A study on perception of "quality" of competitors by male *Drosophila melanogaster* under different levels and quality of sperm competition.

Mehreen Khaleel

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

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Certificate of Examination

This is to certify that the dissertation titled "**A study on perception of "quality" of competitors by male *Drosophila melanogaster* under different levels and quality of sperm competition**" submitted by **Ms. Mehreen Khaleel** (Reg. No. MS08033) 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.

Professor T.R Rao Dr.Sudip Mandal Dr. N. G. Prasad

(Supervisor)

Dated: April 26, 2013

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 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.

Mehreen Khaleel

Dated: April 20, 2013

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)

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In the name of Allah, most Gracious, most merciful

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In the end I dedicate my thesis to my grandparents Professor G.N. Bhat and Mrs. Aisha Bhat. They have always been a constant support to me. I like their thinking of aiming for improvement, and that is what has kept me going.

"Sometimes you wake up. Sometimes the fall kills you. And sometimes, when you fall, you fly."- Neil Gaiman

Mehreen Khaleel

List of Figures

List of Tables

List of Photographs

Contents

Abstract

In promiscuous species, females are known to store sperms from different mating males. Multiple mating leads to sperm competition in males. Males adapt various strategies to compete for both reproductive and fertilization success. *Drosophila*, males are known to show plasticity in reproductive behaviour under different level of sperm competition. Studies suggest male reproductive response to number of competitor, but there is no such study which addresses sperm competition as the quality of the sperm. In the present study, I ask if males can perceive the quality and of competition and what reproductive response do male adapt to compete to this competition. I found a highly significant effect of treatment on the copulation duration of the target males under precopulatory competition. There was no effect on progeny sired by the target male, which were conditioned in early life.

Chapter 1

Introduction

Sperm competition refers to the process of competition between sperms of different males to fertilize an egg from a single female (Parker 1970). Competition takes place when there are more competitors trying to win for a female for a successful mating. Reproductive success of a male depends on the number of matings, i.e., mating success and the average number of progeny sired by the male from each mating, i.e., fertilization success (Bateman 1948). Sperm competition is an evolutionary pressure on males which has lead to adaptation to increase fertilization success. For example, male black winged damsel fly shows adaptation to sperm competition. Female damsel fly mates many times in few hours and can store sperms from all the males in its spermatheca. Males have developed a morphological change in their penis which acts as a brush and literally clears off the sperms from the previously mated male. It is known that 90-100 percent of the competing sperms are removed off (Alcock 1998). Various other defensive strategies have been known to adapt by males under sperm competition. These tactics are physiological, morphological or behavioural. Many organisms like lizards, birds, insects, primates are known to guard their mate by either keeping them in close proximity so that if an opponent male tries to mate he can fight off the rival male and prevent the female from mating with the rival male. Physically blocking the transfer of sperms by depositing a copulatory plug into the female reproductive tract has been known in insects, spiders, reptiles (Stockley 1997). Firebugs are known to engage females in prolonged copulation so that more sperms are transferred to the female and reduce their chance to get engaged in copulation with other males (Schofl 2002). In *Drosophila,* female is also known to store sperms from multiple males which lead to sperm competition. In response to this, male is known to release toxic proteins during copulation which impedes the female from further mating. These toxins are present in the seminal fluid and are known as ACP (accessory gland proteins). ACPs are produced in the accessory gland of the male. This substance acts as an anti-aphrodisiac i.e., a repellent to subsequent mating and also stimulates ovulation and oogenesis (Birkhead 2002). Although these adaptive responses help males in successful mating, they are harmful for the females most of the time. Sperm competition leads to sexual conflict of interest between males and females (Stockley 1997).

Plasticity of male reproductive behaviour under different levels of sperm competition has been previously reported in *Drosophila melanogaster* (Bretman et al 2009, Bretman et al 2010, Nandy et al 2011). These studies suggest that males can adapt to different levels of sperm competition in terms of number of competitor males. The present study takes a different approach to understand the effect of risk on the reproductive behaviour of the male. We define competition in terms of "quality of the competitor" and not just by the number. Quality is defined by the body size of the competitor and we ask if males can perceive different quality and number of sperm competition. Studies suggest that large males produce more number of sperms thereby posing a greater threat and a higher level of sperm competition.

Different body sized males were generated by altering the egg density. It is known that increasing larval density in fruit fly decreases the body size.

The questions we asked in this study were:

1) What is mating success of the target male in presence of a competitor male?
2) How does the presence of a competitor affect the reproductive behaviour of target males?
3) What is the effect of size of the male on the reproductive behaviour in presence of different level of competition than the mating male?
4) Can males perceive the quality of the competitor?
5) How does the risk of competition affect reproductive behaviour and fitness of the target male (under competition and no competition)?

Chapter 2

General experimental method

2.1 Experimental population

The model system used to conduct the studies reported in this thesis is *Drosophila melanogaster* laboratory adapted populations. Two populations were used - 1) LH of *D. melanogaster,* are wild-type red-eyed flies, which were initially derived from 400 mated females, caught from an orchid in central California by Larry Harshman in 1991 (Chippindale et al. 2001) and 2) LH-*st,* a large outbreed population of *D. melanogaster* carrying a recessive marker – scarlet eye. All the individuals in this population are homozygous scarlet eyed. LH-*st* was derived from LH by introgressing autosomal recessive scarlet-eye allele by repeated backcrosses (Prasad at al. 2007). Both these populations are maintained in vials following a 14 day discrete generation cycle on standard cornmeal-yeast-molasses media under laboratory conditions of 12hour light-12hour dark photoperiod at 25°C temperature and ~50% relative humidity. These populations had already adapted to laboratory conditions for >500 generations before the following studies were conducted.

2.2 Culture maintenance

The maintenance procedure for both LH and LH-*st* populations are identical. LH is maintained in 60 vials (Effective population size, N_e>5000) while LH-*st* in 30 vials (N_e>2500). To start the culture, 16 males and 16 females are held in each vial with 8ml cornmeal-yeast-molasses media (with limiting amount of live yeast sprinkled over the surface). After two days the flies are transferred to un-yeasted vials and kept for oviposition for 18 hours. The egg density in these vials is controlled to ~150 eggs per vial (~8ml media) by scooping out excess eggs. These vials then constitute the next generation of the given population. After 12 days, when most of the individuals are fully matured, flies are randomly mixed among vials and redistributed in fresh food vials as mentioned above (16 males and 16 females per vial) to start the adult competition vials in which males compete for females and females compete for limited yeast. The redistribution of the adult flies is done under light CO_2-anaesthesia (exposure time <3 minutes).

2.3 Method of egg collection for the experiments

On 12th day the flies of a given population were transferred into a cage (25cm×20cm×15cm) and were given yeast paste (ad-libitum amount) spread on corn-meal food plate. Two days later an oviposition plate (a small food plate) was provided. Flies were allowed a window of six hours for oviposition. After this period, eggs were carefully collected from these plates and counted on an Agar (1%) strip. Pieces of Agar containing exact number (80/150/300 as will be evident later) of eggs (counted under dissection microscope, Zeiss Stemi 2000) were then transferred to food vials.

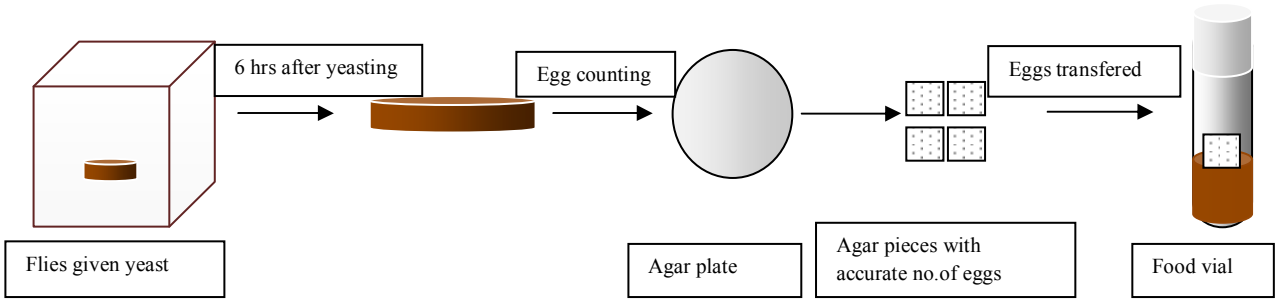


Figure 1: The diagram shows the steps involved in egg collection and transferring to food vials.

2.4 Generation of different body sized experimental males

In *Drosophila*, larval crowding is known to generate body size differences. Under uncrowded conditions flies develop into larger body sized adults compared to when they are grown under crowded condition.

For the purpose of the experiments described in the thesis, only LH flies were crowded to different degrees (i.e., grown under different larval densities) to generate different body-sized individuals. Three different densities - 80, 150 and 300 eggs per 8ml of food in each vial were used to generate large (1.261±0.01), medium and small (1.113±0.01) individuals respectively. LH-*st* flies were grown under normal egg density (i.e., 150 eggs per 8ml food in each vial), at which the stock is maintained.

The LH's are used as "competitor males" for the LH-*st* "target male" in all the experiments. The competitors are collected two days before the LH-*st* flies' eclose so as to negate the factor of age of the competitor male. Flies obtained from eggs of LH-*st* are collected as virgins within every 5hour. As soon as LH-*st* flies start to eclose, they are

kept with competitor males for three days of "conditioning". All the observations are conducted on 12 or 13th day after egg collection.

2.5 Agar media for egg collection

Agar plates are prepared by dissolving 1mg of bacteriological agar in 100ml of water in a beaker. This solution is boiled and mixed properly till no granules are left in the beaker. The solution is poured into Petri-plates and allowed to cool. Solidified agar plates are used for egg collection.

2.6 Method of collecting flies as virgin

Wherever mentioned, the experiment was carried out with virgin flies (males/females) generated in the following procedure. The culture vials (vials where eggs were cultured) were monitored regularly for the sign of pupation. After pupation was completed, on 10th day post-egg collection they were monitored for eclosion. LH-flies usually take about 9-10 days to complete larval development and show a sharp window of maximum eclosion. All the experimental flies were collected as very young ($\leq$ 5 hours post eclosion) during the peak of their eclosion. Such method of collecting young flies ensured the virgin status of the flies. This method of collecting virgin flies is a standard method practiced in this system (Byrne and Rice 2006, Prasad et al. 2007, Nandy and Prasad 2011, Nandy et al. 2012, 2013). Collection was done under light CO_2-anaesthesia. After sorting males and females as virgins they were held either singly or in single sex vials till the day of the experimental mating or the set up of treatment (conditioning).

2.7 Method of behavioural observation

Each mating vial was observed for Mating latency and Copulation duration for all the experiments described in "Experimental procedure section"

Mating latency (ML) is the time a virgin pair (a male and a female) takes to start copulation. For the purpose of the experiments described here, it was calculated as follows:

ML (in minutes) = Observation start time – mating start time

Copulation duration (CD) refers to the duration for which a given copulation has taken place.

During the observation, it was made sure that the vials were not disturbed in anyway. Observation was done during the light phase of the light-dark cycle under ample ambient illumination.

2.8 Data analysis

Data for each trait ML, CD and progeny number were either analysed using a two-factor mixed model analysis of variance (ANOVA) with treatment as fixed factor and block as random factor or a one –factor ANOVA (mentioned wherever applicable). Multiple comparisons were implemented using Tukey's Honestly Significant Difference (HSD) wherever required.

A linear regression of mean progeny sired on mean CD and mean ML to determine the relation between the two for both target males as well as competitor were done.

A separate section is detailed for experiments if the data is analysed with a different method.

Chapter 3

Experiments performed and procedure

3.1. Experiment 1: Effect of male body size on reproductive behaviour and fitness component.

3.1a. Experimental procedure:

The present experiment was done using the Large (L) and Small (S) males from the LH population and Medium (M) sized target males from the LH-*st* population. The method of generation of these males is described in Chapter 2, Section 2.3.

For the purpose of the experiment, one M-males were combined with virgin LH-*st* female and either one L-male (treatment-1) or one S-male (treatment-2).

On 9^{th}-10^{th} day from egg collection LH adult males were separated using CO_2 anaesthesia and held singly in 1.5ml corn-meal for three days. LH-*st* were collected as virgins on 9^{th}-10^{th} day from egg collection and kept individually in vials containing 1.5ml media for three days. On 13^{th} day, different body sized competitors are transferred into mating vials containing freshly prepared media along with one scarlet female and one scarlet male of medium body size.

These mating vials were observed for mating latency, copulation duration (as described in Chapter 2, section 2.6) and the identity of the successful male (male that successfully started copulation) with the help of the eye colour marker. Those females which did not mate were discarded. As soon as the mating was over, female were flipped to 8ml corn-molasses media containing vials and kept for oviposition for a window of 18 hours. After 18 hours female were discarded and the vials were incubated at 25 °C. On 11^{th} day when all the progeny had eclosed, flies were frozen and the progeny was counted. Red-eyed competitor males from the mating vials were separated and frozen at -20 °C. The frozen flies were dried at 60 °C in hot air oven for body weight measurements.

This experiment was run with n=50 replicates for each treatment. So, there were 150 mating vials for each observation.

3.1b Body-weight measurement

To obtain body weight measurements, on 10^{th} day after egg collection when all the competitor flies had eclosed, male flies were immediately frozen in -20 °C refrigerator and dried at 60 °C in hot air oven for at least 48hours. Body weights were measured in a five males at a time using high precision electronic balance (Sartorius CPA225D).

