Effect of environmental stress in *Drosophila melanogaster* evolved under differential levels of sexual selection

A Thesis

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by

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Certificate

This is to certify that this dissertation entitled, "Effect of environmental stress in *Drosophila melanogaster* evolved under differential levels of sexual selection" towards the partial fulfilment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research, Pune represents study/work carried out by Santhosh at Indian Institute of Science Education and Research, Mohali, under the supervision of Dr. N.G. Prasad, Associate Professor, Department of Biology, during the academic year of 2019-2020.

Santhosh

Committee:	Dr. N.G. Prasad	Prof. Sutirth Dey
	Supervisor	TAC

Declaration

I hereby declare that the matter embodied in the report, “Effect of environmental stress in *Drosophila melanogaster* evolved under differential levels of sexual selection” are the results of the work carried out by me at the Department of Biology, Indian Institute of Science Education and Research, Mohali, under the supervision of Dr. N. G. Prasad and the same has not been submitted elsewhere for any other degree.

Santhosh

03-April-2020.

CONTENTS

Abstract

Evolving under biased sex ratios, male- or female-biased, introduces differential levels intersexual conflict between the sexes. While male-biased (M) populations undergo an antagonistic coevolution between the sexes that selects for exaggerated reproductive traits in the males and resistance traits in the females, female-biased (F) populations face a relaxed selection on these traits. Also, variation in the expression of reproductive traits is the raw material for selection to act on, and has an environmental component. It is therefore important to study how a key factor such as environmental variation influences these reproductive traits. In this study, we address this issue in a systematic manner. We investigated the effect of a key environmental factor, Larval density during development, on the male and female *D. melanogaster* from populations evolving under altered operational sex ratios. To do this, we reared flies from the male- and female- biased selection regimes in the control density and a 4-fold high density, and compared various life-history and reproductive trait values. High larval density had a detrimental effect on all the life-history traits studied in both sexes. And, both male-biased and female-biased selection regime flies were affected to similar levels. However, a sex-specific response to larval crowding was observed in the reproductive fitnesses. The female competitive fitness was robust to larval crowding, whereas the male competitive fitness was detrimentally affected by larval crowding. This study concludes that the evolutionary history of sexual selection does not play a crucial role in modulating the responses to developmental environment variation in *D. melanogaster*.

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1. Introduction

Fitness of an Individual can be decomposed into three components- survival, fecundity and mating/fertilization success (Arnqvist and Rowe, 2005). The last component represents the role of sexual selection in determining fitness. Darwin (1871) defined sexual selection as “the advantage which certain individuals have over others of the same sex and species solely in respect of reproduction”. He explained that sexual selection could act through competition within the same sex for access to mates (Intra-sexual selection) and/or mate-choice imposed by one sex on the other. Since then, sexual selection theories have played a crucial role in the attempts to solve the important question of why and how traits, such as bright coloration, large horns and antlers that provide no survival benefits evolve?

Ronald Fisher (1958) proposed the “Run-away” selection, where he suggested that male ornaments may become genetically correlated to the female mate preferences. That is, when the males expressing the preferred trait were selected, the female preference itself evolves with the evolution of the male trait. Given that the trait increases the fitness of the individual, this preference could lead to an increase in the female’s fitness and trait value over generations. Thus, the trait and the female preference for the trait may coevolve. This argument was further developed by saying that the elaborate ornaments and other sexual signals should be costly and can only be afforded by individuals of high genetic quality. This gave rise to the “good genes” hypotheses of sexual selection (Zahavi, 1975; Hamilton & Zuk, 1982; Maynard-Smith, 1991), which stated the traits that females choose are honest indicators of the male’s ability to pass genes that will increase the survival and fitness of her offspring.

And once a trait becomes costly, we expect it to evolve condition-dependence. And, there is a great deal of evidence for the condition-dependence of sexually selected traits. Condition has been popularly defined as the cellular efficiency in acquiring and processing metabolic resources to maintain optimal functionality (Hill, 2011). The genic capture (Rowe & Houle, 1996) posited that sexually selected traits are honest indicators of overall condition as their expression may reflect polygenic variation across large parts of the genome. Such a selection for the condition could target large proportions of the genome, purging of deleterious mutations with pleiotropic effects on survival. While genic capture and many coevolution paradigms focus on the

contribution of “good genes,” Condition is also likely to be emphatically influenced by the environment in which the organism resides. Therefore, in relevance to changing environments, the condition has also been defined as the cellular capacity to withstand environmental challenges (Hill, 2011).

Having said all this, it is still clear through a large body of evidence that the males and females follow different strategies to derive fitness. For instance, the two sexes have different optimum mating frequencies (Bateman, 1948). The fitness of a female majorly depends on the number of eggs she produced, while the fitness of the male depended on the number of females he mated with. This difference between the sexes may give rise to a misalignment in the evolutionary interests of the two sexes, which has been called intersexual conflict or, simply, sexual conflict). This arises from the fact that, although males and females share most of the genome, the selection is often distinct in the two sexes. Therefore, the expression of an allele that is favorable in one sex might be deleterious when expressed in the other sex, resulting in intra-locus sexual conflict (Lande, 1980; Rice, 1984; Arnqvist and Rowe, 2005). And, Intra-locus sexual conflict has been shown to affect a range of fundamental evolutionary processes such as maintenance of genetic variation (Fry, 2010; Arnqvist, 2011) and adaptation to environmental change (Whitlock and Agrawal, 2009) among others.

Also, in promiscuous species, the two sexes may adopt different strategies to maximize their fitness at the expense of the fitness of the opposite sex. This gives rise to the second form of sexual conflict called inter-locus sexual conflict. For instance, males experience intense intrasexual selection in the form of male-male competition. Such competition can occur prior to mating when males compete for mating opportunities (Bateman, 1948) or even post-mating through sperm competition (Parker, 1979; Simmons, 2001; Snook et al., 2003). And male strategies to circumvent such competition have been found to adversely affect the females causing increased mortality and reduced life-time progeny production. These harmful effects arise as by-products of male-benefitting functions of traits and most likely do not benefit the males directly (Rice, 2000; Morrow et al., 2003). For example, male ejaculate has been shown to carry components that enhance the short-term fecundity of the females but reduces their receptivity to mating and longevity (Civetta and Clark, 2000; Wolfner, 2002). As a male’s fitness is expected to be diluted if his mate copulates again with a different male, these are beneficial for the males.

Due to this male induced harm, females are selected to evolve resistance traits against harm-inducing traits (Wigby and Chapman, 2004). Such female counter-adaptations may render the male adaptive traits useless, thereby selecting males to further increase their investment in reproductive activities or evolve different mechanisms to maximize fitness. To conclude, male-male competition initiates intersexual conflict and leads to an antagonistic co-evolution of reproductive traits in the two sexes. Therefore, intersexual conflict can most likely be manipulated by varying the degrees of male-male competition for mating in the population.

In our lab, populations were experimentally evolved under varied operational sex ratios, male-biased (M) and female-biased sex ratios (F). Manipulating the operational sex ratios this way was expected to generate differential levels of male-male competition and, hence, the intersexual conflict between the sexes.

In line with the predictions from theory, the males experiencing high male-male competition (M -regime) evolved to show an increase in competitive fitness and sperm competitively ability, while the males from the low competition (F-regime) evolved lower fitness (Nandy, PhD Thesis). As a result, males from the M-regime also caused increased mortality in females, while the males from the F-regime were found to be significantly benign to female fitness. Furthermore, resistance to mate harm evolved in females as a result of intersexual conflict. The M-females were highly resistant to the male-induced harm, while females from the F-regime were more susceptible as there was a reduction in the longevity after mating interactions. There were significant life-history consequences observed to have evolved under sexual conflict in males and females as well. F-males had evolved reduced susceptibility to starvation, while M-males paid costs in the form of longevity. The M-males exhibited increased locomotor activity and courted females more frequently. In females, F-females evolved increased egg production and lived longer as virgins, possibly due to the reduction in the investment towards resistance traits. Now, the M and F populations have evolved in their respective competition regimes for over 250 generations (Nandy et al., 2013; Nandy, PhD thesis).

results from a balance between the benefits of good genes and the costs of sexual conflict (Matinossi-Allibert, 2018).

