Evolution of Male-Male Aggression in *Drosophila melanogaster* in response to Sexual Conflict

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Certificate of Examination

This is to certify that the dissertation titled "Evolution of Male-Male Aggression in *Drosophila melanogaster* in response to Sexual Conflict" submitted by Ms. Megha Treesa Tom (registration number MS12064) for the partial fulfilment 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 21, 2017

Declaration

The work presented in this dissertation has been carried out by me under the guidance of 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 acknowledgements 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.

Megha Treesa Tom

Dated: April 21, 2017

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.

Dr. N. G. Prasad

(Supervisor)

Acknowledgements

Since this is the only page that most of my dear ones are going to read, let me be as luxurious in writing as possible.

I would like to thank all the people who helped me conduct my experiments smoothly. I would start by thanking Martik and Radhika for not just warning me right at the beginning about how tedious a task taking up this project would be, but simultaneously encouraging me also. Calling this my project would be really unjust as none of the experiments would have been designed or conducted smoothly without the time and effort of all my lab members. So, I thank all my fellow labmates who stayed in the lab the whole day during the experiments, sacrificing their smart-phones so that I could video-tape some flies fighting – Ekta, Neetika, Omal, Bells, Kapila, Kay, TJ, Manas, MC, UdB, JJ, Aparajita, Babua and Nagander Bhaiya for helping me with the experiments. Thanks to my friends Shu and Screw for lending me your phones everytime I asked for it. Thanks to UdB for getting the video camera repaired, though I never used it. And special thanks to the Chemistry Teaching Lab people for letting me use the iron stands.

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Abstract

Intrasexual selection acts on traits that are involved in male-male competition. Adult males fight to access mates either directly or indirectly by acquiring resourceful territories. In the fruit fly *Drosophila melanogaster,* males show aggression while competing for a mate. In this thesis, we try to explore how male-male aggression evolves under sexual selection. For this we use laboratory populations of *Drosophila melanogaster* evolved under different levels of sexual selection by altering the adult sex-ratio. In these populations males under high sexual selection pressure have evolved higher sperm competition ability, higher fitness under competitive environment and higher courtship ability (Nandy *et. al.,* 2013).

To investigate whether higher male aggression has also evolved in populations under high sexual selection pressure, we video recorded pairs of virgin males provided with a common decapitated female and quantified aggression. After completing one replicate of experiment, we found no difference in aggression among males from populations with different intensities of sexual selection.

Chapter 1
Introduction

1.1 Sexual Selection and Aggression

In any population of sexually reproducing species, sexual selection acts on various morphological, physiological and behavioural traits that affect the reproductive fitness of the population. Sexual selection can take two forms: intrasexual selection on traits such as those involved in competition within a sex for access to mates, and intersexual selection on traits affecting mate choice. The strength of this selection varies according to the mating system and increases with increasing deviation from monogamy.

In polygamous most animal species, while the female's reproductive fitness is limited by the number of eggs it can produce and hence on the amount of nutrition it acquires, the male's reproductive fitness depends solely on maximising the number of mates during its lifetime. This pattern was observed in fruit flies by Bateman in 1948 and hence is called the Bateman principle. Thus, male-male competition over access to mates is seen very often in nature. This competition can be either in the form of direct combat between two males or through other mate-attracting tactics such as courtship, song production and visual displays. In certain species e.g. elks and horn beetles specialized 'weapons' like horns, mandibles, tusks and antlers are used for fights among conspecific males. Winning in such fights are often correlated with both territorial and mating success which indicate that the evolution of such 'weapons' in only one sex has been mainly due to sexual selection. Similarly, sex specific traits like song-production in male crickets, bright plumage in several male birds, etc. are also sexually selected traits.

In *Drosophila melanogaster*, which is the model system used in this study, several male competitive traits has been identified and well-studied in the literature such as, courtship activities, sperm competition and male-male aggression (Chen *et. al.* 2002; Fernandez *et. al.* 2013). Aggression among males while courting a common female was first reported by Sturtevent in 1915 during experiments on a different species, *D. ampilophila*. Aggression in *Drosophila* does not involve use of any special weapons or armaments, but rather the use of a complex set of behavioural tactics which are innate but also modified with experience. Both male and female fruit flies show aggression however, the innate behavioural patterns are known to be sex-specific. Female fruit fly shows aggressive

behaviour towards a courting male as a sign of rejection and also towards another female over food resources. Male-male aggression over food is much rarer and of lower intensities compared to aggression elicited by mate cues (Chen *et. al.* 2002; Nilsen *et. al*., 2004).

Aggression and courtship are both important social behaviours and eliciting the right behaviour depends on the male's ability to correctly identify its potential mate and potential opponent. *Drosophila* uses multiple sensory modalities such as visual, olfactory and gustatory to identify the sex, age, nutritional status etc. of the neighbouring individual (Fernandez *et. al.* 2013).

