Drosophila melanogaster as a model organism to study impacts of microplastics on terrestrial organisms

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A dissertation submitted for the partial fulfilment of BS-MS dual degree in Science

Indian Institute of Science Education and Research mohali

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Certificate of Examination

This is to certify that the dissertation titled "*Drosophila melanogaster* as a model organism to study impacts of microplastics on terrestrial organisms" submitted by Mr. Mubarak Jamal (Reg. No. MS16136) for the partial fulfillment of BS-MS dual degree programme of the Institute, has been examined by the thesis committee duly appointed by the Institute. The committee finds the work done by the candidate satisfactory and recommends that the report be accepted.

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Dated: April 30, 2021

Declaration

The work presented in this dissertation has been carried out by me under the guidance of Prof. Dr. N.G. Prasad at the Indian Institute of Science Education and Research Mohali.

This work has not been submitted in part or in full for a degree, a diploma, or a fellowship to any other university or institute. Whenever contributions of others are involved, every effort is made to indicate this clearly, with due acknowledgement of collaborative research and discussions. This thesis is a bonafide record of original work done by me and all sources listed within have been detailed in the bibliography.

Mubarak Jamal

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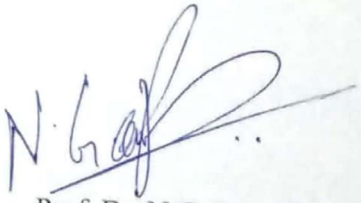
Dated:

In my capacity as the supervisor of the candidate's project work, I certify that the above statements by the candidate are true to the best of my knowledge.



Prof. Dr. N.G. Prasad
(Supervisor)

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Abstract

We investigated the effects of polystyrene microparticles in adult *Drosophila melanogaster* when exposed to both larval and adult stages of their life cycle. Even though there have been plenty of studies done on the impacts of microplastics on aquatic organisms, assessments of the same on terrestrial organisms are very scarce. Two independent studies were able to show intestinal damage, locomotor dysfunction, upregulation of HSP70 and significant changes in the daily activity of *Drosophila melanogaster* upon chronic exposure to polystyrene microparticles. We mixed polystyrene latex beads of size 0.8µm in Drosophila food to examine the effects on mating behavior, fecundity and other reproductive fitness characteristics of both male and female flies in control and microplastic treated flies. Two sets of experiments were done in which the first one was done in a way that drosophila larva was ingested with polystyrene and the adults eclosed from this were observed. In the second set, the adult fly was exposed with polystyrene for two days. Polystyrene ingestion did not cause a change in any of the traits (fecundity, mating behavior, body size, fluctuating asymmetry and sperm defense ability) that we assessed in both the types of experiments.

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Chapter 1

Introduction

Microplastics(MPs) are plastic particles of size less than 5 mm which are commercially synthesized or naturally produced by means of chemical, physical, or photo-degradation of plastic(Kalogerakis et al., 2017). These small particles of varying sizes and shapes get occupied in terrestrial, aquatic, and aerial ecosystems through the wind, waves, etc. Commercially synthesized microbeads or pellets of microplastics are often used as fillers in personal care products and medical products(Hale, et al., 2020). These minute particles are heavily accumulated in the marine ecosystem which is mostly polystyrene, polypropylene, and polyethylene(Andrady, et al., 2009). The increasing accumulation of MPs in diverse food chains including fishes(Lu et al., 2016), mussels(von Moos et al., 2012), zooplankton(Chua et al., 2014), etc makes this study significant.

There have been a lot of publications on the harmful effects of microplastic exposure on animals and plants in the last 10 years. Since microplastics are heavily found in the aquatic ecosystem, most of the studies were conducted on aquatic organisms. In one study on oysters, exposure to polystyrene microparticles negatively affected their reproduction and development (Sussarellu, et al., 2016). An adult zebrafish study of 2019 revealed that microplastic ingestion is able to induce alteration in the expression of immune system genes and behavior (Limonta, et al., 2019). Significant reduction of body size of offspring(Bhagat, et al., 2019), influence on survival, growth, reproduction, and transgenerational toxicity(Eltemsah, et al., 2019) were observed in *Daphnia magna* when exposed to polystyrene microparticles. In Caenorhabditis elegans, shortening of lifespan and alteration of the intestine function(Shang, et al., 2020), generation of oxidative stress(Yu, et al., 2020), and induction of transgenerational neurotoxicity(Chen, et al., 2021), was observed when ingested with microplastic particles. Furthermore, some studies have successfully shown the ability of microplastics to act as potential carriers of chemical contaminants and ions(Browne et al., 2013).

Even though there have been plenty of studies done on the impacts of microplastics on aquatic organisms, assessments of the same on terrestrial organisms are very scarce despite the fact that soil is also one of the dumping places of plastic wastes. In a mice model system, maternal exposure to different sizes of polystyrene microplastics during gestation led to altered levels of metabolic products in their offspring (Luo, et al., 2019). Oral microplastic ingestion led to the accumulation of 4µm and 10µm polystyrene in the testis of mice which induced testicular inflammation. A significant decline in sperm quality & testosterone levels was also observed (Jin, et al., 2021).

There are two published studies on microplastic impact in *Drosophila melanogaster*, and one of them was able to show gut damage using the trypan blue staining method, And they observed locomotor dysfunction by doing a climbing assay (Zhang, et al., 2020). However, this study does not investigate the effects of microplastics on important fitness characteristics, physiological traits, or life-history traits. The second work pointed out that there is little to no effect on mortality, fertility, and development of *Drosophila melanogaster* upon polystyrene exposure(Matthews, et al., 2021). Nevertheless, they were able to show upregulation of HSP70 expression, intestinal damage, and significant changes in locomotion and daily activity after chronic exposure to polystyrene particles.

Drosophila melanogaster, being a holometabolous organism, has 4 stages in its life cycle: zygote, larva, pupa, and adult stage(Figure 1). 18 hrs later to egg-laying, the first instar larva hatches out from the egg. Larval stage progresses through three sub-stages; first, second and third instars.They dig into the food during the larval stage and effectively feed on the available food. After getting into a certain size, the late third instar larva ceases eating, emerges from the food and settles down on a suitable position. 3rd instar larva gets enclosed by a chitinous layer nearly on the 5th day in order to become a pupa. It takes roughly 8-10 days for the culmination of the pre-adult stage which leads to the eclosion of the adult fly. The emerged ones become reproductively mature within nearly 8 hours.