3.1c Data analysis

Data is analysed for;

1) Differences in dried body weight.
2) Mating success, the number of mating by either large or small male,
3) ML for both red and scarlet eyed males,
4) CD for both red and scarlet eyed male,
5) Relation of CD if scarlet male mated in presence of both large or small male and vice-versa.
6) The correlation between CD and the number of progeny sired by the mating male,

For data analysis refer to Chapter 2, section 2.8 for ML, CD and progeny number. A contingency analysis using Pearson's Likelihood test was done to find the χ^2 value for mating success.

3.2. Experiment 2: Do males perceive the risk of sperm competition by co-inhabitant and /or quality?

3.2a <u>Experimental procedure:</u> The experiment was done using the target LH-*st* males (medium sized) and competitor/conditioning males from LH population of three types – L, M and S, described in Chapter 2. The method of generation of the experimental males is described in detail in Chapter 2. LH-*st* female was used as the common female type for all the treatment.

Females are held individually in separate vials whereas Virgin males were randomly assigned to five different treatments that differed in the number and type of the competitor in each vial –NC, 1S, 1L, 4S, 4L, where the numeric figure stands for the number of competitors (viz. 1 or 4) and the letter stands for the type of the competitors (L=large, S=small). NC stands for "No competitor", where a medium sized LH-*st* male (i.e., the Target male) was held in a single vial immediately after it was collected as virgin. The experimental scheme is shown in Figure 2.

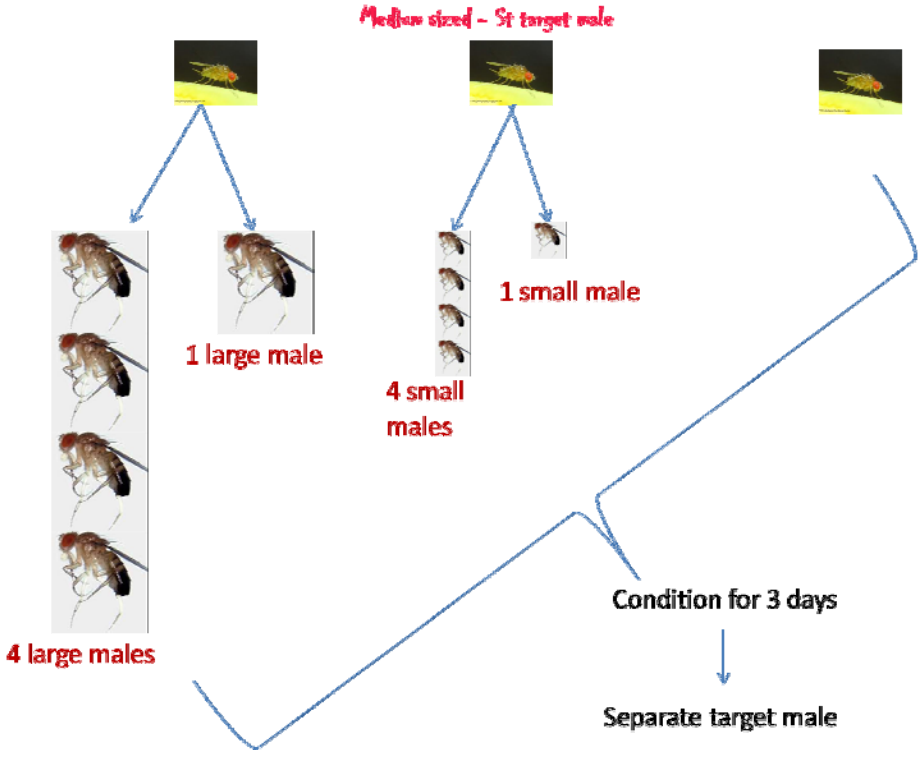


Figure 2: Experimental scheme for conditioning of medium sized target males. There are five different treatments: conditioning with 4 large, 1 large, 4 small, 1 small, and with no competitor. Conditioning is done for three days and on third day target males are separated and combined immediately with females. All the competitors are red eyed and targets are scarlet eyed.

Target males were conditioned for three days and on third day they were separated using mild CO_2 anaesthesia. The competitor (or conditioning) males were discarded. After a recovery time of half an hour the conditioned target males were paired with a three day old LH-$_{st}$ virgin female. Each pair was observed individually for ML and CD. Those females which did not mate were discarded. As soon as the mating is over, females are separated and combined with medium sized (M) red eyed (LH) males and left to interact for 18 hours. The second male was discarded after 18 hours and females were put into individual test-tubes (12mm × 75mm) with food and allowed to oviposit for 18 hours. Twelve days later, the progeny was counted and scored on the basis of their eye colour. The proportion of scarlet eyed flies gave the sperm defence (P1) value of the target males.

In a separate subset of this experiment I investigated the outcome of conditioning on the progeny production of the females when there was no second mating (i.e., no sperm competition). The design of this assay was identical except the part following the first round of mating. Here, after the first round of mating with the target males (i.e., conditioned males), the female was immediately transferred to oviposition vials and allowed to oviposit for 18 hours. Following this 18 hour long window, they were again transferred to another set of oviposition vials and allowed to oviposit for 24 hour.

There were 40 replicates for each treatment. So, there were 200 mating vials for each observation. This experiment was done in two blocks run on two separate days.

3.2b Data analysis

This experiment was done in four separate blocks two of which were run on consecutive days whereas, the other two were run on two separate days, with 40 replicates for each treatment. A total of 240 mating vials were observed for ML, CD in minutes. Data was analysed for:

1) Effect of conditioning on mating latency (ML) of target male.
2) Effect of conditioning on copulation duration (CD) of target male.
3) Effect of conditioning on sperm defence (i.e., P1).

Data for ML and CD was analysed using a two-factor mixed model ANOVA as per section 2.8 of Chapter 2. P1 for each female was calculated using the following definition:

$$P1 = \frac{\text{Progeny produced by female on mating with first male}}{\text{Total progeny produced by that female}}$$

In this experiment, the female was scarlet eyed, the first male (i.e., the target male) was scarlet eyed and the second male was red eyed. Therefore all the progeny from the first male was scarlet eyed and the all the progeny from the second male was red eyed. Hence, calculation of P1 could be done on the basis of progeny count.

The P1 values =1 were discarded as it might indicate the absence of second mating.. The data for P1 is checked for a normal distribution. In case of a non-normal distribution, P1 values are arcsine square-root transformed using the formula;

$$\sqrt[2]{ArcSine(P1)}$$

The transformed P1 values were then analysed using treatment as a fixed factor for ANOVA.

Data from the assay under no-competition was analysed in the following manner:

1) Effect of conditioning on Mating latency (ML) of target male,
2) Effect of conditioning on Copulation duration (CD) of target male.
3) The correlation between CD and the number of progeny sired by the mating male.

For analysis of CD and ML see Chapter 2, section 2.8. A linear regression of mean progeny produced on mean CD was done to determine the relation between the two.

3.3 Experiment 3: Does effect of conditioning have different fitness consequence under different contexts?

3.3a *Experimental procedure:* On 10th day from egg collection competitor males are separated and held as, either 1 L or 1S per vial. For flies from LH-$_{st}$ population virgin males and females are collected on 9th day from egg collection. Virgin females are held singly in 1.5ml food vials, whereas males are randomly assigned to vials containing L, S or held singly with no competitor for conditioning and are kept together for three days. On third day from conditioning, the target male is separated using mild CO_2 anaesthesia and combined with three day old virgin scarlet eyed female. Both the male and female are held in mating vials containing freshly prepared food and labelled according to the status of the conditioned male as "with large male", if target male was conditioned with a large red-eyed male, and "with small male", if it was conditioned with small red-eyed male and "with none", if it was conditioned with no competitor. Immediately after combining, ML and CD are recorded in minutes individually for each target male.

As soon as the mating is over, the females are separated using CO_2 and held to recover for half-an-hour. For each treatment, the females were divided equally to combine with second male. For the females which were initially held with target males conditioned with a large red-eyed male, half the number of them were given large red-eyed male and the other half of the females were given small red-eyed male as the second male. Similar procedure was followed for the rest of the treatments as well. 18 hours after interaction, the second male is discarded and females are put in individual vials with food and allowed to oviposit for 18-20 hours. Twelve days later, the progeny was scored on the basis of the eye colour.

A total of 240 observations with 80 replicates for each treatment were recorded. For the P1 analysis the sample size for each treatment was 40. This experiment was run in one block only.

The experimental scheme is shown in figure 3

3.3b Data analysis

1) Effect of conditioning on ML of target male
2) Effect of conditioning on CD of target male
3) Sperm defence under different status of the second male.

Data for each trait ML and CD is analysed using a one-factor model analysis of variance (ANOVA) with treatment as fixed factor. Multiple comparisons were implemented using Tukey's HSD.

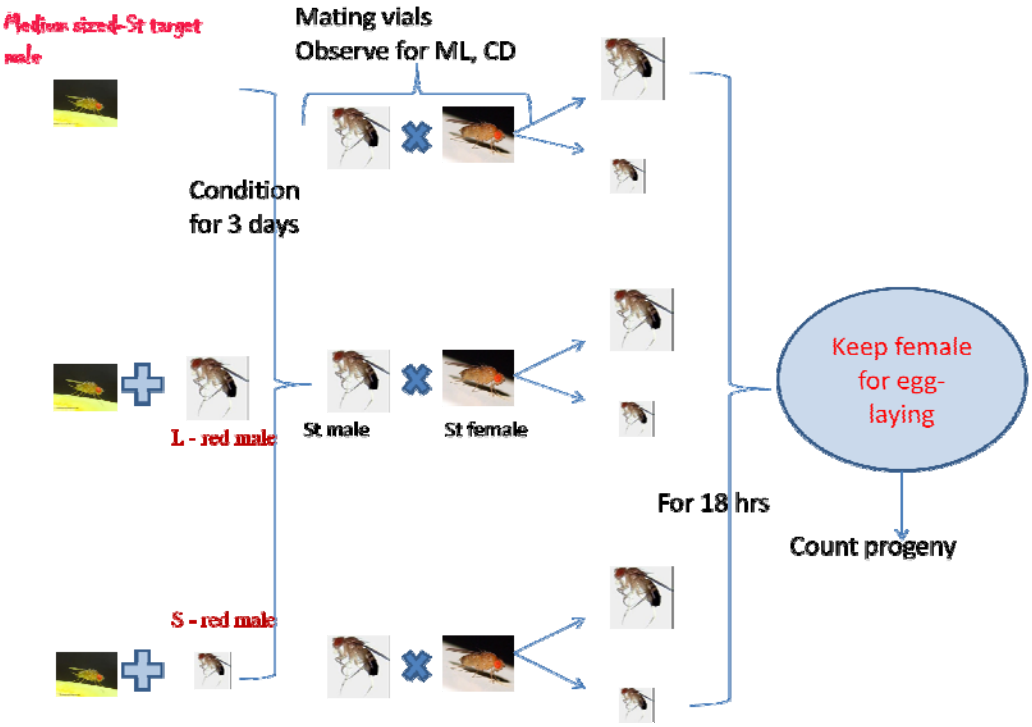


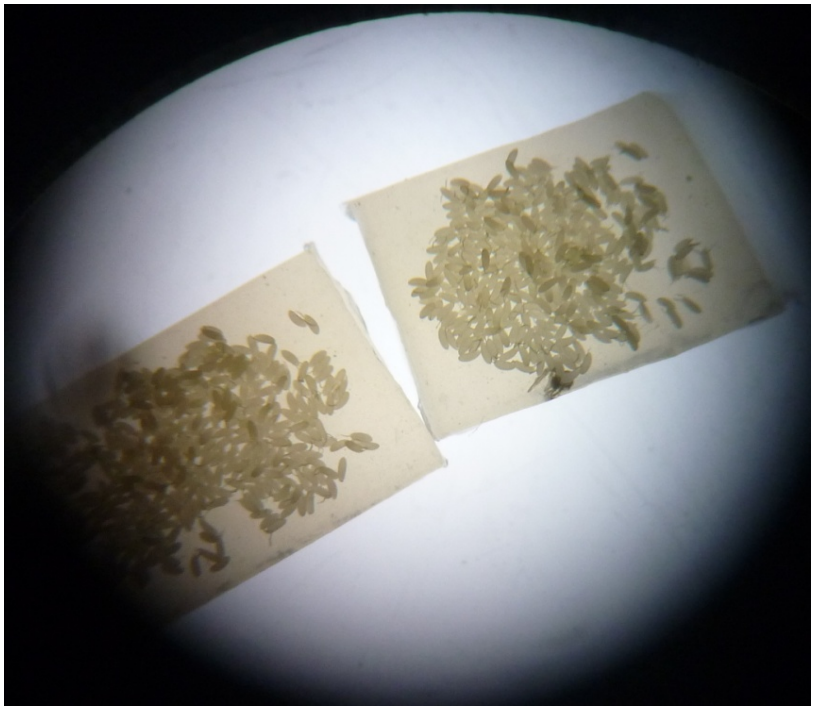
Figure 3: Experimental scheme for Exp3 Targets are conditioned for three days with either large, small or no competitor. After three days males are separated and combined with females for mating. As soon as the mating is over, males are discarded and females are separated and a second male (large or small) is put for second mating.