As males show aggression in presence of female, and the behavioural patterns show sexual dimorphism, we assume that male-male aggression in *Drosophila melanogaster* is a trait that can be under sexual selection. Male-male aggression can have reproductive advantage if males winning territories after fights also gain more mating opportunities. In *D. melanogaster* the correlation between mating success and territorial success has been studied using a number of inbred lines that are different in their aggression levels. However, no consistent pattern for correlation between territorial and mating success was seen and the results depended upon the male and female genotype involved (Cabral et. al. 2008).

Bodhisatta Nandy started the M, C and F laboratory populations in which every generation the operational sex ratio is manually altered to be male-biased (3:1) in M, female-biased (1:3) in F and an equal sex-ratio in C (control). Thus, males in M population are under high male-male competition while males in F population face very low competition. Males in the C population have an intermediate level of competition. Female competition over food has been equalized in all three regimes by standardizing/equalizing the per capita yeast provided in all populations. These populations have been derived from a single ancestral population LHst. Further, three replicates of each of M, C and F are being maintained in the lab. Detailed description of the maintenance protocol of these selection lines is described in the doctoral thesis of Bodhisatta Nandy.

By altering the operational sex-ratio, the three types of populations are being evolved under different intensities of sexual selection and sexual conflict. Thus, M population is under high intensity of conflict, C is under medium intensity of conflict and F has the

lowest intensity of sexual conflict. As a result of this selection, several components of male reproductive fitness including competitive fitness, courtship frequency and sperm competitive ability have increased to significantly greater amount in M males compared to F males (Nandy *et. al.* 2013a). Moreover, F males have evolved decreased sperm competitive ability. Here, we study another potential trait of male-male competition, *i.e.* male-male aggression.

In 2014, Vrinda Ravi Kumar assayed aggression of M and F mated males. She found no difference in the total aggression levels within the M and F populations. However, in fights between M and F mated males, M males showed significantly greater aggression than F males. She concluded that the males were able to judge the 'aggressiveness' of their opponent and gauge aggression accordingly.

1.2 Aim

The aim of this study is to investigate the evolution of male-male aggression in laboratory populations of *Drosophila melanogaster* that have been selected under different intensities of sexual selection/conflict. A major assumption that we make here is that the male mating success in the ancestral line LHst is positively correlated with male aggression.

1.3 Experimental Design and Hypothesis

The experimental design is similar to the experiment conducted by Vrinda. However, here we conducted the experiment with virgin males that were kept in isolation for two days before the assay. This was done to remove all effects of adult experience and monitor the innate levels of aggression across selection regimes. We assessed the male to male aggression both within and between the laboratory populations of *Drosophila melanogaster* selected for high and low levels of sexual conflict. We conducted our experiments on the M and F populations. For the logistic simplification of the experiment we did not use the C population that has medium level of sexual conflict. Further, conducting experiments on the C population was not necessary for answering our questions.