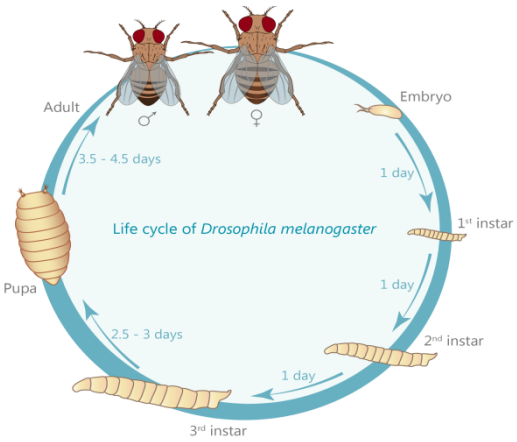

Figure 1.1: Different stages of the life cycle in *Drosophila melanogaster*(source: https://www.walter-lab.com/methods).

In my master's thesis, I have investigated how polystyrene ingestion affects male and female reproductive fitness characters and life-history traits in *Drosophila melanogaster*. Polystyrene particles have been reported in the terrestrial(Scheurer, et al., 2018) and marine ecosystem. Here we assay copulation duration, which is the time taken by the flies to complete just one copulation. Copulation duration is an important indicator of sperm investment in reproduction (i.e., how much sperm a male fly ingests in single copulation) which will clearly give us an idea about what microplastic ingestion does to the reproductive fitness of the flies. A fecundity check reveals the microplastic impact on female reproductive fitness. These experiments were done as two separate sets in a way that microplastic impacts were examined in adults after treating both larvae and adults separately. It is possible that the larva would experience stress during development since both published studies on Drosophila were able to show gut damage upon polystyrene exposure. The wing size difference and fluctuating asymmetry are also checked in flies that were administered polystyrene in the larval stage which can point out the developmental instability due to stress experienced during the developmental stages(Ludwig, 1932). If we get a significant result from these observations, we could proceed with various experiments regarding microplastics with our powerful genetic model system, *Drosophila melanogaster*. Variations in development time and the number of viable larvae could point out severe developmental impacts. Investigating the impacts of microplastic ingestion on multiple generations of Drosophila is one among those interesting future experiments.

Chapter 2

Materials and methods

2.1 Drosophila stock

Typically, a laboratory population is begun with a number of wild captured mated females and allowed to reproduce in the lab. A large group of interbreeding individuals is retained under a more or less defined maintenance regime(temperature, humidity, food, cage, density etc.) from the next generation onwards. These populations are only used for studies after many generations of acclimating to the laboratory conditions.

The LH population (Larry Harshman formed a baseline population two years ago from 400 mated females collected in central California and held at 25 °C with Ne > 5,000 on a two-week generation time) and a derived population – LHst – are used in all of the experiments mentioned in this thesis and a derived population (Chippindale and Rice 2001). Ever since, the population has been held on regular cornmeal-yeast-molasses fly food in a 14-day discrete generation period at 25°C, 60-80 percent relative humidity, and 12-h light / 12-h dark. The population is maintained in 8-dram vials (25mm diameter × 90mm height). The population consists of 60 vials in total. Larvae are cultured at a moderate density per generation (around 150 eggs per 8-10ml of food in 8-dram vials). LHst was derived from LH base population by introducing the recessive-autosomal trait scarlet-eye (‘st’) by repeated back crosses (Prasad et al. 2007). LHst is also held under the same conditions as LH, with the exception that the population comprises 30 vials. LHst is backcrossed with LH on a regular basis to maintain genetic uniformity between the two populations.

2.2 Polystyrene beads

Polystyrene latex beads (0.8µm mean particle size) were purchased from Sigma-Aldrich company as a dispersed solution (10% w/v, 2 mL) and kept under 25 °C.

2.3 Cornmeal molasses food

All the ingredients from 1 to 5 (in Table 1) are mixed very well and heated till it starts boiling. Turn off the stove once the solution becomes a thick suspension. Once the suspension is cooled a bit, add the preservatives Propionic acid and p-Hydroxymethyl benzoate in Ethanol to the suspension. Mix it very well so that the preservatives are uniformly reached everywhere in the food. Then the hot food is poured into vials and kept under a fan for the cooling purpose before the actual use.

Sl. No.	Ingredient	Amount (per litre of food)
1	Water (ml)	1000
2	Agar powder (gm)	14.8
3	Molasses (ml)	100
4	Cornmeal (gm)	100
5	Yeast (gm)	41.2
6	Propionic acid (ml)	8
7	p-Hydroxymethyl benzoate (gm)	2.25
8	Ethanol (ml)	22.5

Table 2.1: Composition of ingredients for cornmeal - molasses food (Nandy, B. 2012).

2.4 Two types of treatments

2.4.1 Polystyrene solution + yeast solution:

Polystyrene latex beads were mixed in a yeast solution with a particular concentration of polystyrene per 300µL yeast solution. The yeast solution contains 1 gram of baker's yeast in 6 mL of water. After properly mixing the whole solution using a vortex machine (Model: Vortex mixer 230V EU PLUG, Brand: Labnet international inc.), this was slowly added on top of normal molasses food in the glass vial. Vials were covered with cotton plugs after drying the vials using a fan.

2.4.2 Yeast solution(control):

The same number of vials were made as mentioned above but without mixing polystyrene in the solution. Just 300µL yeast solution was poured above molasses food and kept under the fan.

2.5 Polystyrene detection

Two studies have already shown that *Drosophila melanogaster* is ingesting polystyrene particles of micrometer size when it is mixed with their food. We mixed food color with the yeast - polystyrene (of the same size range as they used in the mentioned experiments) mix and dissected out their gut using a needle and forceps. Gut parts were

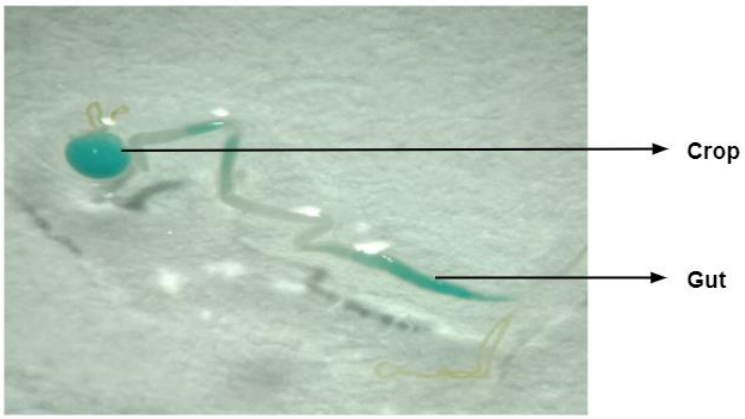

Figure 2.1: Drosophila gut with yeast - PS mix mixed with food color

2.6 Effects of polystyrene upon treating larva

2.6.1 Fecundity, copulation duration and mating latency

Polystyrene solution is mixed in yeast solution with a composition of 15mg polystyrene per 300µL yeast solution. The concentration used was assigned with respect to the concentration information provided in the experiment which was able to show gut damage after polystyrene exposure(Zhang, et al. 2020). 150 eggs per vial are collected from the LH population in both treated and control food vials. A full factorial assay of copulation duration, fecundity and mating latency were done, with all the 4 combinations:-

A → Both male and female from yeast-treated vials.