For sperm defence analysis, P1 values were assigned according to the formula as described in section 3.2b

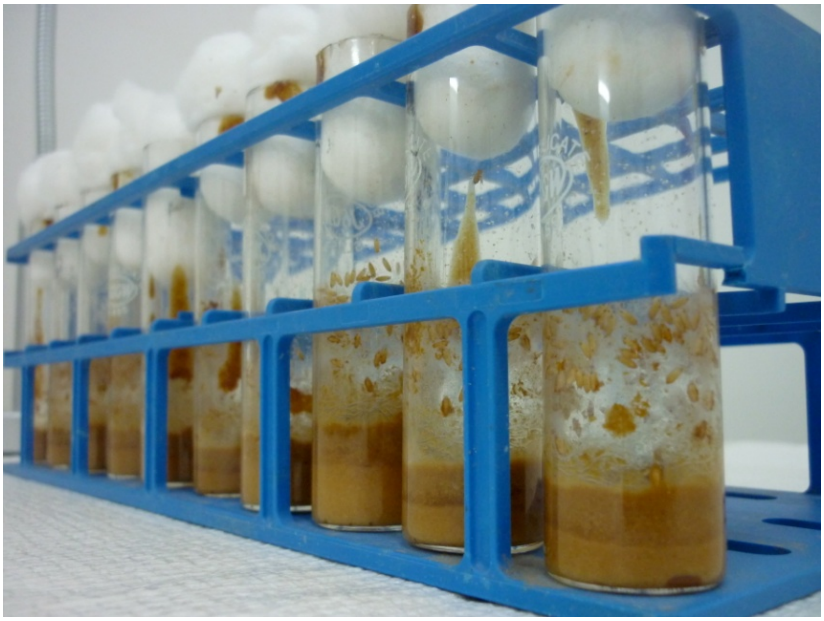
The p1 value =1 are discarded as it means that there is no second mating, hence no sperm defence. The data for p1 is checked for a normal distribution. In case of a non-normal distribution, P1 values are arcsine square-root transformed using the formula;

Now, arcsine transformed p1 values are plotted against treatment as a fixed factor for ANOVA.

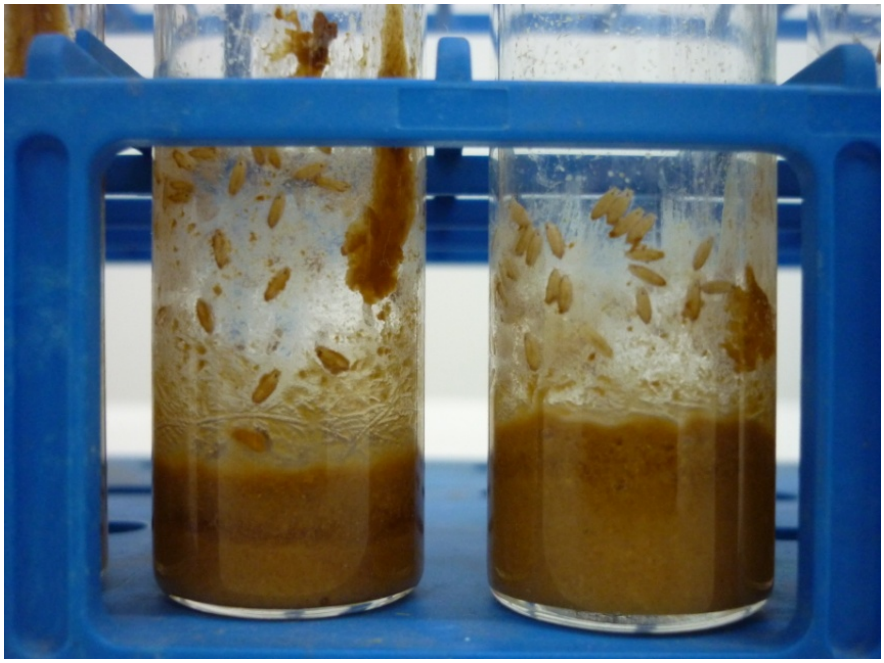
Interaction of treatment and second male yield the effect of second male being either large or small on the proportion of progeny produced by the first male (P1).



Photograph 1: Picture of accurately counted eggs on agar pieces under microscope.

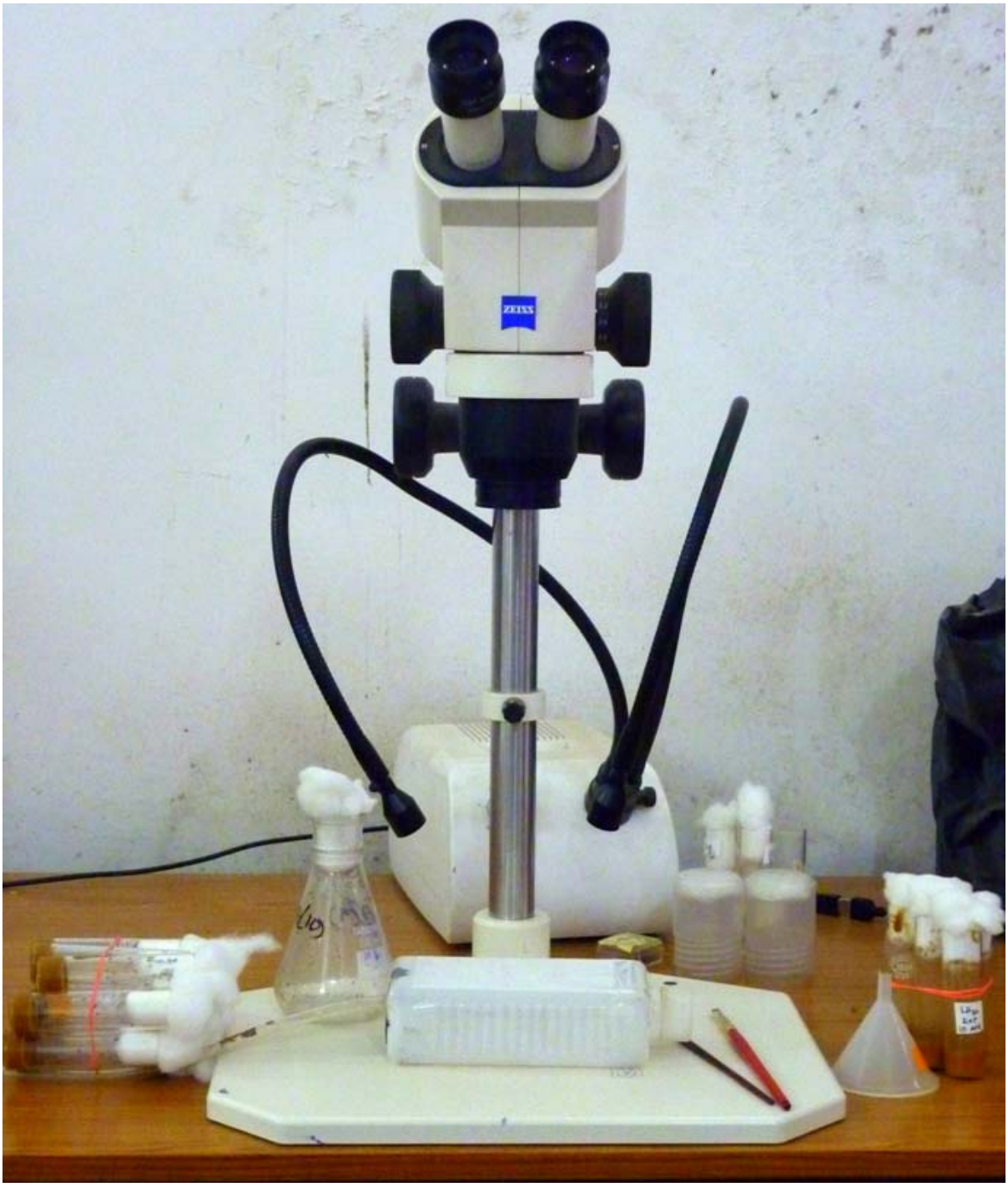


Photograph 2: Vials containing pupated flies on 9th day after egg collection.

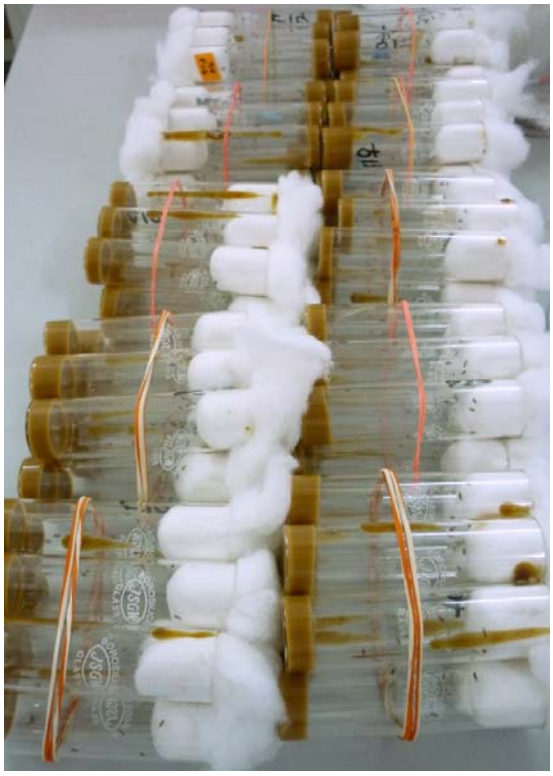


Photograph 3: A closer look on the pupated flies in corn-meal molasses food media.

Photograph 4: Culture vials in incubator maintained at 12hr L: 12hr D photoperiod at 25 °C and 50% relative humidity.



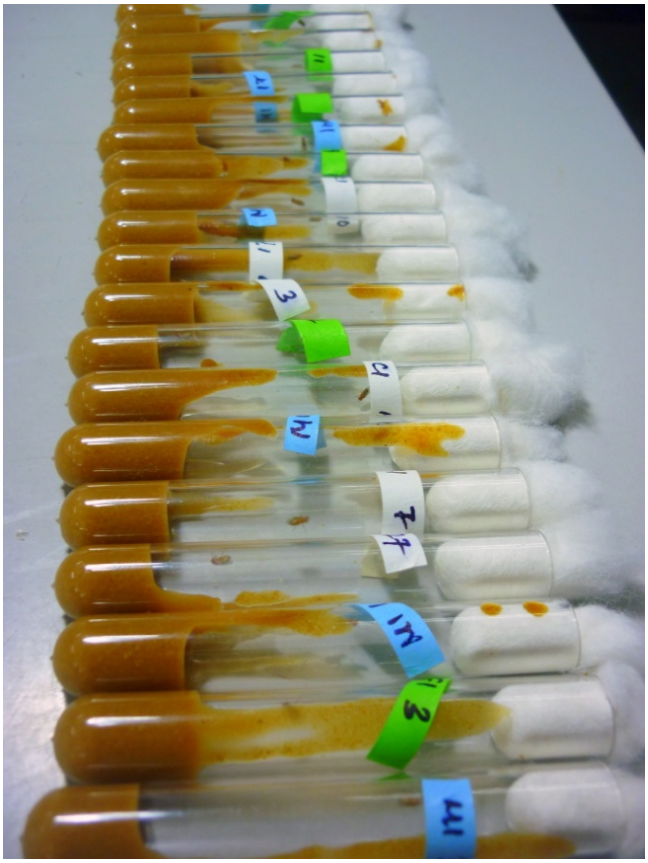
Photograph 5: Gas station to collect adult flies under slow CO_2 anaesthesia.



Photograph 6: Vials containing virgin males and female in separate vials.

Photograph 7: Mating vials (with label). After mating is over females are separated and kept to oviposit in small tubes containing media.

Photograph 8: Separating females after mating using slight CO_2 anaesthesia.



Photograph 9: Females kept in separate tubes with corresponding labels and allowed to oviposit.

Chapter 4

Results

Experiment 1*: Effect of male body size on reproductive behaviour and fitness component.*

1) *Differences in dried body weight.*(Figure *1.1)*

Larval density was found to have a significant effect on the dry body weight of the males ($p<0.0001$, $F=63.798$, $DF=1$, $SS= 0.109$). Males grown under lower density (80 eggs/8 ml foods in each vial) were found to be significantly larger (Figure 1.1) than the males grown in higher density (300 eggs/8ml food in each vial). It should be noted here that from here onwards, "large" males refer to the males grown under low larval density (i.e., 80/vial) and "small" males refer to the males grown under high larval density (i.e., 300/vial).

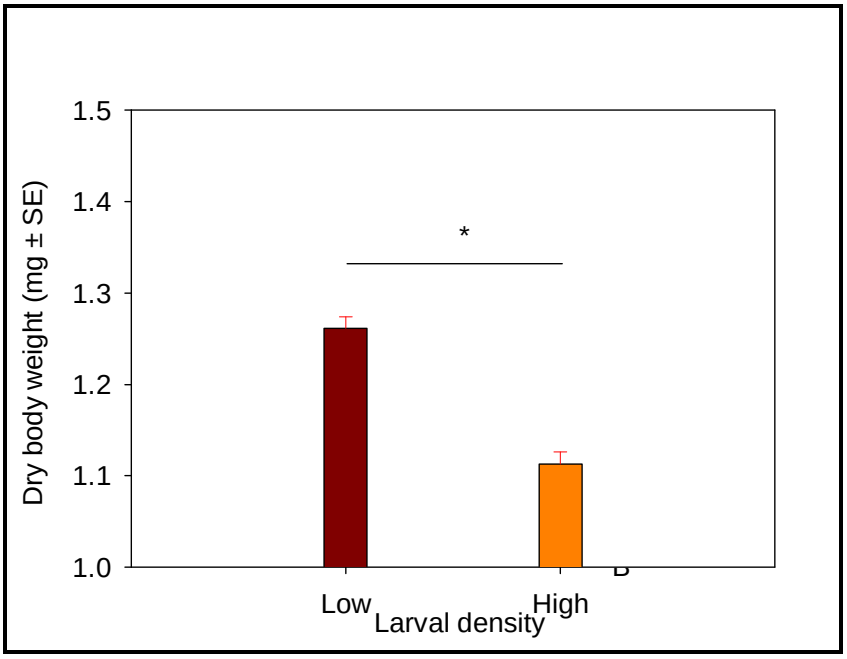


Figure 1.1: Effect of larval density on body-weight. Different densities are low density of 80eggs/vial and high density of 300eggs/vial. Bars connected by single line show they are significantly different.