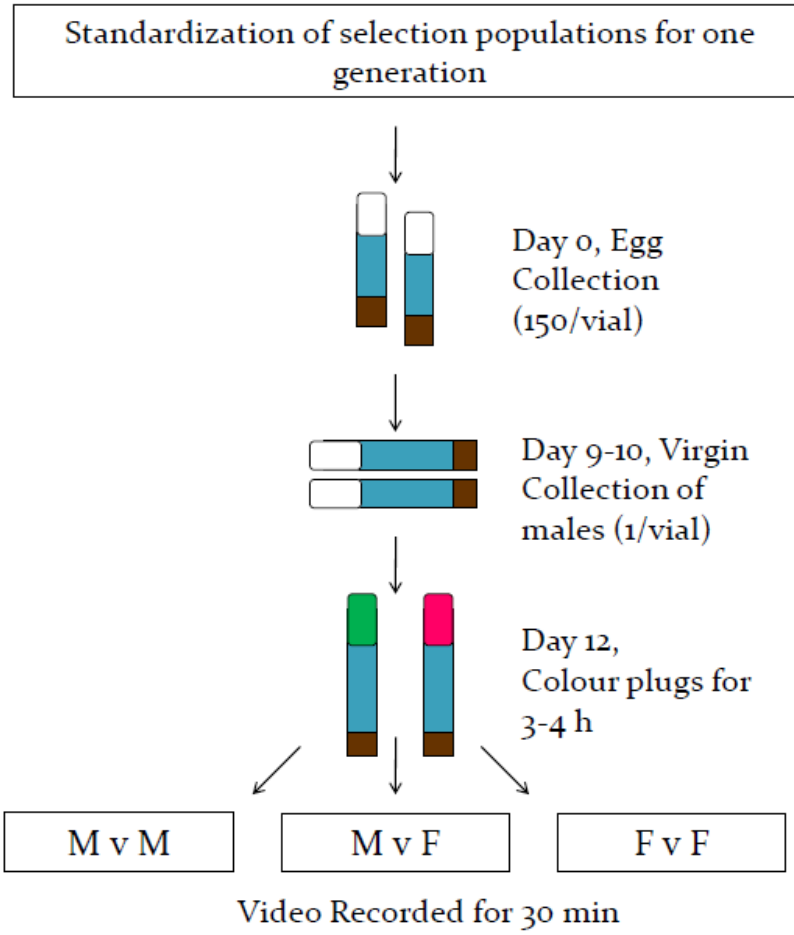

Fig. 1: Schematic of the experimental design

3 Treatments of the experiment were:

1. MvM : An M male was competed with another M male.

2. FvF : An F male was competed with another F male.

3. MvF : An M male was competed with an F male.

In his doctoral thesis, Bodhisatta Nandy showed that M males have evolved higher levels of behavioural traits such as courtship activity, spontaneous locomotory behaviour and also increased competitive reproductive fitness. M males are also seen to have higher mate-harm ability than F males (Nandy *et. al.* 2013b). One way of harming mates could be that M males physically harm their mates upon mating which can also result from being more aggressive in nature. Based on these observations we hypothesized that overall aggression levels within the M population would be greater than within the F

population. Furthermore, on combining M and F males, a hierarchical relationship should form with M males being more aggressive than F male.

Chapter 2
Materials and Methods

2.1 Generation of the experimental flies

While studying evolved traits in experimental evolution, a standard protocol followed is to relax the selection and maintain the different selection lines under similar conditions for one generation. This is to make sure that differences among different populations are due to the differences in their genetic make-up acquired through selection and not due to any immediate non-genetic effects of the selection. In our laboratory, the back-ups for each of the M, C and F selection lines are derived from the respective stock populations every generation. The selection is relaxed in these back-ups and they are reared in similar conditions, *i.e.* 140-150 egg density per large-pour cornmeal-molasses food vial.

On the 12^{th} day in their life-cycle, back-ups of M and F population were transferred from rearing vials to plexiglass cages, 25cm x 20cm x 15cm in size. A food plate with freshly made yeast paste with provided for two days. Yeast increases the egg-production in females. After two-days a fresh food plate is given for 8-9 hours for oviposition. Eggs from this food plate are counted on non-nutritious agar plate and transferred to large-pour food vials at a density of 150 eggs per vial. The vials are kept for rearing at 1-2 ml food at standard conditions of 25˚C, 50-60% relative humidity, and 12h:12h light-dark cycle. Starting from the 9^{th} day post egg collection virgin males from both M and F populations are collected and stored singly in vials at standard conditions till the 12^{th} day. Thus, our experimental males are both virgins and isolated for two days before the aggression assay is conducted. Thus, they had no social contact since sexual maturation (6-8 hours after eclosion).

2.2 Generation of females for the assay

Since our study of aggression is in terms of aggression for mating opportunities, we had to provide them a mate cue. For this we provided the males with decapitated female with a small piece of banana-jaggery food that together formed the territory. Decapitation of the female restricts female behavioural effect on male aggression and also removes female choice component from our results. However, the decapitated female remains alive for about an hour and gives proper olfactory, gustatory and visual cue for the males.

We used banana food as it is light in colour and, therefore, aids in taking proper observations.

We used females from the laboratory baseline population BRB which is unrelated to the selection lines in question. Since the BRB population is equally distant to M and F populations, we expect it to be equally attractive for both selection lines.

Eggs were collected at a density of 60-80 eggs per 6-8ml food vial containing banana-jaggery food. This was done on the same day when M and F eggs were collected. On 11^{th} day post egg collection, adult females were separated under mild CO2 anaesthesia and collected in food vials at a density of 30 flies per vial. These would be used the following day for the experiment.

2.3 Preparation of assay plates

12-well tissue culture plates with low evaporation lid, manufactured by Becton Dickinson Labware, were used for the assays. At a time four cells from each plate were used. With a heated screw-driver, holes were made on the lids, large enough to put the male flies through them. A large piece of tissue paper folded 4-5 times was placed between the plate and lid. This gave a white background for taking observations and kept the lid tightly closed.

2.4 Coding samples

32 samples of each of the three treatments were prepared and video-recorded. To avoid bias during observations the samples were randomly coded and the codes were broken only after all observations of an experimental block were over. Thus, the observer remained blind while taking the observations.

2.5 Colouring the experimental flies

The experiment was conducted on the 12^{th} day from egg collection. Half of the flies from each regime were coloured pink and the other half of each regime was coloured green. For this, on the morning of the day of experiment vials holding the isolated male flies were plugged with plugs dipped in small amount of fluorescent colour dust(EC011 (Aurora Pink) and EC018 (Signal Green) by Day-Glo Color Corp.). These vials were then kept undisturbed for 3-4 hours. After that, flies are flipped into fresh food vials for half an

hour, during which flies remove excess colour from their bodies. The remaining colour on their abdomens serves to identify these flies during observations.