B → Male from yeast treated and female from polystyrene treated vials.

C → Male from polystyrene treated and female from yeast treated vials.

D → Both males and females are treated with polystyrene.

For assaying copulation duration, 40 replicates of each combination were observed for one hour. Mating start time and mating end time are noted down to get the copulation duration. After one hour, male flies were discarded from every vial, and females were kept in the incubator for 18 hours. After 18 hours, females were discarded and vials were kept in the -20℃ freezer to avoid egg hatching. Then the number of eggs in every vial was counted to get the fecundity. Since intestinal damage and locomotor dysfunction are observed due to polystyrene exposure, a change is expected in the feeding efficiency and interactions between mating partners, which is predicted to have an impact on the mating behavior and fecundity of the flies. Observed data were entered in Excel followed by plotting and analysis using R studio software.

2.6.2 Morphometric analysis

PS treated and control flies of both sexes are kept in the -20℃ freezer for 24 hours. Wings are imaged using Future winjoe software after the dissection of left and right wings are with the help of a forcep and a needle. Then their linear measurements are done using Image J software. An image of standard 1 micrometer line on was used as reference for measuring the length.

1) Body size assay: Effects on body size indicate developmental instability due to stress from the environment during developmental stage. Body size is estimated as the mean of L3 vein Length of both right and left wing [(R+L)/2]. L3 vein length starts from the anterior cross vein to the end of the second longitudinal vein (Markow and Ricker, 1992).

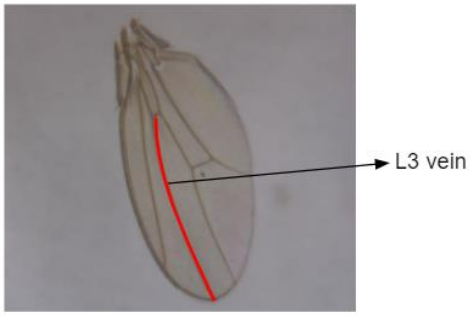

Figure 2.2: L3 vein in *Drosophila melanogaster* wing

2) Fluctuating asymmetry: Effect on fluctuating asymmetry (FA) also indicates developmental instability. FA values were estimated as the variance of difference between the sides [(R-L)] standardized over the mean trait size [(R+L)/2](Shenoi, V. 2016).

2.7 Effects of polystyrene upon treating adult

Polystyrene solution is mixed in yeast solution with a composition of 1mg polystyrene per 300µL yeast solution and this solution is poured on top of cornmeal-molasses food. 10 LH flies/vials of both sexes are collected in both treated and control food vials. The concentration used was assigned with respect to the concentration information provided in the drosophila experiment by Zhang (Zhang, et al. 2020). LHst flies are also collected on cornmeal-molasses food vials to use ase mating partners for focal LH flies in both male and female based experiments.

2.7.1 Female based experiments

Adult females from the LH population were treated with polystyrene mixed in yeast solution for two days after their eclosion. Same number of another set of LH females were given just yeast solution to use as control for the experiment. Then they were mated with LHst males cultured in cornmeal-molasses food. Mating activity was observed for 1 hour to note down the copulation duration and mating latency. After 1 hour, males were discarded, and female flies were kept in an incubator at 25℃ for 18 hours. Then the vials were kept in a -20℃ freezer for three days after removing the females. Number of eggs were counted for both PS treated and just yeast treated females to check effect on fecundity.

2.7.2 Male based experiments

As detailed above, LH males were treated with yeast solution-PS mix and just yeast solution. After placing them with LHst mating partners, copulation duration and mating latency are noted down. Then we start with our next steps to evaluate the effects of microplastics on sperm defense ability (the ability to resist displacement by sperm from other males) of males. After 1 hour of observations, LH males were removed and LHst males (cultured on cornmeal-molasses food) were introduced to these vials with LHst females. Females are collected from these vials after 24

hours and replaced to test tubes with cornmeal-molasses food. They are given 24 hours due to the fact that females will not mate immediately after their first mating. These test tubes are placed in the incubator at 25℃ for the next 11-12 days. The newly eclosed progenies are scored for their eye colors(Scarlet and Red) after putting them in the -20℃ freezer for the ease of directly counting them.

Chapter 3

Results

3.1 Effects of polystyrene upon treating larva

Treatment	MALE	FEMALE
A	Yeast	Yeast
B	Yeast	Polystyrene+Yeast
C	Polystyrene+Yeast	Yeast
D	Polystyrene+Yeast	Polystyrene+Yeast

Table 3.1: Four combinations of distinct treatments (A,B,C & D) on drosophila larva.

3.1.1 Effects of microplastics on copulation duration

Adults from all four combinations showed nearly equal copulation duration. Copulation duration was slightly reduced when female larvae were exposed to polystyrene. However, these differences were not statistically significant (Table 3.2).

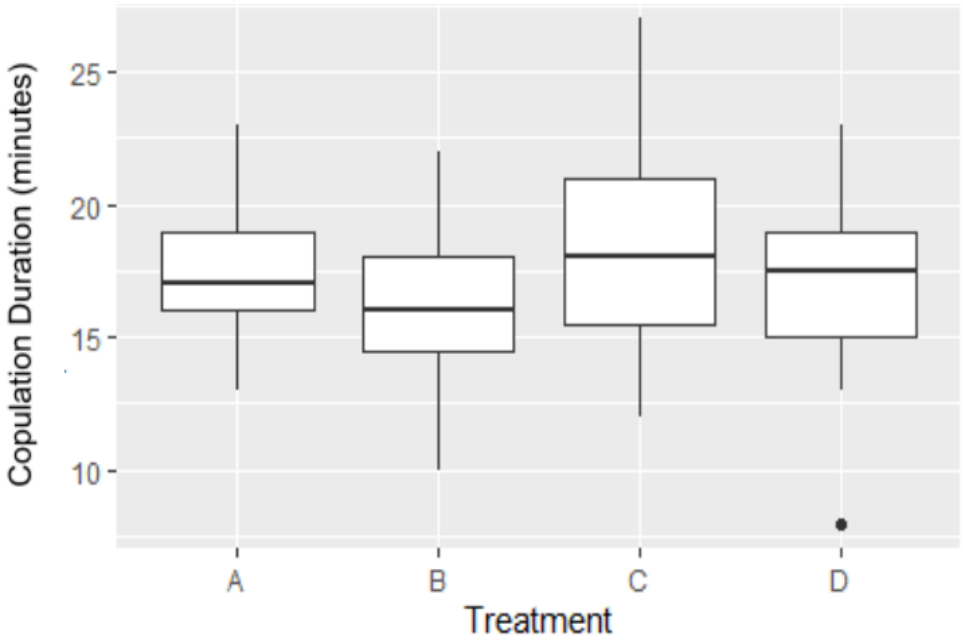

Figure 3.1: Time taken to complete one copulation plotted against different combinations of treatments. The line represents the median and the box the interquartile range.