2) *Mating success, the number of mating by either large or small male.*

Results of the χ^2-test indicated that the mating success of the target and the different competitors are not significantly different (Likelihood ratio and Pearson test: $\chi^2 = 0.196$. This indicates that mating success of the males is not substantially affected by their body size.

3) *Mating latency and copulation duration of target male.* (Figure 1.2 and 1.3)

Results of two-factor mixed model ANOVA on both ML and CD suggested that treatment did not have a significant effect on either of the traits (Table 1.1 for ML and Table 1.2 for CD). However, closer scrutiny of the data revealed that when the target male is in presence of a small body sized competitor male both the mean copulation duration and mean mating latency (±se) is less than when it is in presence of a large competitor (Figure 1.2 for ML, 1.3 for CD). In other words, target males were found to take longer to mate and also mate for longer duration in presence of a large competitor, compared to that in presence of small competitor.

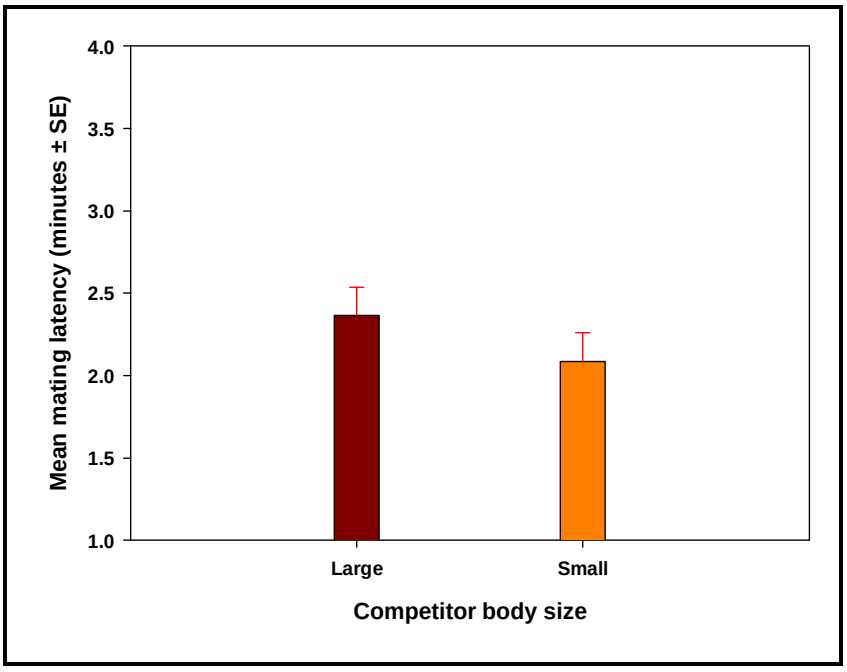


Figure 1.2 Effect of presence of competitors of different body size (large and small) on mean mating latency (in minutes) of the target (scarlet eyed) male. Bars not connected by line are not significantly different.

Table 1.1: Results of two-factor mixed model ANOVA on mating latency (ML) of target male using treatment (body size of the competitor–small/large) as fixed factor and block as random factor

Trait	Effect	SS	MS Num	DF Num	MS Den	DF Den	F	P
ML	Comp. Body size	1.939	1.939	1	1.524	96	1.272	0.262
	Block	6.452	6.452	1	1.524	96	4.234	0.042
	Comp body size × Block	1.405	1.405	1	1.524	96	0.922	0.339

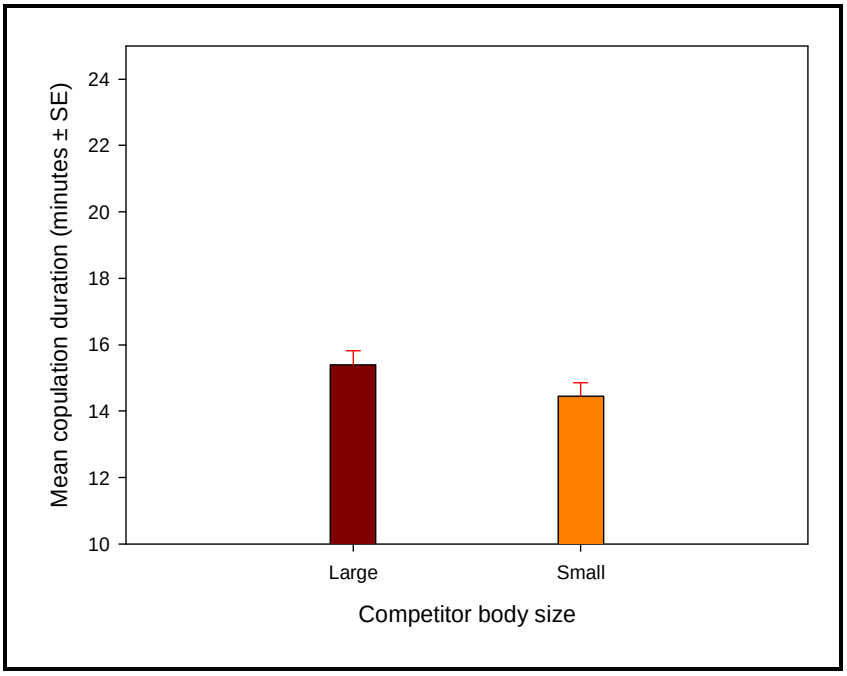


Figure 1.3 Effect of presence of competitors of different body size (large and small) on mean copulation duration (in minutes) of the target (scarlet eyed) male. Bars not connected by single line are not significantly different.

Table 1.2: Results of two-factor mixed model ANOVA on copulation duration (CD) using treatment (body size of the competitor–small/large) as fixed factor and block as random factor

Trait	Effect	SS	MS Num	DF Num	MS Den	DF Den	F	P
CD	Competitor body size	22.863	22.863	1	8.679	98	2.634	0.108
	Block	108.056	108.056	1	8.679	98	12.450	0.001
	Comp. body size × Block	40.435	40.435	1	8.679	98	4.659	0.033

4) *Progeny sired by the target male in presence of the competitor male (large or small).* (Figure 1.4)

There was no significant effect of the treatment on the number of progeny produced by the females (MS=7.54, DF=1, F=0.06, p=0.8).

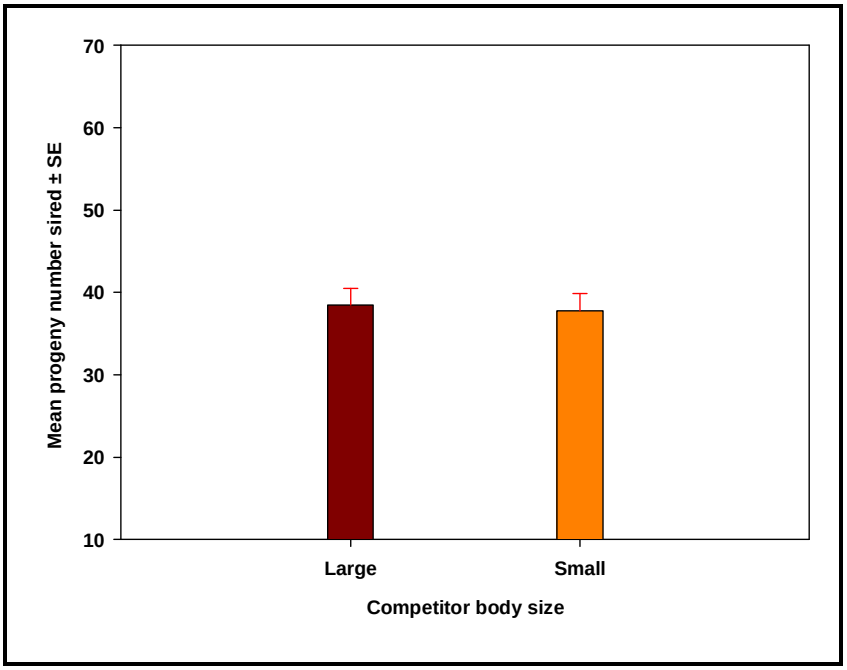


Figure 1.4 Effect of competitors body size (large/small) on the progeny sired by target (scarlet eyed) male. Bars not connected by line are not significantly different.

5) *Correlation between CD and progeny sired by target male in presence of competitor male.* (Figure 1.5).

   a) *CD Vs Progeny sired by target male in presence of large male.* (Figure 1.5)

There was no significant correlation between CD and number of progeny produced by the females when target male is in presence of a large male (slope = - 0.202, $r^2 = 0.003$, p=0.767).

b) *CD Vs progeny sired by target male in presence of small male* (Figure 1.5)

A significant correlation between CD and number of progeny produced by females was found when target male is in presence of a small male. (Slope = - 1.364, r^2 = 0.150, p=0.05*).

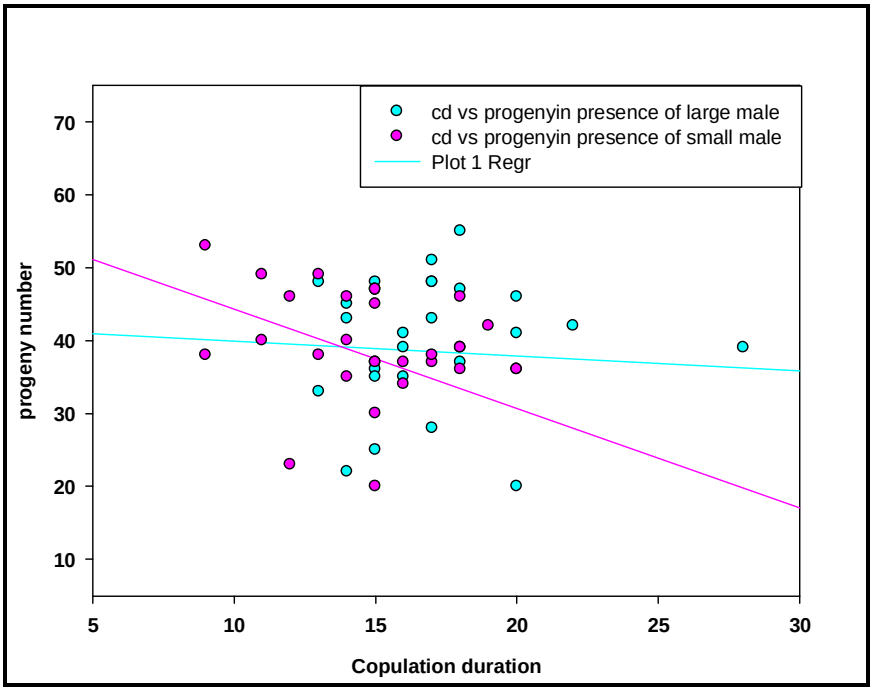


Figure 1.5 correlation of progeny number and copulation duration of target male in presence of large (cyan dots) and small male (pink dots).

6) *Copulation duration and mating latency of competitor male*. (Figure 1.6, 1.7)

The two-factor mixed model ANOVA indicated that body size did not have a significant effect on CD and ML of the males (i.e., competitors, Table 1.3 for ML, Table 1.4 for CD). Although mating latency and copulation duration does not show any significant difference between large or small male, there is a trend which suggests that large males mate for lesser duration compared to small males which mate for longer duration (Figure 1.7).

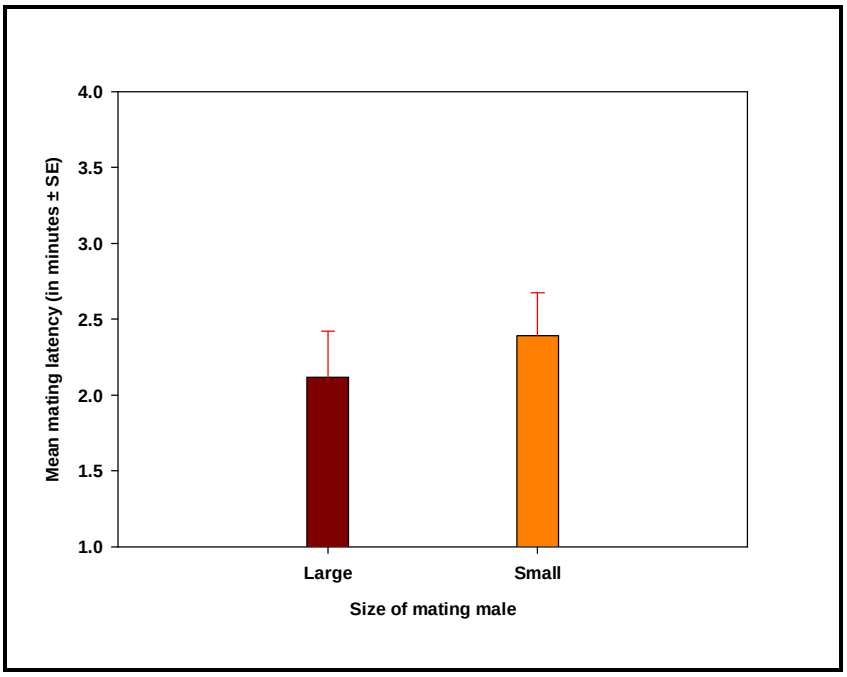


Figure 1.6 Mean mating latency (in minutes) of different body size males (large/small) in presence of medium sized (Scarlet eyed) competitor. Points not sharing common alphabet are significantly different.

