or selection × treatment did not have any significant effects on mean

781 larvae to adult survivals. Open bars represent the FSB and closed bar represent the FCB

782 populations

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