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Treatment	3	70.09	23.3642	2.4302	0.06797
Residuals	135	1297.92	9.6142		

Table 3.2: ANOVA table with polystyrene treatment as independent variable and copulation duration as dependent variable.

3.1.2 Effects of microplastics on fecundity

Number of offspring produced by female flies from all four combinations of fly pairs were nearly equal. I.e, No effect on adult fecundity when larva was treated with polystyrene (Table 3.3).

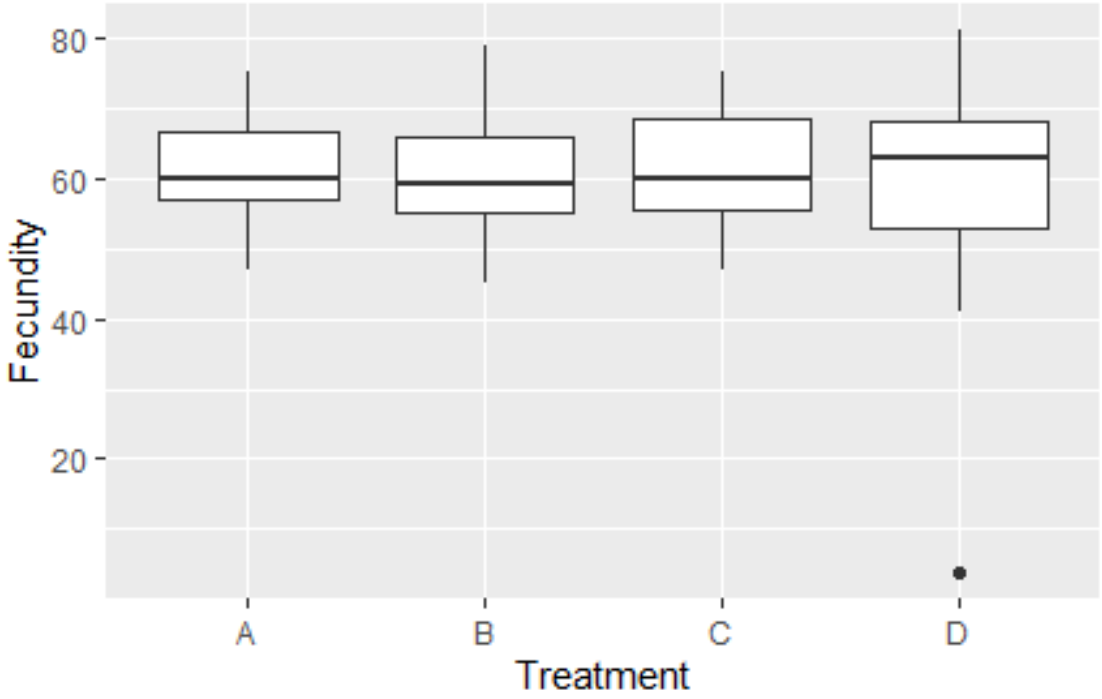

Figure 3.2: Number of offspring produced by all four combinations of fly pairs plotted against different combinations of treatments. The line represents the median and the box the interquartile range.

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Treatment	3	53.3	17.772	0.1866	0.9054
Residuals	133	12666.6	95.237		

Table 3.3: ANOVA table with polystyrene treatment as independent variable and fecundity as dependent variable.

3.1.3 Effects of microplastics on mating latency

Adults from all four combinations showed nearly equal mating latency. There was a slight increase in mating latency when both males and females were collected from polystyrene treated vials. However, these differences were not statistically significant (Table 3.4).

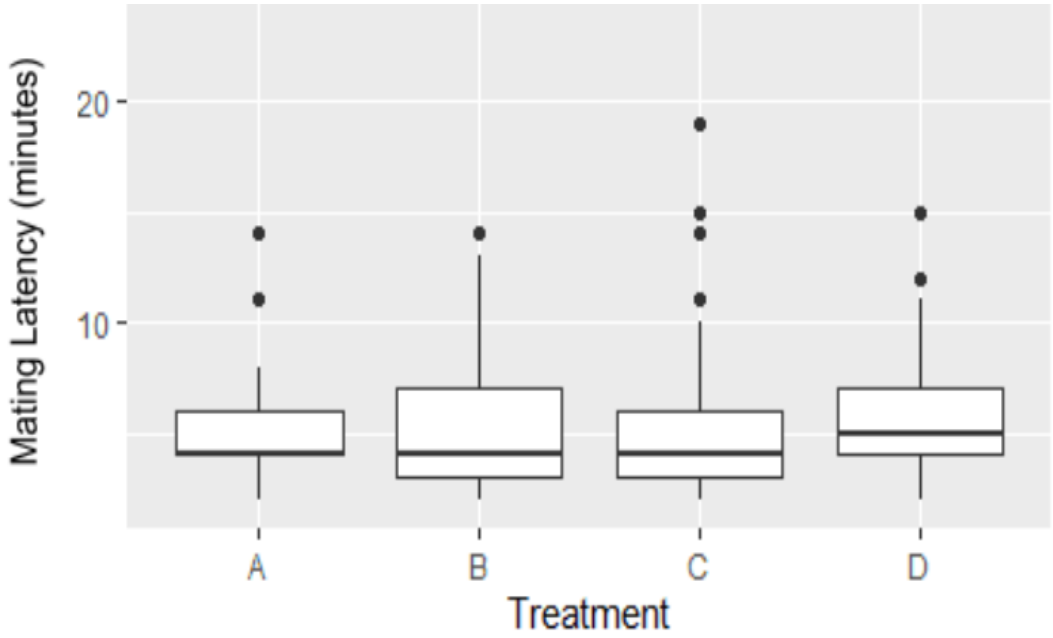

Figure 3.3: Time taken to start mating plotted against different combinations of treatments. The line represents the median and the box the interquartile range.

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Treatment	3	23.13	7.7091	0.514	0.6733
Residuals	135	2024.57	14.9968		

Table 3.4: ANOVA table with polystyrene treatment as independent variable and mating latency as dependent variable.

3.1.4 Effects of microplastics on body size

Body size was slightly lower for the adults eclosed from polystyrene treated larvae. However, these differences were statistically insignificant (Table 3.5).