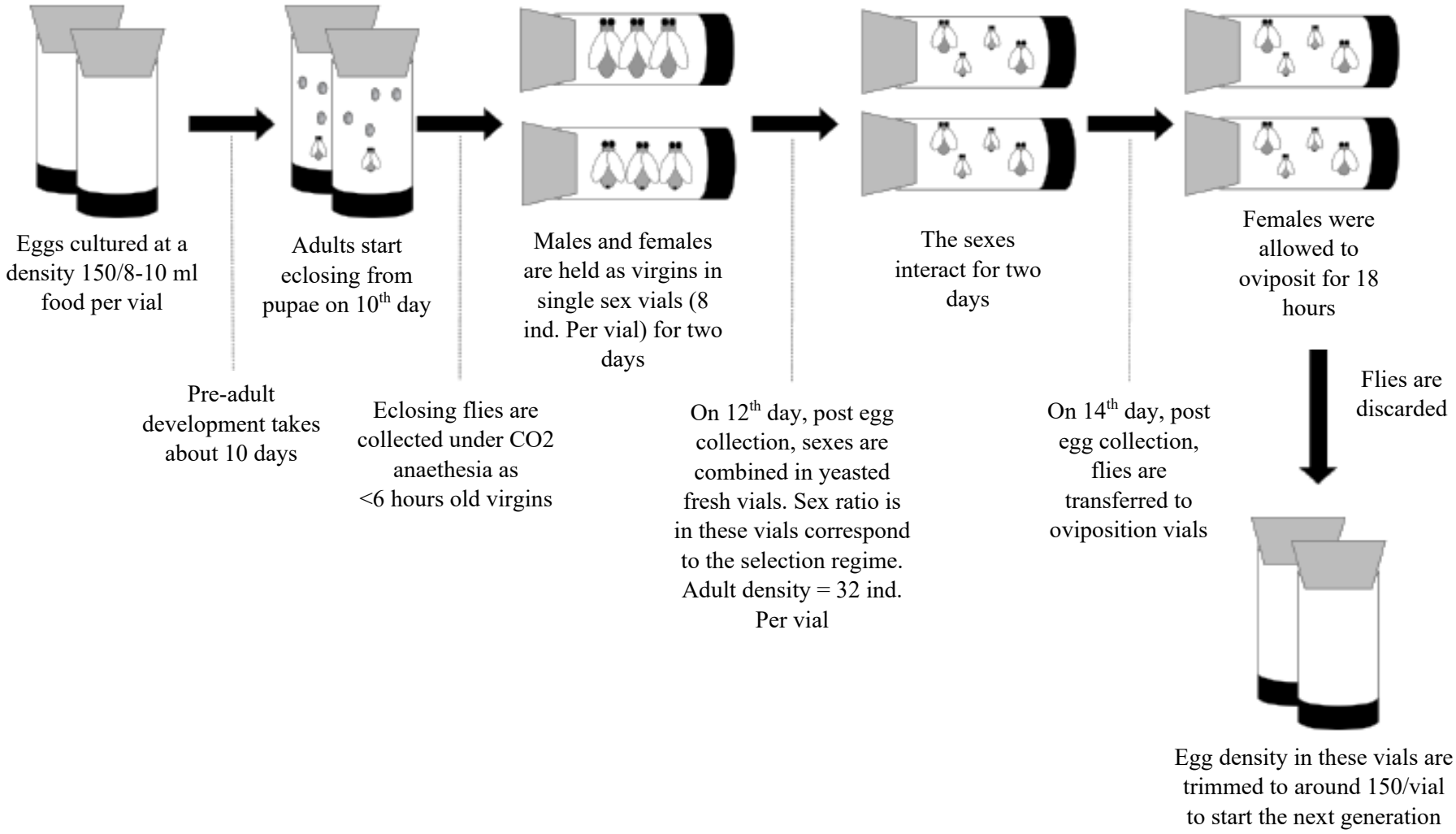

Fig.1. General maintenance protocol for the selected populations (M1-3, F1-3) (Nandy, PhD thesis)

And when these processes are influenced by the change in the environment, the costs and benefits of sexual selection could shift the balance in the maladapted populations. Therefore, it is crucial to assess how these the effects of sexual selection manifest in a novel environment and empirical data addressing these dynamics are scarce. The selected populations, M & F, have been reported to have evolved in response to the differential levels of sexual selection, in different directions and expressed differences in their life-history and reproductive traits. Thus, they provide a suitable platform to address the above-mentioned questions. Addressing this question is essential as it would allow us to elucidate whether sexual selection will help or hinder the adaptation of a species to novel or changing environments. And, Observations from experimental provide evidence for sexual selection to increase adaptation (Fricke & Arnqvist, 2007) as well as impeding it (Chenoweth et al., 2015).

For my thesis, I studied the effect of a key environmental factor, Larval Density, on the fitness and the life-history traits of the flies from the M- and F- populations. The larval environment has also been shown to play a crucial role in the development and survival of larvae. I measured the egg to adult survivorship and the developmental time of the selected flies when reared in the two different densities. In general, larval survival and developmental rate were seen to be negatively correlated to larval density during development (Lyimo et al., (1992); Muriu et al. (2013)). Under crowded conditions, the last instar larvae of the red flour beetle, *tribolium freeman,* fail to pupate, causing mortality (Nakakita, 1982). Even field studies on Damselflies, *C. puella,* revealed a larval density-dependent reduction in adult body size and increased larval mortality (Banks & Thompson, 1987). The responses to larval crowding were also sex-specific for certain traits. Agnew et al. (2000) noted in the house mosquito, *culex pipiens* that, although both males and females took longer to develop into adults, only the female adult body size was sensitive to larval density and the females also faced significantly higher mortality.

In holometabolous insects, the adult body size is fixed at eclosion and determined by the environment during the pre-adult stages (Boggs, 1981; Clancy & Kennington, 2001). An increase in larval density is expected to cause higher competition for the limited resources and hence lead to a reduction in adult body size. And, evidence from numerous studies from a wide range of taxa supports this prediction. Ireland & Turner (2005) showed that more crowded larvae cultures resulted in the production of

undersized larvae and adults in the blowfly, C*alliphora vomitoria.* Larval density was also found to strongly affect body size and survival in the pearl oyster, *Pinctada margaritifera* (Doroudi & Southgate, 2000). Therefore, I studied the body size of the selected flies (males and females) by measuring their dry bodyweight after rearing them in a crowded high larval density.

I also studied the effect of larval density on the female's ability to produce eggs by measuring the fecundity of the females after a single mating. Adult body size often tends to correlate positively with female productivity and male quality. And, female adults reared in crowded larval condition showed a reduction in lifetime fecundity in the beetle, *Dacne picta* (Sato et al. 2004). Larval density was shown to influence reproductive success in the leaf beetles as females reared as isolated larvae laid significantly more eggs than their high-density counterparts (Muller et al. 2016). This reduction in fecundity could be solely attributed to the reduction in the adult body size, which could determine the amount of resources that can be stored and used for various life-processes, including egg-laying.

Although the reproductive success of the females depends on its ability to produce eggs, the reproductive success of males is affected by both pre- and post-copulatory events. In both sexes, the adult body size is often associated with reproductive success. In general, large individuals have been seen to have higher reproductive success. And, this could be because they may be better able to attract, stimulate or coerce potential mates (Simmons, 1988; Crean et al. 2000). But cases where smaller males being more successful are also observed, where agility in competition, particular aspects of courtship are preferred (Vencl & Carlson, 1998; Steele & Partridge, 1998). Also, males of different sizes may adopt different mating strategies. In the water strider, *Gerris lacustris* (Danielsson, 2001)*,* and the dung fly*, Scathophaga stercoraria* (Simmons et al., 1996) larger males were shown to mate more often than the smaller males. Still, the smaller males mated for a longer duration and sired more offsprings per mating. Thus, small males invested more per each mating. This was also seen in *D. melanogaster,* as small males mated longer and ejaculated proportionally more of the stored accessory gland sex peptide resources (Wigby et al., 2015).

Moreover, the female body size plays an important role in determining male investment in mating. Males preferred and mated for significantly longer with larger

females (Lefranc & Bundgaard, 2000). Wigby et al., 2015 also noted that males selectively invested and transferred more seminal fluid proteins and sex peptides to larger females than their smaller counterparts. Hence, I measured the mating latency and copulation duration expressed towards the selected females raised in the different densities. In nature, however, both males and females face socially competitive environments. Males have to compete with other males for access to mates and females compete for limiting resources etc. Edward & Chapman (2012) studied the reproductive fitness of males with different body sizes in a competitive environment and observed that smaller males had substantially reduced share of paternity. I studied the effect of density on the competitive fitness of both males and females when competing for mates (in males) or for limiting resources (in females).

Thus, the experiments done in this thesis attempted to answer three broad question:

i) How does M- & F- selected flies respond to the crowded larval environment in the expression of crucial life-history traits like dry bodyweight, developmental time, fecundity?

ii) How does the larval density interact with the selection regime in determining the reproductive fitness in the M- & F- populations, with different evolutionary histories of sexual selection?

iii) Is there a sexual dimorphism in the responses to high larval density in the life-history and reproductive success?

2. Materials and methods

2.1. Maintenance of populations

The 'M' (Male biased) and the 'F' (Female biased) populations, used in this study, were derived and are maintained as described in Nandy *et al.* 2013. These populations were derived from an outbred laboratory-adapted population of *Drosophila melanogaster,* LHst. The selected populations (M & F) are maintained in a 14-day discrete generation cycle at 25°C, 60% relative humidity and 12:12 hours light/dark regime on cornmeal medium. For the selection regime, flies are reared in a controlled larval density of 140 – 160 per vial on 8-10 ml of standard cornmeal medium in standard vials. These vials are referred to as juvenile competition vials. A total of 20 such vials make a population. Male and female flies are collected as virgins by separating them within 6 hours from eclosion and are held in single-sex vials till the 12^{th} day from egg collection. On the 12^{th} day, the two sexes are combined in adult competition vials in sex ratios corresponding to the selection regime of the population, provisioned with 0.47mg of live yeast per female. The M-regime had a male-biased sex ratio of three males for every female (24 males: 8 females) and the F-regime had a female biased sex ratio of three females for every male (8 males: 24 females). After 48 hours in the adult competition vials, the flies are transferred into fresh vials with ~8-10 ml of the cornmeal medium and are allowed to oviposit for a period of 18 hours. The eggs laid in these fresh vials are trimmed to a density of ~140 – 160 eggs and become the next generation of the population.