Table 1.3: Results of two-factor mixed model ANOVA on mating latency (ML) of the competitor male using treatment (body size of the competitor–small/large) as fixed factor and block as random factor

Trait	Effect	SS	MS Num	DF Num	MS Den	MS Den	F ratio	P
ML	Size of mating male	1.592	1.592	1	3.648	1	0.437	0.628
	Block	13.739	13.739	1	3.648	1	3.766	0.303
	Size of mating male × Block	3.648	3.648	1	3.395	84	1.074	0.303

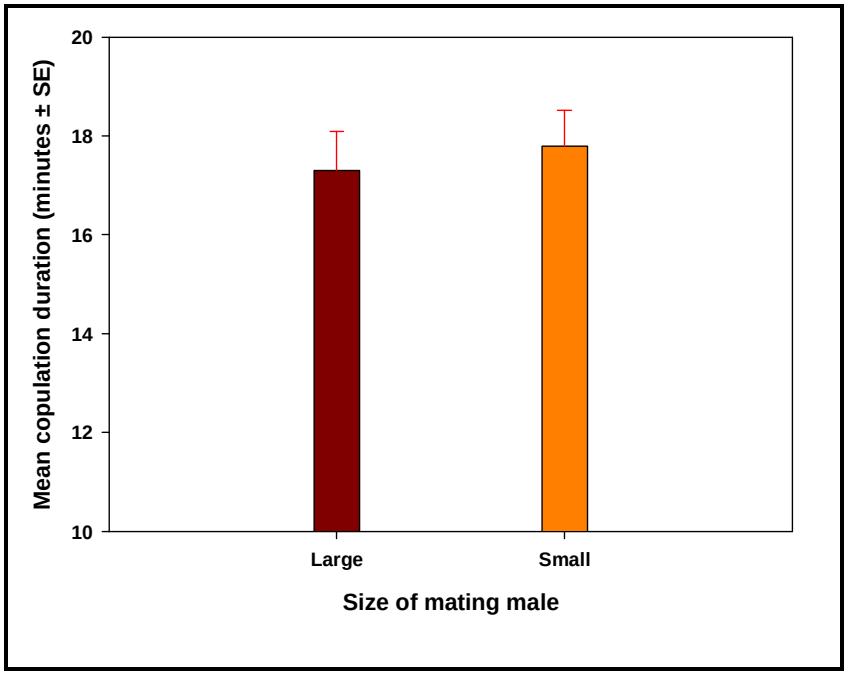


Figure 1.7 Mean copulation duration (in minutes) of different body size males (large/small) in presence of medium sized (Scarlet eyed) competitor. Points not sharing common alphabet are significantly different.

Table 1.4: Results of two-factor mixed model ANOVA on copulation duration (CD) using treatment (body size of the competitor–small/large) as fixed factor and block as random factor

Trait	Effect	SS	MS Num	DF Num	MS Den	DF Den	F	P
CD	Size of mating male	5.166	5.166	1	24.710	1	0.209	0.727
	Block	7.641	7.641	1	24.710	1	0.309	0.677
	Size of mating male × Block	24.710	24.710	1	25.882	85	0.955	0.331

7) *Progeny produced by females on mating with competitor (Large or Smal)l male.* (Figure 1.8)

There is no significant difference between the progeny produced by the large male or the small male (MS=75.390, DF=1, F ratio=0.524, p=0.473). However, large males sired less number of progeny relative to the small males (Figure 1.8).

8) *Correlation of CD, ML on the progeny sired by competitor male.*

a) *For large body sized competitor: CD Vs progeny number* (figure 1.9)

The linear regression analysis yields a non significant negative correlation between copulation duration and progeny number with slope = -3.135, p=0.04, r^2=0.256. This suggests that large males even if mating for lesser duration are more harming to the female as compared to smaller ones.

b) *For small body sized competitor: CDVs progeny number* (figure1.9)

If the male is small the correlation between CD and progeny sired is non-significant positive (slope=0.469, r^2=0.031, p=0.408).

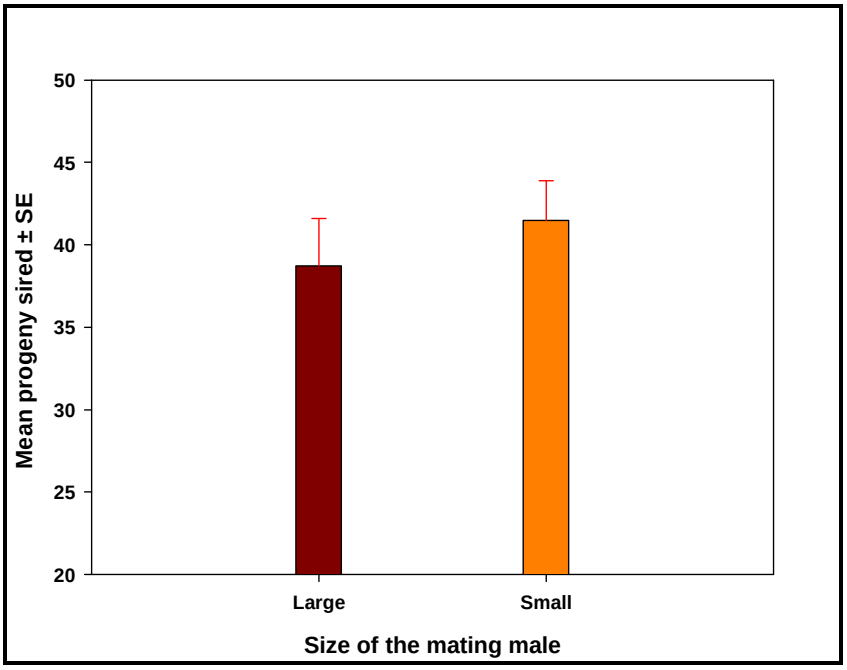


Figure 1.8 Mean progeny sired by males of different body size (large/small) in presence of medium sized (Scarlet eyed) competitor. Bars not connected by single line are not significantly different.

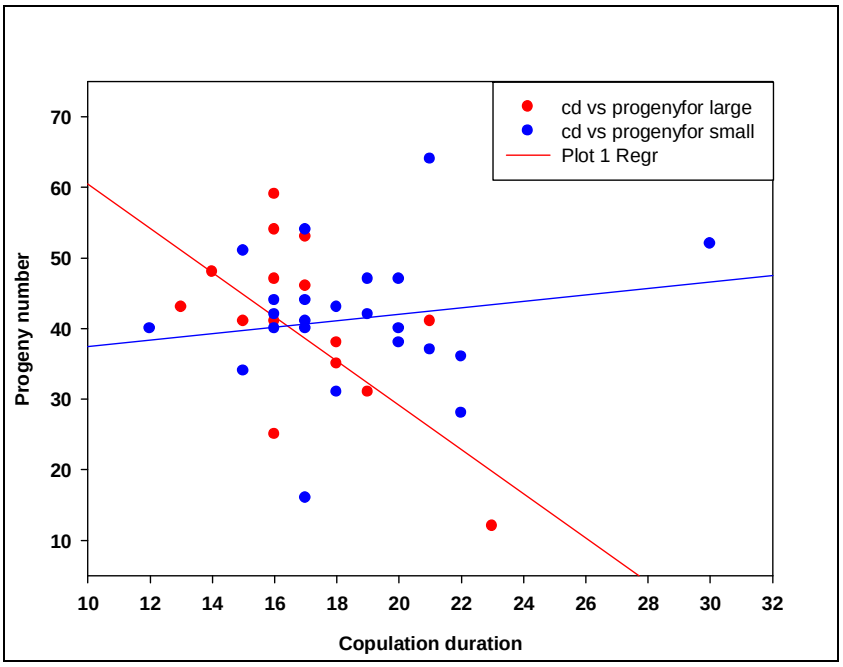


Figure 1.9: Correlation between copulation duration and progeny sired by large (red dots) and small (blue dots).

Experiment 2: ***Do males perceive the risk of sperm competition by co-inhabitant number and/or quality.***

1) *Effect of conditioning on Mating latency of target male* (Figure 2.1)

A two-factor mixed model ANOVA on mating latency using treatment as fixed factor and block as random factor have an effect of conditioning on mating latency of the target male but this effect is not considered as there is a lot of block interaction (Table 2.1). Mean mating latency for targets conditioned with 4L and 4S are not significantly different from each other but both show a significant difference from NC (Multiple comparisons using Tukey's HSD).

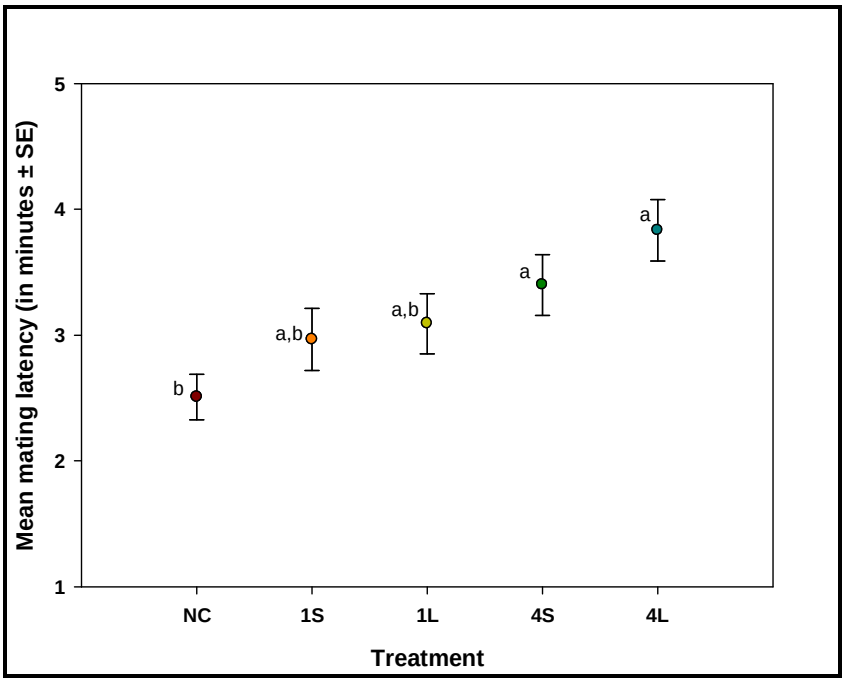


Figure 2.1 Effect of conditioning on mating latency (in minutes) of target (scarlet eyed) males. The target males are conditioned for three days prior observation. (Connotations NC=no competitor, 1S=1 small, 1L=1 large, 4S=4 small, 4L=4 large, represents different treatments under which target (scarlet-eyed) males were kept for conditioning). Points not sharing common alphabet are significantly different (multiple comparison using Tukey's HSD).

Table 2.1: Results of two-factor mixed model ANOVA using treatment as fixed factor and block as random factor

Trait	Effect	SS	MS Num	DF Num	MS Den	DF Den	F	P
ML	Treatment	186.784	46.696	4	8.655	839	5.395	0.0002
	Block	289.567	96.522	3	8.655	839	11.152	<.0001
	Treatment × Block	261.656	21.805	12	8.655	839	2.519	0.003

2) *Effect of conditioning on Copulation duration (CD) of target male.*(Figure 2.2)

Results of two factor mixed model analysis of variance (ANOVA) with treatment as fixed factor and randomized block (Table 2.2) gives a highly significant difference (p=<0.001*) of conditioning on copulation duration of target male. NC is different from 1S, 1L, 4S and 4L. Also 4L and 1S are significantly different (Multiple comparisons using Tukey's HSD). These results suggest that when target males are held in presence of large competitors they mate for longer duration, compared to when they are held with small competitors, prior to mating.