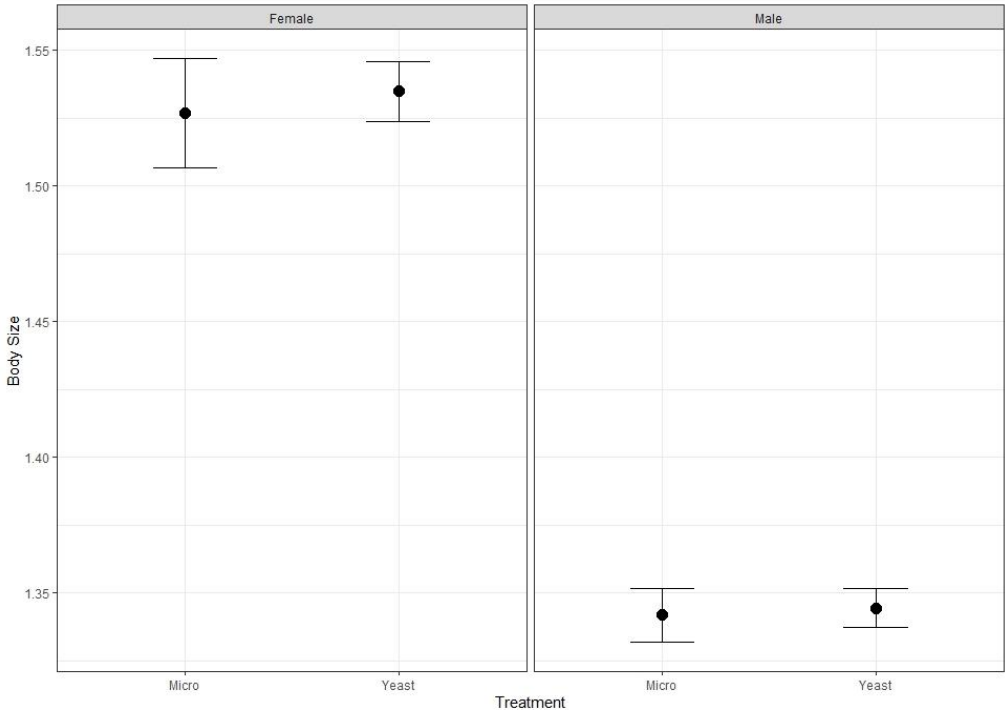

Figure 3.4: Mean of L3 vein Length of both right and left wing plotted against two types of treatments in females and males. Micro stands for microplastic treatment and yeast stands for yeast treatment. The point in the middle represents the median and the line the interquartile range.

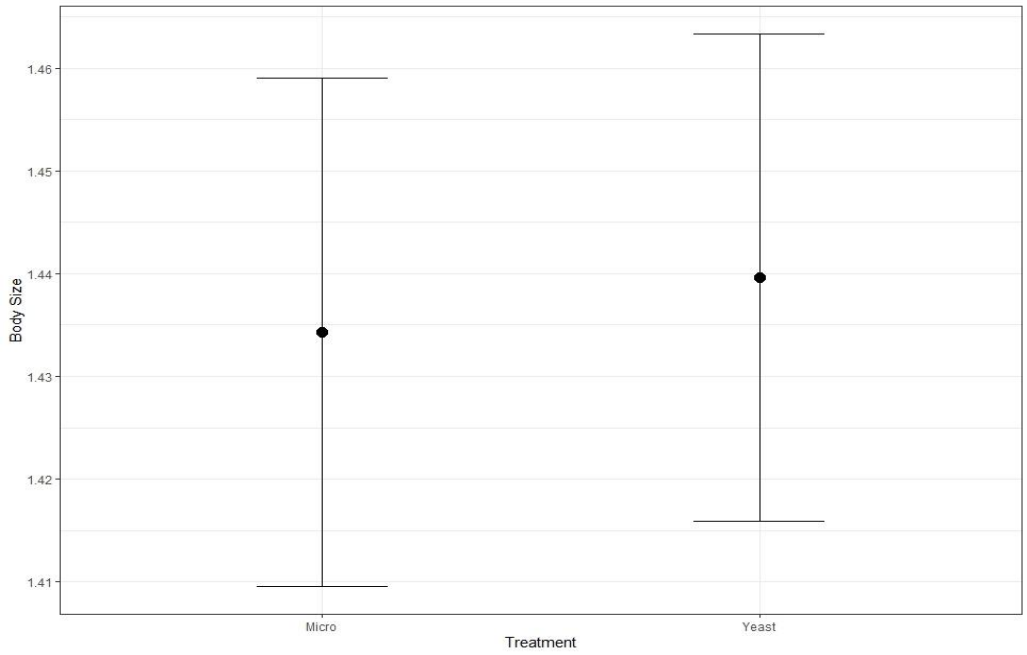

Figure 3.5: Mean of L3 vein Length of both right and left wing plotted against two types of treatments. Micro stands for microplastic treatment and yeast stands for yeast treatment. The point in the middle represents the median and the line the interquartile range.

	Sum Sq	**Df**	**F value**	**Pr(>F)**
Treatment	**0.00100**	**1**	**0.6968**	**0.4053**
Sex	**1.23385**	**1**	**860.5560**	**<2e-16*** **
Treatment:Sex	**0.00025**	**1**	**0.1742**	**0.6771**
Residuals	**0.19499**	**136**		

Table 3.5: ANOVA table with polystyrene treatment and sex as independent variables and body size as dependent variable.

3.1.5 Effects of microplastics on fluctuating asymmetry

Fluctuating asymmetry was nearly equal for the adults eclosed from both polystyrene treated larvae yeast treated larvae. The small differences were statistically insignificant (Table 3.6).