2.6 Setting up the assay

Females were decapitated with a sharp scalpel and forceps under mild CO_2 anaesthesia and transferred immediately into a cell of tissue-culture plate along with a 2cm x 1cm rectangular piece of banana-jaggery food. The plate is closed with its lid and the folded tissue papers as mentioned above. Then, males were aspirated from vials and put into the cells through the holes made and the holes are sealed with transparent cello-tapes.

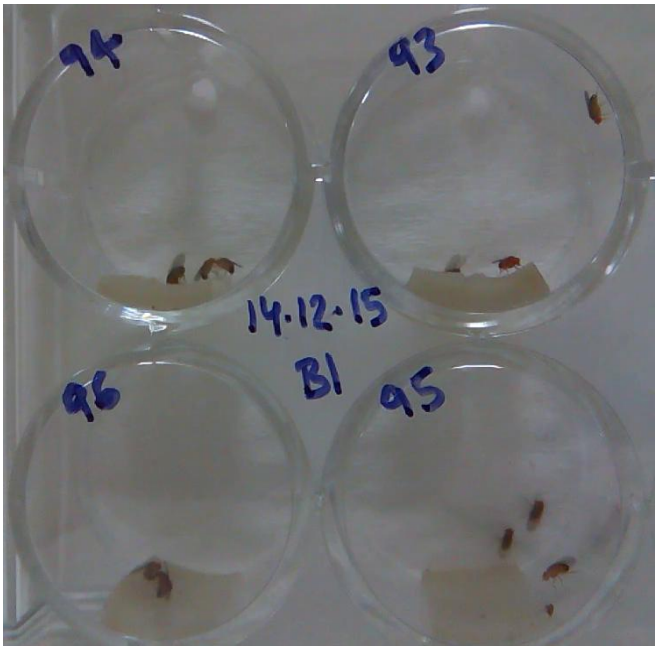

Fig. 2: Picture of an assay plate with the four experimental cells zoomed in. A high level aggressive interaction (boxing) can be seen in cell 94.

For each treatment, one pink and one green coloured male was combined in a single cell. For the MvF treatment, a pink M male and a green F male was used in half of the total samples and the reciprocal for the other half. The plates were kept vertically and 30 minutes long videos were recorded using smart phone cameras clamped to iron stands.

2.7 Observations

The following data was collected for each sample from the recorded videos:

1. Number of encounters: Male-male aggression happens through a series of short encounters that may last for a few seconds to few minutes. End of an encounter was

defined by pauses of 6 seconds or more. In other words, if no aggressive behaviour is observed for more than five seconds, an encounter is considered over. For each fly, we noted down the number of encounters for which it was aggressively active.

2. Initiator of an encounter: The colour of the fly which initiated the encounter is noted. For certain encounters, it was not possible to clearly make out the initiator.

3. Behaviours: For each encounter, all behaviours shown by each of the flies were noted down. We used a slightly modified form of the ethogram of male *Drosophila melanogaster* aggressive behaviours described by Chen *et. al*., 2002.

	Behaviour	**Symbol**	**Description**
1.	Approach	A	One fly walks towards the other at the start of an encounter
2.	Fencing (high-level and low level)	f	One or both flies extend one leg and tap the opponent's leg or body part
3.	Pushing	P	One fly extends both his forelegs and pushes the other fly
4.	Lunging	lg	One fly jumps over the other fly with forelegs extended
5.	Holding	hl	One fly holds the opponent with its forelegs and tries to immobilize
6.	Wing-threat	WT	One fly raises both its wings up at 45˚ angle while facing towards the opponent
6.	Chase-off	ch	One fly runs after the other fly
7.	High intensity behaviours (Boxing, Tussling)	X	Both flies strike or tumble over each other
8.	Retreats (walk, run or fly away, defensive wing-threat)	R	One fly (loser) moves away from the other fly immediately following an encounter, Defensive wing-threat is when fly raises both wings at 45˚ angle while facing away from the opponent

Table 1: Ethogram for taking observations of various aggressive behaviours.

4. Duration: For each encounter, we noted down the exact duration in seconds for each of the flies.

Chapter 3
Results

Aggression assays have been completed for 3 blocks, over generations 182-187. However the analyses of only block is finished and presented here.

We performed Student's t test on each of the treatments to check if colour of the flies has any effect on their behaviour. For this we calculated the parameter $(D_p - D_g)/(D_p + D_g)$, where D_p and D_g are the total duration of aggression by pink and the green fly respectively. We tested if this value was different from zero and found no difference between pink and green coloured flies in any of the treatments (MvM (p=0.1206), FvF (p=0.9039), MvF (p=0.958)). Thus, the colours we used have no effect on the aggression of the flies.

3.1 Total Aggression Levels

The following analysis was done to assess if the overall aggression levels were different across treatments. The software JMP was used for performing ANOVA and SigmaPlot was used for plotting the graphs.

1. The total duration of aggression in every treatment was calculated by summing up the duration of aggression for individual flies. One-way ANOVA was performed and no significant effect of treatment was seen (p=0.2586). Average total aggression in MvF was greater than MvM by 22.8% and FvF by 44.1%. However, due to the large amount of variation in this behavioural trait, the differences were found to be non-significant.