The LH and LHst populations were used to generate flies that were used as mates from a common background for some of the experiments. The LH population (named after Larry Harshman, the founder of the population) is maintained under a 14-day discrete generation cycle in similar standard conditions as M and F populations. A total of 60 vials make the population. LHst population was derived from LH populations by introducing a recessive-autosomal trait, scarlet-eye, by repeated backcrosses (Prasad et al. 2007). LHst is maintained under identical conditions as the LH, except that the population is made up of 30 vials. LHst is periodically backcrossed with LH to maintain the genetic uniformity across the two populations. The detailed description of the maintenance of the LH and LHst populations can be found here(Nandy, PhD thesis).

2.2. Generating experimental flies

To eliminate any non-genetic parental effects, the selected populations were standardized by relaxing selection using common rearing conditions, 1:1 sex ratio in cuboidal fly cages, for one generation. The flies used in the assays were generated by collecting eggs from these standardized populations of M & F. The populations were fed yeast paste for a period of 48 hours. Then, a fresh plate was provided for 6-8-hours, on which the flies laid their eggs. The eggs were scraped off of the food plates using a brush and counted on an agar strip under a microscope.

In a preliminary experiment aimed to standardize the protocol for larval manipulation, I reared the flies from the two regimes, of block-1 only, in four different larval densities (150, 300, 450, 600 eggs per vial) in standard vials with ~8-10 ml cornmeal medium. Then, I studied the effect of these larval densities on the life-history traits of flies from block1.

Then, a density of 600 eggs, counted in exact numbers, per vial was chosen to be the 'high-density' for which the responses of the selected populations were studied. The corresponding control was the standard rearing condition of 150 eggs in a standard vial (hereon referred to as 'low density'), also in the exact number. These vials were incubated in standard culture conditions (25◦C, 60–80% RH, 12 hours–12 hours light / dark cycle and hereafter referred to as "standard conditions") to generate the experimental flies.

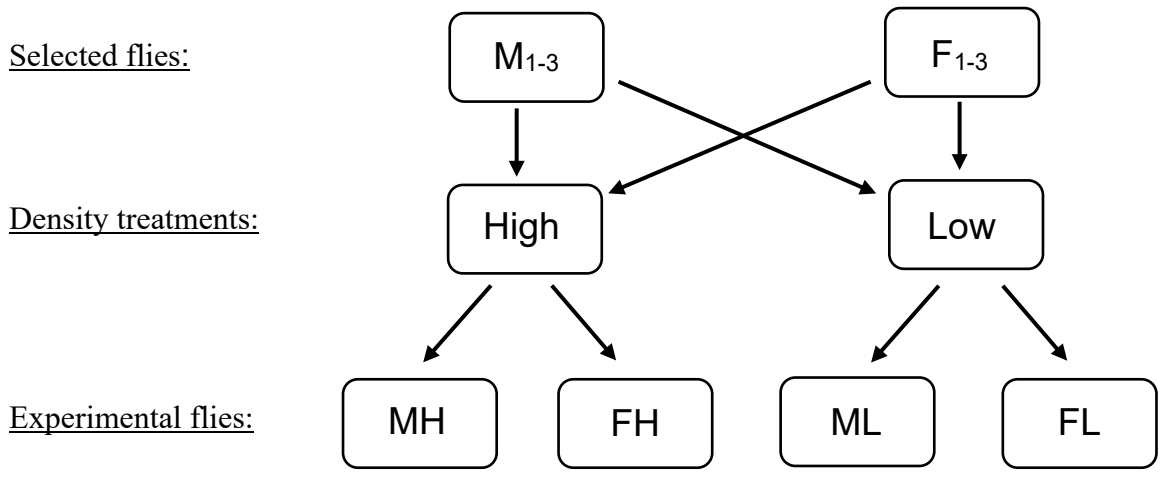

Fig.2. generation of experimental flies for the assays.

2.3. Assays

The experiments decribed below were conducted thrice, i.e. in all the 3 blocks of the M- and F-populations.

2.3.1. Developmental Time

Developmental time was calculated as the time taken for the flies to develop from eggs to adults. Eggs were collected into vials in exact numbers, 150 eggs or 600 eggs per vial for low and high-density conditions, respectively. To synchronize the developmental stages of the eggs, only the eggs laid within a 2-hour window were collected. After 48 hours of yeasting, a fresh plate was provided for a period of 2 hours. This plate is discarded and another fresh food plate is provided for a period of 2 hours. Only eggs laid on the second plate were scraped and collected into standard rearing vials for the assay. 5 such vials were collected into, for each regime and density treatment. These vials were incubated in standard conditions.

Starting from the 7^{th}-day post egg collection, the rearing vials were scanned once every 4 hours for any adult flies. And, the eclosed flies were counted and marked for their sex. Once the majority of the flies had eclosed, the vials were scanned with longer intervals. This process of scanning and counting lasted until the last fly ecloses. And, the difference between the time of egg collection and the time point at which a fly is counted is taken to be the developmental time for that fly.

2.3.2. Survivorship

Survivorship was calculated as the proportion of the eggs that develop into adults. Eggs were collected into vials in the respective ancestral (150eggs/vial) and high (600eggs/vial) densities in a short 2-hour window, as described above for developmental time assay. 5 such vials were collected into, for each regime and density treatment. These vials were incubated in standard conditions. The egg to adult survivorship was measured in five vials per treatment and selection regime. Starting from the 7^{th} day, I counted the number of eclosed flies in each vial every 4 hours. This counting process continued till no more flies eclosed. The sum of all the flies eclosed at each counting timepoint was taken as the total number of flies eclosed. The egg to adult survivorship was calculated by dividing the total number of eclosed flies by the total number of eggs collected in that vial.

2.3.3. Dry Bodyweight

Adult flies reared in the two larval densities, high and ancestral, were collected soon after eclosion on the 9th and the 10th day, post egg collection. The flies were separated by sex and freeze killed at -20°C. On the 12th day, Flies from the two treatments and selection regimes were placed in 1.5 ml centrifuge tubes in groups of 5 flies per tube. Male and female flies are placed separately. In total, ten such tubes are made for each sex from the two treatments and the selection regimes. These tubes are dried in a hot air oven at 60°C for 48 hours.

The dry bodyweights of the flies were scored by emptying the tubes, i.e., groups of 5 flies, onto a weighing scale. The weights were scored to the closest 0.01mg. To account for the variation that might arise due to the weighing scale, the contents of each tube were weighed thrice, resetting the scale after each measurement. The average of the three measurements was taken as the dry bodyweight of the flies in that tube.

2.3.4. Fecundity

Fecundity of the female flies was measured as the number of eggs laid after a single mating in an 18-hour oviposition window. Flies were reared in the two larval densities and females were collected as virgins on the 9th and the 10th-day post egg collection. On the 12th day, the virgin females were combined, singly, with common virgin males reared at standard conditions. Virgin males from the LHst baseline population reared at 150 egg density per vial were used as common males for the experiment. 30 females were used per density and selection regime and vials were manually observed for mating. The males were removed from the vials soon after the flies separated from copula. The females are retained in the same vials for a further 18 hours for egg-laying. After that, the females are discarded and the vials are frozen at -80°C to inhibit hatching. Later, the number of eggs laid on the surface of the food in the vials were counted under a microscope.

2.3.5. Mating Latency and Copulation Duration

Mating latency and copulation duration expressed towards the females from the two larval densities were measured as the time taken by the common males to initiate mating and duration of the first mating event, respectively. Virgin males from the LHst

baseline population reared at 150 egg density per vial were used as common males for the experiment. On the 12th day, virgin females from the two densities were introduced, singly, to virgin males in standard vials with ~2-3ml of food. The vials were observed manually, immediately after the introduction of the pair and the time of the start of observation was noted. Then, the time of the formation of copula and the time at which the pair separated from copula was also noted for each vial.

2.3.6. Competitive Fitness (CFS)

Males - Competitive fitness of the focal males is measured as the proportion of progeny sired by the focal males when competing with ancestral males for females. The common females for the experiment were derived from the LHst baseline population. And, the competitor males were derived from LH baseline population. The flies from the LH populations could be easily distinguished from the LHst and the selected populations (M & F) by their eye colour. The LHst and the selected populations carried a recessive marker for scarlet eyes, while the LH populations expressed red eyes. Flies reared at the two treatment densities were collected as virgins on the 9th and 10th-day post egg collection. On the 12th-day post egg collection, five virgin focal males from the two densities are combined with 10 LH males and 15 LHst females in standard glass vials with 4-5ml of cornmeal medium and few grains of live yeast. 15 interaction vials were created per density and selection regime and left undisturbed for 48 hours.

Then, 7 of the 15 females were sorted under mild CO_2 and were transferred, singly, into test tubes filled ~1/3rd with cornmeal medium for oviposition. After 18 hours of oviposition, the females are discarded. These test tubes are incubated under standard conditions for 11 days for flies to eclose and are frozen at -20°C. Later, the freeze killed adult flies in each tube were counted under light and scored based on their eye colour.