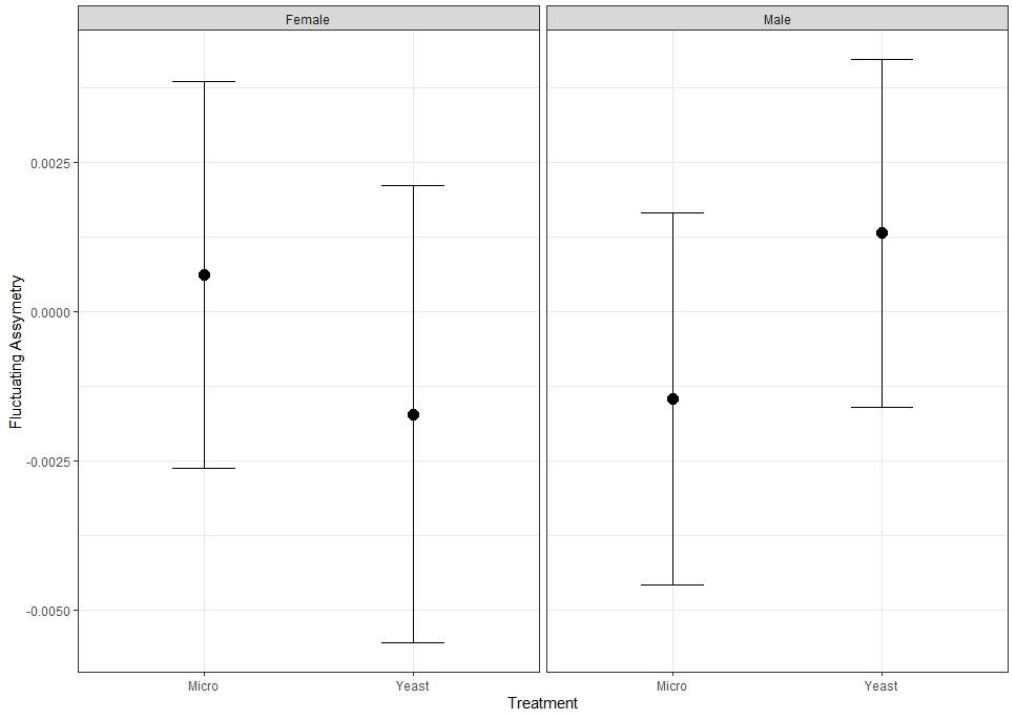

Figure 3.6: Fluctuating asymmetry (FA) values are plotted against two types of treatments in females and males. Micro stands for microplastic treatment and yeast stands for yeast treatment. The point in the middle represents the median and the line the interquartile range.

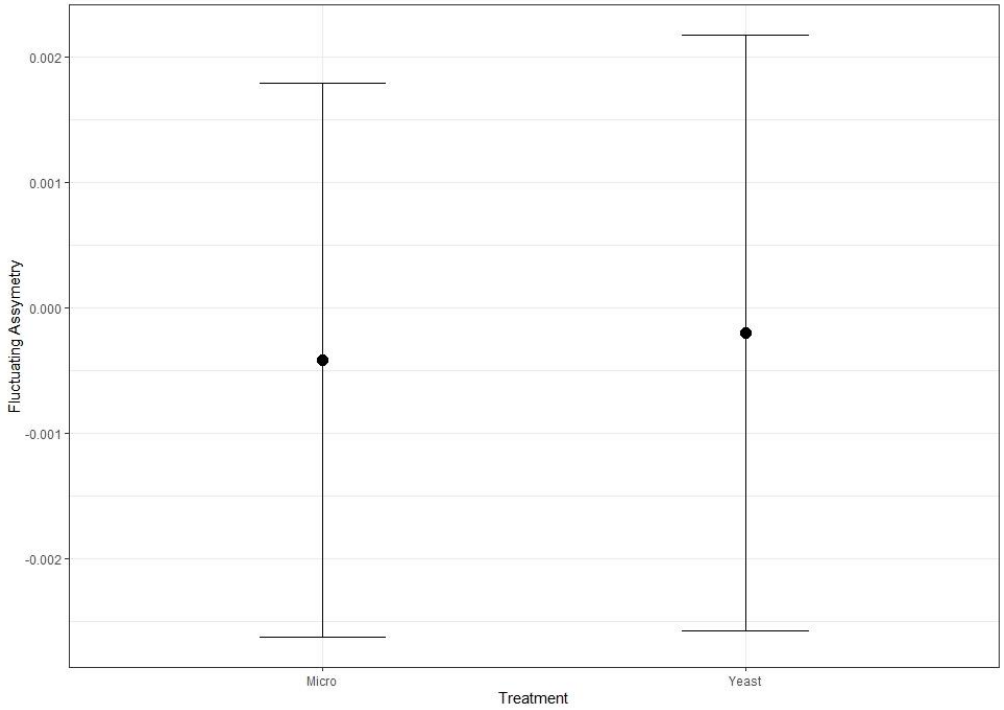

Figure 3.7: Fluctuating asymmetry (FA) values are plotted against two types of treatments. Micro stands for microplastic treatment and yeast stands for yeast treatment. The point in the middle represents the median and the line the interquartile range.

	Sum Sq	**Df**	**F value**	**Pr(>F)**
Treatment	**0.0000956**	**1**	**1.0407**	**0.3095**
Sex	**0.0000760**	**1**	**0.8278**	**0.3645**
Treatment:Sex	**0.0002290**	**1**	**2.4930**	**0.1167**
Residuals	**0.0124936**	**136**		

Table 3.6: ANOVA table with polystyrene treatment and sex as independent variables and fluctuating asymmetry as dependent variable.

3.2 Effects of polystyrene upon treating adult

Female experiment		
Treatment	Male(LHst)	female(LH)
A	Molasses food	Yeast
B	Molasses food	Microplastic
Male experiment		
Treatment	Male(LH)	female(LHst)
C	Yeast	Molasses food
D	Microplastic	Molasses food

Table 3.7: Two types of treatments (A, B, C & D) in both male and female based experiments.

3.2.1 Effects of microplastics on fecundity

Number of offspring produced by female flies of two types of treatments were nearly equal. I.e, No significant effect on adult fecundity when larva was treated with polystyrene (Table 3.7).

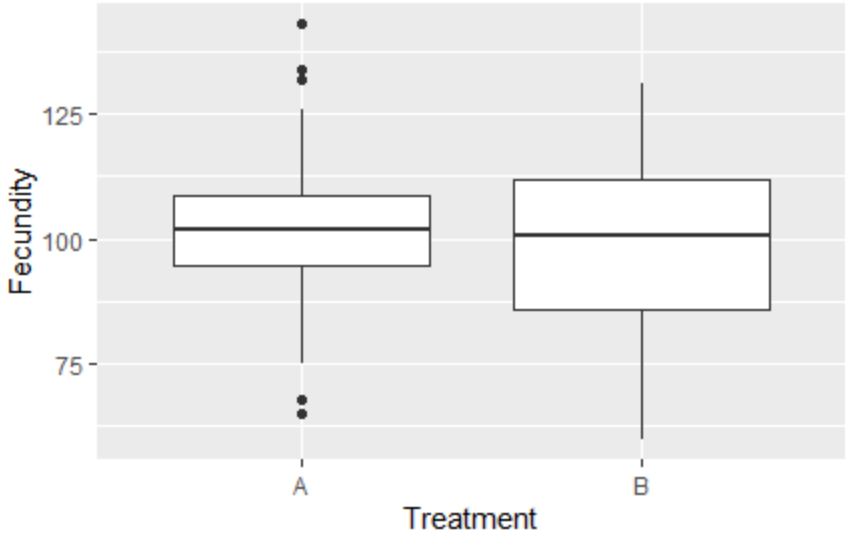

Figure 3.8: Number of offspring produced by females of two types of treatments plotted against different combinations of treatments. The line represents the median and the box the interquartile range.

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Treatment	1	106.2	106.18	0.3415	0.5607
Residuals	76	23628.8	310.90		

Table 3.8: ANOVA table with polystyrene treatment as independent variable and fecundity as dependent variable.

3.2.2 Effects of microplastics on copulation duration

Copulation duration did not show any significant change after polystyrene intake in both males and females (Table 3.8).