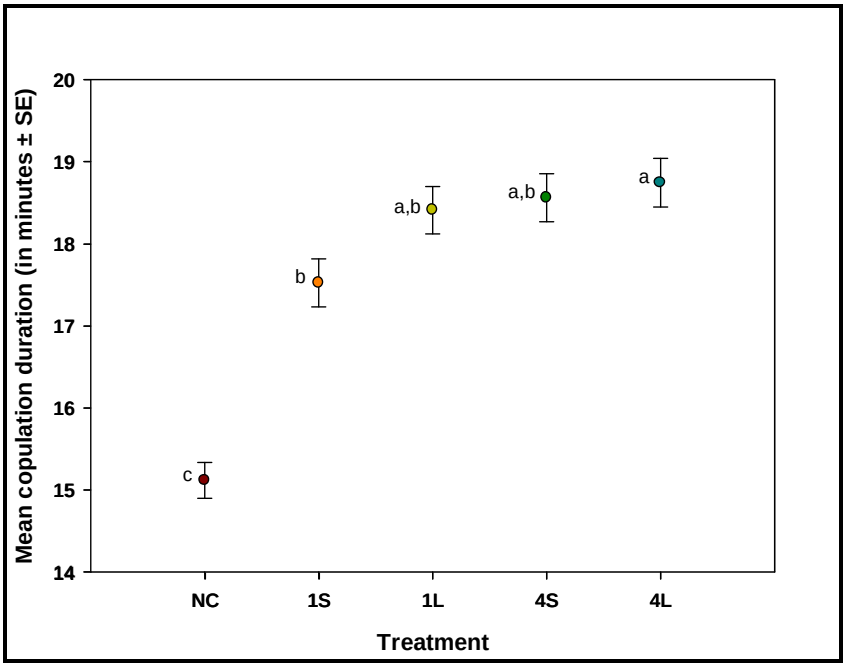


Figure 2.2 Effect of conditioning on copulation duration (in minutes) of target (scarlet eyed) males. The target males are conditioned for three days prior observation. (Connotations NC=no competitor, 1S=1 small, 1L=1 large, 4S=4 small, 4L=4 large, represents different treatments under which target (scarlet-eyed) males were kept for conditioning). Points not sharing common alphabet are significantly different (multiple comparison using Tukey's HSD).

Table 2.2: Results of two-factor mixed model ANOVA using treatment as fixed factor and block as random factor

Trait	Effect	SS	MS Num	DF Num	MS Den	DF Den	F	P
CD	Treatment	2034.671	508.668	4	12.551	839	40.528	<.0001*
	Block	79.792	26.597	3	12.551	839	2.119	0.096
	Treatment × Block	26.597	17.115	12	12.551	839	1.364	0.178

Experiment 2a: ***Effect of conditioning on progeny number under "no competition"***

3) *Effect of conditioning on Mating latency of target male* (Figure 3.1)

A two factor ANOVA on mating latency yields a non significant difference between the treatments (Table 3.1). The trend in the data suggests that whenever target males are conditioned with large male(s) the mean mating latency is lower than when they are conditioned with small male(s).

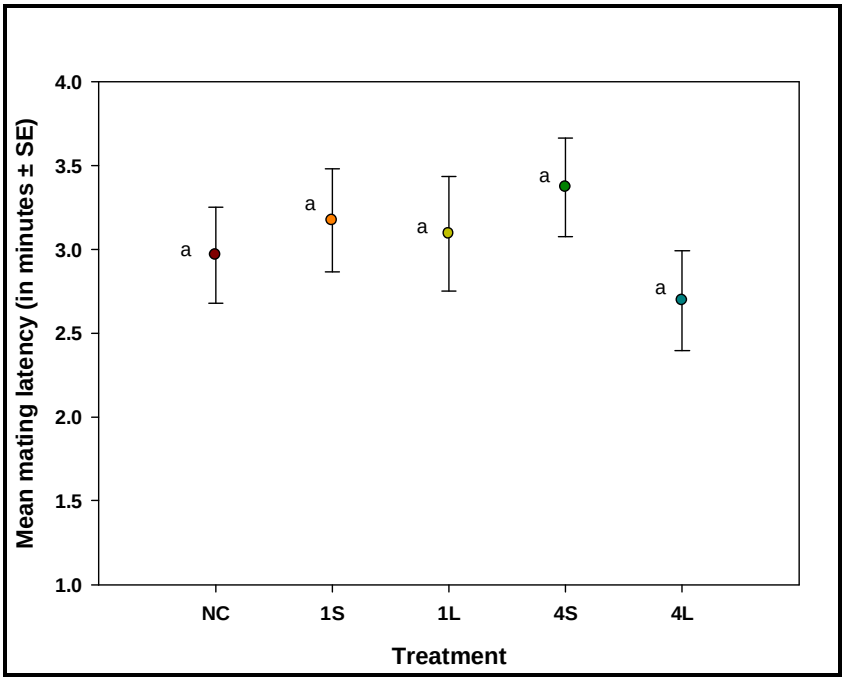


Figure 3.1 Effect of conditioning on mating latency (in minutes) of target (scarlet eyed) males. The target males are conditioned for three days prior observation. (Connotations NC=no competitor, 1S=1 small, 1L=1 large, 4S=4 small, 4L=4 large, represents different treatments under which target (scarlet-eyed) males were kept for conditioning)

Table 3.1 Results of two-factor mixed model ANOVA using treatment as fixed factor and block as random factor

Trait	Effect	SS	MS Num	DF Num	MS Den	DF Den	F	P
ML	Treatment	16.435	4.109	4	5.890	301	0.698	0.594
	Block	6.045	6.045	1	5.890	301	1.026	0.312
	Treatment × Block	33.217	8.304	4	5.890	301	1.410	0.231

4) *Effect of conditioning on Copulation duration (CD) of target male.* (Figure 3.2)

The copulation duration results for this experiment are highly significant (Table 3.2) and are in line with the results from experiment 2. The Tukey's HSD shows no significant difference between copulation duration for 1S, 1L, 4L, 4S treatments but a similar trend exists.

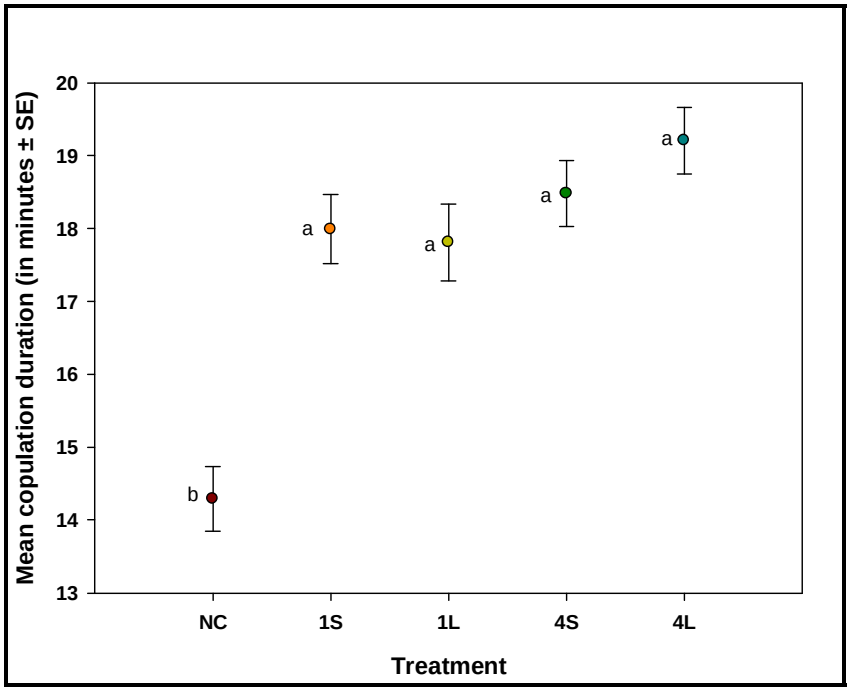


Figure 3.2 Effect of conditioning on copulation duration (in minutes) of target (scarlet eyed) males. The target males are conditioned for three days prior observation. (Connotations NC=no competitor, 1S=1 small, 1L=1 large, 4S=4 small, 4L=4 large, represents different treatments under which target (scarlet-eyed) males were kept for conditioning).

Table 3.2: Results of two-factor mixed model ANOVA using treatment as fixed factor and block as random factor

Trait	Effect	SS	MS Num	DF Num	MS Den	DF Den	F	P
CD	Treatment	949.775	237.444	4	13.960	301	17.009	<.0001*
	Block	24.216	24.216	1	13.960	301	1.735	0.189
	Treatment × Block	88.732	22.183	4	13.960	301	1.589	0.177

5) *Progeny sired by males conditioned for early adult life.*

a) Progeny after 18 hours (Figure 3.3a)

Data was analysed by one factor ANOVA on mean progeny sired by target male. Mean progeny produced by the target male within an 18 hour window has no difference when the target is exposed to different body sized males (p=0.203, MS=312.776, DF=4, F ratio=1.509). The mean progeny produced by the target males which were held without any competitor has an increased mean progeny number.

b) Progeny after 24hours (figure 3.3b)

The mean progeny produced by females 24 hours after first egg laying did not have any significant difference (MS=145.253, DF=4, F=0.583, p=0.675). An increased trend in mean progeny produced by females when they mated with target males which were conditioned for no competitor is observed.

c) Total progeny sired by the target male (figure 3.3)

One factor ANOVA suggested no significant difference between treatments. (DF=4, SS=2989.725, F=1.076, p=0.371)

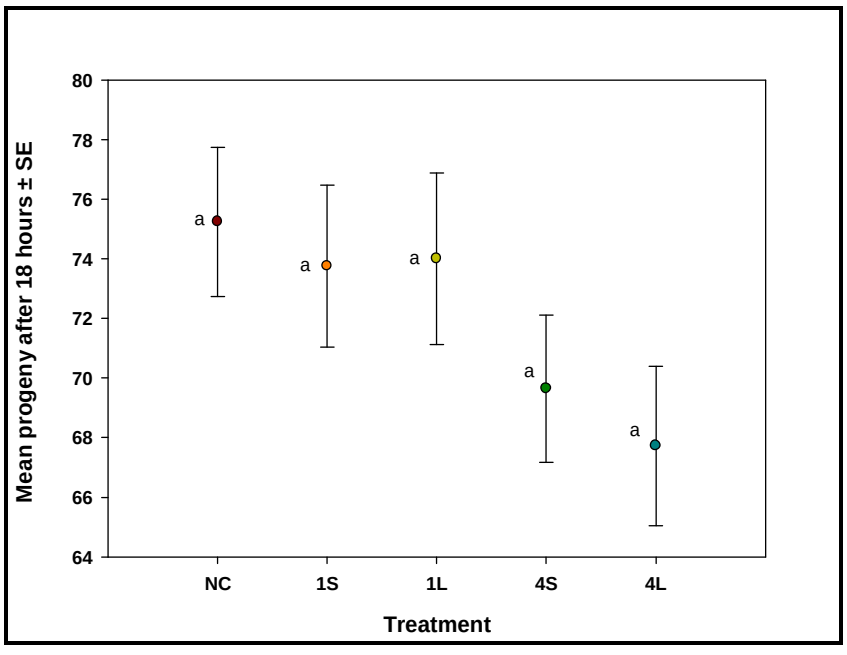


Figure 3.3a Effect of conditioning on progeny sired (after 18 hours) by target (scarlet eyed) males. The target males are conditioned for three days prior observation. (Connotations NC=no competitor, 1S=1 small, 1L=1 large, 4S=4 small, 4L=4 large, represents different treatments under which target (scarlet-eyed) males were kept for conditioning)

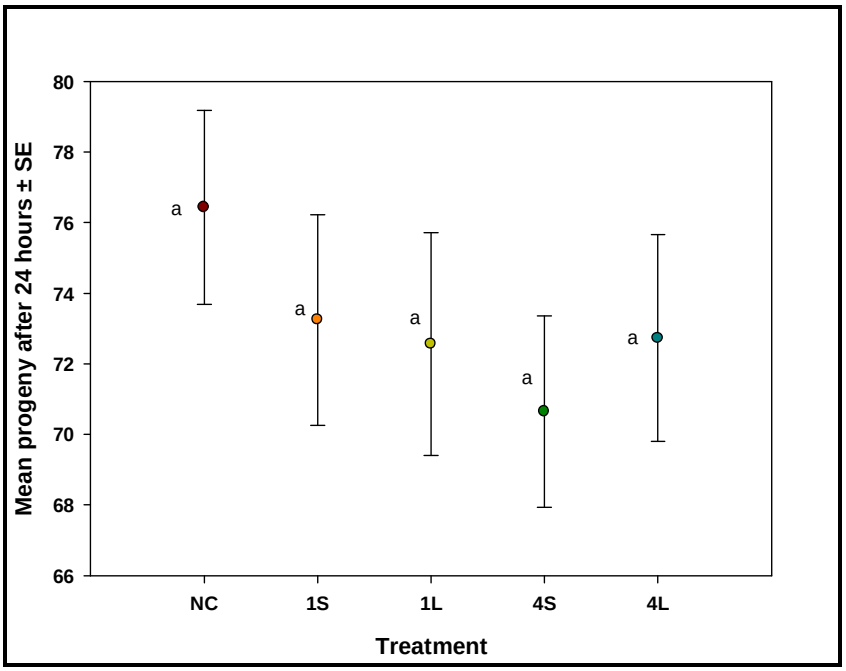


Figure 3.3b Effect of conditioning on progeny sired (after 24 hours) by target (scarlet eyed) males. The target males are conditioned for three days prior observation. (Connotations NC=no competitor, 1S=1 small, 1L=1 large, 4S=4 small, 4L=4 large, represents different treatments under which target (scarlet-eyed) males were kept for conditioning.