Females – Competitive fitness of focal females is measured using a setup similar to that for the males. Competitive fitness was calculated as the total number of progeny produced when competing with females from the LH populations for limiting amounts of yeast. The common males used in the experiment were derived from the LHst baseline populations. All the flies were collected as virgins on the 9th and 10thday post egg collection. On the 12th day, Focal females are combined with 10 LH females and 15 LHst males in standard vials with 4-5ml of cornmeal medium and limiting amounts

of yeast (100 µl/vial of 0.07mg/ml yeast solution). 15 such interaction vials were created and left undisturbed for 48 hours.

Then, all the focal females were sorted under mild CO_2 and transferred into test tubes with cornmeal medium for oviposition. After 18 hours of oviposition, the females are discarded. These test tubes are incubated under standard conditions for 11 days for flies to eclose and are frozen at -20°C. Later, the total number of adult flies in each tube were counted under white light.

2.4. Statistical analysis:

For the developmental time assay, the median developmental time of the flies from each vial was calculated. And, these medians were used as the unit of analysis. For the CFS assays, the proportions (for male CFS) and the number (for female CFS) of offspring of all the focal flies from the same interaction vials were averaged to obtain the mean fitness per vial. These vial means were used as the unit of analysis.

Analysis of all the assays was performed in Rstudio (version:1.2.5033). Males and females were analyzed separately for the developmental time and dry bodyweight assays because an effect of sex is established for these traits in the *Drosophila melanogaster* system.

A mixed model was built using the '*lmer*' function of the *lme4* package with "selection regime" and "density" treatment as fixed factors, and "block" as a random factor. An ANOVA was performed on the model using the "*anova*" function of the *stats* package. For significant main effects, the estimated marginal means were obtained using the "*emmeans*" function from the *emmeans* package of the model. And, pairwise comparisons of the treatments were performed through a post-hoc Tukey's HSD test using the "*pairs"* function from the *graphics* package on the EMMgrid generated by the "emmeans" function.

In assays where there was a significant effect of block, the blocks were analyzed separately. A two-factor linear model was built using the "*lm*" function from the *stats* package with the selection regime and the density treatment as fixed factors. And an ANOVA was performed on the linear model using the "anova" function of the stats package in Rstudio.

3. Results

The test statistic and the P-values of all the experimental results described below are given in tables 1 & 2.

3.1. Survivorship

Analysis of the survivorship data revealed a significant effect of block. Therefore, the different blocks were analyzed separately. All the three blocks exhibited a significant main effect of the density treatment with higher egg to adult survivorship in the low density than the high density. Blocks 1 & 2 showed a significant main effect of the selection regime. And, pairwise comparisons showed higher survivorship in the F-regime compared to the M-regime vials.

A significant selection regime x density interaction effect was also observed in block 2 and the pairwise comparisons showed a significantly higher survivorship of F-regime in the ancestral density, while there was no difference between the regimes in the high-density treatment.

3.2. Developmental Time

As there exists a dimorphism in the developmental time between the sexes in *D. melanogaster*, data from the males and females were analyzed separately.

3.2.1. Males

There was a significant effect of the random factor, block. Therefore, the blocks were analyzed separately. All three blocks revealed a significant main effect of the selection regime and the density treatment. The high-density caused a significant delay in eclosion. A significant selection regime x density treatment was also observed in all the three blocks.

Pairwise comparisons revealed that M-males took longer to eclose compared to the F-males in the high-density treatment in blocks 1 & 2. But in block 3, M-males took longer to eclose than F-males in the ancestral density treatment and no difference was observed in the high-density.

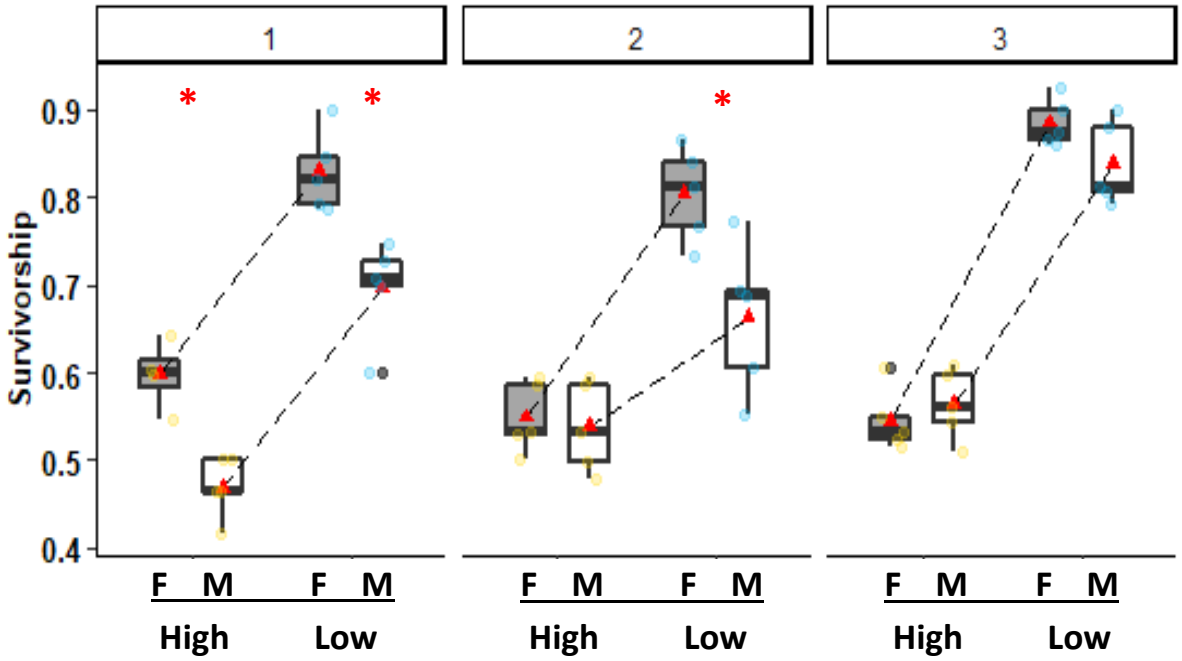

*Fig.3. Survivorship: Proportion of eggs that develop to adults in the low-density (L) and the high-density (H). The blocks are separated as facets. The grey boxes represent the F-regime and the white boxes represent the M-regime. * represents significant difference between the selection regimes in that density.*

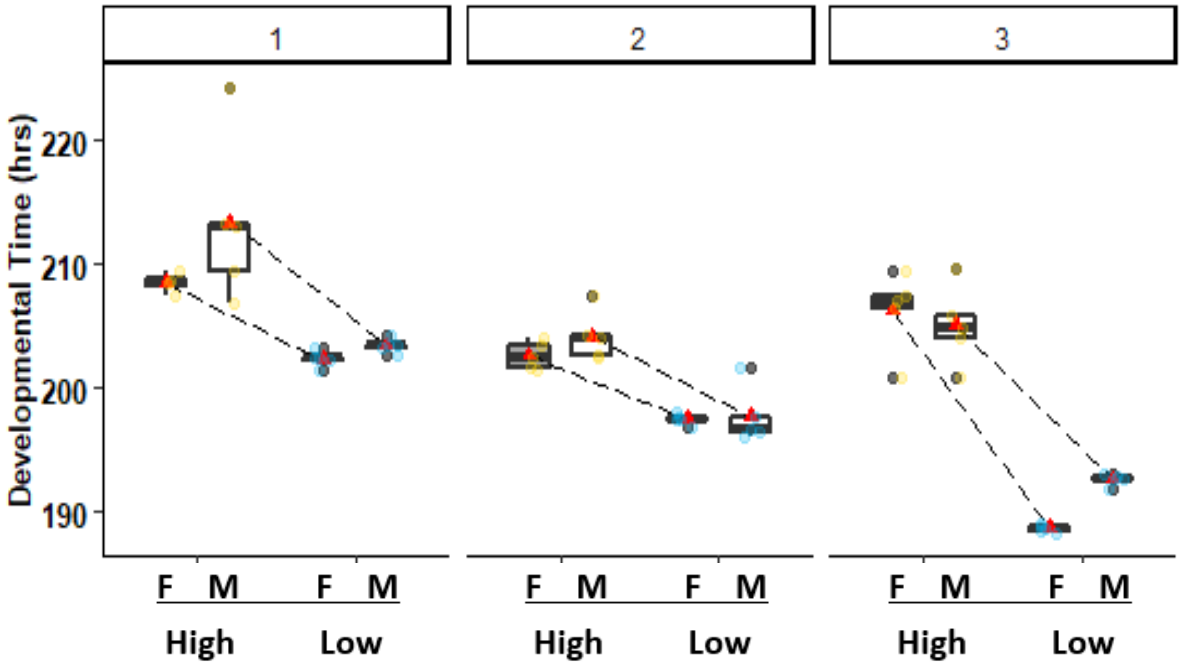

*Fig.4. Developmental Time: The time taken (in hours) for the female flies to develop from eggs to adults in the low-density (L) and the high-density (H). The blocks are separated as facets. * represents significant difference between the selection regimes in that density.*