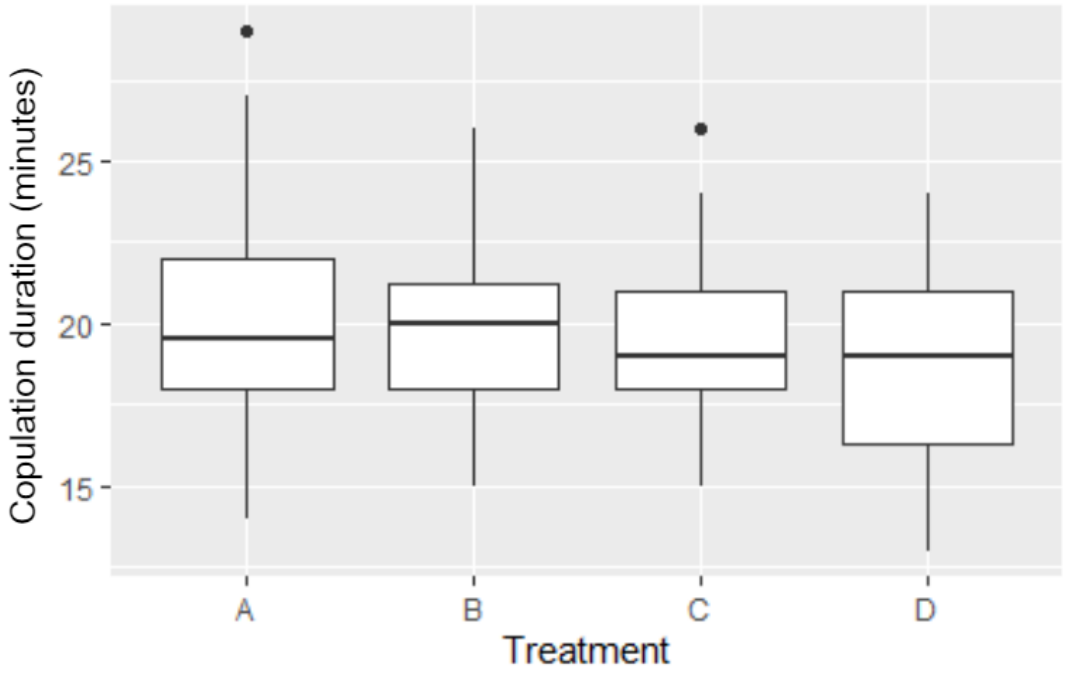

Figure 3.9: Copulation duration of fly pairs plotted against different combinations of treatments. The line represents the median and the box the interquartile range.

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Treatment	3	38.47	12.8249	1.5615	0.2011
Residuals	153	1256.65	8.2134		

Table 3.9: ANOVA table with polystyrene treatment as independent variable and copulation duration as dependent variable.

3.2.3 Effects of microplastics on mating latency

Adults from male-treated combinations showed nearly equal mating latency. Adults from all four combinations showed nearly equal. Mating latency was higher for females which were treated with PS compared to control. However, these differences were not statistically significant (Table 3.9).

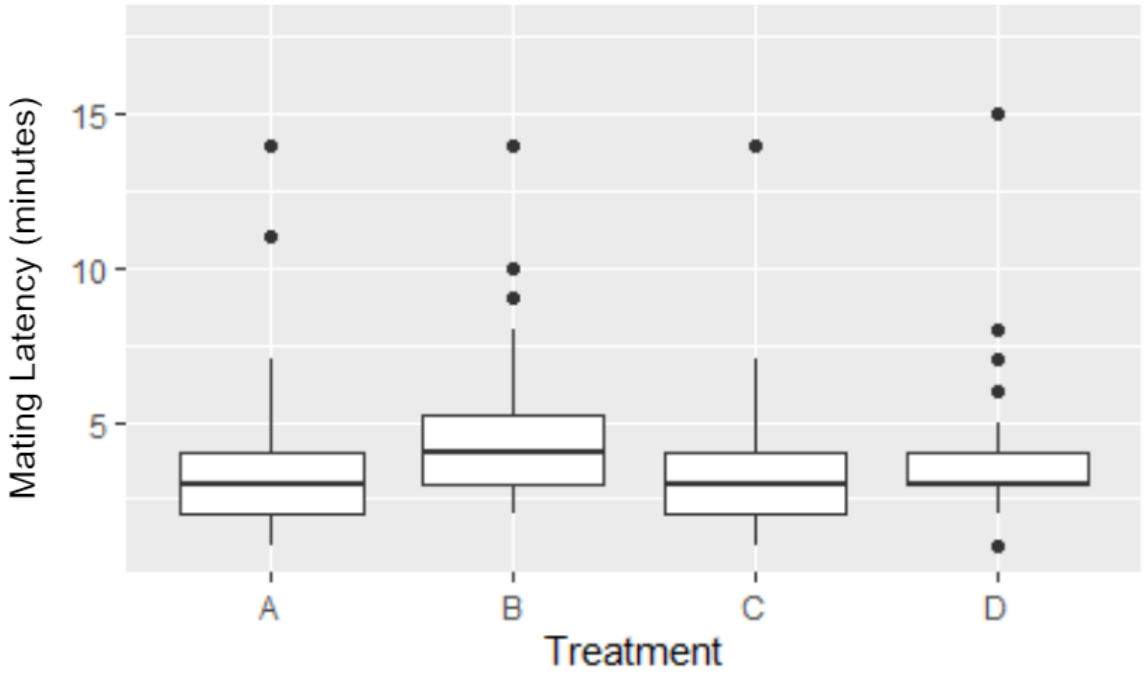

Figure 3.10: Mating latency plotted against distinct treatments of male based and female based experiments. The line represents the median and the box the interquartile range.

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Treatment	3	47.42	15.8081	2.0747	0.1059
Residuals	151	1150.55	7.6195		

Table 3.10: ANOVA table with polystyrene treatment as independent variable and mating latency as dependent variable.

3.2.4 Effects of microplastics on sperm defense

Scarlet eyed offsprings produced by PS treated were slightly lesser compared to the control. However, these differences were not statistically significant (Table 3.10). Number of Red eyed progenies produced by PS treated were nearly equal to that of control. This data explains that Sperm defense ability is not affected by PS exposure.