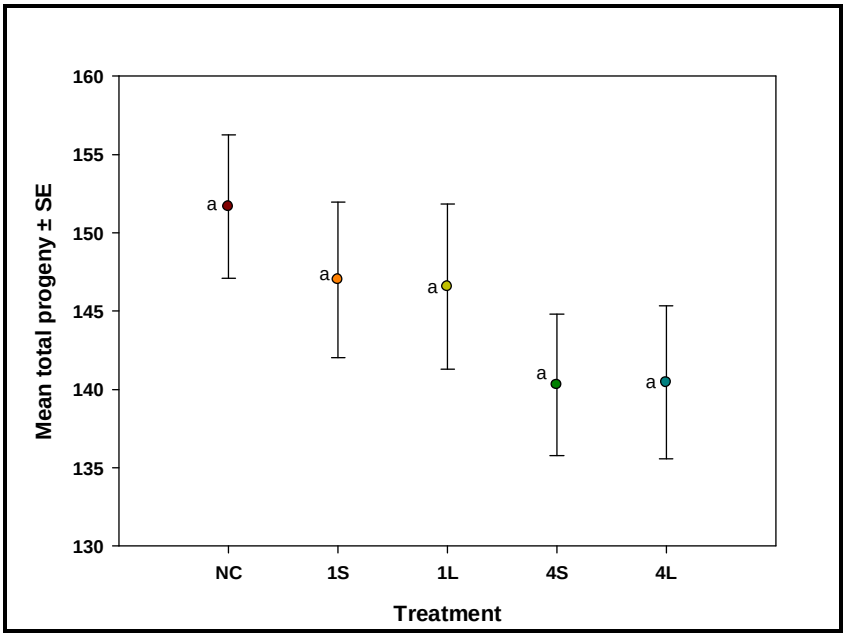


Figure 3.3 Effect of conditioning on total progeny sired by target (scarlet eyed) males. The target males are conditioned for three days prior observation. (Connotations NC=no competitor, 1S=1 small, 1L=1 large, 4S=4 small, 4L=4 large, represents different treatments under which target (scarlet-eyed) males were kept for conditioning)

6) The correlation between CD and the number of progeny sired by the mating male.

There was no significant difference between CD and number of progeny sired by the mating male. This was consistent with all the treatments.(figures 3.4, 3.5, 3.6).

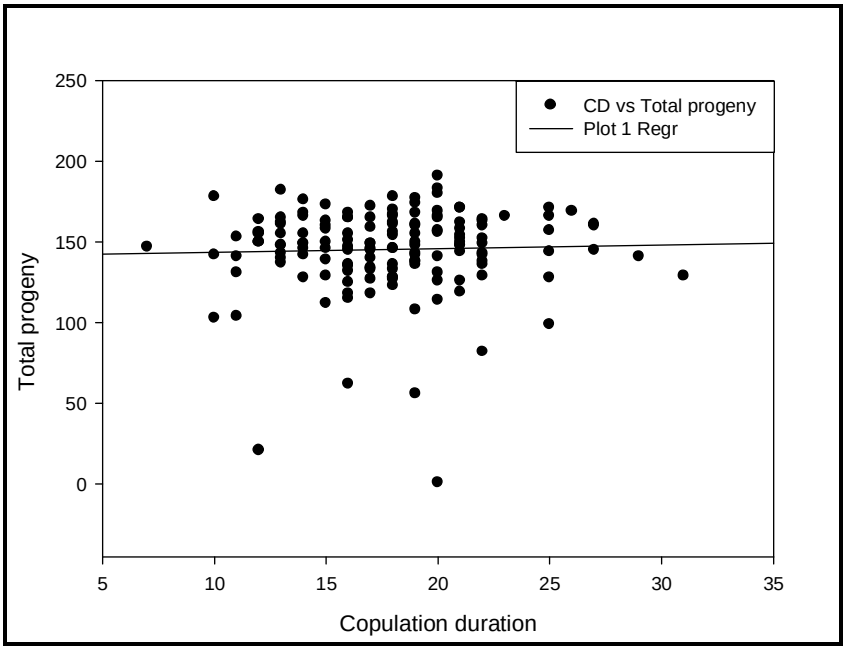


Figure 3.4: Correlation between copulation duration and the total progeny produced by the female on mating with target (scarlet-eyed) conditioned male.

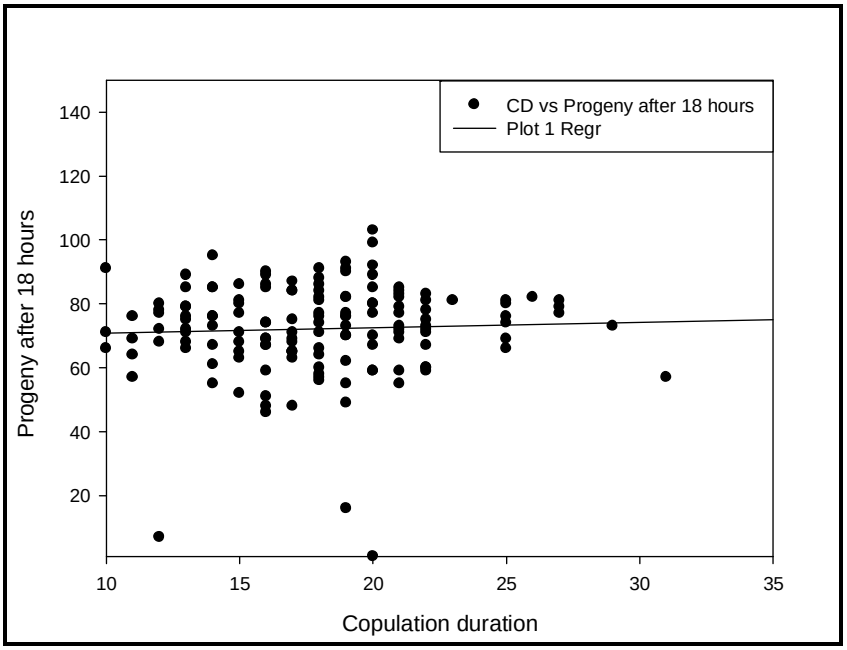


Figure 3.5: Correlation between CD and progeny produced by females on mating with target conditioned males after 18 hours

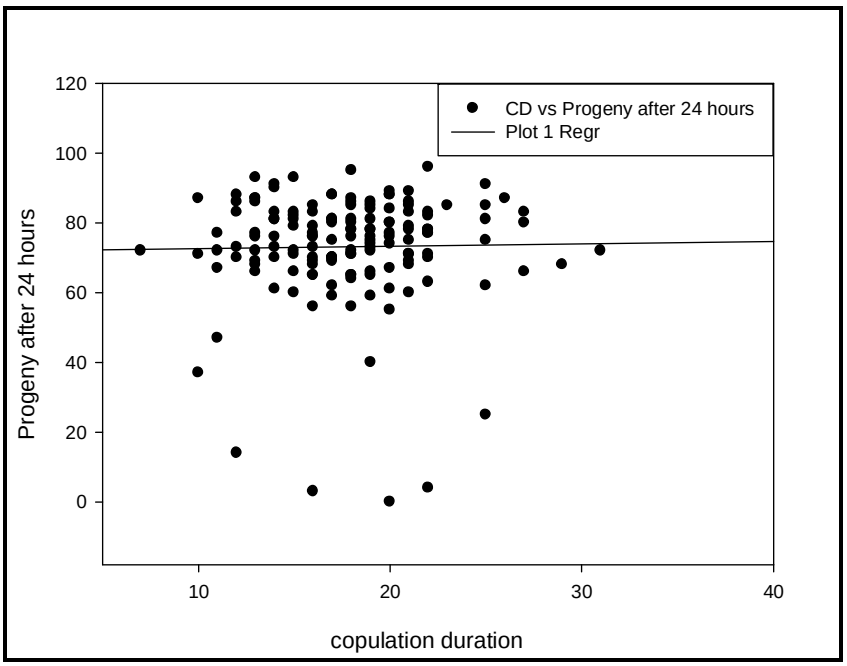


Figure 3.6 Correlation between CD and progeny produced by females on mating with target conditioned males after 24 hours from first egg laying.

Experiment 3*: Does effect of conditioning have different fitness consequence under different contexts?*

1) *Effect of conditioning on Mating latency of target male* (Figure 4.1)

One factor ANOVA on mean mating latency showed an increased ML for the males conditioned with small males, whereas, target males when conditioned with large males took longer time to start copulation. The ML data is not significant (DF=2, SS= 35.263, F= 1.991, p= 0.139) it could be that since ML is measured in minutes we are not able to pick up slight differences in ML.

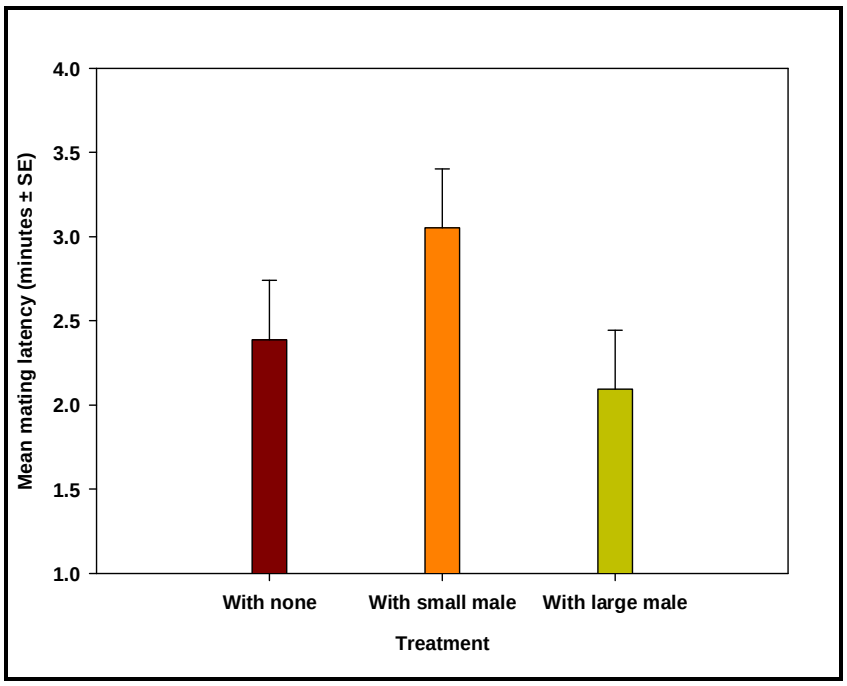


Figure 4.1 Effect of conditioning (with large or small or none) on mean mating latency (in minutes) of the target (scarlet eyed) male. Bars not connected by single line are not significantly different (multiple comparisons using Tukey's HSD).

2) *Effect of conditioning on Copulation duration of target male* (Figure 4.2)

One factor ANOVA yields a highly significant difference (DF=2, SS=501.849 F=23.16, p<0.0001*) of treatments on copulation duration of the target male. Although there is no significant difference between CD for target male when conditioned with either Large or Small, but both are statistically different from the mean CD of target male held singly (no competition) (Multiple comparisons using Tukey's HSD).

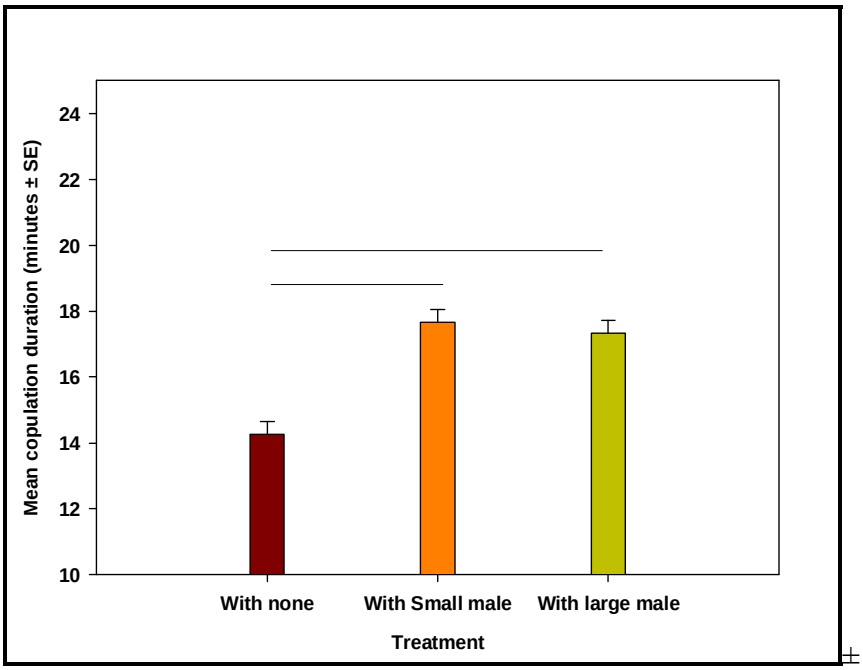


Figure 4.2: Effect of conditioning (with large or small or none) on mean copulation duration (in minutes) of the target (scarlet eyed) male. Bars connected by a single line are significantly different (multiple comparisons using Tukey's HSD).

3) *Comparison of Mean P1 values for the first male.* (Figure 4.3)

One factor ANOVA yields a non significant effect of treatment on sperm defence by conditioned target male (DF=2, SS=0.114, F= 1.465, p=0.234). Figure 4.3 shows the mean P1 values for first male. For target males when held with no competitor, mean P1 values are larger than the p1 values for target under conditioning.