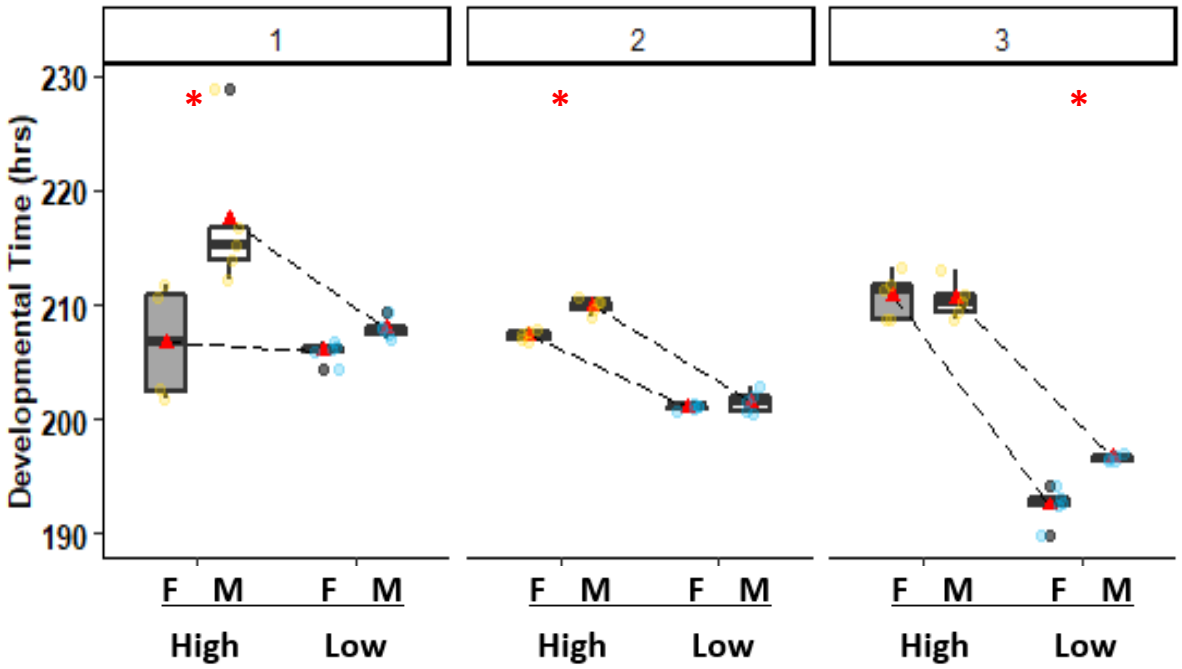

*Fig.5. Developmental Time: The time taken (in hours) for the male flies to develop from eggs to adults in the low-density (L) and the high-density (H). The blocks are separated as facets. * represents significant difference between the selection regimes in that density.*

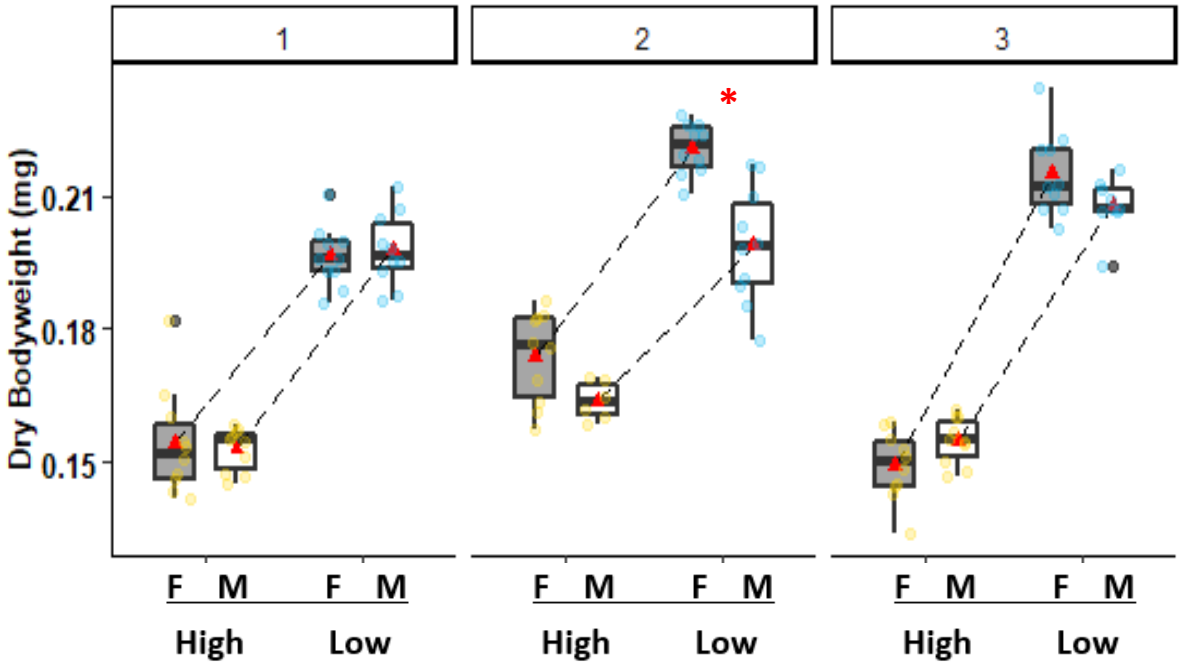

*Fig.6. Dry Bodyweight: The dry bodyweight (in mg) of the male flies reared in the low-density (L) and the high-density (H). The blocks are separated as facets. * represents significant difference between the selection regimes in that density.*

3.2.2. Females

Analysis of the female data also revealed a significant effect of block. On analysing the blocks separately, a significant main effect of treatment was observed in all three blocks with high-density treatment causing a significant delay in eclosion compared to the low-density. There was also a significant selection regime x density treatment interaction effect in block 3. But pairwise comparisons did not reveal any significant differences within the density treatments.

3.3. Dry Bodyweight

As there exists a dimorphism in the body sizes between the sexes in *D. melanogaster*, the data from males and females were analysed separately

3.3.1. Males

There was a significant effect of the random factor, block. Therefore, the blocks were analysed separately. All three blocks showed a significant main effect of density treatment with high density, causing a significant reduction in body weight.

A significant main effect of selection regime was observed in block-2. The pair-wise comparison revealed that the F-males had a higher bodyweight in the ancestral treatments. Also, the block-3 showed a significant selection regime x density interaction. But pairwise comparison did not show any differences within the treatments.

3.3.2. Females

As there was a significant effect of block, data from the different blocks were analyzed separately. A significant main effect of density treatment was observed in all three blocks, with high-density females having lower bodyweights. A significant main effect of selection regime was observed in block-2. And, block-2 & -3 exhibited a significant selection regime x density interaction. And, pair-wise comparison in block-2 revealed that F-females being significantly bigger than M-females in the low-density treatment.

In block-3, however, M-females were significantly bigger than F-females in the high-density treatment.

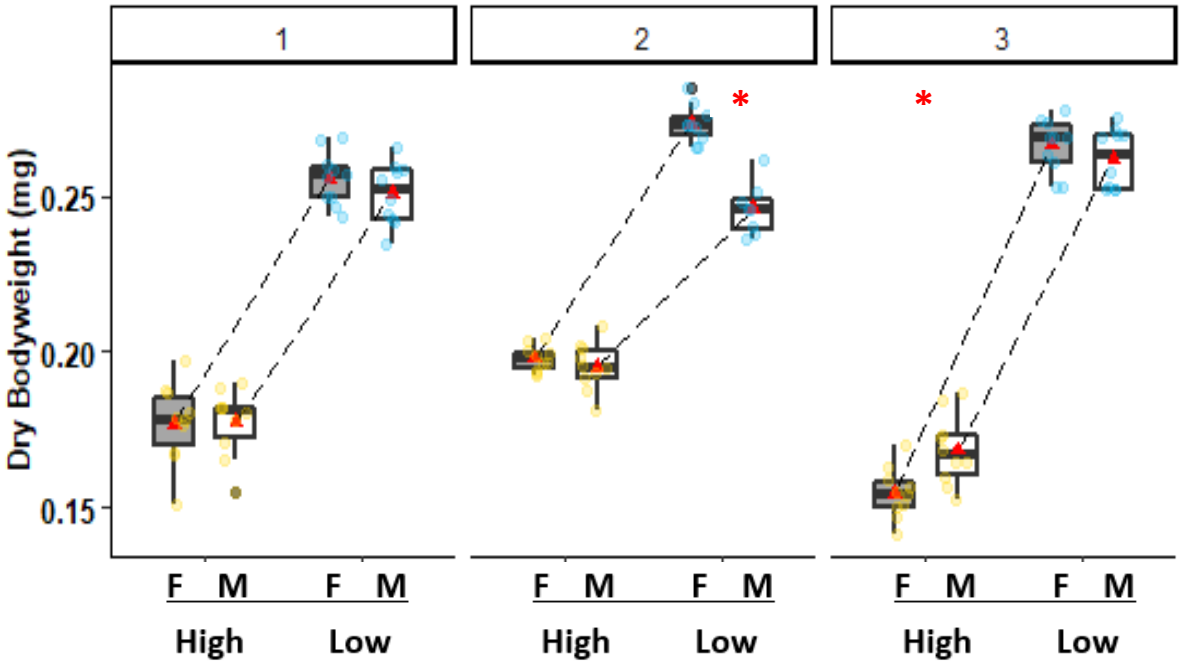

*Fig.7. Dry Bodyweight: The dry bodyweight (in mg) of the female flies reared in the low-density (L) and the high-density (H). The blocks are separated as facets. * represents significant difference between the selection regimes in that density.*