Source	**DF**	**Sum of Squares**	**Mean Square**	**F-Ratio**	**Prob > F**
Treatment	2	70029.2	35014.6	1.3751	0.2586
Error	82	2087946.1	25462.8		
C.Total	84	2157975.3			

Table 2: Table obtained from One-way ANOVA on the total duration of aggression.

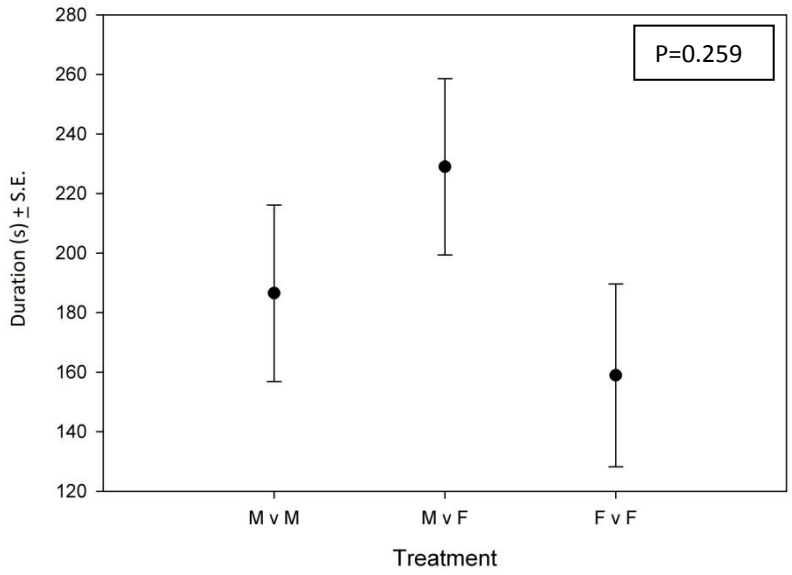

Fig. 3: Total duration of aggression (y-axis) versus treatments (x-axis).

2. Sum of the numbers of active encounters for each fly. By doing so, the encounters common for both flies are counted twice, and so combinations/treatments where both flies are aggressive will have a higher score than those where only one fly is aggressive. One-way ANOVA was performed and results showed no significant difference between treatments (p=0.2590).

Source	**DF**	**Sum of Squares**	**Mean Square**	**F-Ratio**	**Prob > F**
Treatment	2	1144.640	572.320	1.3736	0.2590
Error	82	34166.184	416.661		
C.Total	84	35310.824			

Table 3: Table obtained from One-way ANOVA on the total number of active encounters.

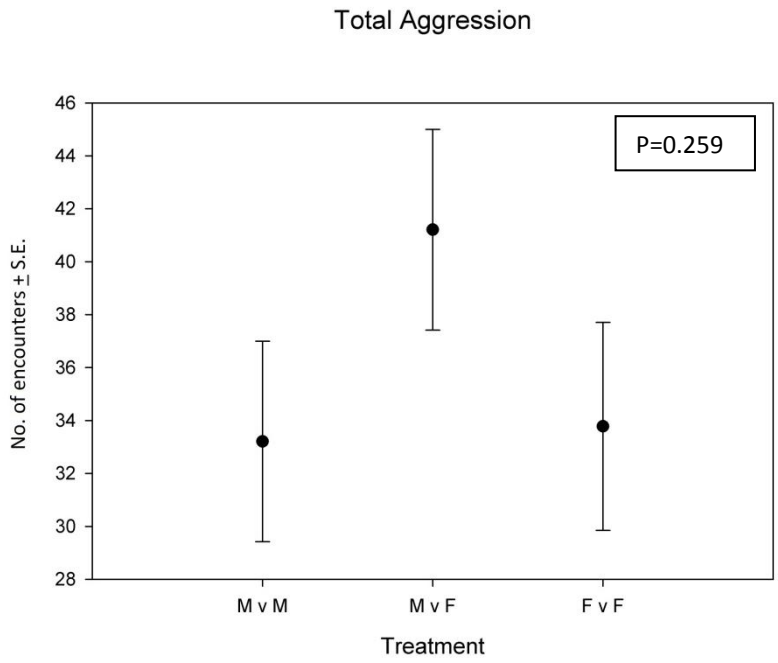

Fig. 4: Total number of active encounters (y-axis) versus treatments (x-axis).

3. Sum total of the number of lunges taken by each fly was calculated. One-way ANOVA was performed and results showed no significant difference between treatments(p=0.253).

Source	**DF**	**Sum of Squares**	**Mean Square**	**F-Ratio**	**Prob > F**
Treatment	2	7999.47	3999.74	1.3990	0.2527
Error	82	234443.52	2859.07		
C.Total	84	242442.99			

Table 4: Table obtained from One-way ANOVA on the total number of lunges.