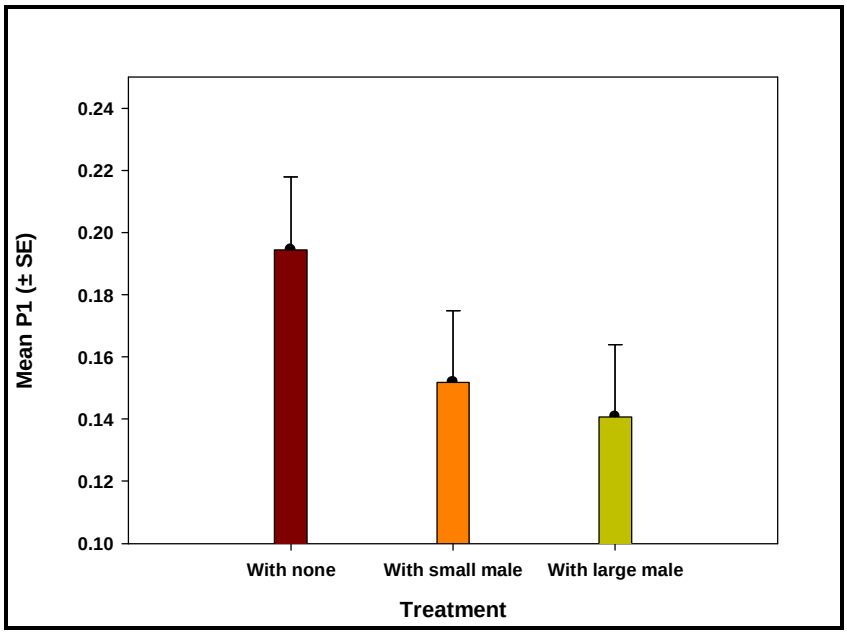


Figure 4.3: Mean p1 for the target male conditioned with large, small or no competitor male. Bars not connected by lines are not significantly different.

4) *Comparison of Mean P1 values for the second male.*(Figure 4.4)

The differences are not significant (DF=1, SS=0.049, F=1.267, p=0.262) for effect of competitor body size on P1. The mean P1 value for the second male when it is small is more than when the second male is large.

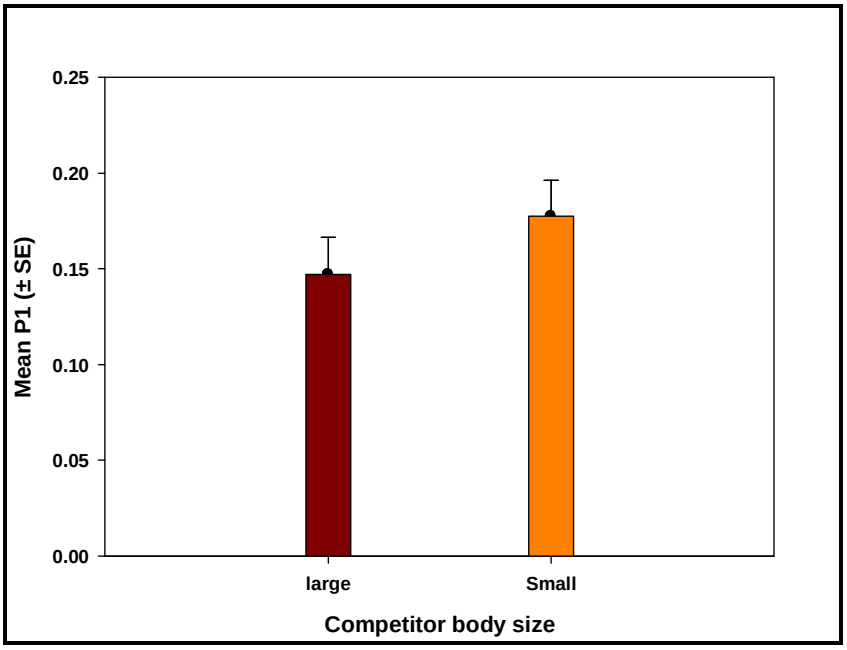


Figure 4.4 Effect of competitor body size (large and small) on the progeny sired by first male. Bars not connected by a single line are not significantly different.

5) *Effect of status of second male on the progeny of first male. (Treatment × second male interaction)* (Figure 4.5)

One factor ANOVA on P1 yields a non significant result (Table 4.5). The trend in figure 4.5 suggested when target males are not held in presence of any competitor during early life, the second male slightly has an increased effect on mean P1 values. Similar was the trend of target males earlier conditioned with small body sized males. There was no effect of second male on the proportion of progeny produced from first mating when the target males were conditioned with large body sized competitor males.

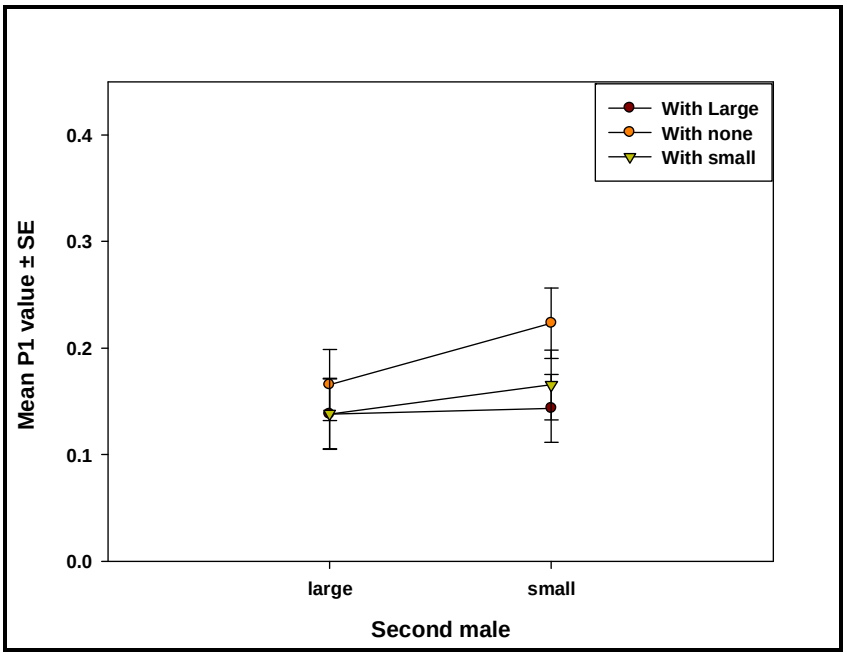


Figure 4.5: Effect of status of the second male (large or small) on the progeny sired by target (scarlet) conditioned male.

Table 4: Results of ANOVA on the effect of status of second male on sperm defence by target conditioned male.

	DF	SS	F RATIO	P>F
TREATMENT	2	0.114	1.465	0.234
SECOND MALE	1	0.049	1.267	0.262
TREATMENT × SECOND MALE	2	0.025	0.319	0.727

Chapter 5: Discussion

The present study addressed the perception of "quality" of sperm competition by male *Drosophila melanogaster* and how does potential levels of sperm competition affect the reproductive behaviour of the males when conditioned in their early reproductive life. The first experiment enlightens us with the fact that flies generated under different larval densities have significant effect on the body size of the males. Results suggest that mating success and reproductive behaviour (ML, CD) are not significantly affected by the body size of the competitor present in the mating vial. The important finding is that although large males invest little in mating by showing lower copulation duration, it is negativity correlated to the progeny sired. These results are in line with previous study (Pitnick and Garcia-Gonzalez, 2002) which shows that large males are more harming to the females. This implies that large body sized males are potent competitors in terms of sperm competition. In light of these results, all of the experiments were designed in such a way that target males were conditioned in their early life with a potent competitor either large or small.

Another important result of this study is that males alter their ejaculate investment, in terms of copulation duration according to different levels to Sperm competition. This study is different from previous reports (Bretman et al 2010, Nandy et al 2011) in a way that here we have represented a different aspect to sperm competition which is in terms of 'quality' and 'number' of competitor males. Quality is defined as "quality of the sperm" by altering egg densities and generating different body sized males (see chapter 2 for procedure).

a) *Effect of larval crowding on body size*

Different larval densities of 80eggs/vial and 300eggs/vial produced different body sized flies. We used dried body weight as the measure to identify body size. Our results are in line with earlier works where body size is known to decrease with increasing egg and larval density (Atkinson 1979, Wilkinson 1987, Imroze et al 2011).

Since the stocks of LH and LH-st populations used in the experiments are maintained at an egg density of ~150eggs/vial, changes in larval density produces a constraint of resources as there are many other larvae competing for same resources. Under crowded condition the amount of resources available to the developing flies is low thereby causing

them to grow up to small body sized adults. Conversely, when there is ample amount of resource available per individual, they tend to grow larger in size.

b) *Effect of presence of competitor on reproductive behaviour of target males*

This study shows that the presence of competitor affects the reproductive behaviour in terms of copulation duration of the target male. In Experiment 1, in presence of a large competitor male, target males mate for longer duration as compared to the presence of small competitor. Other component of reproductive behaviour ML shows an increase when mating is taking place in presence of large male. Since ML was recorded in minutes and not in seconds there is a high possibility that the differences are not significant due to poor resolution of observations.

In presence of large body sized competitor males, target males are able to perceive the threat That is why mating for longer will ensure that more sperms are transferred to the female and this is correlated by slightly negative slope which is consistent with the above argument of higher mating duration has negative correlation with progeny produced.

c) *Reproductive behaviour of competitor male*

Our results are consistent with the previous study that large males mate for short duration and produce less progeny because they tend to harm females more as compared to smaller males. Larger size for males is an advantage in inter-specific competition for mates, 'bigger is better' is considered to be a better outcome (Darwin 1871, Anderson 1994). Differences in body size cause physical changes to the fly such as changes in wing length, thorax length, testis size. Testis size is known to scale with body size in butterflies (Gage, 1994), bats (Wilkinson and McCracken, 2003) as well. It is known that large body size leads to increased male genitalia hence large sperm production. Large sperm production is a threat to competing male which is of medium body size. So, in order to compete and win the war of competition, competing males are known to device reproductive strategies to make sure that the maximum progeny sired is his (Bretman et al 2009). This plasticity of male mating behaviour has been reported in various species of *Drosophila*.

We found a negative correlation between the progeny produced and the copulation duration of the competitor male which is consistent with the report of increasing male size harm females more (Pitnick and Garcia-Gonzalez et al 2002). The trend in effect of body

size on the mean mating latency suggests that smaller males are taking longer to start mating. Moreover, it was found that mean ML has a positive relation on mean progeny produced.

d) *Males perceive the risk of sperm competition by coinhabitant number and quality.*

Exposure of males to competition in early adult life causes changes in their reproductive behaviour, copulation duration in particular. CD is an index to measure male ejaculate investment (Friberg 2006, Bretman et al 2009). On an average CD lasts for ~20min (Gilchrist and Patridge 2000) with some exceptions from the current study when the mating pair took more than 30mi to separate. In Drosophila sperm transfer usually takes between first 6-8min. From the first male along with sperms present in the seminal fluid there are proteins which are known to act as a switch or signal for the second male that the female is mated. Earlier reported by Nandy et al 2011 suggests that the males conditioned with different number of competitor males shows an increase in CD and sperm defence. This increase peaks till a certain level of competition (when the number is increased from 2 to 16) and as the competition is higher than 16 males the mean CD tends to decrease. The decrease might be due to the physical inability of the organism to produce large number of sperms beyond a certain limit. From the results of experiment 2 when target male is conditioned with different number and quality of the competitor male they showed differences in mean ML and CD. The target males when conditioned with 1S have decreased ML and CD. This decrease is more than when it is not conditioned with any competitor and held singly. Results from experiment 1 and 2 conclude that target males invest less when either a small male is in the mating arena or they have been previously exposed to a smaller male. Similar are the results when the targets are exposed to large males in their early life or in the mating arena, the mean CD is higher. The significant difference of 1S from other treatments (multiple comparison using tukey's HSD) suggests that somehow target males are able to perceive the quality and number and can alter their reproductive investment according as 1S and 4L treatments show very significant results.

When exposed to smaller body sized competitor males the mean copulation duration falls as compared to when exposed to larger body sized competitor. The different body sized competitor males' means different levels of sperm competition. More availability of

resources gives rise to large flies, which means more production of seminal fluid. This fluid in Drosophila is known to contain sperms as well as ACP's (Accessory gland proteins) which inhibit the sperms from other male to fertilize the eggs inside the reproductive tract of the female fly. Thus ACP's provide a barrier for other sperms as well as assurance for the first male that the progeny sired will be its. A direct inference could be that when a medium sized target male is kept in different conditions of sperm competition it is able to adapt to such condition (conditioning period of three days) and regulate its levels accordingly.

e) Effect of Conditioning on progeny number under no competition

Since the differences in copulation duration from experiment 2 suggests that targets can perceive and adapt according to the competition, it is expected to have an effect on progeny number. As one would expect that those males which are less harming would produce more offspring and vice-verse we did not find any correlation in experiment2b between progeny produced and the treatment. A consistent increase of mean progeny number when the mating male is held under no conditioning could be easily observed but it was not significant.

f) Effect of conditioning on the fitness consequence of target male depending on the status of the second male.

In order to resolve the difference of conditioning between Large and small we reduced the conditioning part to these treatments only. The CD data does not yield a significant difference between these two treatments even though they are different from CD of target males held with no competitor (Tukey's HSD). We expected a decrease in mean CD when the targets were held with large but it was different from the results obtained.

An important component of male fitness is the sperm defence ability. We did not find any affect of the status of the second male on the mean P1 that is the proportion of progeny sired by the first male.

Conclusion and Future outlook

In conclusion this study shows that male *D. Melanogaster* can alter its reproductive behaviour in particular, copulation duration according to the quality of competitors experienced in early adult life as well as to the presence of competitors. There is no effect of conditioning on the progeny produced by females on mating with conditioned males.

Since, sperm defence is an important aspect of fitness I look forward to analysis the data for the same.

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