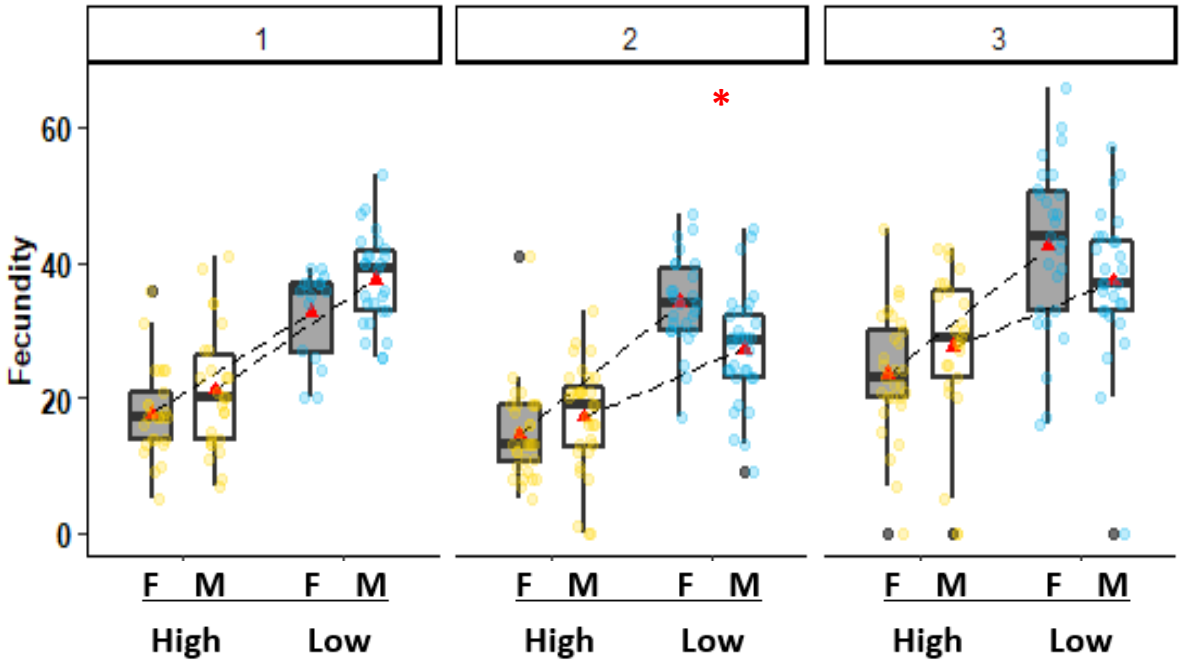

*Fig.8. Fecundity: The number of eggs laid by the female flies reared in the low-density (L) and the high-density (H), after a single mating. The blocks are separated as facets. * represents significant difference between the selection regimes in that density.*

3.4. Fecundity

The blocks were analyzed separately, as there was a significant effect of block in the data. The density treatment had a significant effect on fecundity, with high-density females having significantly lower fecundity than the low-density in all the three blocks.

In block-1, there was a significant main effect of selection regime. But, the pairwise comparisons did not show any significant difference within the treatments. Block-2 revealed a significant selection regime x density treatment interaction with the F-females having significantly higher fecundity than M-females in the ancestral density, but no difference was observed in the high-density treatment.

3.5. Competitive Fitness (CFS)

3.5.1. Male competitive fertilization

The male competitive fitness assay revealed a significant main effect of density treatment. The males from the high-density produced a significantly lower proportion of progeny, when competing with LH males, compared to the males from the low-density. But, there was no significant effect of the selection regime or the selection regime x density interaction on the male competitive fitness. Pairwise comparisons did not reveal any significant differences within the treatments. No effect of selection was observed in the ancestral density, which is surprising as I expected the M-males to exhibit a higher competitive fitness, as reported earlier (Nandy et al., 2013).

3.5.2. Female competitive fitness

The female data revealed a significant effect of block. Therefore, the data from the blocks were analyzed separately. In block-1, both the main effects of selection regime and density treatment were significant with high-density causing a significant reduction in the number of progenies produced. The block-1 females also showed a significant selection regime x density interaction with the M-females producing significantly more progeny than the F-females in the high-density treatments.

However, block-2 & -3 did not show any significant effect of the selection regime or the density on the number of progenies produced.

3.6 Mating Latency

The mating latency data did not reveal any significant main effects of the selection regime or the density treatments in any of the blocks. There was no significant interaction effect of the selection regime x density treatment either in any of the three blocks. The common LHst males readily mated with the females irrespective of the selection regime or the density treatment from which the females came from.

3.7. Copulation Duration

A significant effect of the block was observed in the data. Therefore, the blocks were analysed separately. The common LHst males mated for a longer duration with the females from the low-density treatment compared to the high-density females revealing a significant main effect of the density treatment. A significant effect of selection was also observed in blocks 1 & 3, with the common males mating longer with the M-females from both the density treatments. There was no significant effect of the selection regime x density treatment observed in any of the blocks.

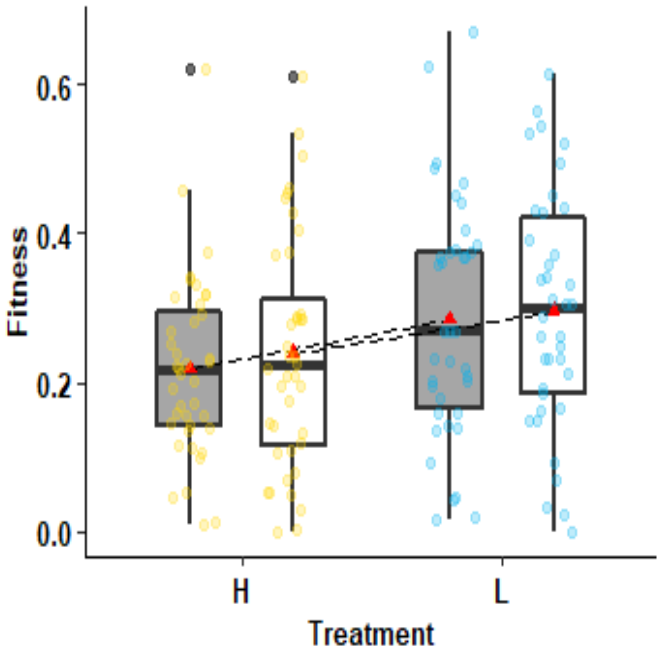

Fig.9. CFS: The proportion of progeny sired by the male flies reared in the low-density (L) and the high-density (H) in a competitive environment. The blocks are separated as facets.

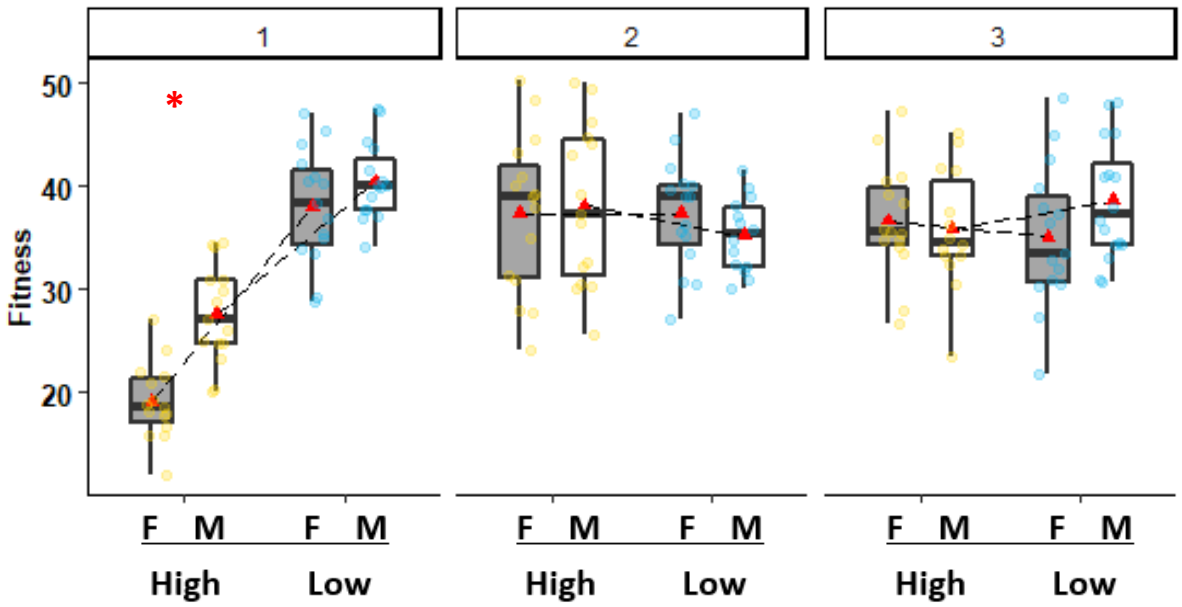

*Fig.10. CFS: The number of progeny produced by the female flies reared in the low-density (L) and the high-density (H) in a competitive environment. The blocks are separated as facets. * represents significant difference between the selection regimes in that density.*

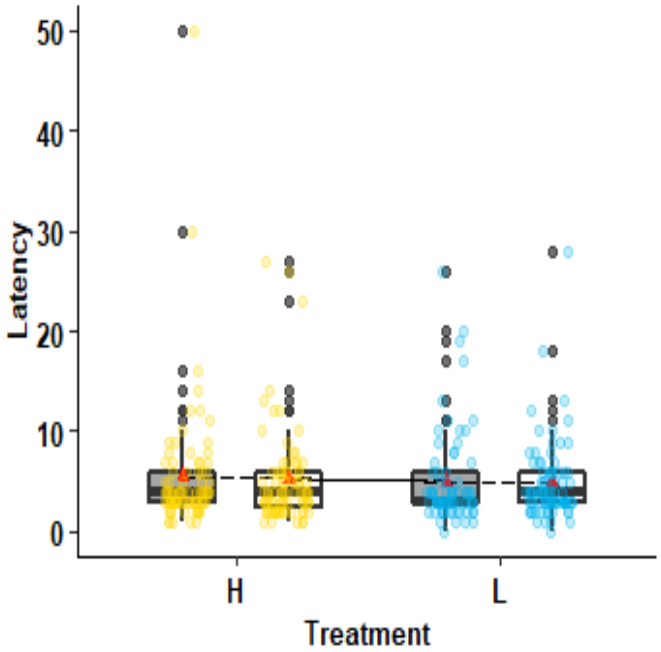

Fig.11. Mating Latency: The time taken by the common males to initiate mating with the female flies reared in the low-density (L) and the high-density (H) in a competitive environment. The blocks are separated as facets.