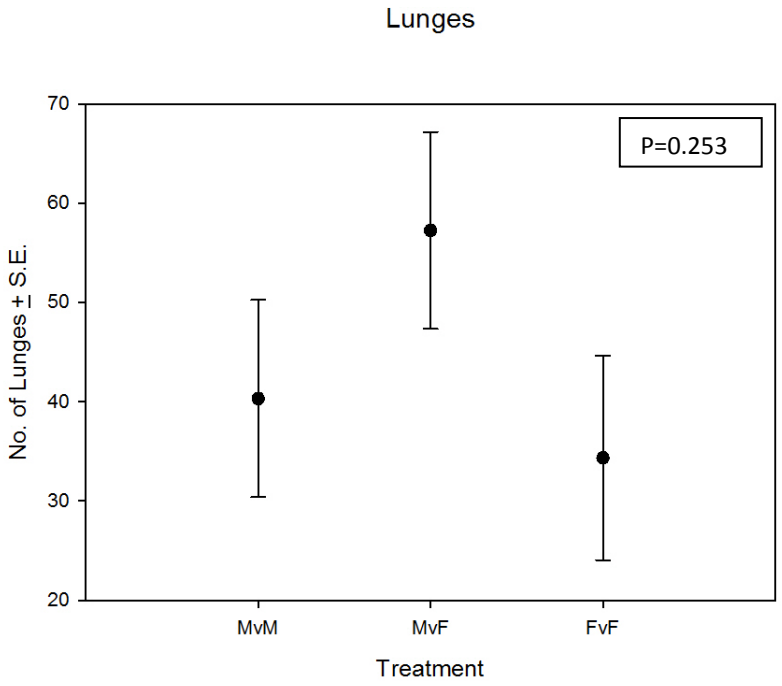

Fig. 5: Total number of lunges (y-axis) versus treatments (x-axis).

3.2 M vs F treatment

The following analysis was done to assess the aggressiveness of the M and F males when combined together to fight for a female.

1. Number of encounters where a given fly was active was counted for each fly as E_M for M males and E_F for F males. Total number of encounters was counted as $E_{tot.}$ The parameter $(E_M - E_F)/ E_{tot}$ was calculated. Using Student's t test, this value was not found to be different from zero (p=0.124).

2. Number of encounters initiated by each male type was counted as I_M for M male and I_F for F male, whenever identifying the initiator was possible. The parameter $(I_M - I_F)/E_{tot}$ was calculated (where E_{tot} is the total number encounters) and found to be not different from zero by doing Student's t-test (p=0.268).

3. The total duration of aggression by M male (D_M) and the F male (D_F) was measured. To compare proportion of time spent in aggression by each male we calculated $(D_M - D_F)/(D_M + D_F)$, where D_M and D_F are respectively. By performing Student's t-test, this value was not found to be significantly different from zero (p=0.8454).

4. To analyse individual behaviours, we compared the following values: number of lunges by a single male, chase score: number of encounters where a male chases its opponent atleast once, retreat scores: number of encounters where a male retreats at the end of the encounter. No difference was found between total number of lunges (p=0.278), chase-scores (p=0.849) or retreat scores (0.6333) of M male and F male. Paired t-test was performed for the analysis.

3.3 Effect of opponent on aggression

Next, we sought to see if the males behaved differently in presence of different opponents. We compared behaviour of one of the males from MvM treatment (pink from half and green from other half) with the behaviour of M male from MvF treatment. Upon performing 2-sample Student's t-test no difference was found for number of active encounters (p=0.166), duration (p=0.364), number of lunges (p=0.258), chase scores (p=0.207) and retreat scores (p=0.9).

Similarly, one male from FvF treatment was compared with the F male from MvF treatment. 2-sample Student's t-test no difference was found for number of active encounters (p=0.792), duration (p=0.445), number of lunges (p=0.862), chase scores (0.724) and retreat scores (p=0.683).