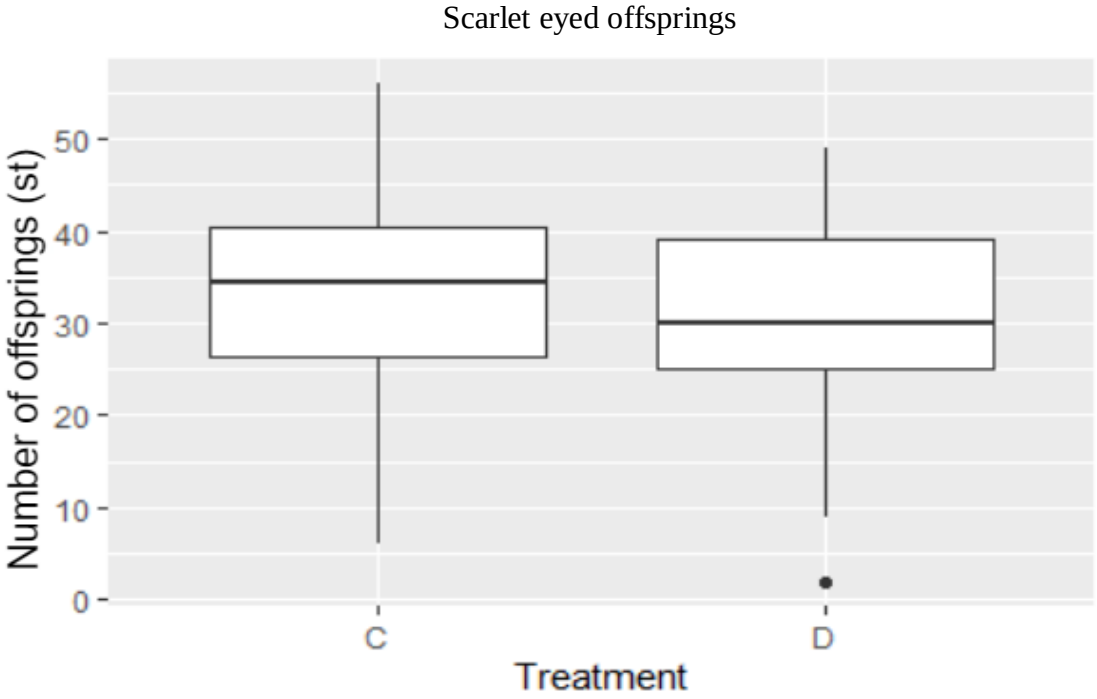

Figure 3.11: Number of scarlet eyed offsprings produced by flies from both PS and yeast treatments plotted against different treatments. The line represents the median and the box the interquartile range.

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Treatment	1	212.3	212.31	1.5677	0.2154
Residuals	60	8125.6	135.43		

Table 3.11: ANOVA table with polystyrene treatment as independent variable and number of offsprings as dependent variable.

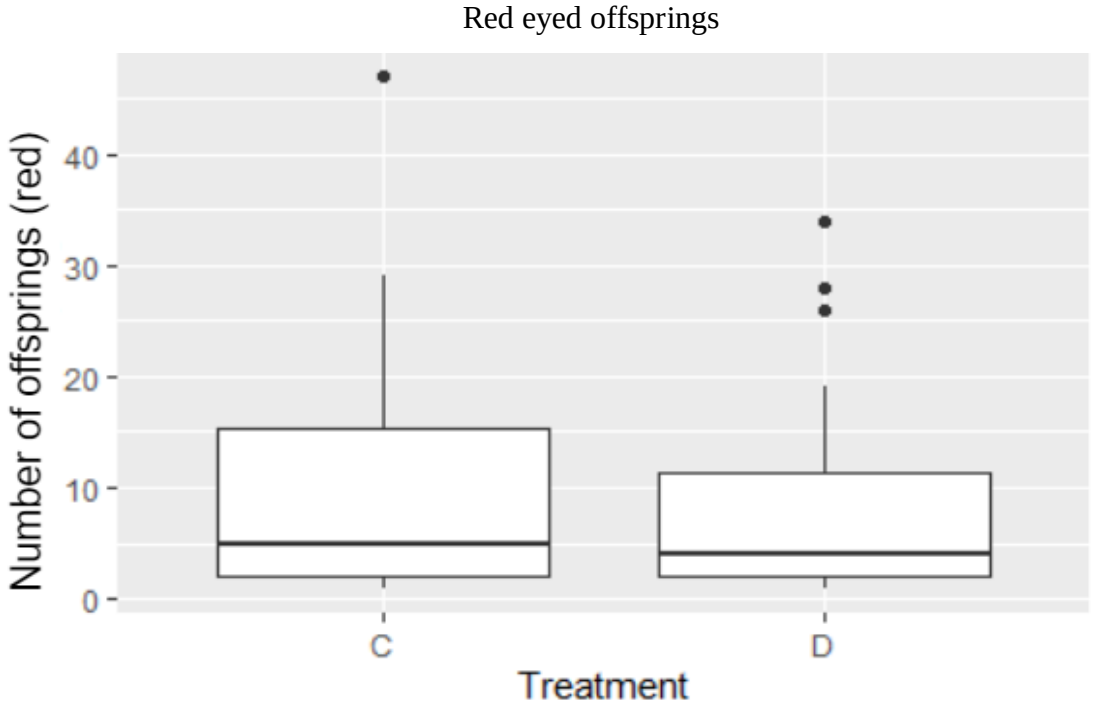

Figure 3.12: Number of red eyed offsprings produced by flies from both PS and yeast treatments plotted against different treatments. The line represents the median and the box the interquartile range.

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Treatment	1	36.5	36.535	0.3037	0.5844
Residuals	44	5292.9	120.294		

Table 3.12: ANOVA table with polystyrene treatment as independent variable and number of offsprings as dependent variable.

Discussion

In this thesis project, I investigated the effects of polystyrene intake on life history traits, fitness characters and development of *Drosophila melanogaster*. In the first set of experiments, I checked the effects in adults which were ingested with microplastics in the larval stage. In this set, fecundity, copulation duration and mating latency of adults remained normal even after the ingestion of microplastics. Body size and fluctuating asymmetry of frozen flies was assessed which showed no significant difference with polystyrene. In the second set of experiments, I checked the effects in adults which ingested microplastics in the adult stage itself for two days. In this set, I did male based and female based experiments. In the male based experiments, I got neutral results when I examined sperm defence ability, copulation duration and mating latency after polystyrene exposure. In the female based experiments, fecundity, copulation duration and mating latency did not show any noteworthy difference upon microplastic uptake.

Overall, polystyrene does not show any impact on the traits that we assessed (fecundity, mating behavior, body size, fluctuating asymmetry and sperm defense ability) in *Drosophila melanogaster* model system. Chemically inert plastic particles might not be having any effect on these traits of fruit fly. Or, it could be the case that the flies and larva have excreted the tiny plastics along with the food. I did the complete experiments in one particular concentration which might not be enough to create a prominent change in the traits that we examined. There are several other traits which we haven't examined like immunity, development time, etc that might show some changes upon polystyrene exposure. Expanding the experiment to multiple generations also could reveal some significant results. In short, future experiments yet to be explored are greater compared to the works that have already been done on *Drosophila melanogaster*.

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