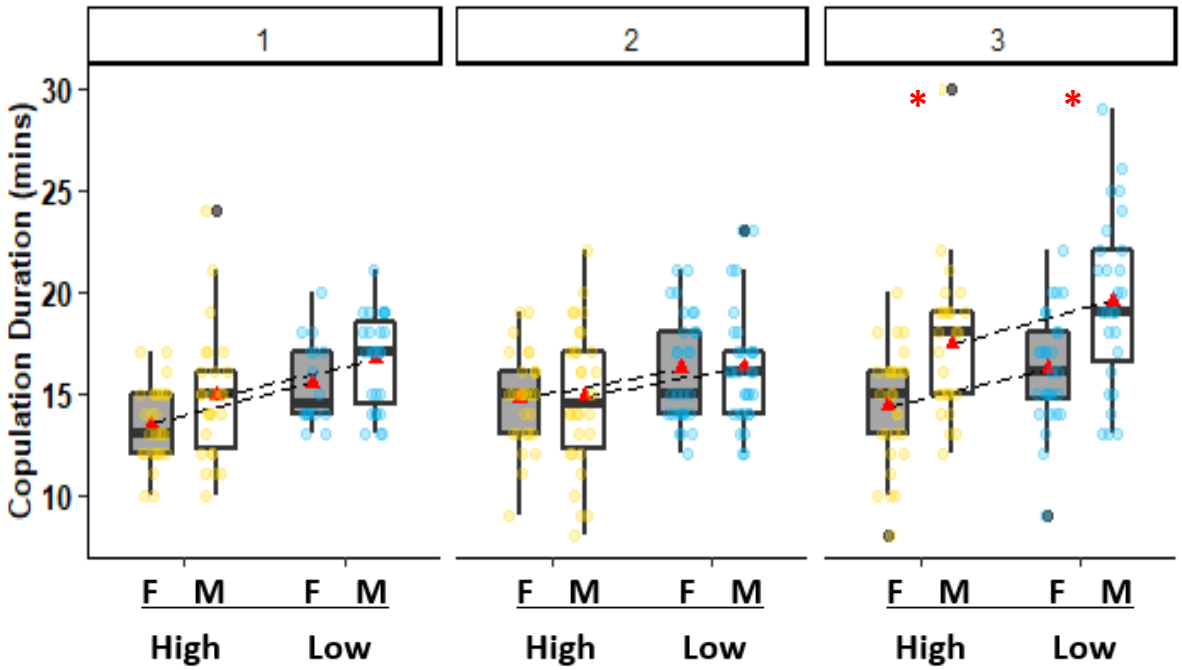

Fig.12. Copulation Duration: The time spent by the common males mating with female flies reared in the low-density (L) and the high-density (H) in a competitive environment. The blocks are separated as facets. * *represents significant difference between the selection regimes in that density.*

Assay	Sex	Block	p-value			F			Pairwise TukeyHSD p-value		Sample Size (n)	df (effect, error)
			Selection	Treatment	Selection x Treatment	Selection	Treatment	Selection x Treatment	M (H) - F (H)	M (L) - F (L)		
Survivorship	**-**	1	5.0E-06	1.4E-08	9.0E-01	47.65	120.85	0.02	0.004	0.002	5	(1, 15)
		2	1.2E-02	2.5E-06	2.6E-02	8.08	50.34	5.97	0.992	0.009	5	(1, 16)
		3	4.2E-01	5.8E-12	8.0E-02	0.69	315.96	3.50	0.881	0.263	5	(1, 16)
Developmental Time (hrs)	**M**	1	2.4E-03	1.0E-02	3.6E-02	13.72	8.72	5.38	0.004	0.628	5	(1, 14)
		2	9.8E-05	3.0E-14	2.4E-03	26.48	624.16	12.93	0.000	0.697	5	(1, 16)
		3	1.2E-02	8.0E-14	4.6E-03	8.04	550.88	10.86	0.988	0.003	5	(1, 16)
Developmental Time (hrs)	**F**	1	5.2E-02	3.5E-07	4.7E-01	4.51	80.67	0.54	0.247	0.699	5	(1, 14)
		2	2.7E-01	5.6E-07	3.6E-01	1.33	63.84	0.90	0.468	0.999	5	(1, 16)
		3	2.0E-01	1.1E-10	2.5E-02	1.80	214.31	6.14	0.852	0.068	5	(1, 16)
Dry Bodyweight (mg)	**M**	1	1.0E+00	3.9E-18	5.8E-01	0.00	263.38	0.32	0.977	0.979	10	(1, 36)
		2	1.2E-03	3.6E-14	8.0E-02	12.67	164.98	3.27	0.211	0.000	10	(1, 32)
		3	3.4E-01	5.1E-24	1.1E-02	0.92	634.86	7.15	0.363	0.166	10	(1, 35)
Dry Bodyweight (mg)	**F**	1	5.2E-01	7.8E-23	4.4E-01	0.42	508.96	0.61	1.000	0.744	10	(1, 36)
		2	1.1E-09	7.1E-26	1.7E-06	68.62	878.25	33.40	0.828	<.0001	10	(1, 34)
		3	1.2E-01	4.0E-29	4.9E-03	2.61	1181.53	8.97	0.013	0.764	10	(1, 36)

Table.1. The p-values and test statistic values (F-values) for main effects and interaction in the "lm" function in Rstudio. Sample sizes of the various assays conducted on the M- & F-flies reared in the two densities. For p-vlaues, Red indicates p<0.05 (Significant). M: Males; F: Females.

Assay	Sex	Block	p-value			F			Pairwise TukeyHSD p-value		Sample Size (n)	df (effect, error)
			Selection	Treatment	Selection x Treatment	Selection	Treatment	Selection x Treatment	M (H) - F (H)	M (L) - F (L)		
Fecundity		1	3.1E-04	2.8E-16	6.2E-01	14.08	100.07	0.25	0.401	0.176	30	(1, 90)
		2	3.9E-01	6.9E-21	1.6E-04	0.74	131.75	15.16	0.241	0.003	30	(1, 117)
		3	3.1E-01	4.0E-11	8.5E-02	1.06	54.85	3.02	0.328	0.872	30	(1, 101)
Competitive Fitness	**M**		3.5E-01	4.5E-03	6.9E-01	0.87	8.34	0.17	0.238	0.799	15	(1, 149)
Competitive Fitness	**F**	1	1.7E-05	1.7E-19	1.1E-02	22.08	186.92	6.91	<.0001	0.466	15	(1, 56)
		2	7.1E-01	3.9E-01	4.1E-01	0.14	0.74	0.70	0.990	0.820	15	(1, 55)
		3	3.5E-01	7.1E-01	1.8E-01	0.88	0.14	1.81	0.988	0.386	15	(1, 56)
Mating Latency (mins)	**-**		8.1E-01	8.7E-01	5.2E-01	0.06	0.03	0.41	--	--	30	(1, 320)
Copulation Duration (mins)	**-**	1	2.4E-03	3.0E-04	8.0E-01	9.72	14.13	0.06	0.780	0.441	30	(1, 93)
		2	9.0E-01	7.7E-03	9.9E-01	0.02	7.36	0.00	0.711	0.938	30	(1, 114)
		3	3.2E-06	3.4E-03	8.1E-01	24.18	9.00	0.06	0.008	0.010	30	(1, 107)

Table.2. The p-values and test statistic values (F-values) for main effects and interaction in the "lm" function in Rstudio and in the "lmer" function in cases of insignificant effect of block. Sample sizes of the various assays conducted on the M- & F-flies reared in the two densities. For p-vlaues, Red indicates p<0.05 (Significant). M: Males; F: Females.

6. Discussion

My results are in agreement with previous studies in establishing that that the larval density has a significant detrimental effect on the life-history traits in *D. melanogaster* (Lints and Lints, 1971; Agnew *et al.*, 2000; Baldal *et al.*, 2005). However, the trait values and the responses to larval density differed significantly between the blocks. This observation could be a result of the fact that these populations have been evolving independently for more than 250 generations. And, this might lead to the populations evolving different trait values.