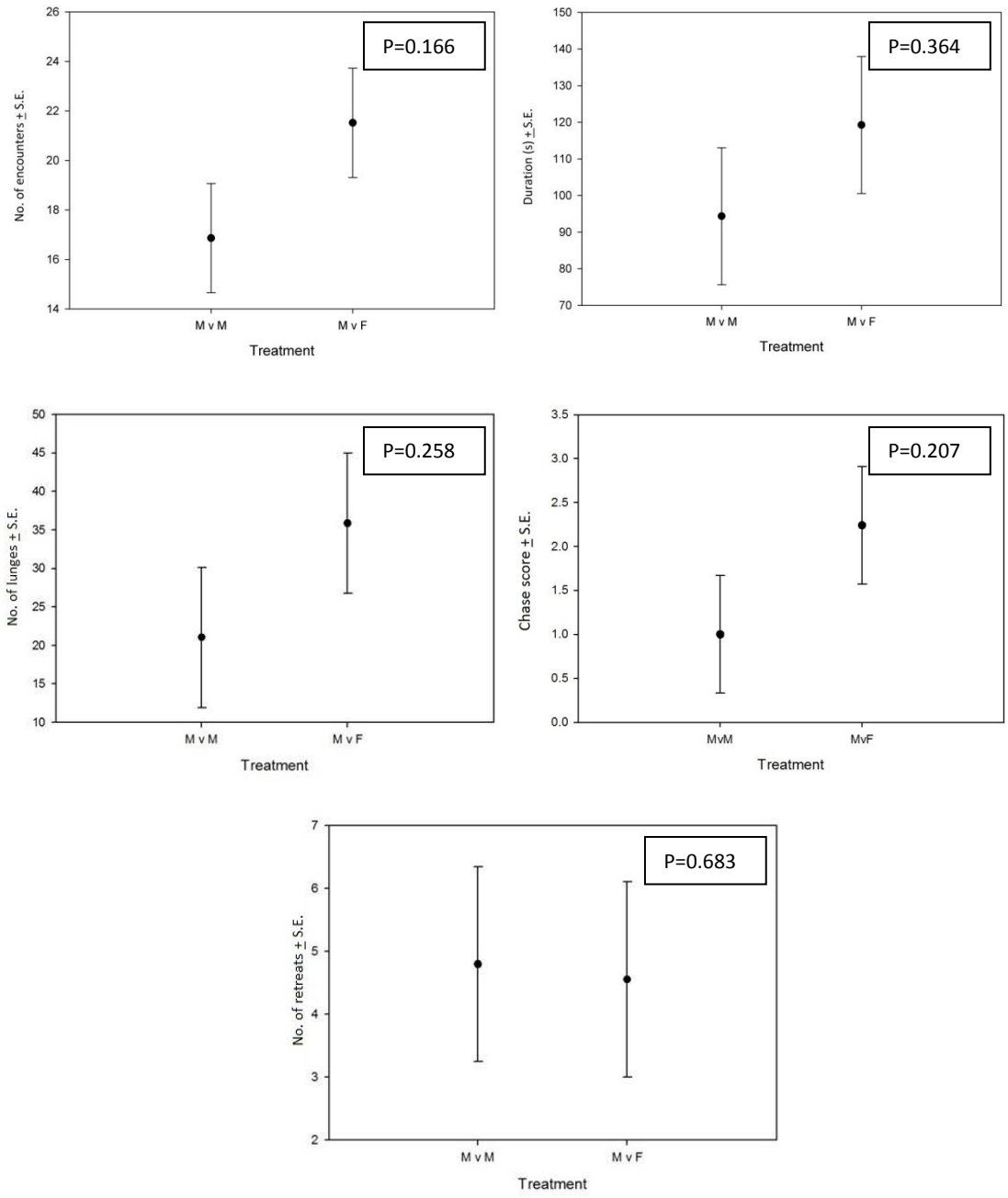

Fig. 6: Graphs for M male in MvM and MvF treatments showing a). number of active encounters, b). duration of aggression, c). no.of lunges, d). chase scores, and e). no. of retreats on the y-axis versus treatment on the x-axis.