When the blocks were analyzed separately, the crowded larval environments had a significant impact on the life-history traits studied. It caused a detrimental reduction in the pre-adult survivorship, dry bodyweight, and the female fecundity in both the selection regimes. The adults of both the sexes took longer to eclose when reared in high larval densities than in the ancestral density. Also, the duration that the common males mated with the females was influenced by the density at which the female was reared in. However, I did not observe the selection regime to play a crucial role in determining the expression of these traits. The larval density affected the reproductive success of the males as the smaller males from both the regimes sired a lesser proportion of progeny than their counterparts from the ancestral density. In the females, however, neither larval density nor the selection regime had any effect on reproductive success in two of the three blocks suggesting sex-specific responses to environmental stress.

Development under a crowded environment is a common phenomenon in nature and could be a principal agent of selection (Bakker, 1961; Roper et al. 1996). Hence, the developmental environment can play a crucial role in determining adult life-history traits and the individual's fitness (West-Eberhard, 2003). And incidentally, a significant impact of larval density has been observed and established in *Drosophila* and other species as well.

In my experiments, the high larval crowding was accompanied by increased mortality during the pre-adult stages in these populations. I observed this response in all three blocks and the selection regimes suffered equally worse mortalities in high-larval densities. This increased pre-adult mortality could, possibly, arise due to a scarcity of food in the high-density environment (Scheiring et al. 1984). As a result, a fewer

number of larvae are able to acquire enough resources to attain the critical bodyweight needed for pupa formation. Additionally, the high-density environment is known to have increased concentrations of waste products like uric acid and urea (Botella et al. 1985), which at high concentrations might cause larval mortality.

In the ancestral larval density, however, I observed higher larval mortality in the M-regime compared to the F-regime, although the difference was significant only in blocks 1 and 2. These results were in contrast to the earlier data of pre-adult survival on the same populations when they were 52-55 generations old, where the two selection regimes had similar amounts of pre-adult survivorship.

Among the flies that eclosed, the median developmental time to eclosion was longer when reared in the high-larval density, for both the sexes. Larval density has been shown to affect the developmental rate negatively in the *Drosophila* system earlier as well. Lints and Lints (1971) studied the effect of a wide range of pre-imaginal densities and temperatures. They observed a gradual increase in the duration of development from 8 to 16 days with increasing densities. Within their larval densities, the females from the two selection regimes did not differ in the time taken to develop in any of the blocks. In the males, however, the developmental time responses were varied between the blocks. The selection regime interacted with the density treatment and the developmental times of F-males were more robust than M-males to the larval density stress in the blocks 1 & 2.

Nandy (PhD Thesis, 2013), in contrast, reported a significant effect of the sexual selection regime with the F-flies taking significantly longer to eclose than the M-flies in both the sexes. But, it is essential to note that even the baseline developmental time of the M- an F-population has evolved. Adult flies emerged earlier by roughly a day than they had during the earlier study and the effect of the sexual selection regime has also disappeared since. As virgins are collected soon after the eclosion begins, the selection regime could represent an indirect selection for the earlier eclosion in the M- and F- population that might cause this decrease in developmental time.

Nandy (PhD Thesis, 2013) attributed the delay in eclosion of the F-flies to the evolution of increased body size, as he observed that the F-flies were also bigger than the M-flies. And, larger flies have been shown to take longer to finish larval development (Robertson, 1963). In the current study, this trend in the controls was observed only in

block-2. As expected, the M- & F- populations produced smaller individuals when reared in a crowded larval condition, indicated by the lower dry bodyweight in both the sexes. The bodyweight was measured after collecting adult flies, drying the flies at 60°C to avoid any confounding effects that could arise from feeding after eclosion. The lower dry bodyweight can be attributed to a scarcity of food late in development, when exoskeletal developmental might be accomplished with all the available food. And, there is not sufficient food for building up internal reserves (Murdie, 1969). Or the scarcity of food results in a larger energy expenditure in search of food that is paid at the expense of size and weight (Lints & Lints, 1971). And, the M- & F- flies expressed no differences in their dry bodyweights when reared in crowded larval conditions. The result from block-2, in both sexes, suggests that the severity of the stress caused by larval crowding maybe eliminating any effect of the selection regime expressed in the ancestral density.

Generally, body size has been found to correlate with the female fecundity positively. Both M- and F-populations suffered a significant reduction in fecundity when reared in crowded larval densities. This relationship could be explained by the observation that starvation during larval development reduces the ovariole number in *D. melanogaster* females (Thomas-Orillard, 1972). Our measure of fecundity, i.e., the number of eggs laid after single mating with a common male, ensured that the fecundity is attributed to the female's ability to produce eggs and less so the male's fertility. Nandy et al. (2013) showed that the heavier F-females expressed higher fecundity after a single mating. Only block-2 revealed this effect of selection regime in the ancestral density. Also, the significant interaction effect in block-2 indicates higher robustness in the fecundity of the M-females to the larval environmental stress. In blocks 1 & 3, however, the selection regimes did not differ in their responses. In a similar experiment, Martinossi-Allibert et al. (2018) revealed that both sexual selection regimes (monogamy vs. polygamy) exhibited decreased fertility when exposed to elevated temperature stress.

In addition to the reduction in body size, the lower fecundity of the smaller females can also be attributed to the male's preference and higher reproductive investment towards larger females. Byrne and Rice (2006) showed in *D. melanogaster* that males expressed adaptive mate choice towards larger females indicating better quality. Given that there is mate choice, the males also copulated for a longer duration with

larger females (Lefranc and Bundgaard, 2000) and invested more, by transferring higher amounts of ejaculate and seminal fluid proteins, towards the larger females (Wigby et al. 2016). The common males in my experiments, readily mated with the females irrespective of the female's size and the selection regime of origin. This behaviour could just be an artifact of the experimental design itself, as the latency to mating was measured in a no choice paradigm. And in line with the above studies, the common males invested a longer copulation duration with larger females irrespective of the selection regime of the female.

In natural populations, the competitive ability of an individual could play a crucial role in determining fitness, as individuals have to compete for food resources, access to mates, available space, etc. In *Drosophila*, males compete for access to mates and females compete for limited food resources or egg-laying grounds. Here, I accessed the effect of larval environment on the reproductive success of males and females in adult competitive environments. The males raised in the high- and low- larval densities competed with the common males from an ancestral background for access to mates. And, our experimental design allows for both pre-copulatory and post-copulatory competition to occur and the proportion of progeny sired by the focal males is considered the reproductive success of the males. The females competed for a limiting amount of yeast, which is a protein source that is known to influence the female fecundity in *D. melanogaster*.

The male CFS data revealed that larval density had a detrimental impact on male fitness. The smaller males from the crowded larval environment sired a lesser proportion of progeny compared to the larger males. Edward & Chapman (2012), studying the larval density effects on reproduction, observed a similar reduction in the males' share of paternity. However, the two selection regimes did not show any differences in their reproductive fitness in either density. This result is surprising as I expected the M-populations to show higher competitive fitness, at least in the ancestral density, as reported earlier (Nandy, PhD Thesis). This deviation from the expectation could arise due to the subsampling of the females from the experiment.

As the females mate only twice in a 48-hour window, all the females may not have mated with a focal male and, hence, won't sire any scarlet progeny. Therefore, the absence of scarlet progeny could either mean no mating with a focal male has

occurred or failure of post-mating fertilization. Hence, subsampling 7 out of 15 females gives rise to noise in the male competitive fitness data, which could explain the insignificant effect of selection.

My results are in contrast with a very similar study exploring the effect of a different environmental stress, temperature, on competitive fitness (Matinossi-Allibert, 2018). Their study revealed a higher fitness of individuals evolving in the presence of sexual selection and male-female coevolution. The primary differences between these studies are they manipulated the level of sexual selection by rearing populations of the seed beetle, *callosobruchus maculatus,* either in the presence (polygamy) or in the absence (Monogamy) of sexual selection. Whereas, our study was conducted on populations evolving in differential levels of sexual selection through altered operational sex ratios.

Also, their study revealed a sex specificity in the responses as female fitness was, in general, more robust to the elevated temperatures while male fitness was affected negatively. The insignificant effect of the high-larval density on the fitness of the females from blocks 2 & 3 was in support of the above observation. I observed a significant reduction in the fecundity of as well as the copulation duration towards the smaller females from the crowded larval densities. However, block-1 showed a contrastingly different trend. The crowded larval environment caused a reduction in the fitness of the females. And, the higher fitness of the M-female can be attributed to the higher resistance to mate harm in the M-females evolved in higher sexual conflict.

Thus, the effect of the sexual selection on life-history traits that were observed early in the experimental evolution (~50-55 generations) was found to have disappeared in my study (~250-255 generations). This observation could be arise due to fecundity selection that could later have become a significant agent of selection, leading to similar trait values in these populations. And, the insignificant differences in the fitness of M- & F-populations suggests that the “good-genes” effects resulting from sexual selection may dissipate after many generations of selection. Moreover, the consequential impact of sexual selection on the population demography in a novel environment could depend on the environmental change itself. The results and conclusions obtained from this study could be specific to larval density change and could be varied for a different change. Another environmental stress that could play a

crucial role in the sexual selection dynamics is temperature, which is yet to be studied in these populations. This study is only the second such study, that I have come across, addressing the responses to environmental change in populations evolved in various sexual selection regimes. And, our study concludes that the selection regime may not be playing a crucial role in determining the responses the developmental environmental change, High larval density, in *Drosophila melanogaster*.

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