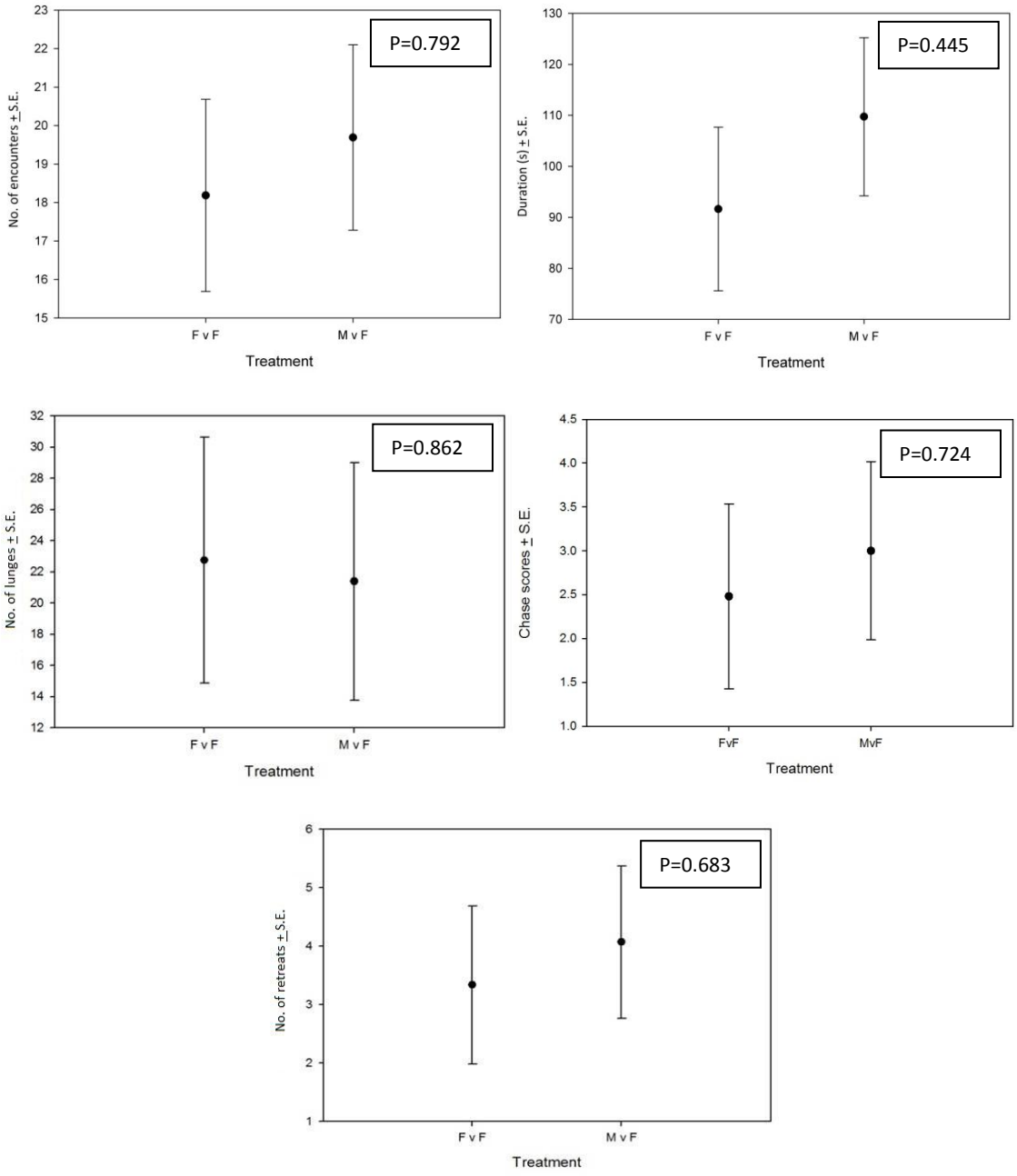

Fig. 7: Graphs for F male in FvF and MvF treatments showing a). number of active encounters, b). duration of aggression, c). no.of lunges, d). chase scores, and e). no. of retreats on the y-axis versus treatment on the x-axis.

3.4 High Intensity Aggression

In every treatment, we counted the number of samples in which high intensity aggressive interactions such as boxing and tussling was seen. We then performed a chi-square goodness of fit test. Although, we high intensity aggression was seen twice as much in MvF treatment than in the other treatments, the result was not statistically significant (χ^2 =3.974, df=2, p=0.137).

	High Intensity Aggression Present	**High Intensity Aggression Absent**	**Total Samples**
MvM	5	24	29
FvF	4	24	28
MvF	10	19	29
	19	67	86

Table 5: 3x2 contingency table for the high intensity aggressive interactions in different treatments.

Chapter 4
Discussion

Our hypothesis was that males from male-biased selection regime would show greater level of aggression towards their opponents than males from female-biased selection regime. However, we found that the aggressive interactions between two M males were no different from that between two F males. Even when an M male is competed with an F male, they both show equal levels of aggression. In other words, we did not see any effect of sexual selection on male-male aggression in our selection lines.

One reason for why aggression has not evolved in these lines could be that it is an energetically expensive trait. Involvement in aggressive interactions can cause physical damage to the fighters and thereby decrease fitness. Such a costly trait may not get selected even if there are some reproductive benefits or may evolve at a very slow rate and only to a certain value. Thus, selection of higher sperm competition, courtship and mate-harm abilities in the male-biased selection regime did not parallel with a positive selection on aggression.

Males gauge their aggression based on the assessment of their opponent's abilities. If all males in a population are equally aggressive, aggression would not increase to very high levels. That is, if all M males were equally aggressive and all F males were equally aggressive, the overall level of aggression in MvM was same as that of FvF treatment.

We found that in MvF combination the mean total aggression greater than MvM by 22.8% and greater than FvF by 44.1%. However, this was not statistically significant. This result is similar to what was found earlier in Vrinda's thesis. However, contrary to her results M males are not more aggressive than there F competitors in MvF treatment. Our analyses show that M and F males are both equally aggressive. The overall increase in aggression levels seen in the M v F treatment was because both M and F males showed an increase in aggression. There was also no difference in the number of encounters initiated by a given male-type. We found high intensity aggression more often in the MvF treatment than in any other treatments, although it was not statistically different. Escalation of the fights to such high intensity means that both male types were willing to show higher levels of aggression. A possible explanation for this could be simply that fruit fly males tend to show more aggression towards unrelated males or males from a

different population. Since high levels of aggression can be harmful, suppressive cues may evolve in a population which could keep the levels of aggression low. Since our selection lines have been separated for more than 180 generations before the experiment, it is possible that there chemical cues have diverged. Aggression was, therefore, not suppressed when males from different populations were combined. This hypothesis can also be tested by conducting aggression assays between males from different replicates of the same selection regime.

However, these are results from only one block. For any conclusive results to be obtained, the remaining two blocks should also be analysed.

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